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Type 2 diabetes-related proteins derived from an *in vitro* model of inflamed fat tissue

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ABSTRACT

Currently, there is a worldwide increase of patients with type 2 diabetes (T2D). During the progression of healthy obese to T2D status, there is an influx of immune cells, in particular macrophages, into visceral adipose tissue, accompanied by an increase of inflammatory cytokines, such as, IL6, TNFα and Hp. To get a better insight in the underlying mechanisms, we performed a quantitative LCMS analysis on a modified *in vitro* assay, combining 3T3L1 adipocytes and activated RAW264.7 macrophages, thus mimicking inflamed adipose tissue. Clinically known proteins, *e.g.* IL6, TNFα, AdipoQ, complement factor C3, B and D were identified, thus confirming the assay. In addition, we found 54 new proteins that can potentially be used for research into the mechanism of T2D. Comparison of our results to a study on human visceral fat of obese non-diabetic and obese diabetic subjects, indicated that AUH, NAGK, pCYT2, NNMT, STK39 and CSNK2A2 might indeed be linked to insulin resistance in humans. Moreover, the expression of some of these genes was also altered in human blood samples at early or later stages of insulin desensitization. Overall, we conclude that the direct contact co-culture of 3T3L1 adipocytes with activated macrophages could be a mechanistically relevant and partially translational model of inflamed visceral adipose tissue.

1. Introduction

In many Westernized cultures there is an increase in obesity, which may develop into metabolic syndrome and type 2 diabetes (T2D) [1,2]. In obese subjects, an increase of lipid storage by adipocytes is observed [2]. In addition to lipid storage, adipose tissue can also have important endocrine and immune-regulatory functions [2–4] and most of these inflammatory factors, that can regulate insulin resistance, are produced by visceral adipose tissue rather than by subcutaneous adipose tissue [3,4].

In the progression of healthy obese to a T2D status the increase of the influx of immune cells, especially macrophages, into visceral adipose tissue is mediated by adipocyte-derived chemokines and this influx is accompanied by inflammatory cytokines such as Tumor Necrosis Factor α (TNFα) and Interleukin 6 (IL6) [5,6]. Although the role of IL6, C-reactive protein (CRP), Haptoglobin (Hp) in insulin resistance is not clear, there are clear indications on the mechanism of insulin resistance caused by the macrophage-derived cytokine TNFα [7]. Upon activation of the TNFα receptor, the adaptor molecule Insulin Receptor Substrate 1 (IRS1) will become phosphorylated on a serine residue, thereby inhibiting tyrosine phosphorylation of this adaptor molecule. Tyrosine phosphorylation of IRS1 is required for relaying the signal from the insulin receptor towards Glucose transporter type 4 (GLUT4), which will subsequently, translocate to the plasma membrane and allow uptake of glucose [7]. Indeed, studies in human patients suggest that fasting serum levels of glucose can be lowered by preventing TNFα receptor activation using an inhibitory anti-TNFα antibody [8].

Macrophages can produce and secrete TNFα after activation of the Toll-like receptor 4 (TLR4) by lipopolysaccharide (LPS). Interestingly, TLR4 knockout mice are less prone to insulin resistance after a high fat diet than their wildtype littermates, suggesting that TLR4 is involved in insulin resistance, possibly by inducing TNFα [9,10]. Whether this is due to TLR4 stimulation by LPS is still under debate and other agonist for TLR4 might be involved. For instance, Free Fatty Acids (FFA) bound to the lipid carrier protein Fetuin A (FetA) are also described to efficiently activate TLR4 and regulate insulin resistance [11,12].

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In order to better understand the role of inflamed adipose tissue in T2D and identify novel proteins or pathways involved in insulin desensitization, we used a direct co-culture system of 3T3L1 adipocytes and RAW264.7 macrophages and analyzed protein expression by quantitative LCMS analysis. We then verified the identified proteins in existing, published human patient data sets. This approach allowed us to identify new proteins/genes which may help us to better understand the pathways that can lead to T2D.

2. Results

2.1. Direct contact versus indirect contact between adipocytes and macrophages

Currently, most publications on 3T3L1 adipocytes in co-culture with RAW264.7 macrophages describe indirect co-cultures by using transwell systems separating the adipocytes and the macrophages with a semi-permeable membrane that allows diffusion of cytokines and other cellular products, but prevents direct cellular contact [14,15]. *In vivo*, adipocytes and macrophages engage in direct cell-cell interactions and we postulate that this is important for the inflammatory response [16]. To test this, we prepared co-cultures of 3T3L1 adipocytes with RAW264.7 cells in regular cell-culture plates (allowing cell-cell contact) or indirectly by using a transwell (no contact between macrophages and adipocytes). Cells were then stimulated with LPS (1 μg/ml) to activate TLR4. First we tested the secretion of IL6 and TNFα, which are well-known upregulated proteins in RAW264.7 after LPS stimulation [17]. Indeed, single cultures of RAW264.7 started to produce IL6 and TNFα after LPS exposure, however, for IL6 we observed a higher increase of IL6 in the presence of 3T3L1 cells, whereas TNFα appeared to be lower in the presence of 3T3L1 cells (Fig. 1A), suggesting that the macrophages are the main source of TNFα in this co-culture. Moreover, we observed significantly higher levels of IL6 in the LPS-stimulated direct contact co-cultures compared to the transwell co-cultures (Fig. 1A). This was also observed for IL1α mRNA expression (Fig. 1B), suggesting that direct contact between RAW264.7 cells and 3T3L1 cells enhances the production and secretion of pro-inflammatory cytokines IL6 and IL1α, whereas TNFα expression appears to be independent of cell-cell contacts. In addition, AdipoQ expression decreased after LPS in both direct and indirect co-cultures to similar levels (Fig. 1C), suggesting that this decrease is independent of cell-cell contact as well.

2.2. Identification of novel proteins involved in inflamed adipose tissue

The established direct-contact co-cultures of adipocytes and macrophages were tested for changes in protein expression when stimulated with LPS for 18 h. After exposure, cells were lysed and analyzed by LCMS (Fig. 2A). We expected to see an increase in Hp, AdipoQ, TNFα, IL6, CFB, SAA3 and many other inflammatory proteins [1,18–22], however, due to the sensitivity of the assay, we observed only 54 significantly changed proteins among which Hp, CFB and SAA3, were increased (Fig. 2B).

Next, we performed micro-array analyses and compared the mRNA and protein expression of the 54 proteins identified in the LCMS (Fig. 2C). IL6 and TNFα were included as controls for the LPS activity and AdipoQ as a marker for adipogenesis (Fig. 2C). Although the changed expression of many proteins correlated with the changed expression of their mRNA, *e.g.* Hp, SAA3, CFB, there was a clear subset of genes that changed protein expression without changing their mRNA expression, e.g. SigmaR, Csnk2b, NNMT and pCYT, or had an inverse correlation, where protein expression increased and mRNA expression actually decreased. This indicates that LCMS allows us to identify new proteins and possible signaling pathways (Figure S2) that could not have been identified when using micro-array analyses.

2.3. CFB, CFD and C3 expression in 3T3L1 cells is regulated by TNFα

To test whether the increased Hp protein expression was produced by 3T3L1 or RAW264.7 cells, we performed QPCR analyses and observed an increase in Hp mRNA expression when adipocytes were stimulated with LPS, which appeared to be independent of macrophages (Fig. 3A). CFB was observed to be increased in LPS-stimulated co-cultures of adipocytes and macrophages (Fig. 2). This fits with human data showing that CFB expression is increased in patients with T2D [18–22]. In these patients, the expression of Complement factor 3 (C3) is also increased, whereas expression of CFD is decreased [18]. To test whether our *in vitro* system reflects the patient situation we tested the mRNA expression of CFB, C3 and CFD and found that CFB mRNA expression only increased in LPS-stimulated co-cultures, whereas C3 mRNA expression was already present in LPS-stimulated single cultures of adipocytes (Fig. 3B). This suggests that the increase of C3 mRNA observed in the co-cultures was most likely produced by the adipocytes.

When adipocytes were exposed to either macrophages or LPS, there was no significant decrease of CFD observed on mRNA level. However, when adipocytes in direct co-culture with macrophages were stimulated with LPS we observed a significant decrease of CFD mRNA (Fig. 3B).

These observations suggest that a macrophage-derived factor is responsible for the reduced expression of CFD, possibly TNFα and/or IL6. Therefore, we tested whether IL6 or TNFα themselves can alter the expression of CFB, CFD and C3 to the same level as we observed in LPS stimulated co-cultures of 3T3L1 and RAW264.7 cells. Indeed, when 3T3L1 adipocytes were stimulated with TNFα, there was an increase of CFB and C3, and a decrease of CFD to similar levels as observed for LPS-stimulated co-cultures, whereas IL6 did not affect the expression of these complement factors (Fig. 3C). This suggests that TNFα is a key player in the regulation of complement expression by adipocytes.

2.4. Visceral adipose tissue in healthy obese versus T2D obese women

Next, the objective was to check whether identified molecules in our *in vitro* model could be of relevance for future T2D studies. To this end we analyzed a publicly available human gene expression data set of visceral adipose tissue of 10 Indian obese women [19]. Five of these subjects were healthy obese and five were indicated as having T2D. Both groups were matched for BMI. Expression of all 54 genes of the proteins which we identified in our LCMS analysis as important, with a notable difference between healthy obese and T2D obese subjects are shown in Fig. 4.

A decreased expression for VPS26A, VPS26B, pCYT2, AUH, STK39 and CSNK2A2 and an increase for VCAM1, EFHD2 and SAA1 (human orthologue for mouse SAA3) are in agreement with our LCMS data (Figs. 4 and 2). However, NAGK and NNMT mRNA showed an increase in expression *in vivo,* whereas, we observed a decreased expression *in vitro* on protein level. Of note, not all of the 54 newly identified proteins, whose expression changed in our *in vitro* model, showed an altered gene expression in the T2D obese subjects compared to healthy obese subjects in the *in vivo* study on obese Indian women [19].

Currently, visceral fat is considered to have a larger impact on the development of T2D than subcutaneous fat [20]. To test whether the observed differences in gene expression were specific for visceral fat or can also be observed in subcutaneous fat from subjects suffering from T2D, we analyzed the expression of the genes from Fig. 2A to a published study on human subcutaneous adipose tissue data sets from healthy and T2D donors [21]. Whereas we observed changes in gene expression levels upon T2D development in visceral fat, no differences in gene expression were observed in subcutaneous fat from healthy obese versus T2D obese patients (Figure S3). This was the case for all 54 newly identified genes (Figure S3) and AdipoQ (Figure S3), suggesting that the *in vitro* co-culture reflects visceral adipose tissue rather than subcutaneous adipose tissue (Figure S3).

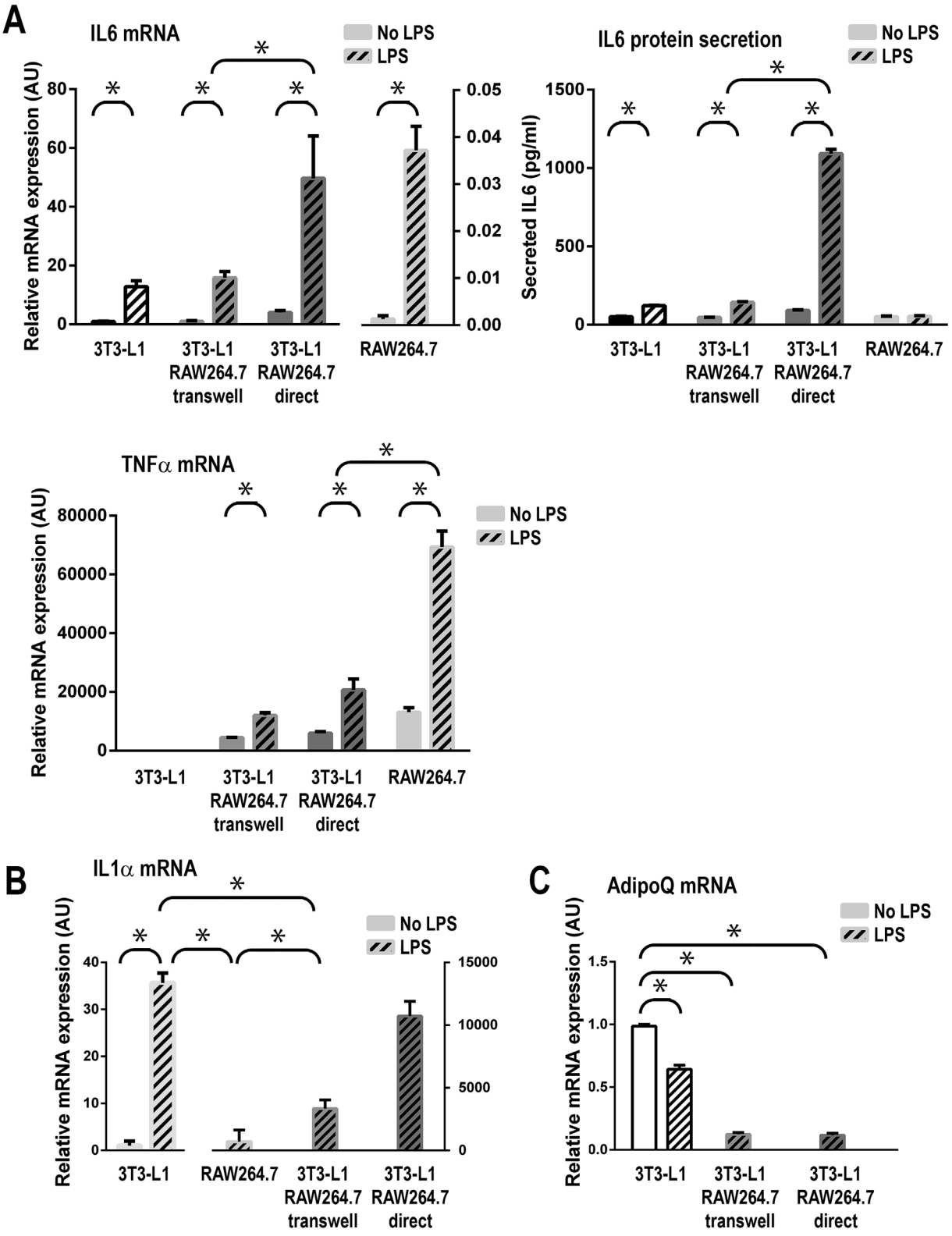
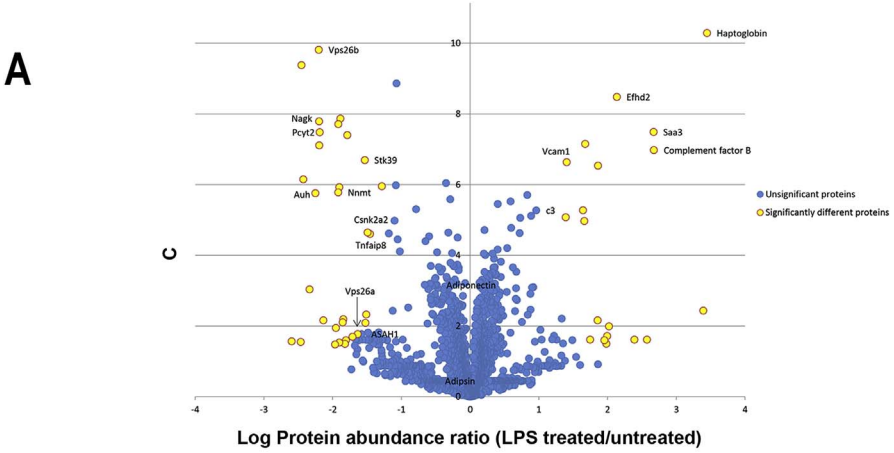


Fig. 1. Direct contact between 3T3L1 adipocytes and RAW264.7 macrophages have enhanced inflammatory response.
3T3L1 adipocytes and RAW264.7 cells were either co-cultured in direct contact or separated by a transwell membrane. RAW264.7 cells were seeded in the upper compartment and the adipocytes in the lower compartment of the transwells. Cells were co-cultured and stimulated with LPS for 18 h (A) IL6 protein and mRNA and TNFα mRNA were normalized to untreated adipocytes. (B) IL1α and AdipoQ mRNA were determined by QPCR and normalized to untreated 3T3L1 adipocytes. All experiments were performed 3 times.
(* indicates p-values which are lower than 0.01 with unpaired parametric Welch's *t*-test).

2.5. Some significant visceral adipose markers are reflected in blood

When testing the relevance of our newly identified proteins and their role in T2D it would be less invasive for patients to test these proteins on blood samples rather than visceral adipose tissue samples. The proteins, observed in our study, can be either produced by



B

Upregulated proteins				
Protein ID	Gene names	-Log t-test p-value	Log Protein abundance ratio (LPS/untreated)	
Q3UBS3	Hp	10.29	3.45	
E9Q5U3	Gm10358	2.43	3.39	
B8JJN0	CfB	6.98	2.68	
P04918	Saa3	7.49	2.67	
B7ZCQ3	Hmgn2	1.61	2.57	
Q6ZQH8	Nup188	1.61	2.39	
Q8C845	Efhd2	8.48	2.14	
B2RTB0	Pdap1	1.99	2.02	
Q9Z1G4	Atp6v0a1	1.72	2.00	
Q66JR8	Ptms	1.50	1.98	
A0JNY3	Gphn	1.59	1.96	
P15116	Cdh2	6.54	1.86	
B1B079	Zc3h15	2.16	1.86	
P17095	Hmga1	1.61	1.75	
Q3UGJ5	Rasa3	7.15	1.67	
P99027	Rplp2	4.97	1.66	
P54987	Irg1	5.27	1.64	
P29533	Vcam1	6.64	1.41	
Q6IRU2	Tpm4	5.08	1.39	
Downregulated proteins				
Q541Z2	Fnta	4.62	-1.18	
A2AVJ6	Commd9	5.96	-1.28	
D3Z312	Tnfaip8	4.61	-1.46	
O54833	Csnk2a2	4.64	-1.49	
Q9D967	Mdp1	2.32	-1.51	
Q8R3Q6	Ccdc58	2.09	-1.52	
A2AQL0	Stk39	6.70	-1.53	
Q3TWT5	Asah1	1.77	-1.58	

Downregulated proteins			
Protein ID	Gene names	-Log t-test p-value	Log Protein abundance ratio (LPS/untreated)
Q6P069	Sri	1.77	-1.64
P67871	Csnk2b	1.61	-1.66
P40336	Vps26a	1.62	-1.67
Q3UBR8	Timm17b	1.70	-1.71
Q8VE96	4930471M23	7.40	-1.79
Q9JKV1	Adrm1	1.60	-1.81
Q924D0	Rtn4ip1	1.49	-1.82
P50428	Arsa	2.19	-1.84
A2AN96	Rbbp9	2.10	-1.85
Q3TA37	Tsfm	7.87	-1.89
Q01149	Col1a2	5.93	-1.90
B1B0W8	Hprt	1.53	-1.90
E9Q272	Hook3	7.71	-1.92
O55239	Nnmt	5.78	-1.92
Q7TNL5	Ppp2r5d	1.94	-1.95
Q542Y0	Nqo1	1.48	-1.96
P20918	Plg	2.16	-2.13
Q3USD5	Pcyt2	7.48	-2.19
Q99LY9	Ndufs5	7.11	-2.19
Q9D997	Nagk	7.79	-2.20
Q8C0E2	Vps26b	9.81	-2.20
Q9JLZ3	Auh	5.76	-2.25
Q3UWG5	Cd81	3.04	-2.34
A2AMS1	Sigmar1	6.15	-2.43
P61082	Ube2m	9.38	-2.45
A3E4B1	ATP8	1.55	-2.46
O70456	Sfn	1.57	-2.60

C

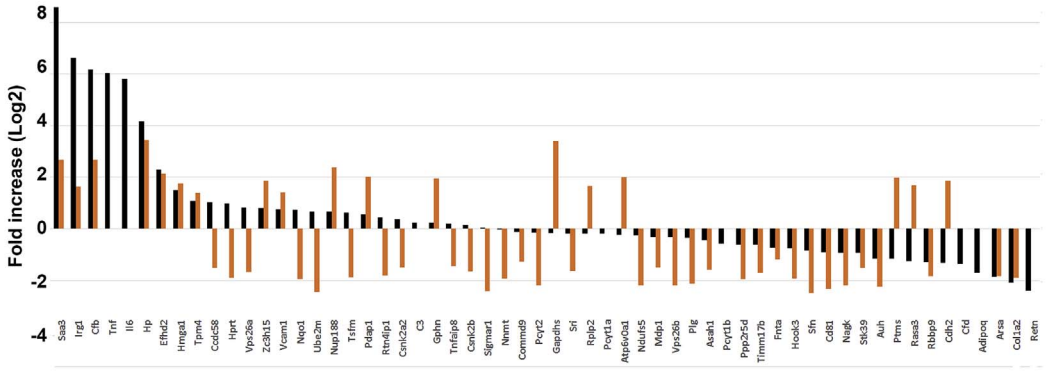


Fig. 2. Quantitative LCMS and mRNA analysis of LPS-stimulated co-cultures of 3T3L1 adipocytes and RAW264.7 macrophages.
3T3L1 adipocytes and RAW264.7 cells were co-cultured in direct contact and stimulated with LPS (1 μg/ml) for 18 h. (A) Vulcano plot of identified proteins in quantitative LCMS, showing proteins that become up-regulated upon LPS treatment on the positive right side and down-regulated proteins on the negative left hand side in the figure. T-test p-values are shown on the y-axis as "–Log p-value". (B) Table showing proteins (with their gene names) that have changed their expression after LPS stimulation as determined by quantitative LCMS. Proteins that change their mRNA expression in humans are indicated in bold. (C) Fold increase of mRNA (black) and protein (orange) expression of proteins identified in quantitative LCMS, in single adipocytes cultures versus co-cultures of adipocytes and RAW264.7 macrophages which were stimulated with LPS (1 μg/ml) for 18 h. All experiments were performed 3 times (* indicates p-values which are lower than 0.01 with unpaired parametric Welch's *t*-test). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

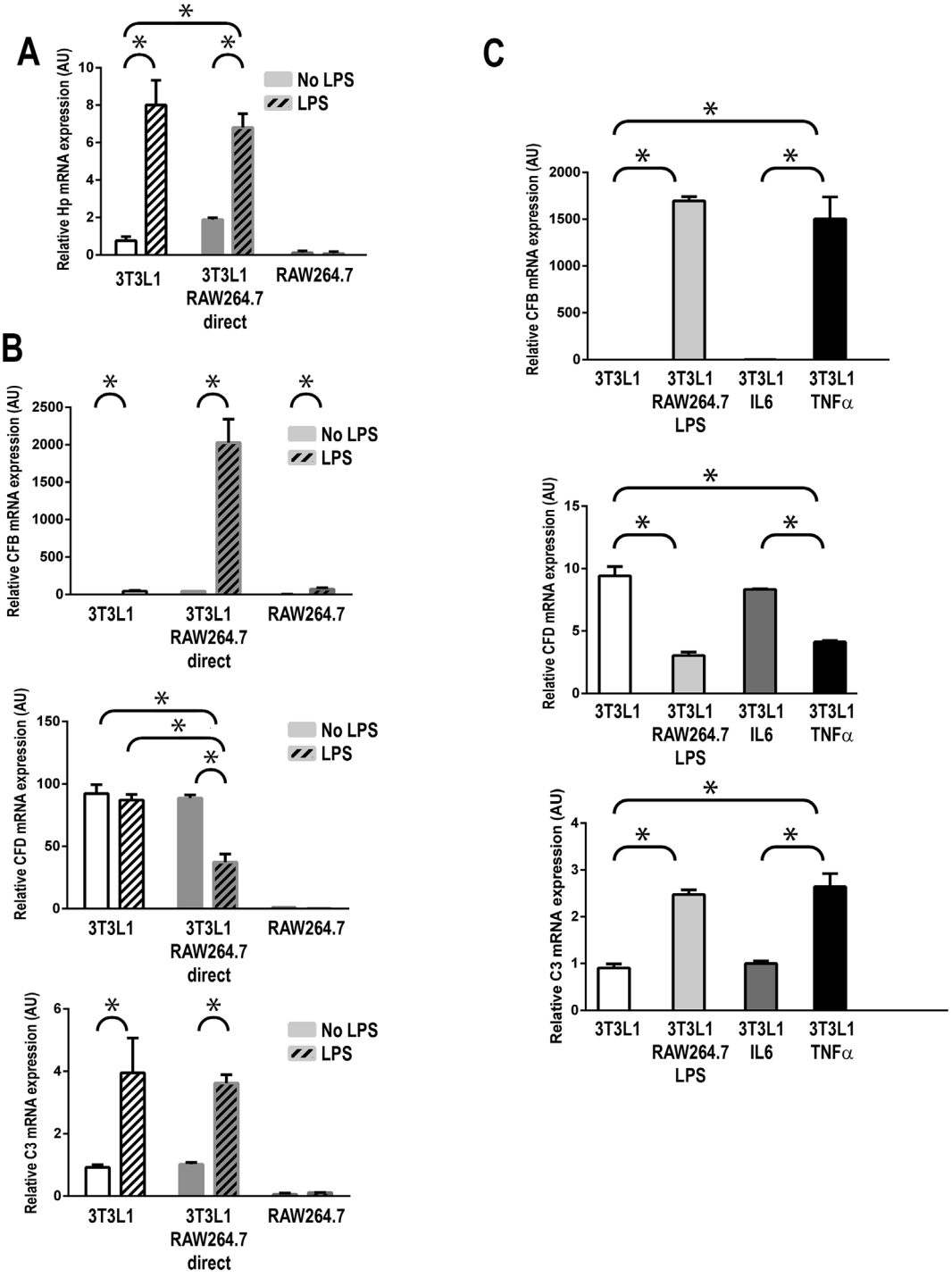


Fig. 3. TNFα is a key-player in complement expression by 3T3L1 adipocytes and RAW264.7 macrophages.
3T3L1 adipocytes and RAW264.7 cells were co-cultured and stimulated with LPS (1 μg/ml) or TNFα (1 ng/ml) for 18 h where indicated. (A) Hp mRNA was determined by QPCR and normalized to untreated adipocytes. (B–C) CFB, CFD and C3 mRNA were determined by QPCR and normalized to untreated 3T3L1 adipocytes. All experiments were performed 3 times (* indicates p-values which are lower than 0.01 with unpaired parametric Welch's *t*-test).

adipocytes, macrophages or both. Although adipocytes will, most likely, not easily enter the blood or lymphatic system, the more motile macrophages can. Therefore, we analyzed in another publicly available dataset [22] whether the expression of the genes from Fig. 2 is significantly changed in blood samples from obese male adult human subjects having either a normal fasting glucose level, an impaired fasting glucose (IFG) level or diagnosed as T2D patient. As a control, we first checked whether the expression of currently used biomarkers, such as AdipoQ and Hp [2,23–26], were changed in the blood cell analyses of that study. Indeed, expression of AdipoQ and Hp decreased and increased, respectively, in subjects with decreased insulin sensitivity or T2D (Fig. 5). Although not all genes in blood changed their expression as compared to our analysis on visceral fat, we noted a downregulation of pCYT2 (expressed highly by RAW264.7), STK39, NAGK and SAA1 and a slight up-regulation of NNMT and AUH mRNA in blood of subjects with IFG and T2D (Fig. 5). This suggests that these genes could be interesting druggable targets to improve insulin sensitivity (Figure S4).

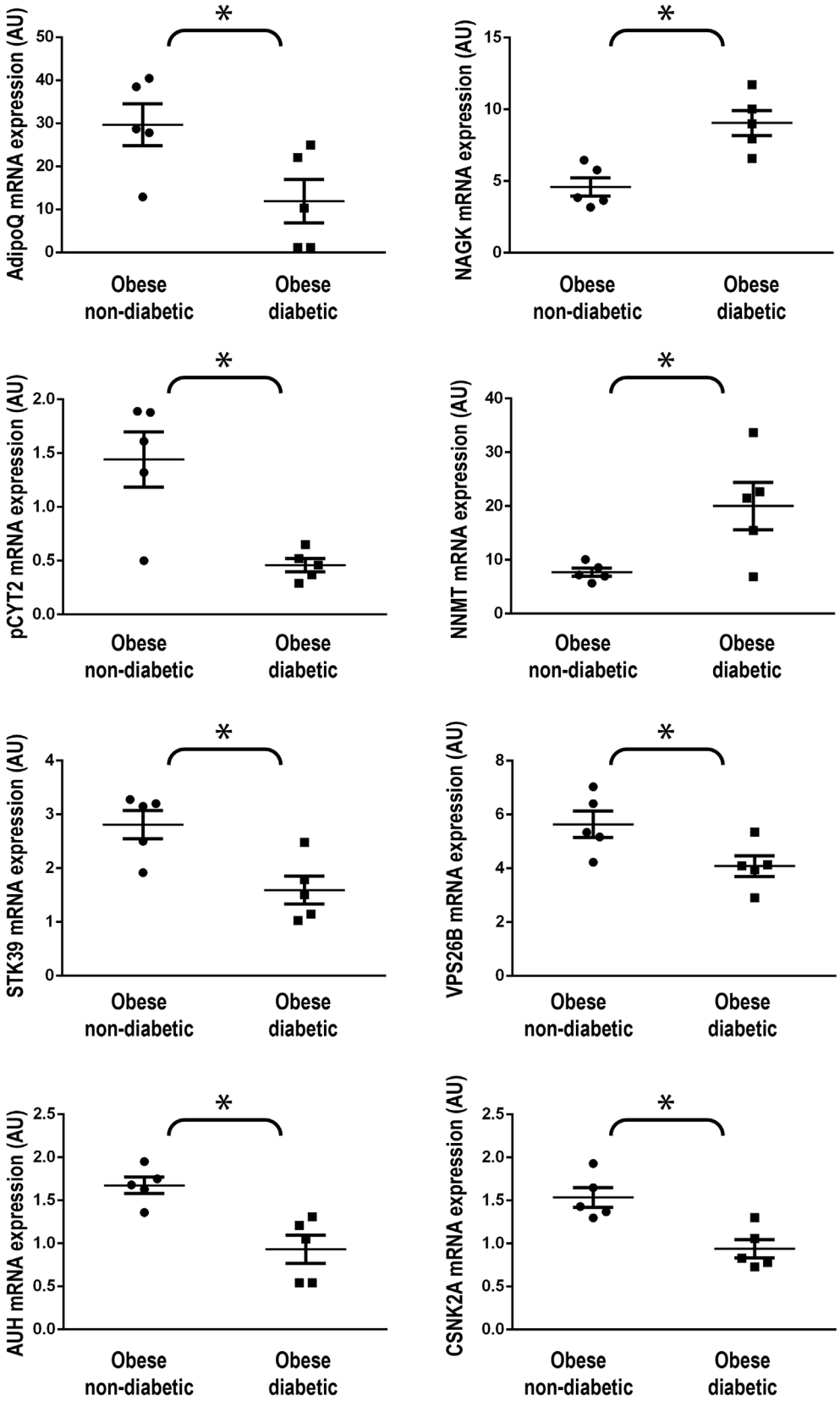


Fig. 4. mRNA expression of genes identified in quantitative LCMS in obese non-diabetic and obese diabetic Indian women.
Comparison of mRNA expression of genes, identified through proteomics, in 5 obese non-diabetic and 5 obese diabetic women. Every data point represents 1 subject (* indicates p-values which are lower than 0.05 with non-parametric *t*
-test according to Mann-Whitney).

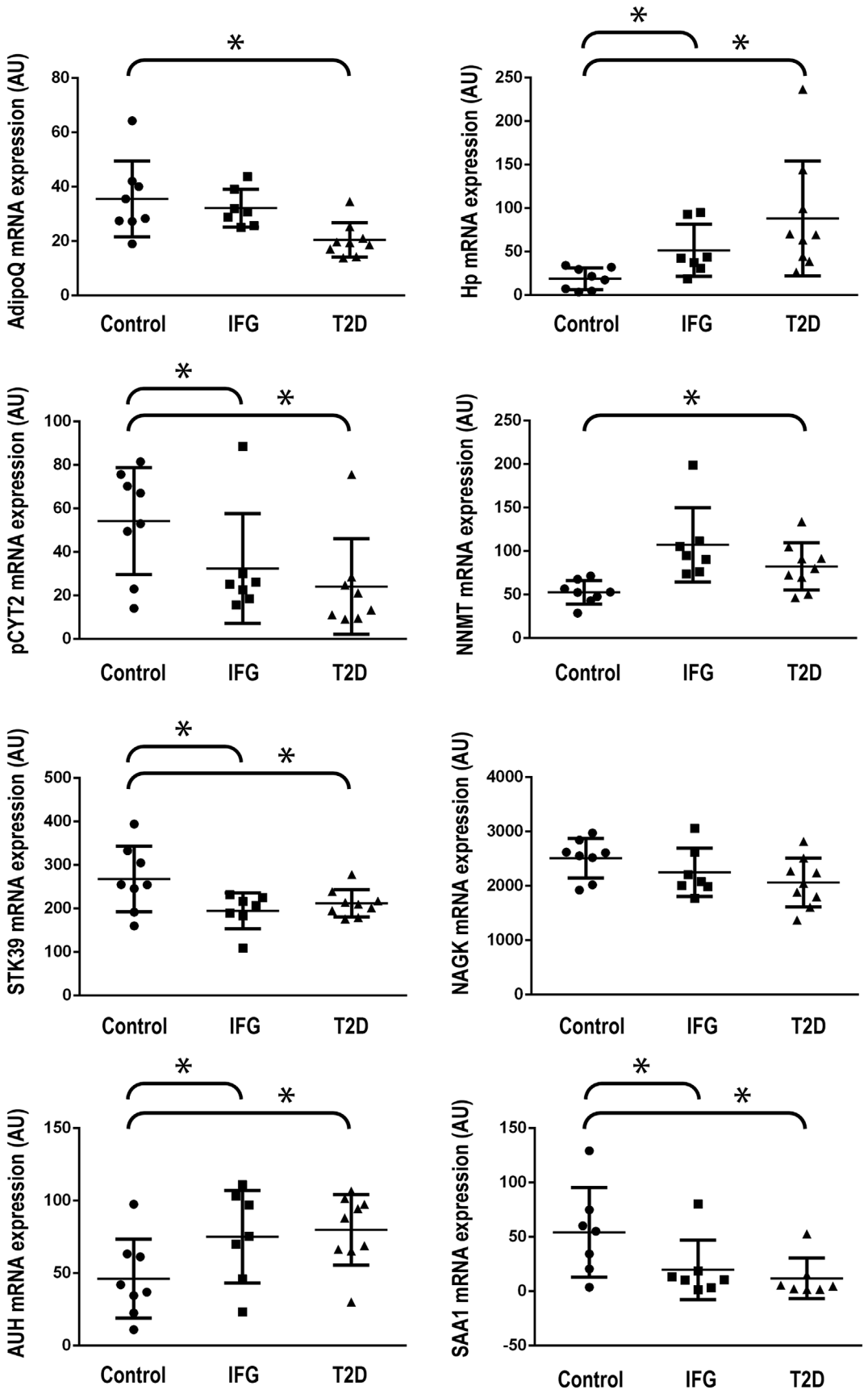


Fig. 5. mRNA expression in blood samples of healthy obese, IFG and T2D subjects of genes identified in quantitative LCMS.
Comparison of the mRNA expression of genes identified through proteomics, in blood samples which were obtained from obese male adult human subjects (21–70 years of age) with normal fasting glucose (< 6.1 mmol glucose/L), impaired fasting glucose (IFG) (6.0 mmol glucose/l - 7.0 mmol glucose/L) and T2D (> 7.0 mmol glucose/L) [22]. Every data point represents 1 subject (* indicates p-values which are lower than 0.05 with non-parametric *t*-test according to Mann-Whitney).

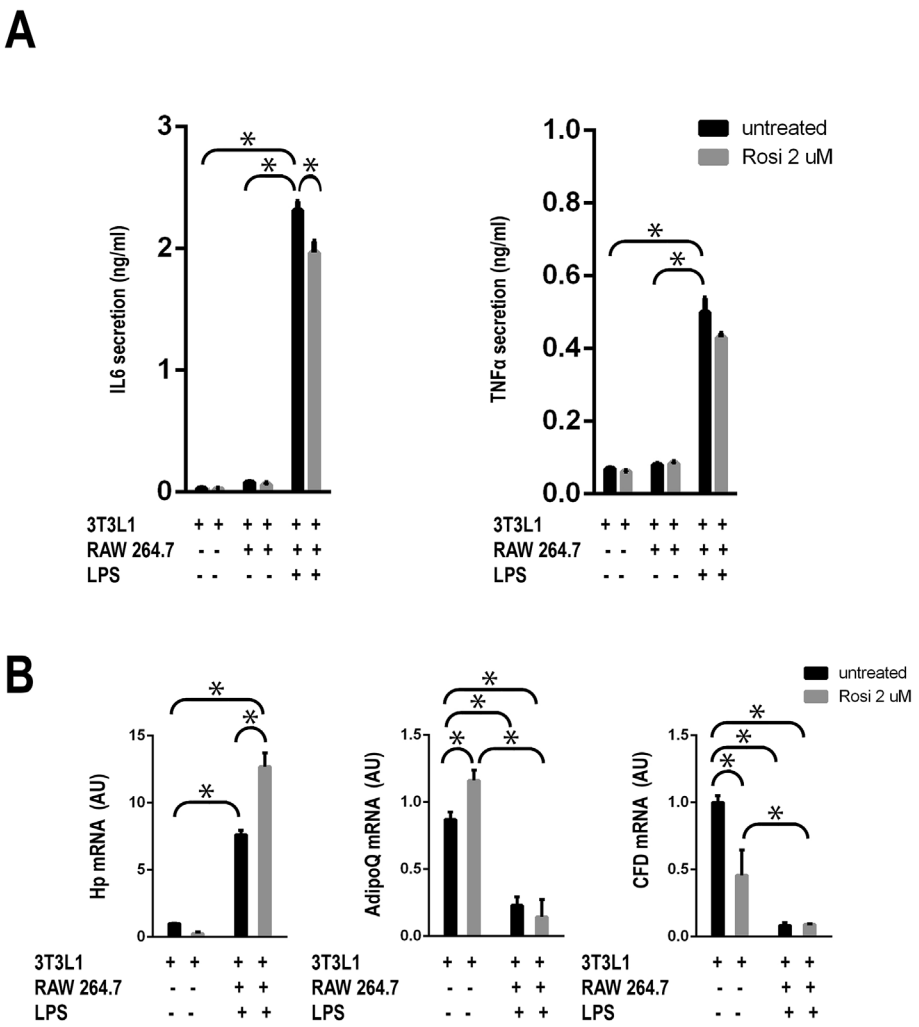


Fig. 6. Involvement of PPARγ agonist in adipocyte inflammation.
3T3L1 adipocytes and RAW264.7
cells were co-cultured and stimulated with LPS (1 μg/ml) for 18 h where indicated. (A) IL6 and TNFα protein concentrations were determined by ELISA and normalized to untreated adipocytes. (B) Hp, AdipoQ and CFD mRNA expression was determined by QPCR normalized to untreated adipocytes (* indicates p-values which are lower than 0.01 with unpaired parametric Welch's *t*-test).

2.6. Effect of rosiglitazone in co-culture of adipocytes and macrophages

To test whether our *in vitro* model can be used for drug screening we tested rosiglitazone. Rosiglitazone is known for its beneficial effect on insulin sensitivity in humans by activating PPARγ and increasing AdipoQ [27]. To test whether rosiglitazone can affect the expression of IL6, TNFα, Hp, AdipoQ or CFD *in vitro* co-cultures, we exposed the adipocytes and macrophages to rosiglitazone in the absence or presence of LPS. After an overnight exposure, ELISAs for IL6 and TNFα (Fig. 6A) indicated that rosiglitazone inhibited LPS-induced IL6 and TNFα secretion, which points to a beneficial effect for insulin sensitivity.

The rosiglitazone-samples were tested for mRNA expression of Hp, AdipoQ and CFD by QPCR (Fig. 6B) and we noticed that, similar to what is observed in humans [27], rosiglitazone can increase AdipoQ mRNA expression in single adipocyte cultures (Fig. 6B). However, this beneficial effect of rosiglitazone on AdipoQ is completely abolished when adipocytes were co-cultured with macrophages in the presence of LPS. In addition, rosiglitazone caused a higher Hp mRNA expression but could not prevent the LPS-induced decrease of CFD (Fig. 6B). This suggests that rosiglitazone can affect T2D through multiple pathways.

3. Discussion

Through LCMS analysis on an *in vitro* co-culture of macrophages and adipocytes we have shown that this model might be helpful in investigating some aspects of T2D development, especially the inflammatory aspect of T2D, as indicated by the altered expression of proteins known to be involved in T2D, such as AdipoQ, Hp, TNFα and IL6 [2,7,26,28]. Newly identified proteins in our study (pCYT2, NNMT, STK39, NAGK, SAA1, VPS26A&B and AUH) were confirmed by comparison to a published study on human mRNA expression. The observed proteins can be linked to signaling pathways for a better understanding of the development of T2D and to design specific therapeutic approaches (Fig. 7 and Figure S3). Here, we will discuss the potential roles in the development of T2D of the proteins found to have an altered expression *in vitro* and *in vivo* (Fig. 7). First, we will discuss key regulator TNFα followed by other proteins identified in this study but with a hitherto less well known role in T2D development.

3.1. TNFα

A key molecule in insulin desensitization is TNFα, well known to inhibit insulin receptor signaling pathways by blocking IRS1 function and subsequently GLUT4-mediated glucose transport across the plasma

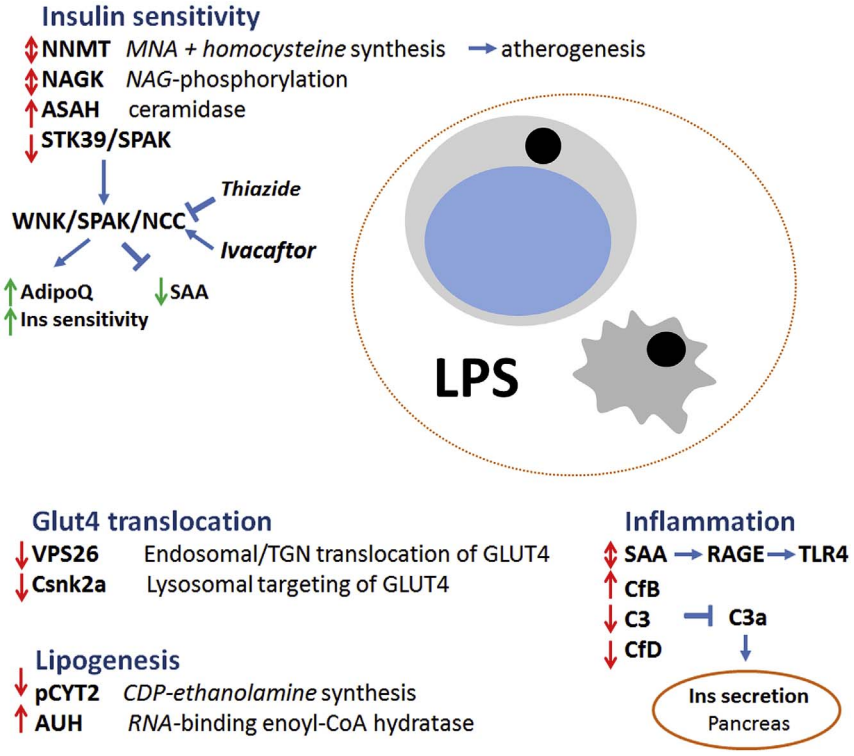


Fig. 7. Schematic overview of identified proteins. Inflamed adipose tissue is in this model represented by co-culture of adipocytes and macrophages, stimulated with LPS. Red arrows indicate the change in expression after LPS exposure. Green arrows indicate the change in expression when the WNK/STK39/SPAK/NCC is activated. Blue arrows indicate downstream pathways. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

membrane [3]. Besides its effect on IRS1 functioning, it is possible that TNFα has other ways of affecting diabetes. Indeed, the presented data shows that TNFα regulates the expression of CFB, C3, and CFD in 3T3L1 adipocytes (Fig. 3). CFD from adipocytes, which is downregulated by TNFα, is involved in the production of C3a from C3, the first being a recently newly identified secretatogue for insulin [29]. Although we observed an increase of C3 mRNA, we found a decrease in CFD mRNA, suggesting that less C3a will be produced after TNFα exposure. This indicates that TNFα is not only involved in desensitizing the insulin receptor but also, via CFB/CFD/C3a, in regulating insulin secretion by the pancreas.

3.2. pCYT2

One of the proteins that was down-regulated upon LPS stimulation of adipocyte/macrophage co-cultures was pCYT2, an enzyme involved in *de novo* biosynthesis of phosphatidylethanolamine (PE) from ethanolamine and diacylglycerol [30]. pCYT2$^{+/-}$ mice, containing less of this enzyme, show elevated lipogenesis and lipoprotein secretion, hypertriglyceridemia, obesity, and insulin resistance [31]. This suggests that decreased expression of pCYT2 as a result of inflamed adipose tissue, could be one of the underlying mechanisms involved in T2D development.

3.3. NNMT

Another protein whose expression decreased after LPS treatment *in vitro* was NNMT (Fig. 2). The mRNA expression of NNMT, on the other hand, was increased in obese non-diabetic *vs.* obese diabetic Indian women (Fig. 4). Recently, the importance of NNMT for T2D-related health effects was demonstrated by showing that NNMT expression was markedly increased in adipose tissue of GLUT4$^{-/-}$ mice [32]. Moreover, NNMT knockout (NNMT$^{-/-}$) mice were protected against diet-induced obesity and showed improved glucose tolerance [32]. The finding in the human study with Indian women is in agreement with the data observed in NNMT$^{-/-}$ mice. The discrepancy of these *in vivo* findings with the observed decreased NNMT expression in our *in vitro* model cannot be explained by a difference between mRNA and protein, because the NNMT mRNA expression in our LPS-stimulated *in vitro* co-cultures did not change compared to non-stimulated co-cultures (Fig. 2). Perhaps, the decreased expression found *in vitro* is a result of a short-term TLR4 stimulation, as cells may behave differently when cells are exposed to TLR4 agonists, such as LPS or FetA/Free Fatty Acids complex [11], for a longer period, *e.g.* weeks or months, which might be the case *in vivo*.

3.4. STK39

A third protein we found down-regulated after treating adipocyte/macrophage co-cultures with LPS was STK39/SPAK (Figs. 2 and 4). The serine/threonine kinase STK39/SPAK is part of the WNK (With-No-Lysine)/SPAK/NCC pathway that regulates sodium and potassium transport across the plasma membrane [33,34]. Besides changes in expression of STK39/SPAK, it has been observed that STK39/SPAK and its substrate NCC (Na-Cl co-transporter), are highly phosphorylated in hyperinsulinemic db/db mice compared to non-diabetic db/db mice [35], indicating a role for STK39 and NCC in the development of T2D. In addition, clinical studies with the NCC-specific inhibitor thiazide, used to prevent high blood pressure, showed that patients with diabetes increase their fasting glucose and decrease their insulin sensitivity after administration of thiazide, indeed suggesting that the WNK/SPAK/NCC-pathway is involved in increasing insulin resistance [36,37]. Besides affecting NCC, STK39/SPAK can also inactivate the chloride-transporter CFTR, which is involved in Cystic Fibrosis [38]. This raises the question whether this transporter might be involved in diabetes. Recently, a new CFTR-activating drug (Ivacaftor) was developed, which is currently used as a treatment for a sub-population of CF patients. Preliminary data with Ivacaftor show that CF patients suffering from the first stage of diabetes revert their fasting glucose levels to normal when treated with this drug [39]. This indeed suggests that the WNK/STK39/NCC pathway might be involved in T2D development and can be a potential druggable target [39].

3.5. NAGK

NAGK was found to be upregulated after LPS treatment *in vitro* and *in vivo* (Figs. 2 and 4) but has never been functionally linked directly to T2D. NAGK is a kinase that phosphorylates N-acetyl-glucosamine (GlcNac) [40,41] and it has been described that increased O-linked beta-N-acetylglucosamine (O-GlcNAc) is associated with insulin resistance in muscle and adipocytes [42,43]. In addition, GlcNac might be involved in insulin receptor signaling by regulating the interaction between PI3K and the Insulin Receptor Substrates 1 and 2 (IRS1 and IRS2) [44,45], suggesting that GlcNac and perhaps its kinase NAGK might play an important role in insulin signaling.

3.6. SAA

The human acute-phase serum amyloid A protein 1 (hSAA1) is produced in adipose tissue [46] and, in agreement with our data, the expression of this inflammatory adipokine correlates with metabolic complications, such as atherosclerosis and insulin resistance [47]. In our study, we used LPS-mediated activation of TLR4 as a trigger for insulin resistance, however, *in vivo* the expression of mSAA3 (homologue of hSAA1) can also be triggered by a high fat-diet (HFD) and the expression of mSAA3 correlates with the onset of insulin resistance [48]. Moreover, exposure of 3T3L1 adipocytes to SAA3 caused alterations in gene expression, which closely resembled those observed in HFD-mice [48].

3.7. VPS26B

VPS26A and B are part of the prototypical heteropentameric mammalian retromer (sorting nexin (SNX)-BAR retromer) complex that regulates the translocation of GLUT4 from endosomes to the Trans-Golgi-Network (TGN) [49]. When expression of VPS26 is lost, GLUT4 is no longer transported to the TGN but rather redirected to lysosomes, where it will be degraded [49]. This redirection is dependent on CSNK2-activity and inhibition of CSNK2 blocks the lysosomal targeting and degradation of GLUT4 [49]. In our study, both VPS26 and CSNK2 are down regulated in protein and mRNA expression *in vitro* (Fig. 2) and *in vivo* (Fig. 4), suggesting that VPS26 and CSNK2 might be important factors in reduction of glucose transport in inflamed adipose tissue.

3T3L1 adipocytes have been used in many studies for unraveling the pathways of adipogenesis, insulin signaling and T2D development. Most of these studies focus on known targets or try to identify new genes, based on mRNA expression profiles by microarray-based techniques [51–53], whereas only a few papers have been published with respect to proteomics on adipose tissue [13,54]. In this paper, we show that many relevant genes change their protein expression without significantly changing their mRNA levels (Fig. 2). In addition, the co-cultures of adipocytes and macrophages have expression profiles that are more comparable to visceral than subcutaneous adipose tissues (Fig. 4, Figure S3) and, as such, are very interesting as an *in vitro* model for drug screening (Fig. 6) and research on signaling pathways involved in insulin sensitivity (Figs. 1, 2 and 7). In addition, we identified genes that are known factors in development of atherosclerosis (NNMT and SAA1), indicating that this *in vitro* model might also help to clarify the role of inflamed adipose tissue in other aspects of the metabolic syndrome and might not be restricted to diabetes.

4. Materials and methods

4.1. Cell culture and 3T3L1 differentiation

For all cultured cells, we used DMEM F12 + Glutamax medium (Gibco 31,331–028) supplemented with 10% Fetal Calf Serum (Gibco 10,082–147), 1% Pen/Strep (Gibco 15,070–063), and 1 mM sodium pyruvate (Gibco 11,360–039). 3T3L1 (ATCC® CL-173™) and RAW264.7 (ATCC® TIB-71™) cells were split 1:30 every 3–4 days. 3T3L1 pre-adipocytes were differentiated into adipocytes as described previously (55,56). Briefly, cells were grown to confluency in a T75 bottle after which medium was replaced with fresh culture medium containing 1 μM dexamethason (Sigma D4902), 10 μg insulin/ml (Sigma I0516) and 0.5 mM IBMX (Sigma 1–5879). After 3 days, medium was replaced with culture medium supplemented with 10 μg insulin/ml. Cells were allowed to differentiate for an additional 5 days after which cells were reseeded in collagen-coated (Advanced Biomatrix 5005-B; plates were coated for 18 h at 4° with 100 μg collagen/ml 0.01 N HCl) 6 well plates at a concentration of 500.000 cells per well. Cells were allowed to attach and grow for 2 additional days before addition of RAW264.7 cells (50.000 cells per well) and administration of E coli-derived LPS (Sigma L4391, 1 μg/ml) were started. Cells were co-cultured for another 18 h.

4.2. RNA isolation and real-time PCR quantification

Briefly, RNA from each sample was isolated by using a quick RNA isolation kit according to the manufacturers protocol (Zymo Research R1055) and 200 ng of RNA was used for cDNA synthesis by reverse transcription (Bio Rad 170-8891). Real-time PCR reactions included 5 μl of diluted RT product (1:6 dilution), 10 μl FAST SYBR Green buffer (AB 4385614) 4,8 μl H2O and 0.5 μM forward and 0.5 μM reverse primer. Reactions were incubated in an Applied Biosystems 7500 Fast Real-Time PCR system in 96-well plates. The primers used are described in Figure S1. Relative mRNA expression was determined by ddCT with HPRT mRNA expression as control.

4.3. IL6 and TNFα ELISA

Supernatants of exposed cells were collected and diluted 1:10 and 1:50 in ELISA dilution buffer according to manufacturer's protocol (R& D cat nr DY206-05). Briefly, plates were coated with capture antibody for 18 h, blocked for 1 h, washed 3× and exposed for 2 h to 100 μl diluted supernatant from exposed cells. Then plates were washed 3× and detection antibody was added for another 2 h after which the plates were washed again for 3× and streptavidin-HRP was added for 30 min. Plates were washed again and HRP substrate (TMB, Sigma T4444) was added for 10–30 min after which the reaction was stopped by adding 50 μl of 1 M H2SO4. Colorimetric determination was performed in an ELISA plate reader at 450 nm wavelength.

4.4. LCMS

Four replicates of 3T3L1 adipocytes and RAW264.7 cells (untreated or LPS treated) were washed twice with PBS and then lysed in 250 μl of 2× concentrated Laemli sample buffer. Samples were sonicated for 30 min. Precast gels of 12% of Tris-glycine sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) (Thermoscientific) were used for the separation of the proteins with gel electrophoresis. In each slot 35 ul of sample-buffer (×5) diluted and boiled samples were added. The gel was stained with colloidal Coomassie blue (Colloidal blue staining kit, Thermoscientific). The gel was destained by washing twice with water. Each lane was cut into 5 equal pieces and placed in low protein-binding clean eppendorf tubes and were processed for in-gel digestion. Cysteine reduction was performed by adding 100 μl of 50 mM dithiotreitol (DTT) in 50 mM NH4HCO3 and incubated for 1 h at 60 °C with gentle shaking. Alkylation followed by replacing DTT with 100 μl of 100 mM iodoacetamide (IAA) in 50 mM NH4HCO3 and incubating for 1 h at room temperature with gentle shaking. The enzymatic digestion was performed by adding 100 ul of 10 ng/μl diluted in 50 mM NH4HCO3 and incubated overnight at room temperature. For the extraction of the proteins, samples were sonicated for 30 s and centrifuged shortly. Supernatants were transferred to clean low protein-binding tubes. The pH was fixed between 2 and 4 by adding 10% of TFA. The samples were analyzed by injecting 18 μl sample over a 0.10 * 32 mm Prontosil 300-

5-C18H (Bischoff, Germany) pre-concentration column (prepared in-house) at a constant pressure of 270 bar (normally resulting in a flow of ca. 7 μl/min). Peptides were eluted from the pre-concentration column onto a 0.10 * 250 mm Prontosil 300-3-C18H analytical column (prepared in-house) with an acetonitril gradient at a flow of 0.5 μl/min with a Proxeon EASY nanoLC. The gradient consisted of an increase from 9 to 34% acetonitril in water with 1 ml/l formic acid in 50 min followed by a fast increase in the percentage acetonitril to 80% (with 20% water and 1 ml/l formic acid in both the acetonitril and the water) in 3 min as a column cleaning step. A P777 Upchurch microcross was positioned between the pre-concentration and analytical column. An electrospray potential of 4.5 kV was applied directly to the eluent via a stainless steel needle fitted into the waste line of the microcross. Full scan positive mode FTMS spectra were measured between m/z 380 and 1400 on a LTQ-Orbitrap (Thermo electron, San Jose, CA, USA) in the Orbitrap at high resolution (60,000). CID MSMS scans of the four most abundant multiply charged peaks in the FTMS scan were recorded in data dependent mode in the linear trap (MS/MS threshold = 5.000).

4.5. LCMS analyses

LCMS runs with all MS/MS spectra obtained were analyzed with MaxQuant 1.3.0.5 [57] using default settings for the Andromeda search engine [57,58] except that extra variable modifications were set for deamidation of N and Q as it was mentioned in previous work [1]. A mouse database downloaded from Uniprot (http://www.uniprot.org) was used together with a contaminants database that contains sequences of common contaminants such as: BSA (P02769, bovine serum albumin precursor), Trypsin (P00760, bovine), Trypsin (P00761, porcine), Keratin K22E (P35908, human), Keratin K1C9 (P35527, human), Keratin K2C1 (P04264, human) and Keratin K1CI (P35527, human). The “label-free quantification” as well as the “match between runs” (set to 2 min) options were enabled. De-amidated peptides were allowed to be used for protein quantification and all other quantification settings were kept default. Filtering and further bioinformatics analysis of the MaxQuant/Andromeda workflow output and the analysis of the abundances of the identified proteins were performed with the Perseus v.1.3.0.4 module (available at the MaxQuant suite). Accepted were peptides and proteins with a false discovery rate (FDR) of less than 1% and proteins with at least 2 identified peptides of which one should be unique and one should be unmodified. Reversed hits were deleted from the MaxQuant result table as well as all results showing a LFQ value of 0 for both LPS-treated and untreated cells. Zero values for one of the two LFQ columns were replaced by a value of 4.5 to make sensible ratio calculations possible. Relative protein quantitation of sample to control was done with Perseus v1.3.0.4 by applying a 2-sided two sample *t*-test using the normalized “LFQ intensity” columns obtained for LPS-treated and untreated 3T3-L1 adipocytes and RAW264.7 cells with a FDR threshold of 0.05 and S0 = 1. P-Values obtained were used uncorrected.

4.6. Data analysis

In vitro experiments were performed in triplicate and repeated at least three times in independent experiments. Data are presented as means with SD. Statistical analyses were performed on the data as depicted in the figure legends.

4.7. Microarray hybridization and analysis

One hundred nanogram of RNA was used for Whole Transcript cDNA synthesis (Affymetrix, inc., Santa Clara, USA). Hybridization, washing and scanning of Affymetrix GeneChip Mouse Gene 1.1 ST arrays and Affymetrix GeneChip Porcine Gene 1.1 ST Arrays was carried out according to standard Affymetrix protocols. Arrays were normalized using the Robust Multi-array Average method [59,60]. Probe sets were assigned to unique gene identifiers, in this case Entrez IDs. The probes on the Mouse Gene 1.1 ST arrays represent 21,213 Entrez IDs. Array data were analyzed using an in-house, on-line system [59].

Conflicts of interest

The authors declare that they have no conflicts of interest with the contents of this article.

Author contributions

JPTK designed, performed and analyzed the data and wrote the paper. AS and SB performed LCMS analysis. JV, SV and RP coordinated the project. All authors reviewed and approved the final version of the manuscript.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.abb.2018.03.003.

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