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ORIGINAL ARTICLE

ADAM28 is elevated in humans with the metabolic syndrome and is a novel sheddase of human tumour necrosis factor-α

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Metalloproteinases are implicated in cleaving numerous proinflammatory mediators from the cell surface. Interestingly, the elevated levels of tumour necrosis factor-α (TNF-α) have been associated with the metabolic syndrome. We aimed to ascertain whether the human metalloproteinase ADAM28 correlates with parameters of the metabolic syndrome and whether ADAM28 is a novel sheddase of human TNF-α. To identify novel metalloproteinases associated with the metabolic syndrome, we conducted microarray studies on peripheral blood mononuclear cells from a well characterised human cohort. Human ADAM28 and TNF-α were overexpressed and ADAM28 expression or activity was reduced with small-interfering RNA (siRNA) or pharmacological inhibition. TNF-α levels were measured in cell supernatant by enzyme-linked immunosorbent assay. We also conducted ADAM28 inhibition studies in human THP-1 macrophages. Human ADAM28 expression levels were positively correlated with parameters of the metabolic syndrome. When human ADAM28 and TNF-α were overexpressed in HEK293 cells, both proteins co-localised, co-immunoprecipitated and promoted TNF-α shedding. The shedding was significantly reduced when ADAM28 activity was inhibited or ADAM28 expression was downregulated. In human THP-1 macrophages, endogenous ADAM28 and TNF-α were co-expressed and TNF-α shedding was significantly reduced when ADAM28 was inhibited by pharmacological inhibition or siRNA knockdown. Our data suggest a novel mechanistic role for the metalloproteinase ADAM28 in inflammation, obesity and type 2 diabetes.
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The prevalence of adult obesity has increased approximately 75% in the last quarter century with an alarming increase in overweight and obese children in both developed and developing countries.[1] An abundant number of disorders directly correlate with obesity. These include a number of cancers, glucose intolerance, dyslipidemia, cardiovascular disease and insulin resistance, which may ultimately culminate in pancreatic beta cell failure and type 2 diabetes (T2D). The understanding of novel mechanisms underlying the pathogenesis of T2D and the development of new strategies to treat 'metabolic disease' is most warranted.

Growing evidence suggests that during T2D, a state of chronic inflammation exists in metabolically active tissues such as the liver, adipose tissue and skeletal muscle. A number of studies have elegantly demonstrated the critical role that inflammatory cascades have in the development of obesity and T2D. Serine threonine kinases such as c-Jun terminal amino kinase (JNK) or IκB kinase β/ε and protein tyrosine phosphatases (PTP) such as PTP1B, which attenuate insulin signalling, may be activated by the production of fatty acid metabolites within insulin responsive tissues.[2–6]

Soluble proinflammatory cytokines including interleukin-6 and granulocyte macrophage colony-stimulating factor have exhibited profound beneficial metabolic effects. These include promotion of fat oxidation and insulin sensitivity and reductions in food intake and body weight, respectively.[7,8] The adipokines, adiponectin and leptin may also have a positive effect on body weight regulation.[9,10] In addition, overexpression of interleukin-10 protected mice from diet-induced inflammation and insulin resistance in skeletal muscle.[11] In contrast, a vast amount of research has implicated a number of

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soluble factors such as tumour necrosis factor-α (TNF-α) in the aetiology of obesity and insulin resistance in rodents and humans.[12–16] However, it should be highlighted that numerous proinflammatory cytokines may work in concert to promote an insulin-resistant phenotype in metabolic cell types.

Cell membrane TNF-α may be cleaved by the metalloproteinase TNF-α-converting enzyme (TACE), otherwise known as ADAM17, a member of a disintegrin and metalloproteinase (ADAM) gene family. Another metalloproteinase, ADAM28 is expressed in numerous cell types including lymphoid cells, and has two isoforms: (i) a membrane-type form (ADAM28m) and (ii) a secreted soluble form (ADAM28s). Our previous study demonstrated that the 65 kDa pro-ADAM28 is processed by matrix metalloproteinase-7 to 40 and 42 kDa forms, which results in an active form of ADAM28.[17] We and others have previously identified a number of novel substrates for ADAM28, which include insulin-like growth factor binding protein-3 (IGFBP-3), connective tissue growth factor, proteoglycans, myelin basic protein and CD23.[17–21] Interestingly, a number of synthetic peptides containing the authentic TNF-α shedding site were shown to be cleaved by ADAM28.[21] This was a very exciting finding that reinforced the notion that ADAM28 may be a novel sheddase of one of the major proinflammatory cytokines involved with the metabolic syndrome.

In this study, we hypothesised that ADAM28 is associated with obesity and T2D in a human cohort and that ADAM28 may be a novel sheddase of TNF-α, which may promote metabolic dysfunction. The outcomes of this study will provide new insights into the metalloproteinase-mediated pathogenesis of obesity and T2D.

RESULTS

ADAM28 expression in human blood mononuclear cells is a novel marker of the metabolic syndrome

Here, we use a large human cohort well characterised for T2D and cardiovascular disease that has also been extensively genetically analysed and carries full genome-wide gene expression profiles on 1240 individuals. This set was filtered to identify novel metalloproteinases involved in the development of obesity and T2D. We have found that high-level expression of ADAM28 in blood mononuclear cells from the San Antonio Family Heart Study (SAFHS) cohort correlated strongly with parameters of the metabolic syndrome and in particular body mass index and relative fat (Table 1). These highly significant clinically relevant observations suggest a role for ADAM28 in the regulation of human metabolism.

Table 1 Parameters of the metabolic syndrome are associated with high ADAM28 expression in human peripheral blood mononuclear cells

Parameter	*P-value*	*Direction of correlation*
Fasting insulin	0.0033	Positive
BMI	8.8×10^{-6}	Positive
HDL cholesterol	0.0079	Negative
Relative fat	1.4×10^{-9}	Positive
HOMA-IR	0.0024	Positive

Abbreviations: BMI, body mass index; HDL, high-density lipoprotein.

ADAM28 is a novel sheddase of the proinflammatory cytokine TNF-α in HEK293 cells

Numerous studies have demonstrated that levels of circulating TNF-α positively correlate with body mass index. Prior reports have also suggested that ADAM28 is able to cleave synthetic peptides possessing the TNF-α shedding site.[21] In initial experiments, we tested the hypothesis that ADAM28 may be a novel sheddase of cell membrane TNF-α protein. When human ADAM28 and TNF-α were co-expressed in human HEK293 cells, both proteins co-localised (a critical requirement for shedding) (Figures 1a–c). In addition, co-immunoprecipitation experiments in these same cells highlighted that human ADAM28 co-precipitated with the 27 kDa membrane-bound pro-TNF-α (Figure 1d).

Significantly, co-transfection of human TNF-α with human ADAM28 expression vectors in HEK293 cells promoted TNF-α shedding four-fold (Figure 2a). We previously demonstrated that the synthetic inhibitor KB-R7785 significantly reduced ADAM28 activity. When the ADAM28 inhibitor was added to HEK293 cells co-transfected with human TNF-α and human ADAM28 expression vectors, we were able to reduce the levels of soluble TNF-α in the cell-free culture supernatant to the basal levels, which existed when the TNF-α vector was transfected alone (Figure 2b). Immunoblotting of the cell lysates of cells co-transfected with human TNF-α and human ADAM28 expression vectors with and without KB-R7785 also showed that there were markedly lower levels of mature shed TNF-α (17 kDa) when the ADAM28 inhibitor was added. This also supports that the shed TNF-α may bind to the TNF-α receptor on the cell surface of the HEK293 cells (Figure 2c), which is of critical importance for later experiments (Figures 4a and b). These data provide compelling evidence that ADAM28 is a major metalloproteinase involved in TNF-α cleavage; however, we could not exclude the possibility that ADAMs other than ADAM28 also have a role in TNF-α shedding in the transfectants. As molecular knockdown of ADAM28 should provide definitive evidence of its role in TNF-α shedding, we conducted ADAM28 small-interfering RNA (siRNA) experiments in a HEK293 overexpression system where all cells were transfected with human TNF-α and human ADAM28 expression vectors. We chose to use a scrambled Cy3-labelled siRNA as our negative control. This indicated that we were able to obtain $>90\%$ transfection efficiency. We also used three different siRNA sequences directed at human ADAM28 mRNA. Importantly, all three ADAM28 siRNAs were able to significantly downregulate the ADAM28 mRNA to less than one quarter of those levels observed for the scrambled siRNA control (Figure 3a). In addition, both the pro-form (62 kDa) and the active form (42 kDa) of the human ADAM28 protein were significantly reduced with all three siRNAs (Figure 3b, c and d). As the active form of ADAM28 was also reduced to less than one quarter of those levels observed for the scrambled siRNA control (Figure 3d), we sought to determine whether the level of TNF-α shedding was also reduced with ADAM28 siRNAs. All three ADAM28 siRNAs significantly reduced TNF-α cleavage compared with the scrambled siRNA control (Figure 3e; top panel). Importantly, the cell membrane-bound form of TNF-α (pro-TNF-α) was elevated in ADAM28 siRNA-transfected cells compared with cells transfected with scrambled siRNA (Figure 3e; bottom panel).

ADAM28-cleaved TNF-α is bioactive and upregulates ADAM28 protein levels

To ascertain whether the ADAM28-cleaved TNF-α was bioactive, we analysed critical signalling pathways downstream of TNF-α binding. We examined phosphorylation of JNK. Interestingly, the cellular protein lysates from HEK293 cells transfected with both TNF-α and

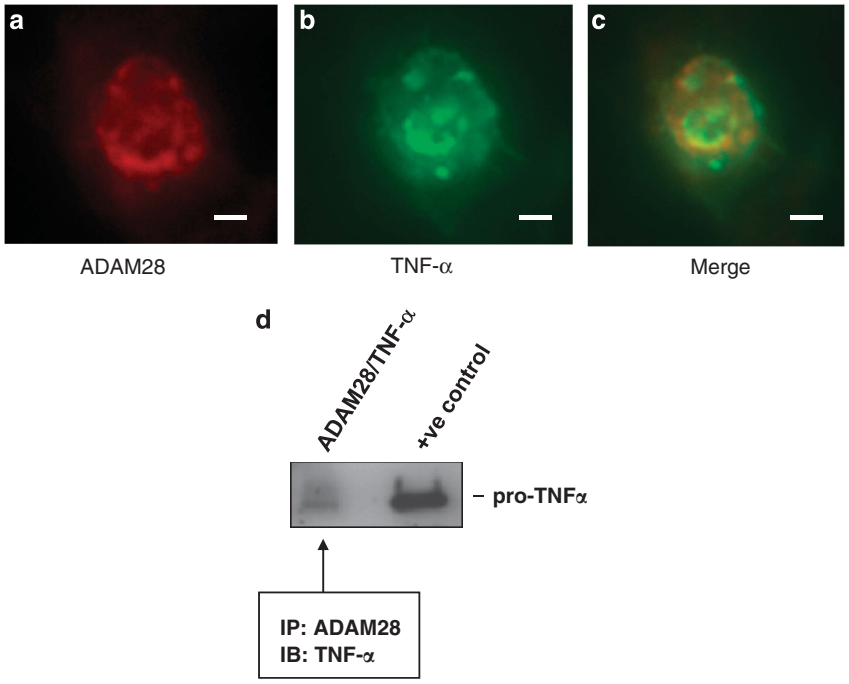

Figure 1 Human ADAM28 and human TNF-α co-localise and co-immunoprecipitate in transfected HEK293 cells. Double staining for ADAM28 (**a**), TNF-α (**b**) and both proteins merged (**c**). Bar = 10 μm. ADAM28 co-immunoprecipitated with TNF-α in double-transfected cells (**d**).

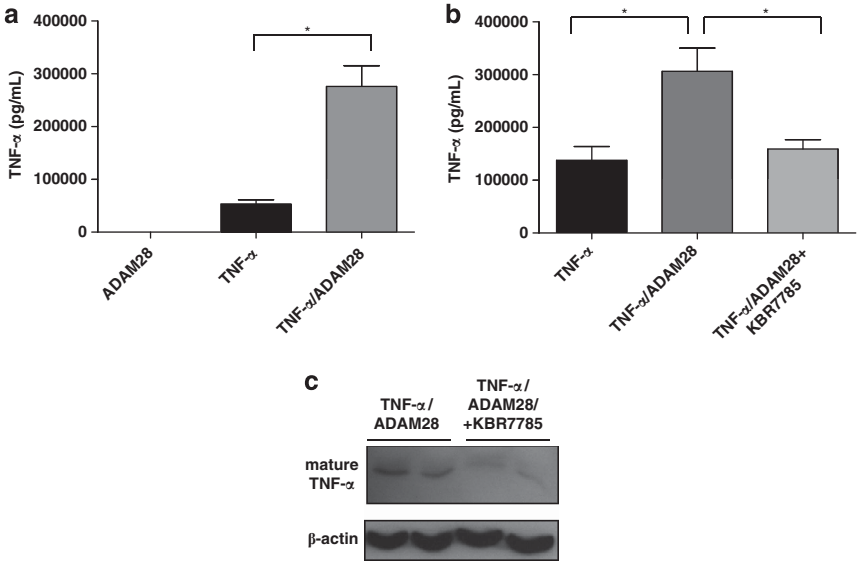

Figure 2 Human ADAM28 sheds human TNF-α in transfected HEK293 cells. TNF-α cleavage was promoted with ADAM28 co-transfection. Two micrograms of each plasmid was transfected and empty vector was used when ADAM28 or TNF-α were alone (**a**). The ADAM28 inhibitor, KB-R7785 was used at 1 μM for 48 h and TNF-α cleavage was measured by enzyme-linked immunosorbent assay (**b**) or immunoblotting (**c**). Data expressed as mean ± s.e.m. $^*P<0.05$ for indicated differences; $n = 3$–6 from at least two independent experiments.

ADAM28 expression vectors displayed a considerable elevation in JNK phosphorylation, suggesting that the elevated TNF-α protein was bioactive (Figure 4a and b). In an effort to determine whether the TNF-α shedding by ADAM28 may result in inflammation in metabolically relevant cell types, we continued with HEK293 conditioned media experiments in L6 skeletal myotubes. When we treated L6 myotubes with either cell-free conditioned media from HEK293 cells transfected with either empty and TNF-α vectors (TNF-α) or TNF-α and ADAM28 vectors (TNF-α/ADAM28), we observed significantly elevated phosphorylation of JNK when the cells were treated with TNF-α/ADAM28 conditioned media (Figures 4c and d).

We next aimed to determine whether ADAM28-mediated TNF-α release may instigate a positive feedback loop, whereby ADAM28 levels are elevated with TNF-α stimulation. Using an ADAM28

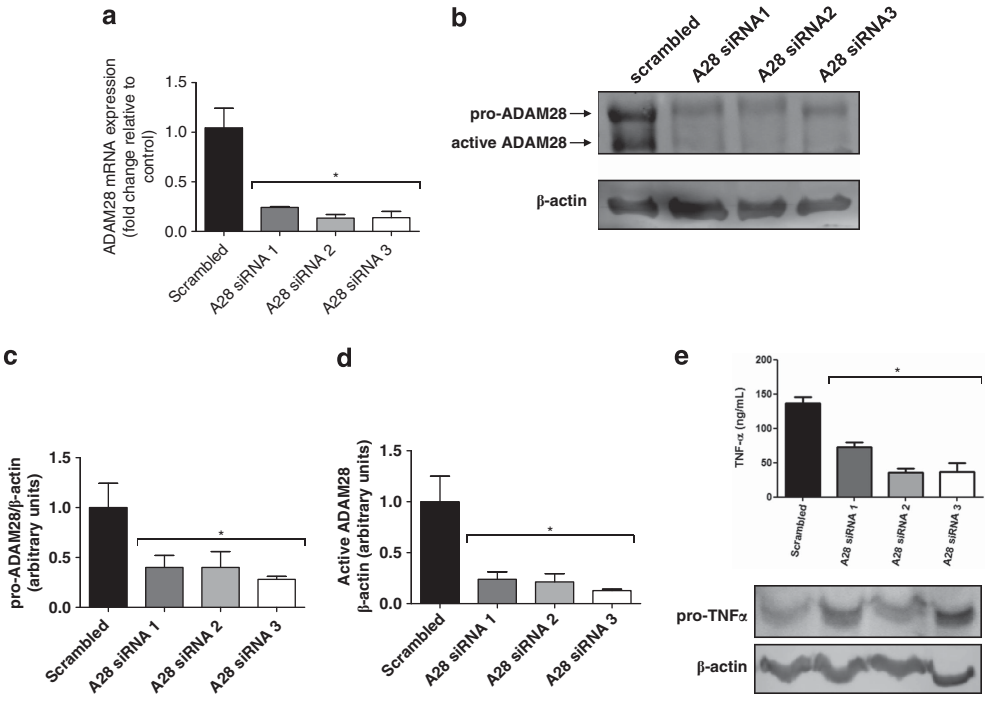

Figure 3 Molecular knockdown of human ADAM28 protein levels diminishes TNF-α shedding in HEK293 cells. HEK293 cells were co-transfected with TNF-α and ADAM28 vectors in addition to either scrambled siRNA or three different human ADAM28 (A28) siRNA. After 48 h, ADAM28 mRNA (**a**), ADAM28 protein (**b**–**d**), TNF-α shedding (**e**; top panel) and membrane bound pro-TNF-α protein levels (**e**; bottom panel) were determined. Data expressed as mean ± s.e.m. (**a**–**e**). *$P<0.05$ compared with scrambled siRNA (scrambled); $n=3$–6 from at least two independent experiments.

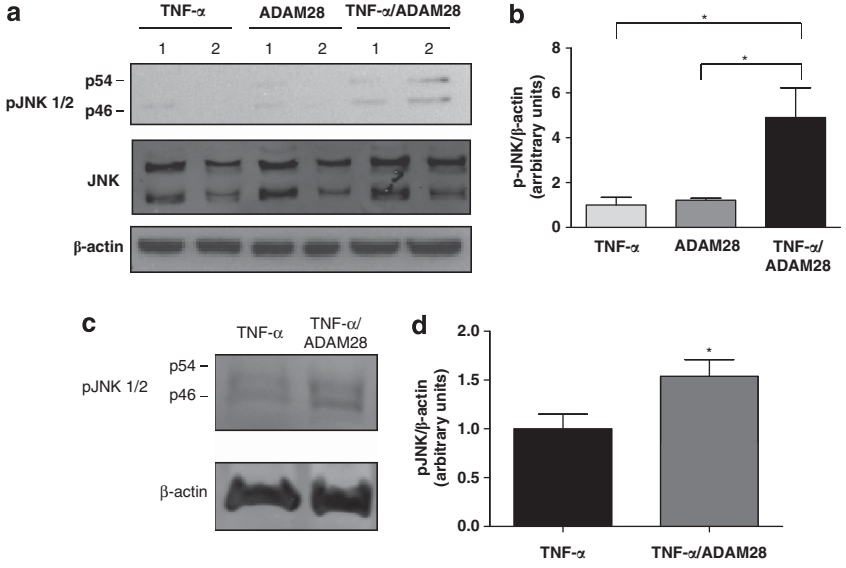

Figure 4 ADAM28 shed TNF-α promotes activation of stress kinases in HEK293 and L6 skeletal muscle cells. HEK293 cells were transfected with TNF-α vector, ADAM28 vector or co-transfected and phosphorylation of JNK was measured (**a**, **b**). L6 myotubes were treated with conditioned media from HEK293 cells transfected with TNF-α vector (TNF-α) or TNF-α and ADAM28 vectors for 48 h and then examined for phosphorylation of JNK (**c, d**). Data expressed as mean ± s.e.m. (**b**, **d**). *$P<0.05$ for indicated differences; $n=3$–6 from at least two independent experiments.

enzyme-linked immunosorbent assay, we demonstrated that ADAM28 levels were increased approximately four-fold when TNF-α and ADAM28 vectors were co-transfected (2 μg each) compared with when ADAM28 and empty vectors were co-transfected (2 μg each) into HEK293 cells (Figure 5a). This eludes to a mechanism by which ADAM28-mediated TNF-α shedding may be maintained. We also conducted TNF-α stimulation experiments in THP-1 monocytes and assessed endogenous human ADAM28 mRNA levels. Consistent with the HEK293 studies, TNF-α stimulation resulted in elevated ADAM28 mRNA levels (Figure 5b).

Inhibition of ADAM28 activity inhibits endogenous TNF-α shedding in phorbol 12-myristate 13-acetate-induced human macrophages and lipopolysaccharide-stimulated human monocytes

It has previously been reported that treatment of human THP-1 monocytes with phorbol 12-myristate 13-acetate (PMA) results in differentiation to macrophages and increases the expression of ADAM28 mRNA after 24 h of treatment. As we were interested in upregulating ADAM28 and TNF-α protein expression, we treated THP-1 monocytes with PMA for 48 h. We definitively demonstrated that the cells became adherent and displayed characteristic macrophage morphology. Using this established human macrophage cell culture model in which endogenous ADAM28 (Figure 6a) and TNF-α (Figure 6b) are co-expressed, we then aimed to determine whether inhibition of ADAM28 activity with the pharmacological inhibitor, KB-R7785, may influence TNF-α shedding. Consistent with our experiments in HEK293 cells, when we treated THP-1-differentiated macrophages with KB-R7785, TNF-α shedding was significantly reduced (Figure 6b). As we have previously shown that we may successfully knockdown ADAM28 expression utilising siRNA technology (Figure 3), we next aimed to utilise siRNA to reduce endogenous ADAM28 expression in THP-1 monocytes. We

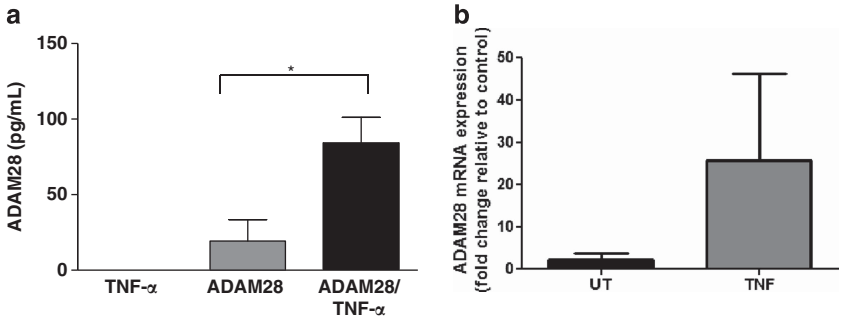

Figure 5 TNF-α stimulation promotes expression of ADAM28 in HEK293 cells and THP-1 monocytes. Cells were transfected with TNF-α vector, ADAM28 vector or co-transfected and cellular ADAM28 levels were then measured by enzyme-linked immunosorbent assay (**a**). THP-1 cells were stimulated with 10 ng ml^{-1} rhTNF-α for 24 h and ADAM28 mRNA levels were assessed (**b**). Data expressed as mean ± s.e.m. *$P<0.05$ for indicated differences; $n=3$–6 from at least two independent experiments.

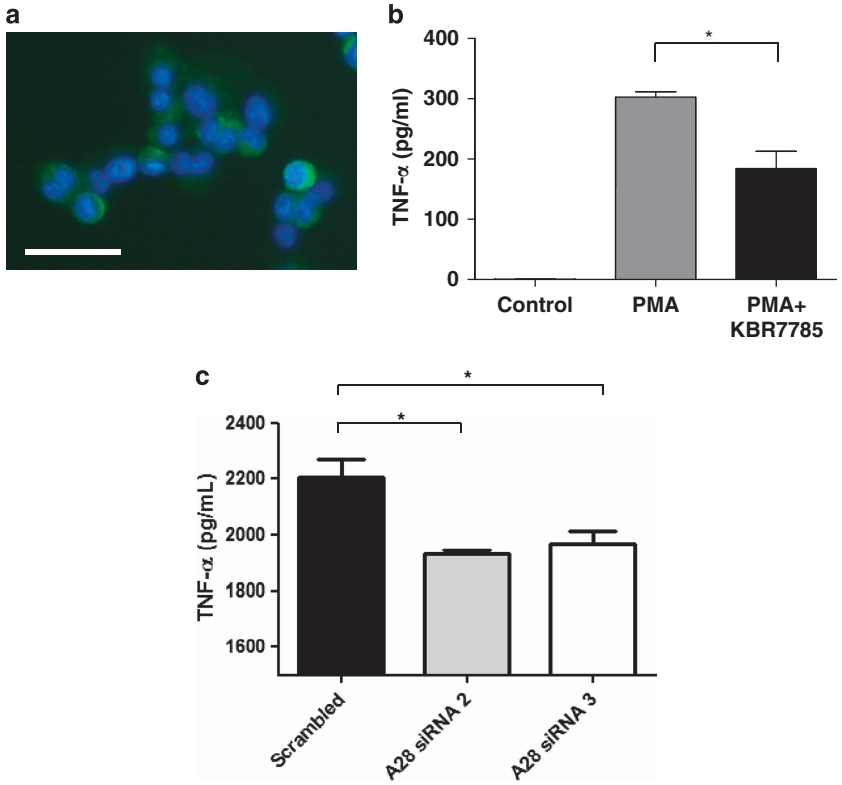

Figure 6 Inhibition of endogenous ADAM28 activity inhibits TNF-α shedding in human PMA-differentiated and lipopolysaccharide stimulated THP-1 monocytes. ADAM28 expression (green) after PMA-induced differentiation of THP-1 cells. Nuclei are stained blue with 4′,6-diamidino-2-phenylindole (**a**), inhibition of ADAM28 activity with KB-R7785 (5 μM) decreases PMA-induced TNF-α shedding (**b**) and inhibition of ADAM28 with siRNA reduces lipopolysaccharide-stimulated TNF-α shedding (**c**). Data expressed as mean ± s.e.m. (**b**, **c**). *$P<0.05$ for indicated differences; $n=3$–6 from at least two independent experiments; Bar = 10 μm.

were able to reduce ADAM28 expression levels by at least 50%. To promote TNF-α expression in THP-1 monocytes, we stimulated cells with 1 μg ml^{-1} lipopolysaccharide for 48 h. In agreement with our previous findings, we now demonstrate that shedding of endogenously expressed human TNF-α may be significantly reduced by knockdown of endogenously expressed human ADAM28 (Figure 6c).

DISCUSSION

We have shown for the first time that ADAM28 expression levels in peripheral blood mononuclear cells isolated from a large human cohort are strongly correlated with parameters of the metabolic syndrome and that ADAM28 promotes the shedding of the cytokine TNF-α, which has been implicated in the metabolic syndrome.

An obesogenic role for the metalloproteinase TACE has been concluded from knockout mouse studies. Interestingly, heterozygous TACE knockout mice are significantly protected from obesity and insulin resistance.[22] Conversely, ablation of tissue inhibitor of metalloproteinases 3 (Timp3), which is the physiological inhibitor of TACE, promotes hyperglycaemia, hyperinsulinemia, glucose intolerance and insulin resistance in mice fed a high-fat diet.[23] Furthermore, it appears hyperglycaemia may be the stimulus for elevated TACE expression in obesity as exposure of aortic smooth muscle cells to high glucose increased TACE activity and promoted shedding of TNF-α via protein kinase C-δ.[24] TACE is currently considered a therapeutic target in osteoarthritis, rheumatoid arthritis and certain cancers.[25] The attenuation of TACE activity *in vivo* by use of siRNA,[26] targeted deletion[27] or pharmacological inhibition[28] has improved cardiac hypertrophy and the survival rates during endotoxic shock and pneumococcal meningitis. Notably, administration of TACE and matrix metalloproteinase inhibitors to humans in clinical trials has proven to effectively decrease inflammatory mediators[29] and diminish disease activity.[30]

We have already documented that there are a multitude of mechanisms by which ADAM28 may promote inflammation and ultimately metabolic dysfunction. In addition to its proteinase functions, the disintegrin domain of ADAM28 also has an important role in inflammation. The disintegrin domain may serve as a ligand for the integrin alpha(4) beta(1), which is expressed on a number of leukocyte populations including macrophages, which are a major source of the proinflammatory cytokine TNF-α.[31] The implications of the interaction of ADAM28 with alpha(4) beta(1) are two-fold: (i) it may promote inflammation due to the accumulation of lymphocytes with other leukocyte populations and (ii) second, it may function to target the active protease to substrates at the site of cell–cell contact. Further supporting this concept of ADAM28 in inflammation, we have recently elegantly demonstrated that the disintegrin-like domain of ADAM28 binds the extracellular portion of P-selectin glycoprotein ligand-1 (PSGL-1) on leukocytes.[32] This enhances PSGL-1/P-selectin-mediated cell adhesion to endothelial cells, promoting leukocyte rolling and adhesion to blood vessel endothelial cells and subsequent migration into tissues, therefore facilitating inflammation.

We have previously illustrated that one of the major substrates of ADAM28 is IGFBP-3.[17] This finding may have major clinical relevance as IGFBP-3 has been implicated in the enhancement of adipogenesis.[33] Furthermore, mice overexpressing IGFBP-3 showed increased epididymal fat pad size.[34] This trait is believed to be due to the elevated insulin-like growth factor levels in the transgenic mice. Using overexpression systems and cells in which ADAM28 and TNF-α are expressed endogenously, we have shown in our current study that human ADAM28 is a novel sheddase for human TNF-α. Using molecular and pharmacological techniques, we highlighted that a direct consequence of ADAM28 inhibition is decreased cleavage of cell membrane-bound TNF-α. Hence, based on our results it is plausible that therapeutic ADAM28 inhibition may reduce inflammation due to diminished TNF-α shedding and binding of its disintegrin domain to integrins and PSGL-1. Worley and colleagues[35] aimed to ascertain whether treatment of human monocytes with low-density lipoprotein (LDL) from T2D would alter metalloproteinase expression. They discovered that LDL from T2D patients significantly increased mRNA levels for ADAM17, ADAM15 and, importantly, ADAM28. This study suggested that the LDL-induced increases in metalloproteinase expression may lead to increased release of adhesion molecules and TNF-α release. We have now formally demonstrated the latter in our current study.

Our project is most significant as it has demonstrated for the first time, the importance of ADAM28 in the metabolic syndrome and further supports that metalloproteinase inhibition is a potential therapeutic target for anti-obesity agents. Importantly, there is the potential for ADAM28-mediated TNF-α cleavage to be implicated in numerous other immunopathological conditions.

METHODS

Human microarray for the identification of metalloproteinases involved in obesity and T2D

RNA samples (from peripheral blood mononuclear cells) were obtained from the SAFHS, a study of risk factors for cardiovascular disease in Mexican Americans living in and around San Antonio, Texas.[36] The SAFHS is a large family-based genetic epidemiological study including 1431 individuals from 42 extended families at baseline. Individuals from large randomly selected, multigenerational pedigrees were sampled independent of their phenotype or the presence or absence of disease. All participants in the SAFHS provided informed consent. The study and all protocols were approved by the Institutional Review Board at the University of Texas Health Science Centre at San Antonio (San Antonio, TX, USA).

Cell culture experiments

L6 myoblast cells, HEK293 and THP-1 cells (<6 passages) were purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured at 37 °C, 5% CO_2 in a humidified chamber. L6 myoblasts were seeded in six-well culture plates and grown in Dulbecco's modified Eagle medium (DMEM) (low glucose; Gibco, Mulgrave, VIC, Australia) + 10% fetal bovine serum + 1% penicillin/streptomycin. Differentiation of the myoblasts was induced by transferring cells to medium containing 2% fetal calf serum (FCS) when the myoblasts were ~90% confluent. Experimental treatments commenced after 7 days of differentiation when nearly all myoblasts had fused to form myotubes. Upon the day of experimentation, cells were serum starved for 4 h and treated with cell-free HEK293 conditioned media for 48 h. HEK293 cells were cultured in DMEM (low glucose; Gibco) + 10% FCS and 1% penicillin/streptomycin. THP-1 human monocytes were cultured in DMEM (low glucose; Gibco) + 5% FCS and 1% penicillin/streptomycin. To differentiate the THP-1 cells to macrophages, when cells were confluent, 5 ng ml^{-1} of PMA was added and cell-free supernantant was collected after 48 h.[37] In other experiments, undifferentiated THP-1 cells were stimulated with 10 ng ml^{-1} recombinant TNF-α (R&D Systems, Gymea, NSW, Australia) for 24 h before RNA extraction. The ADAM inhibitor (KB-R7785)[32,38] was used at 1 μM for HEK293 cells and 5 μM for THP-1 cells to inhibit ADAM28 activity.

Immunoprecipitation

Twenty four hours post transfection, HEK293 cells were washed with ice-cold phosphate-buffered saline and then lysed with immunoprecipitation buffer (50 mM Tris, 130 mM NaCl, 5 mM EDTA, 1% NP-40, 1 mM phenylmethanesulfonylfluoride, 1 mM NaF) containing protease and phosphatase inhibitors (Roche, Mannheim, Baden-Wurttemberg, Germany). After protein determination, 200 μg of protein was cleared with a mixture of washed protein A/protein G (1:1) sepharose beads (Roche) for 1 h. After centrifugation, the

protein solution was added to freshly washed sepharose beads and mouse anti-human ADAM28 antibody (297-2F3; 3 μg ml^{-1}) and incubated O/N at 4 °C with mixing. The following day, the beads were washed three times with ice-cold phosphate-buffered saline and then 40 μl of laemmeli sample buffer was added and boiled for 10 min The supernatant was then subjected to immunoblotting for human pro-TNF-α (27 kDa).

Transfections

HEK293 cells were seeded into six-well cell culture dishes and transiently transfected with either empty pCMV-Tag4a vector or vectors containing the cDNA for human proADAM28[38] or TNF-α using Lipofectamine reagent (Life Technologies, Mulgrave, VIC, Australia). Cell-free culture supernatants were collected after 48 h. Human TNF-α in the cell-free culture supernatant and ADAM28 in the cell lysate collected from transfected HEK293 cells were measured using commercially available enzyme-linked immunosorbent assay kits (TNF-α; R&D Systems, DY210) and (ADAM28; USCN LifeSciences Inc, Wuhan, China, E99202Hu). In all siRNA experiments, HEK293 and THP-1 cells were seeded into 12-well cell culture dishes and transfections were conducted using X-tremeGENE HP DNA transfection reagent (Roche) and incubated for 48 h. THP-1 cells were stimulated with 1 μg ml^{-1} lipopolysaccharide for the duration of the transfection. A Cy3 DS transfection control (scrambled siRNA; Integrated DNA Technologies (IDT)) was used at 10 nM as a negative control. ADAM28 was knocked down using Silencer Select siRNA at 10 nM (A28 siRNA 1 = s21321; A28 siRNA 2 = s21323; A28 siRNA 3 = s21322; Applied Biosystems, Mulgrave, VIC, Australia).

Immunofluorescence

Cells were fixed with ice-cold methanol:acetone and were preincubated in 0.3% H_2O_2 to quench endogenous peroxidase, followed by incubation in 10% FCS to block nonspecific background staining. Sections were incubated overnight at 4 °C with rabbit anti-human TNF-α antibody diluted 1:100 (Cell Signalling Technologies, Beverly, MA, USA; #3707) or mouse monoclonal anti-human ADAM28 (297-2F3) antibody (10 μg ml^{-1}).[32,38] It should be noted that rabbit and mouse IgG isotype control antibodies were used as negative controls (BD Biosciences, North Ryde, NSW, Australia). The TNF-α primary antibody was detected with anti-rabbit 488 (Invitrogen, Mulgrave, VIC, Australia; A11034) and the ADAM28 antibody was detected with either anti-mouse 546 (Invitrogen; A11003) or anti-mouse 488 (Invitrogen; A11029) for 30 min at room temperature. Nuclei were stained with 4′,6-diamidino-2-phenylindole (Sigma, Castle Hill, NSW, Australia; D9542) and then the cells were visualised using an Olympus IX71 microscope (Olympus, Mt. Waverly, VIC, Australia).

Determination of gene and protein expression

RNA was extracted using Trizol reagent (Invitrogen) and cDNA synthesis was performed using the high-capacity RNA-to-cDNA kit (Applied Biosystems). Real-time PCR to determine the mRNA abundance of human ADAM28 and 18S (house-keeper gene) was performed using a Rotor-gene real-time PCR machine (Qiagen, Chadstone, VIC, Australia) using predeveloped TaqMan probe (FAM-labelled) and primer sets for human ADAM28 (Hs00248020_m1) and human 18S (Hs03928990_g1) from Applied Biosystems. Quantitation was conducted as previously described.[39]

For western blotting analyses, cells were washed in ice-cold phosphate-buffered saline and then lysed using cytosolic extraction buffer (10 mM hydroxyethyl piperazineethanesulfonic acid; 3 mM $MgCl_2$; 14 mM KCl; 5% glycerol; 0.2% IGEPAL) containing phosphatase and protease inhibitors (Roche). Cell lysates were cleared and protein concentration calculated using protein assay solution (Bio-Rad, Hercules, CA, USA). Protein lysates were solubilized in Laemmeli sample buffer and boiled for 10 min, resolved by SDS–polyacrylamide gel electrophoresis on 10% polyacrylamide gels, transferred by semi-dry transfer to polyvinylidene difluoride membrane, and then blocked with 5% milk powder. Membranes were then incubated overnight at 4 ºC in Odyssey blocking buffer containing either primary antibody against phospho JNK (Thr183/Tyr185) (Cell Signaling #4668), total JNK (Cell Signalling; #9252), TNF-α (Cell Signalling; #3707), β-actin (Abcam, Cambridge, UK; ab6276) or human ADAM28 antibody (297-2F3) (1 μg ml^{-1})[32,38] using recommended dilutions. Membranes were washed three times in washing buffer and incubated for 60 min at room temperature with either IRDye 680LT-conjugated goat anti-mouse IgG, IRDye 800CW-conjugated goat anti-mouse IgG or IRDye 800CW-conjugated goat anti-rabbit IgG secondary antibodies (LI-COR Biosciences, Lincoln, NE, USA) at a 1:10 000–20 000 dilution, followed by three times 5 min washes in washing buffer. The protein bands were detected and quantified using the Odyssey infrared imaging system (LI-COR Biosciences).

Statistics

Associations between ADAM28 lymphocyte-derived gene expression and clinical parameters in the Mexican American pedigrees were determined using a linear model allowing for residual non-independence among family members as a function of their genetic relationships. A likelihood ratio statistic was utilised to test whether ADAM28 expression significantly predicted a relevant standard clinical parameter (fasting insulin, body mass index, high-density lipoprotein cholestrol, relative fat mass, and the homeostasis model assessment of insulin resistance (HOMA-IR) index of insulin resistance). Other primary covariates were simultaneously controlled for including sex and age. All analyses were performed using the statistical package, SOLAR (Texas Biomedical Research Institute, San Antonio, TX, USA).

In the cell culture experiments all data were analysed from three independent experiments and paired *t*-tests were used to statistically analyse the data where appropriate.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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