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An inflammatory micro-environment promotes human adipocyte apoptosis

Authors: Michaela Keuper¹, Matthias Blüher², Michael R. Schön³, Peter Möller⁴, Anna Dzyakanchuk⁵, Kurt Amrein⁵, Klaus-Michael Debatin⁶, Martin Wabitsch¹, Pamela Fischer-Posovszky¹

Affiliations: ¹ Division of Pediatric Endocrinology, Diabetes and Obesity Unit, Department of Pediatrics and Adolescent Medicine, Ulm University, Germany, ² Medical Department III, University of Leipzig, Leipzig, Germany, ³ Städtisches Klinikum Karlsruhe, Clinic of Visceral Surgery, Karlsruhe, Germany, ⁴ Institute of Pathology, Ulm University, Germany, ⁵ Hoffman-La Roche, Basel, Switzerland, ⁶ Department of Pediatrics and Adolescent Medicine, Ulm University, Germany

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Correspondence / reprint requests:

Prof. Dr. Martin Wabitsch & Dr. Pamela Fischer-Posovszky, Pediatric Endocrinology and Diabetes, Department of Pediatrics and Adolescent Medicine, Ulm University, Eythstr 24, 89075 Ulm, Germany, tel.: +49 731 500 57400, fax.: +49 731 500 57412

email: martin.wabitsch@uniklinik-ulm.de; pamela.fischer@uniklinik-ulm.de

Abstract

Obesity-associated macrophage infiltration into adipose tissue is responsible for both local and systemic inflammation. Recent findings suggest fat cell apoptosis as an initiator of macrophage recruitment.

Here, we investigated the effects of an inflammatory micro-environment on fat cells by using human THP-1 macrophages and SGBS adipocytes. Macrophage-secreted factors induced insulin resistance, inhibited insulin-stimulated Akt phosphorylation, and induced apoptosis of adipocytes. The apoptosis-inducing effect was even more pronounced in direct co-cultures of adipocytes and macrophages. Our data suggest a link between insulin resistance and apoptosis sensitivity. Accordingly, pharmacological and genetic inhibition of insulin signaling at the level of Akt2 sensitized adipocytes to apoptosis induction by macrophage-secreted factors.

In conclusion, we describe here a novel interaction of macrophages and fat cells, i.e. induction of apoptosis. Our data suggest a feed-forward cycle in which macrophages further drive the inflammatory process by inducing insulin resistance and concomitant apoptosis of adipocytes.

Key words: inflammation, fat cells, macrophages, apoptosis, insulin resistance

1. Introduction

Obesity is one of the major health problems in the western world. The excessive accumulation of adipose tissue gives rise to a state of chronic, low-grade inflammation and is associated with severe co-morbidities including type 2 diabetes mellitus, liver steatosis and atherosclerosis (Shoelson et al., 2007; Baker et al., 2007).

It is now well accepted that adipose tissue inflammation is an important early event in the pathogenesis of obesity complications, especially insulin resistance (Xu et al., 2003). In both humans and rodents, macrophages accumulate in adipose tissue with increasing body weight (Xu et al., 2003; Weisberg et al., 2003). Several rodent studies demonstrate that an increase in adipose tissue macrophages worsens insulin sensitivity (Hirasaka et al., 2007; Kamei et al., 2006). Likewise, a decrease in adipose tissue macrophages (ATM) results in a decrease in adipose tissue inflammation and inhibits obesity-induced insulin resistance (Patsouris et al., 2008). Furthermore, genetic ablation of macrophage signaling by knockout of I κ B kinase-beta (IKK- β) or c-Jun N-terminal kinase 1 (JNK1) protects mice from diet-induced insulin resistance (Arkan et al., 2005; Solinas et al., 2007).

The pathogenic mechanisms leading to ATM accumulation in the obese state are still not completely understood. There is increasing evidence that adipocyte hypertrophy and local hypoxia are implicated in macrophage recruitment since both conditions cause an increased production of inflammatory cytokines and chemoattractants (Skurk et al., 2007; Wood et al., 2009). In general, macrophages play an important role in the immune system and tissue homeostasis by sequestering and clearing pathogens, and old and dead cells (Weigert et al., 2009). Measuring the integration of ^{14}C derived from nuclear bomb tests in genomic DNA by accelerator mass spectrometry, Spalding et al. recently showed that there is a continuous turnover of adipocytes with approximately 10 % of fat cells being renewed annually (Spalding et al., 2008). Tissue homeostasis includes supply of new cells from progenitors followed by differentiation into specialists. Indeed, it has long been known that a large pool of preadipocytes exists in human adipose tissue (Fischer-Posovszky et al., 2007). The finding of a pool of early committed white adipocyte precursors in the mural cell compartment of the adipose vasculature supplies an additional putative source for new adipocytes (Tang et al., 2008).

On the other hand, old cells must be eliminated to keep the number of adipocytes in a certain regulated range. There are several distinct types of cell death (Galluzzi et al., 2009), and necrosis of adipocytes has been described (Cinti et al., 2005). However, apoptosis is considered the predominant way cells die in multicellular organisms, including humans (Weigert et al., 2009). Apoptosis of adipocytes was shown in several disease states accompanied by loss of adipose tissue including tumor cachexia (Prins et al., 1994), autoimmune lipodystrophy (Fischer-Posovszky et al., 2006) and in HIV patients under highly active anti-retroviral therapy (Domingo et al., 1999). Most interestingly, not only a pro-apoptotic gene expression profile, but also an increase in apoptosis was detected in mice under a high fat diet (Alkhoury et al., 2010). Blocking the function of pro-apoptotic Bid resulted in a significant decrease of fat cell apoptosis thereby reducing obesity-associated macrophage infiltration into adipose tissue and preventing insulin resistance (Alkhoury et al., 2010). This suggests an important role of fat cell apoptosis in the progression of obesity-induced disorders.

Taken together, recent findings strongly imply a local interplay between ATM and fat cells. We hypothesize that the local pro-inflammatory micro-environment which is accompanied by insulin resistance enhances adipocyte apoptosis. We developed an *in vitro* model system of inflamed adipose tissue to study macrophage-mediated apoptosis induction in human fat cells. To this end, we either incubated SGBS adipocytes with conditioned media from THP-1 macrophages or directly co-cultured SGBS adipocytes with macrophages.

2. Material and Methods

2.1. Cell culture, adipocyte and macrophage differentiation

SGBS preadipocytes were cultured and differentiated into adipocytes as described elsewhere (Fischer-Posovszky et al., 2006). THP-1 cells purchased from ATCC (Wesel, Germany) were cultured in RPMI containing 100 μ M non-essential amino acids, 2 mM L-glutamine, 1 mM sodium pyruvate, antibiotics (basal medium), and 10% FCS. Differentiation of THP-1 cells into macrophages was induced by incubation with 125 ng/ml phorbol myristate acetate (PMA) in serum-free basal medium containing 0.5% BSA for 48 h. Expression of surface molecules was analyzed by flow cytometry using the following antibodies: CD11c-PE (clone B-Ly6), and isotype-PE (all from BD Pharmingen, Heidelberg, Germany). Data were analyzed using CellQuest software (BD Biosciences, Heidelberg, Germany) and ratio of mean fluorescent intensity (MFI) of CD11c-PE to MFI of isotype-PE was calculated. Macrophage-conditioned media (MacCM) were collected after additional 48 h in serum-free basal medium containing 0.5% BSA and cleared by centrifugation. Cytokine concentrations in conditioned media were assessed using Bio-Plex System (Bio-Rad Laboratories, Hercules, CA). Serum-free basal medium containing 0.5% BSA in corresponding concentrations was used as vehicle control in all further treatments. *In vitro* differentiated THP-1 macrophages were added to cultures of SGBS preadipocytes or adipocytes at increasing rates (THP-1:SGBS 1:10, 1:2, 1:1, 2:1).

2.2. Adipogenic differentiation assay

SGBS preadipocytes were incubated with MacCM (10, 20, 50 %) or vehicle during adipogenic differentiation. At day 10 cells were analyzed. Rate of adipogenic differentiation was assessed by direct counting the number of differentiated cells in the monolayers using a net micrometer.

2.3. Basal and insulin-stimulated glucose uptake and *de novo* lipogenesis

After washing twice with PBS, *in vitro* differentiated SGBS adipocytes (differentiation rate > 80 %) were incubated in serum-free medium supplemented with indicated concentrations of MacCM or vehicle for 24 h. For glucose uptake, cells were stimulated with indicated doses of insulin (0 - 1000 nM) for 15 min and then [14 C]-2-deoxy-D-glucose (0.2 μ Ci/well) was added for another 15 min. For lipogenesis, insulin and [14 C]-D-glucose (0.1 μ Ci/well) were added for 24 h. After incubation with glucose, cells were

washed twice in ice-cold PBS, harvested with 100 mM NaOH and incorporation of [¹⁴C]-2-deoxy-D-glucose or [¹⁴C]-D-glucose was measured on a β -counter.

2.4. Preparation of RNA and quantitative RT-PCR

Total RNA was prepared using RNeasy kit or RNeasy Lipid tissue kit (Qiagen, Hilden, Germany). cDNA synthesis was performed as described previously (Fischer-Posovszky et al., 2006). qPCR was carried out on a LightCycler[®] (Roche Diagnostics, Mannheim, Germany). The mRNA levels of target genes were normalized to those of Glycerinaldehyd-3-phosphat-Dehydrogenase (GAPDH) and succinate dehydrogenase complex subunit A (SDHA) using the Δ Ct method. The sequences of gene-specific primers can be found in Table A.2.

2.5. Western blot analysis

Western blot analysis was performed as described previously (Fischer-Posovszky et al., 2006). Enhanced chemiluminescence was used for detection (Amersham Bioscience, Freiburg, Germany). Following antibodies were used: rabbit anti-phospho Akt, rabbit anti-phospho p38 MAPK, mouse anti-phospho ERK1/2, rabbit anti-panAkt, mouse anti-p38 MAPK (all from Cell Signaling, Beverly, MA, USA), rabbit anti-Akt2 (R&D Systems Inc, Minneapolis, USA), rabbit anti-ERK1/2 (Sigma, Munich, Germany), mouse anti- β -actin (Sigma). HRP-conjugated goat anti-mouse IgG and goat anti-rabbit IgG were obtained from Santa Cruz Biotechnology, INC., Europe.

2.6. Induction and quantification of apoptosis after treatment with MacCM

Preadipocytes and adipocytes were stimulated with MacCM (10, 20, 50 %) or corresponding vehicle control. Quantitative determination of apoptosis according to Nicoletti was performed as described elsewhere (Nicoletti et al., 1991, Fischer-Posovszky et al., 2004). In all experiments the percentage of specific apoptosis was calculated as follows to normalize data: $100 \times [\text{experimental apoptosis (\%)} - \text{spontaneous apoptosis in vehicle (\%)}] / [100\% - \text{spontaneous apoptosis in vehicle (\%)}]$. Externalization of phosphatidylserine was measured by Annexin V staining (Roche, Grenzach, Germany) according to the manufacturer's instructions and analyzed by flow cytometry.

2.7. Induction and quantification of apoptosis in macrophage-adipocyte co-cultures

In vitro differentiated THP-1 macrophages were added to cultures of SGBS preadipocytes or adipocytes as described above. THP-1 macrophages are morphologically clearly distinguishable from SGBS cells. This allowed assessing the number of adherent preadipocytes and adipocytes by direct counting the number of cells in the monolayers using a net micrometer after three days of co-culture. Externalization of phosphatidylserine was measured by Annexin V staining after 24 h of co-culture. CD221-PE staining was used to identify preadipocytes in the co-culture system. Adipocytes were separated from macrophages according to their FSC/SSC profile as previously published (Lee et al., 2004; Schaedlich et al., 2010). For immunofluorescence, SGBS cells were grown and differentiated on chamberslides. At day 8 of adipogenic differentiation and after 24 h co-culture with THP-1 macrophages, monolayers were washed, and fixed with 4% PFA followed by staining with CD11c-PE antibody (BD Pharmingen, Heidelberg, Germany) and BODIPY 493/503 (1 µg/ml, Invitrogen, Karlsruhe, Germany). Finally, cells were mounted with DAKO fluorescence mounting medium (DAKO, Hamburg, Germany) containing DAPI (0.5 µg/ml, Invitrogen). Slides were viewed using an Olympus AX70 fluorescence microscope equipped with appropriate filters (Olympus Optical Co., Hamburg, Germany). Images were captured with a CC12 digital camera (Soft Imaging System, Münster, Germany) using cell[^]P software (Olympus, Hamburg, Germany).

2.8. Experimental subjects

Paired human visceral (visc) and subcutaneous (sc) fat tissue samples were obtained from the Leipzig (Germany) cohort fat tissue bank described in previous studies (Harman-Boehm et al., 2007). Fat tissue samples were obtained from 54 Caucasian obese and lean men (n = 30) and women (n = 24) who underwent open abdominal surgery for elective cholecystectomy or explorative laparotomy. Patients with malignant diseases or acute or chronic inflammatory disease have not been included. Mean age of patients was 62 years (range 31 to 84 years) and their mean BMI was 30 kg/m² (range 29 to 42 kg/m²). All procedures were performed in accordance with the Declaration of Helsinki guidelines and approved by the ethics committee of the University of Leipzig. Patients gave a written informed consent in advance.

2.9. Immunohistochemistry

The sc and visc fat samples were fixed at room temperature in 4% formaldehyde and embedded in paraffin. 3 μ m serial sections were mounted on glass slides, deparaffinized in xylol, and stained for CD11c (Clone BU15, mouse IgG₁, Immunotech, Marseille, France) or cleaved caspase-3 (rabbit polyclonal, Cell Signaling, Beverly, MA, USA) using the Envision detection system (Dako, Glostrup, Denmark). Macrophages were identified in the adipose parenchyma (CD11c⁺ within blood vessels were excluded) when staining for CD11c was present along with an identifiable mononuclear nucleus. Data are presented as the number per 100 adipocytes (% macrophages, % apoptotic cells). Counting was performed blinded to sample data and was confirmed by a certified pathologist (P.M.).

2.10. Lentiviral-mediated over expression of shRNA

Specific shRNA sequences targeting the human Akt2 sequence (#1: start 1423: 5'-ggttcttcctcagcatcaact-3'; #2: start 679: 5'-gcaaggaaccttggcaaag-3') and a nonsense shRNA sequence with no corresponding part in the human mRNA (5'-gatcatgtagatagctca -3') were cloned into the pENTRTM/H1/TO entry vector using the BLOCK-iT Inducible H1 RNAi Entry Vector Kit (Invitrogen, Karlsruhe, Germany) and subsequently transferred into the pLenti4/BLOCK-iTTM-DEST expression vector by targeted recombination using BLOCK-iT Inducible H1 Lentiviral RNAi system (Invitrogen) according to manufacture's protocol. The pLenti expression constructs were transfected with packaging plasmids by LipofectamineTM 2000 (Invitrogen) in the 293FT producer cell line using ViraPowerTM Lentiviral Expression Systems (Invitrogen) according to the manufacturer's protocols. Three days after transfection the viral supernatant was harvested, filtered through a 0.45 μ m PVDF filter, and stored at -80°C. SGBS cells were infected at MOI = 1 in the presence of 8 μ g/ml polybrene. Stable bulk cultures were generated by selection with 0.2 mg/ml zeocin for 14 d, and down-regulation of Akt2 protein was controlled by Western blot. Experiments were performed with cells from three independent lentiviral infections.

2.11. Statistical analysis

If not otherwise stated, data from three independent triplicate experiments were expressed as mean \pm standard error of the mean (SEM). For statistical comparison, Student's two-tailed paired t-test was used. $p < 0.05$ was considered statistically significant in comparison to the respective control cells. For correlation analysis spearman's correlation was performed using SigmaStat version 3.5 statistical analysis software (Systat Software GmbH, Inc. San Jose, California, US).

3. Results

3.1. Establishment of an *in vitro* model system for human inflamed adipose tissue

In order to study interactions of macrophages and adipocytes on a cellular level, we designed an experimental setup to mimic adipose tissue inflammation *in vitro*. To this end, we differentiated human THP-1 monocytes into macrophages. Increased mRNA expression of CD11c (Fig. 1A) and the up-regulation of CD11c surface expression (Fig. 1B, C) demonstrated successful macrophage differentiation. Furthermore, the expression of inflammatory cytokines like TNF α , IL-1 β and IL-6 (Fig. 1D-F) was increased after differentiation into macrophages, and cytokines accumulated in the medium supernatants (TNF α ~ 39 ng/ml, IL-1 β ~ 390 pg/ml, IL-6 ~ 2100 pg/ml). Macrophage-conditioned media (MacCM) were used in the *in vitro* setting together with cultures of human preadipocytes and adipocytes (SGBS cells) to mimic the inflammatory micro-environment found *in vivo*.

3.2. Macrophage-secreted factors inhibit insulin-dependent processes in human adipocytes

To examine whether macrophage factors interfere with insulin sensitivity in SGBS adipocytes we performed a panel of experiments related to insulin function. First, we investigated the insulin-dependent differentiation of preadipocytes into mature, functional adipocytes. We found an inhibition of adipogenesis upon stimulation with MacCM as seen by determination of adipogenic differentiation rate (Fig. 2A) and mRNA expression of the adipocyte marker gene FABP4 (fatty acid binding protein 4) (Fig. 2B). Furthermore, we studied if macrophage-secreted factors interfere with insulin-stimulated biochemical functions of mature fat cells, i.e. glucose uptake and *de novo* lipogenesis. When we pre-incubated adipocytes with MacCM, the insulin stimulated uptake of [¹⁴C]-2-deoxy-D-glucose was robustly decreased (fold change 100 nM insulin vs. basal, 0% MacCM: 3.91 ± 0.47 ; 50% MacCM: 1.37 ± 0.12) (Fig. 2C and 2D). Likewise, the insulin stimulated *de novo* lipogenesis determined by [¹⁴C]-D-glucose incorporation into cellular lipids was decreased when adipocytes were incubated with MacCM (Fig. 2E and 2F). Inhibitory effects of macrophage factors on insulin sensitivity were also observed in 3T3-L1 and human primary preadipocytes (Constant et al., 2006) demonstrating that our model system is well suitable for studying the effects of macrophage-secreted factors on fat cell function.

3.3. *Macrophage-secreted factors inhibit insulin signaling in adipocytes*

We next aimed at identifying molecular changes induced by macrophage-secreted factors. Knowing that MacCM inhibits insulin-dependent processes (Section 3.2.), we hypothesized that macrophage-secreted factors interfere with insulin signaling pathways of adipocytes. Thus, we pre-treated SGBS adipocytes with MacCM for 24 h, stimulated the cells with 10^{-7} M insulin for 15 min and subjected protein samples to a protein array to study the phosphorylation state of insulin-stimulated down-stream kinases. Out of several kinases studied, phosphorylation of Akt kinases was most robustly inhibited by macrophage-secreted factors in both the basal and the insulin-stimulated state (Fig. A.1). Likewise, phosphorylation of Akt down-stream targets, e.g. glycogen synthase kinase 3 alpha/beta (GSK3- α/β) and 70-kDa S6 kinase (p70S6K) was down-regulated. Other signaling pathways including c-Jun N-terminal kinases (JNK), p38 MAPK (p38) and p44/42 MAPK (ERK) were either not or only slightly affected (Fig. A.1). Additional Western blot analysis (Fig. 3A) to validate these findings demonstrated that macrophage-secreted factors abolished basal phosphorylation of Akt (Fig. 3B) and down-regulated insulin-stimulated phosphorylation by ~ 65 % (Fig. 3C). The up-regulation of p38 phosphorylation by macrophage-secreted factors was not detected in the protein array.

The data on insulin-stimulated fat cell function (Section 3.2.) together with the insights on phosphorylation of insulin-stimulated downstream targets (Section 3.3.) demonstrates that factors secreted from macrophages provoked insulin resistance in human fat cells.

3.4. *Macrophage-secreted factors induce apoptosis in preadipocytes and lipid laden, mature adipocytes*

To investigate the susceptibility of preadipocytes to enter into apoptosis induced by macrophage-secreted factors, we treated SGBS preadipocytes with escalating doses of MacCM. We found a time- and dose-dependent induction of DNA fragmentation (50% MacCM, 48h: $20.11\% \pm 1.16$ specific apoptosis), which is known as a hallmark of apoptosis induction (Fig. 4A and 4B). Externalization of phosphatidylserine to the cell surface, which served as an eat-me-signal for macrophages (Weigert et al., 2009), was demonstrated by Annexin V staining (Fig. 4C). This method is another gold standard for demonstrating apoptosis. We then extended our experiments to mature, lipid-laden adipocytes. Similar to preadipocytes, macrophage-secreted factors induced apoptosis in a dose- and time-dependent manner in

adipocytes (50% MacCM, 48h: $15.43\% \pm 0.76$ specific apoptosis) (Fig. 4D and 4E). This set of experiments clearly demonstrates that macrophage-secreted factors induced apoptosis in human preadipocytes and adipocytes.

3.5. Macrophages induce apoptosis in human preadipocytes and adipocytes

We next aimed to study the role of direct cell-cell contacts between fat cells and macrophages in apoptosis induction. We therefore extended our studies to a direct co-culture system of both cell types.

After 24 h of co-culture we detected a significant increase in phosphatidylserine externalization in both CD221⁺-preadipocytes (Fig. 5A) and mature, lipid-laden adipocytes (Fig.5B). Most interestingly, we observed a robust decrease in the cell number of adherent preadipocytes after co-culture with macrophages for 72 h (decrease by $43.0\% \pm 9.7$ at ratio THP-1:SGBS 2:1) (Fig. 5C). Also the number of mature adipocytes was significantly decreased upon co-culture with macrophages (decrease by $53.1\% \pm 13.5\%$ at ratio THP-1:SGBS 2:1) (Fig. 5D). Along with the observation of no dead, detached cells swimming in the culture supernatants, this set of data suggests that macrophages induce apoptosis in preadipocytes and adipocytes and directly phagocytose them. An immunofluorescence approach labeling macrophages by CD11c and intracellular lipid droplets by BODIPY further corroborated this finding (Fig. 5E). CD11c⁺-macrophages surrounded lipid-filled adipocytes and we even detected BODIPY-stained lipid droplets inside of macrophages. Notably, our *in vitro* observation (Fig. 5E) highly resembles the *in vivo* situation (Fig. 5F). It has long been proposed that macrophages localize around dead adipocytes (Cinti et al., 2005). However, the knowledge on fat cell apoptosis in different human adipose tissue depots is still limited. We therefore performed immunohistochemistry to detect macrophages by CD11c along with apoptotic adipocytes by staining the active fragment of caspase-3 in both the subcutaneous and the visceral adipose tissue depot of 54 subjects. As expected, apoptotic adipocytes were surrounded by CD11c⁺-macrophages in crown-like structures (Fig. 5F, details about tissue distribution are given in Table A.1). A subsequent correlation analysis performed with the data obtained in the patients having positive cells for both CD11c and active caspase-3 (sc: n = 19; visc: n = 30) revealed that the percentage of apoptotic adipocytes strongly correlated with the percentage of macrophages in both subcutaneous (Fig. 5G) and visceral (Fig. 5H) adipose tissue depots (sc: $r = 0.888$; $p < 0.001$; visc: $r = 0.560$, $p = 0.003$).

Our data unambiguously show that *in vivo* macrophages were closely located to apoptotic adipocytes, further supporting our *in vitro* data on the direct connection between macrophages and fat cell apoptosis.

3.6. *Pharmacological and genetic inhibition of Akt signaling sensitizes adipocytes for apoptosis induced by macrophage-secreted factors*

We have previously shown an anti-apoptotic function of insulin-like growth factor I (IGF-1) in human fat cells (Fischer-Posovszky et al., 2004). Insulin mediates survival in several cell types (Díaz et al., 1999; Tanaka et al., 1995) and has anti-apoptotic effects comparable to IGF-1 in brown adipocytes (Navarro et al., 1998; Tseng et al., 2002). This leads us to hypothesize that insulin resistant adipocytes, where insulin is no longer capable of exerting its survival effects, are sensitive to apoptosis induction. Thus, insulin resistance induced by macrophage-secreted factors would sensitize primarily apoptosis resistant adipocytes to apoptosis induction.

In our experiments, phosphorylation of Akt, the key molecule in the insulin signaling pathway (Kohn et al., 1995), was most robustly inhibited by MacCM (Section 3.3.) suggesting that inhibition of Akt is involved in insulin-mediated anti-apoptosis. To further support our hypothesis, we blocked insulin signaling upstream of Akt in human SGBS adipocytes by using the pharmacological inhibitor LY294002, a small molecule inhibitor targeting phosphatidylinositol 3-kinase (PI3K). Inhibition of Akt phosphorylation was achieved at 20 μ M of LY294002, while phosphorylation of other kinases including p38 and ERK was not affected (Fig. 6A). Inhibition of PI3K/Akt signaling resulted in a significant increase in apoptosis induction by a low concentration of MacCM (10%) in these cells (MacCM + vehicle: $7.05\% \pm 1.11$, MacCM + LY $19.48\% \pm 2.15$) (Fig. 6B). We additionally inhibited Akt by lentiviral-mediated overexpression of shRNA. Out of three existent Akt isoforms, Akt2 (also known as PKBB) is most highly expressed in insulin-sensitive tissues (Brazil and Hemmings, 2001). We designed and cloned two different shRNA constructs targeting human Akt2. We achieved a down-regulation of Akt2 expression by $> 80\%$ with both constructs compared to expression of a nonsense shRNA (Fig. 6C). In line with the pharmacological inhibition, knock-down of Akt2 caused a significant increase in apoptosis induction by macrophage-secreted factors compared to nonsense shRNA control cells (nonsense: $12.23\% \pm 1.27$; Akt2#1: $33.05\% \pm 3.85$; Akt2#2: $24.56\% \pm 3.62$) (Fig. 6D).

4. Discussion

In the present study, we demonstrate that macrophages induce apoptosis in human fat cells by interfering with the anti-apoptotic insulin signaling pathway at the level of Akt. Studying human subcutaneous and visceral adipose tissue samples we found a co-localization of CD11c⁺-macrophages and apoptotic adipocytes indicating a causal relationship of macrophage infiltration and apoptosis of fat cells.

Previous studies of our group showed that human fat cells are protected from death receptor induced apoptosis although expressing all components of the apoptosis signaling pathway (Fischer-Posovszky et al., 2004). The insulin-like growth factor I (IGF-I)/IGF-I-receptor autocrine circuit is responsible for this apoptosis resistance (Fischer-Posovszky et al., 2004). It is known that insulin/IGF-I act as survival factors in several cell types, including cancer cells (Valentinis and Baserga, 1996) as well as brown adipocytes (Navarro et al., 1998). Therefore, we hypothesized that white adipocytes are resistant to apoptosis as long as they are insulin sensitive. Upon overweight and obesity, macrophages infiltrate human adipose tissue and promote both local and systemic inflammation as well as the development of obesity-related disorders (Weisberg et al., 2003; Xu et al., 2003). It is well accepted that the insulin sensitivity of fat cells is decreased upon inflammation (Olefsky and Glass, 2010). In line with our hypothesis this loss of insulin sensitivity seems to be accompanied by increased apoptosis sensitivity. Fat cell apoptosis has been reported in diseases associated with insulin resistance and inflammation including cancer (Prins et al., 1994), HIV-associated lipodystrophy (Domingo et al., 1999) or autoimmune lipodystrophy (Fischer-Posovszky et al., 2006).

To test our hypothesis on a cellular level, we established an *in vitro* model of adipose tissue inflammation. Macrophage-secreted factors inhibited insulin-stimulated processes such as adipogenic differentiation, glucose uptake and *de novo* lipogenesis accompanied by decreased phosphorylation of insulin-stimulated intracellular kinases. Kinases important for the insulin signaling including Akt were robustly inhibited by the macrophage-secreted factors assumable by cytokines such as IL-6 and/or IL-1 β . Both cytokines inhibit phosphorylation of Akt in human primary adipocytes and 3T3-L1 cells (Lagathu et al., 2006; Xie et al., 2010). Under normal physiological conditions human fat cells are protected from apoptosis induction by death ligands (Fischer-Posovszky et al., 2004). Notably, macrophage-secreted factors induced apoptosis in both preadipocytes and adipocytes in a dose-dependent manner. This effect

was even more pronounced in macrophage-adipocyte co-cultures. We conclude that the apoptosis inducing effect of macrophages is mediated in a paracrine manner and is not dependent on direct cell-cell interaction, but cell contact ameliorates the process. Our findings demonstrate that macrophage-secreted factors increased apoptosis sensitivity in human adipocytes by blocking insulin signaling. Obviously, an inflammatory micro-environment abrogates the cellular survival system. In line, we demonstrated earlier that the loss of adipose tissue in autoimmune lipodystrophy is due to adipocyte apoptosis induced by a combination of cytokines together with death ligands, i.e. $\text{INF}\gamma$, $\text{TNF}\alpha$ and CD95L (Fischer-Posovszky et al., 2006).

Pharmacological or genetic inhibition of insulin signaling at the level of Akt sensitized human fat cells for apoptosis induction further supporting a causal connection between insulin resistance and apoptosis sensitivity. Akt is a key molecule in the insulin signaling pathway (Kohn et al., 1995). Among the three isoforms of Akt, Akt2 is the most abundant isoform in insulin sensitive tissues (Brazil and Hemmings, 2001) and it is the major isoform responsible for insulin action (Brazil and Hemmings, 2001). Most interestingly, Akt2 has already been linked to loss of adipose tissue, most likely by apoptosis of fat cells in mice and in humans. Along with insulin resistance, Akt2 knockout mice display an age-dependent loss of adipose tissue (Garofalo et al., 2003). Likewise, a family with a mutation in the Akt2-gene shows an autosomal dominant inheritance of severe insulin resistance and lipodystrophy (George et al., 2004). We conclude from our data that macrophages inhibit Akt2 action thereby diminishing the insulin survival signal and rendering fat cells sensitive to apoptosis triggers.

It has to be mentioned that treatment of 3T3-L1 cells with conditioned media from J774 macrophages protected fat cells from apoptosis induction (Molgat et al., 2011). This apparent discrepancy to our findings can be explained by differences in apoptosis sensitivity between murine 3T3-L1 cells and human fat cells. 3T3-L1 preadipocytes and adipocytes are sensitive to apoptosis induction by serum deprivation (Magun et al., 1998), whereas human SGBS preadipocytes and adipocytes are characterized by a general resistance to apoptosis stimuli including serum withdrawal (Fischer-Posovszky et al., 2004). This resistance to apoptosis stimuli has also been shown for primary human preadipocytes and adipocytes (Fischer-Posovszky et al., 2004). We suggest that 3T3-L1 cells benefit from macrophage-secreted factors such as PDGF (Molgat et al., 2009) which are known to rescue cells from apoptosis. In

addition, it has been shown that macrophage media stimulate Akt phosphorylation in 3T3-L1 cells (Molgat et al., 2011), further supporting the differences between murine and human cells.

Our results show that the number of macrophages correlates with the number of apoptotic adipocytes in visceral as well as in subcutaneous human adipose tissue samples. In our study we used immunohistochemistry to detect cleaved caspase-3, which is a specific marker for apoptosis induction *in situ*. We identified apoptotic adipocytes in the center of CD11c⁺ crown-like structures. Apoptosis is associated with typical biochemical events such as externalization of phosphatidylserine and cleavage of caspases (Kroemer et al., 2009). These characteristics are not found in other forms of cell death such as necrosis (Kroemer et al., 2009). Cinti et al. detected signs of necrotic cell death with crown-like structures in murine and human adipocytes as assessed by rupture of the plasma membrane and dilatation of the endoplasmic reticulum (ER) via electron microscopy (Cinti et al., 2005). The relative absence of neutrophils usually involved in necrosis-induced inflammation led the authors to suggest that the adipocyte cell death might share features of both apoptosis and necrosis (Cinti et al., 2005). It is well conceivable that features of necrosis occur as late, secondary events in the process of adipocyte death after a primary induction of apoptosis.

Interestingly, a recent study showed that blocking the function of Bid, a key pro-apoptotic molecule, resulted in a significant decrease in fat cell apoptosis, which prevented macrophage recruitment into adipose tissue and protected against development of insulin resistance in mice (Alkhoury et al., 2010). In this experimental setting the primary event leading to apoptosis induction is unknown. Potential triggers of apoptosis include stress responses such as endoplasmic reticulum (ER) stress (Hotamisligil, 2010; Schenk et al., 2008) and hypoxia (Ye, 2009; Regazzetti et al., 2009). Both of these pathological conditions have been shown to be implicated in the development of metabolic disease (Wood et al., 2009; Hotamisligil, 2010).

5. Conclusion

We elucidated how macrophages affect apoptosis sensitivity of human fat cells during adipose tissue inflammation *in vitro*. The close neighborhood of both cell types in human adipose tissue strongly suggests biological interaction *in vivo*. We propose a model where macrophages contribute to maintain low grade inflammation in human adipose tissue in a feed-forward cycle. Macrophages, which have already infiltrated human adipose tissue upon stimuli discussed above, affect two, presumably associated, characteristics of functional fat cells – insulin sensitivity and apoptosis resistance. In particular, secreted cytokines block Akt2 in neighboring fat cells thereby inducing insulin resistance. Simultaneously, macrophages produce death ligands such as TNF α and CD95L (Xu et al., 2003; Weigert et al., 2009). This mechanism enables macrophages to induce apoptosis of fat cells that became insulin resistant and therefore apoptosis sensitive. The increased number of apoptotic adipocytes in turn attracts more macrophages, thereby accelerating the inflammatory process within human adipose tissue.

In conclusion, the discovery that macrophages induce apoptosis of human fat cells during adipose tissue inflammation is novel and has important implications for future investigations in the field of obesity-related metabolic disorders.

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Disclosure Statement

AD and KA are employed by Hoffman-La Roche. Remaining authors declare no conflict of interest.

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Figure legends**Fig. 1:****Expression of surface molecules and inflammatory cytokines of THP-1 macrophages**

Differentiation of human THP-1 cells into macrophages was induced by incubation with 125 ng/ml phorbol myristate acetate (PMA) for 48 h. (A) mRNA-expression of CD11c was analyzed by qPCR. Data are expressed as mean + SEM of three independent experiments. * $p < 0.05$. (B) CD11c surface expression was analyzed by flow cytometry. Dotted line: isotype; black line: monocytes; black-filled surface area: macrophages. One representative experiment out of three performed is shown. (C) Mean fluorescence intensity (MFI) of CD11c surface molecules was related to MFI of isotype control. Data are expressed as mean + SEM of three independent triplicate experiments. * $p < 0.05$. Additionally, we analyzed expression levels of the inflammatory cytokines TNF α (D), IL-1 β (E), and IL-6 (F) by qPCR. Data are expressed as mean + SEM of three independent experiments. * $p < 0.05$.

Fig. 2:**Macrophage-secreted factors inhibit adipogenic differentiation and induce insulin resistance in human adipocytes**

SGBS preadipocytes were incubated with conditioned media from THP-1 macrophages (MacCM) during adipogenic differentiation. After 10 days cells were analyzed. (A) Rate of adipogenic differentiation was determined as described in methods. (B) The expression of the fatty acid binding protein 4 (FABP4) was analyzed by qPCR. (C) SGBS adipocytes were pre-incubated with 50% MacCM or vehicle for 24 h. [^{14}C]-2-deoxy-D-glucose uptake was determined with different insulin concentrations (0 - 1000 nM). (D) Adipocytes were pre-incubated with escalating doses of MacCM (0 - 50%) for 24 h. [^{14}C]-2-deoxy-D-glucose uptake was determined with or without insulin (100 nM). (E) SGBS adipocytes were incubated with vehicle or 50% MacCM. After 24 h basal and insulin-stimulated *de novo* lipogenesis was assessed by incorporation of [^{14}C]-D-glucose into lipids in the presence of different insulin concentrations (0 - 1000 nM). (F) After eight days in adipogenic medium SGBS adipocytes were incubated with escalating doses of MacCM (0 - 50%). After 24 h basal and insulin-stimulated *de novo* lipogenesis was assessed by incorporation of [^{14}C]-D-glucose into lipids in the presence or absence of insulin (100 nM). All data are the mean \pm SEM of three independent experiments. For (C) - (F) results are presented as fold change of insulin-stimulated to basal glucose uptake or glucose incorporation. * $p < 0.05$ vs. vehicle.

Fig. 3:**Macrophage-secreted factors inhibit basal and insulin-stimulated Akt phosphorylation**

(A) After seven days in adipogenic medium SGBS adipocytes were pre-incubated with 50% MacCM or vehicle. After 24 h cells were treated with indicated insulin concentrations for 15 min before lysis. Cell lysate (100 µg protein/lane) was separated by 4-12% gradient SDS-page. Phosphorylation of Akt, ERK, and p38 (pAkt, pERK and pp38) was analyzed by Western blot analysis. One representative experiment is shown. Densitometric analysis for the basal phosphorylation (B) or insulin-stimulated phosphorylation (100nM, C) of Akt, ERK and p38 are presented as mean + SEM of three independent experiments. Phosphorylation of the vehicle control was set to one. * $p < 0.05$ vs. vehicle.

Fig. 4:**Macrophage-secreted factors induce apoptosis in human preadipocytes and lipid-laden adipocytes**

(A) SGBS preadipocytes were incubated with vehicle or 50% MacCM. After 24, 48 and 72 h, the content of hypodiploid DNA was determined by flow cytometry. (B) SGBS preadipocytes were incubated with increasing concentrations of MacCM (0 - 50%) for 48 h. The content of hypodiploid DNA was determined by flow cytometry. (C) SGBS preadipocytes were incubated with vehicle or 50% MacCM. After 24 h cells were stained with Annexin V followed by flow cytometric analysis. (D) After seven days in adipogenic medium, SGBS adipocytes were incubated with vehicle or 50% MacCM for 24, 48 and 72 h. The content of hypodiploid DNA was determined by flow cytometry. (E) After seven days in adipogenic medium, SGBS adipocytes were incubated with increasing concentrations of MacCM. After 48 h, the content of hypodiploid DNA was determined by flow cytometry. For (A), (B), (D) and (E) specific apoptosis was calculated as described in Methods (Section 2.6.). All data are expressed as mean ± SEM of three independent, triplicate experiments. * $p < 0.05$ vs. vehicle.

Fig. 5:**Macrophages induce apoptosis in human preadipocytes and adipocytes**

THP-1 cells were differentiated into macrophages as described in Methods and added in increasing rates to cultures of (A, C) SGBS preadipocytes or (B, D) adipocytes. (A) After 24 h cells were stained with anti-CD221-PE and Annexin V-FITC. CD221⁺-preadipocytes were gated and analyzed for Annexin V expression. (B) Adipocytes were gated according to their FSC/SSC profile and analyzed for Annexin V.

For (A) and (B) data are presented as % of control and as mean + SEM of three independent experiments. * $p < 0.05$ vs. control. (C, D) After 72 h of co-culture the number of adherent SGBS preadipocytes (C) or adipocytes (D) was assessed as described in methods. Data are expressed as % of control and presented as mean + SEM of four independent experiments. * $p < 0.05$ vs. control. (E) SGBS adipocytes grown on chamber slides were co-cultured with THP-1 macrophages (ratio 1:1). After 24 h cells were stained with anti-CD11c-PE (macrophages, red), BODIPY (lipid droplets, green), and DAPI (nuclei, blue). Cells were analyzed with a fluorescence microscope. One representative experiment is shown. (F) Microphotographs of CD11c and active caspase-3 staining of serial sections from human fat tissue of one representative patient (black circles: positive cells for indicated staining, magnification: 10x (left panel), 40x (right panel)). For the relationship between CD11c and active Caspase-3 positive cells, macrophages and apoptotic cells in adipose sections of 54 patients were quantified by counting the number of positive cells in 12 random microscopic fields. Spearman's correlation analysis was performed between percentage of macrophages and apoptotic fat cells for subcutaneous (G) and visceral adipose tissue (H). Data were natural-log transformed before correlation analysis. Positive cells for both staining were detected in subcutaneous fat tissue of 19 patients and in visceral fat tissue of 30 patients (details see Table A.1).

Fig. 6:

Insulin resistance sensitizes human fat cells to apoptosis by macrophage-secreted factors

(A) Decreased phosphorylation of Akt by the pharmacological inhibitor of the PI3-kinase (LY294002) was controlled by western blot analysis. SGBS adipocytes were incubated for 1h with or without 20 μ M LY294002. One representative out of three independent experiments performed is shown. (B) After seven days in adipogenic medium SGBS adipocytes were incubated with 20 μ M LY294002. LY was either supplied in vehicle or 10% MacCM. After 48 h content of hypodiploid DNA was determined by flow cytometry. * $p < 0.05$ vs. LY or MacCM. (C) Lentiviral mediated knockdown of two different Akt2 shRNA constructs in SGBS preadipocytes was controlled by western blot analysis. One representative experiment out of three performed is shown. (D) Nonsense, Akt2#1 and Akt2#2 cells were incubated with 50% MacCM or vehicle. After 48 h, the content of hypodiploid DNA was determined by flow cytometry. * $p < 0.05$ vs. nonsense. For (B) and (D), specific apoptosis was calculated as described in Methods (Section 2.6.) and data of three independent, triplicate experiments are expressed as mean + SEM.

Highlights

- We established an *in vitro* model of inflamed human adipose tissue
- Macrophages surround apoptotic adipocytes *in vitro* and *in vivo*
- Macrophages as well as macrophage-secreted factors induce apoptosis of adipocytes
- Macrophages increase apoptosis sensitivity by decreasing insulin sensitivity
- By inducing fat cell apoptosis, macrophages might accelerate adipose inflammation

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Figure 1

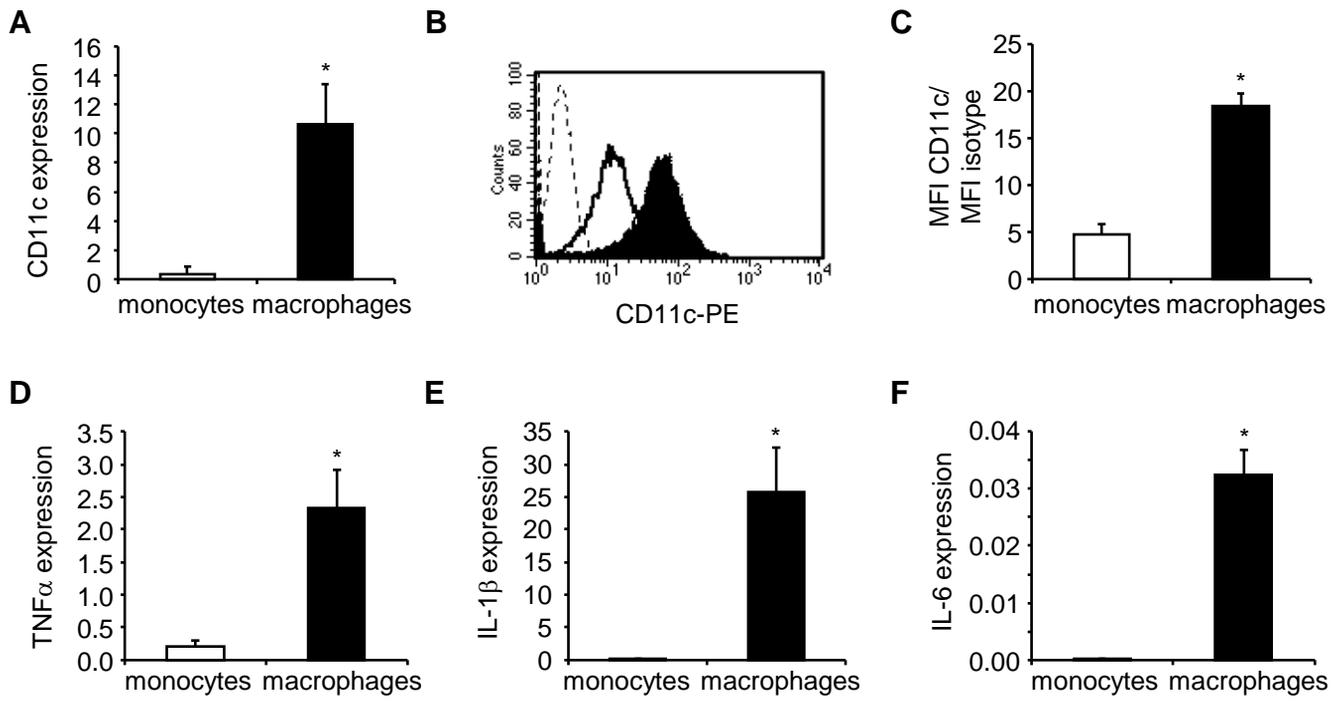


Figure 2

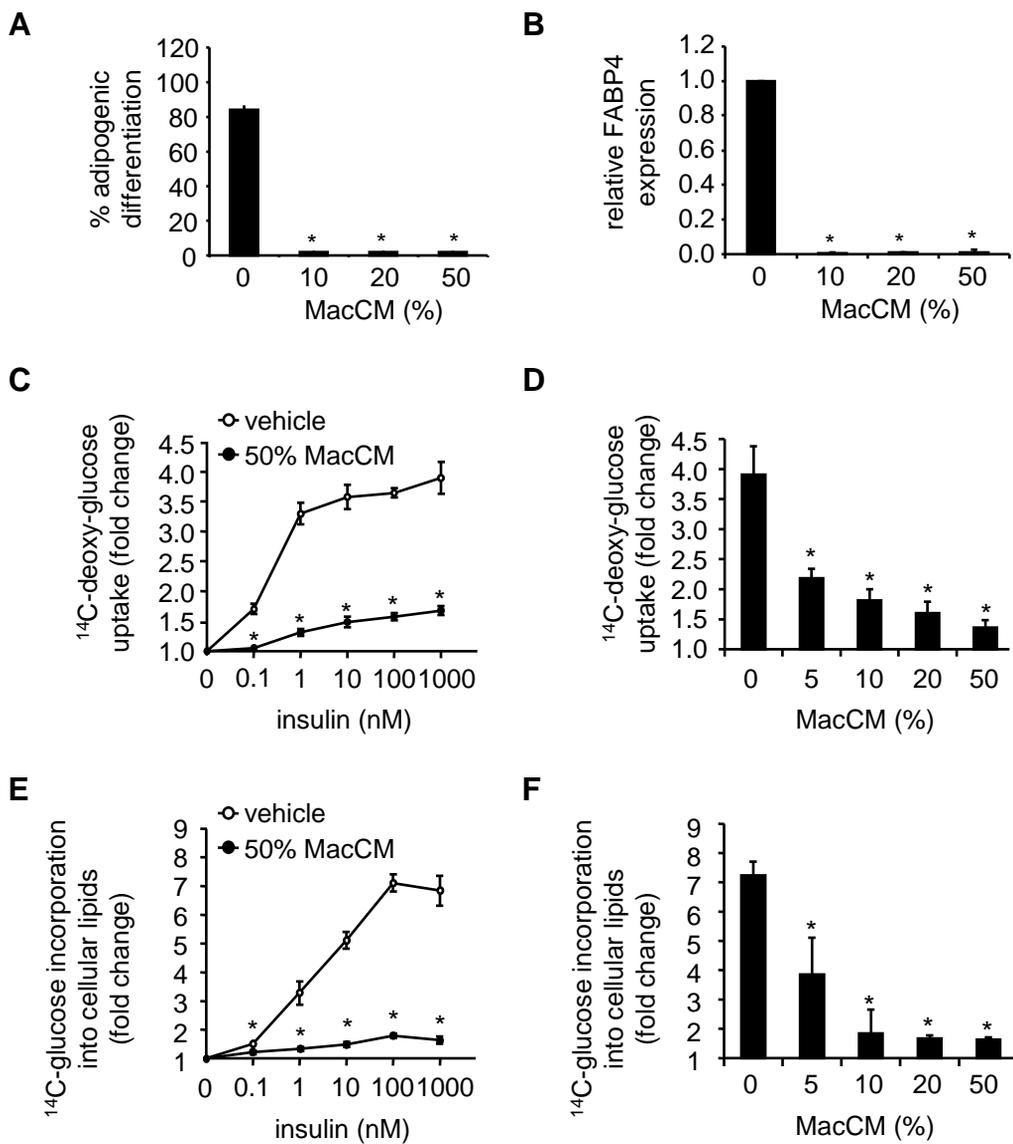
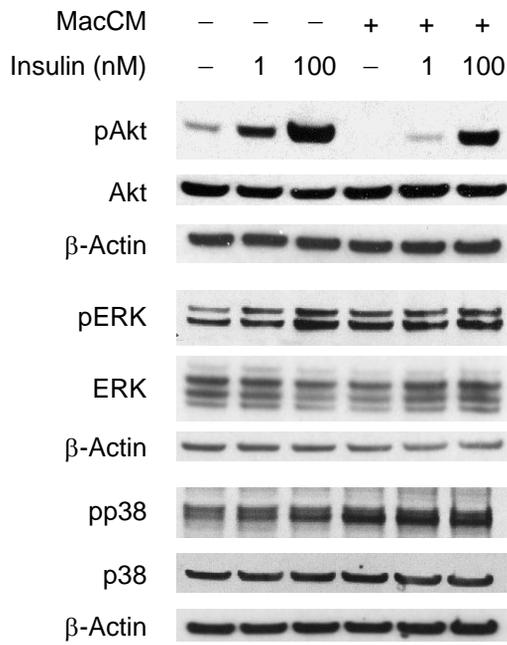
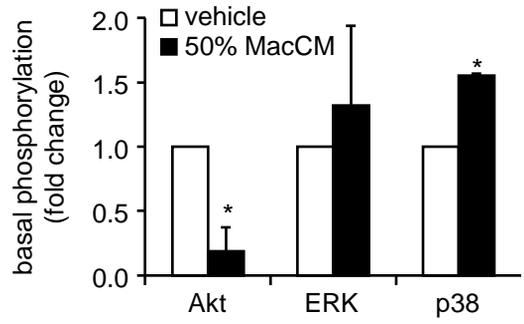


Figure 3

A



B



C

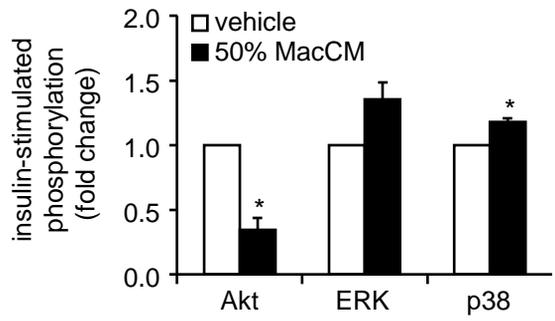


Figure 4

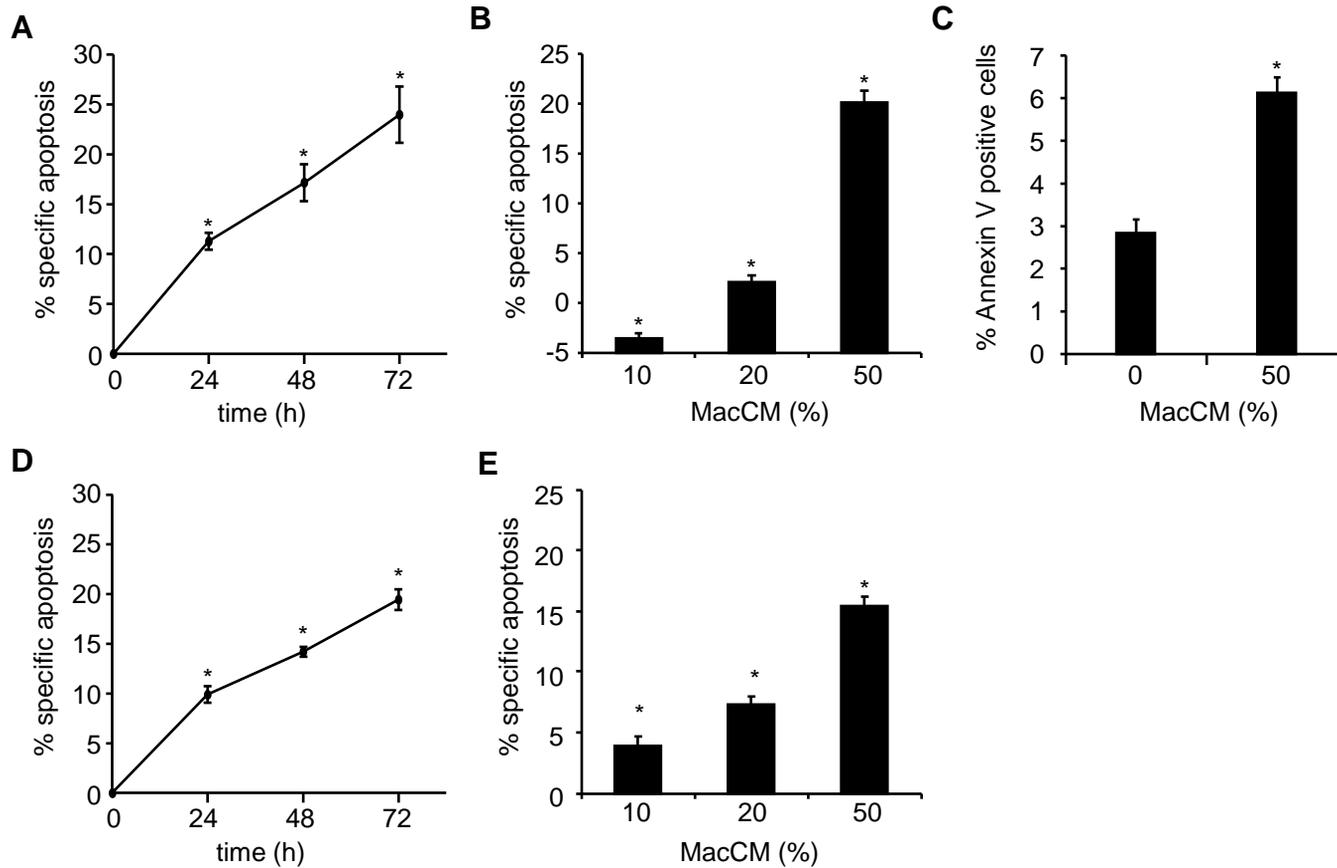


Figure 5

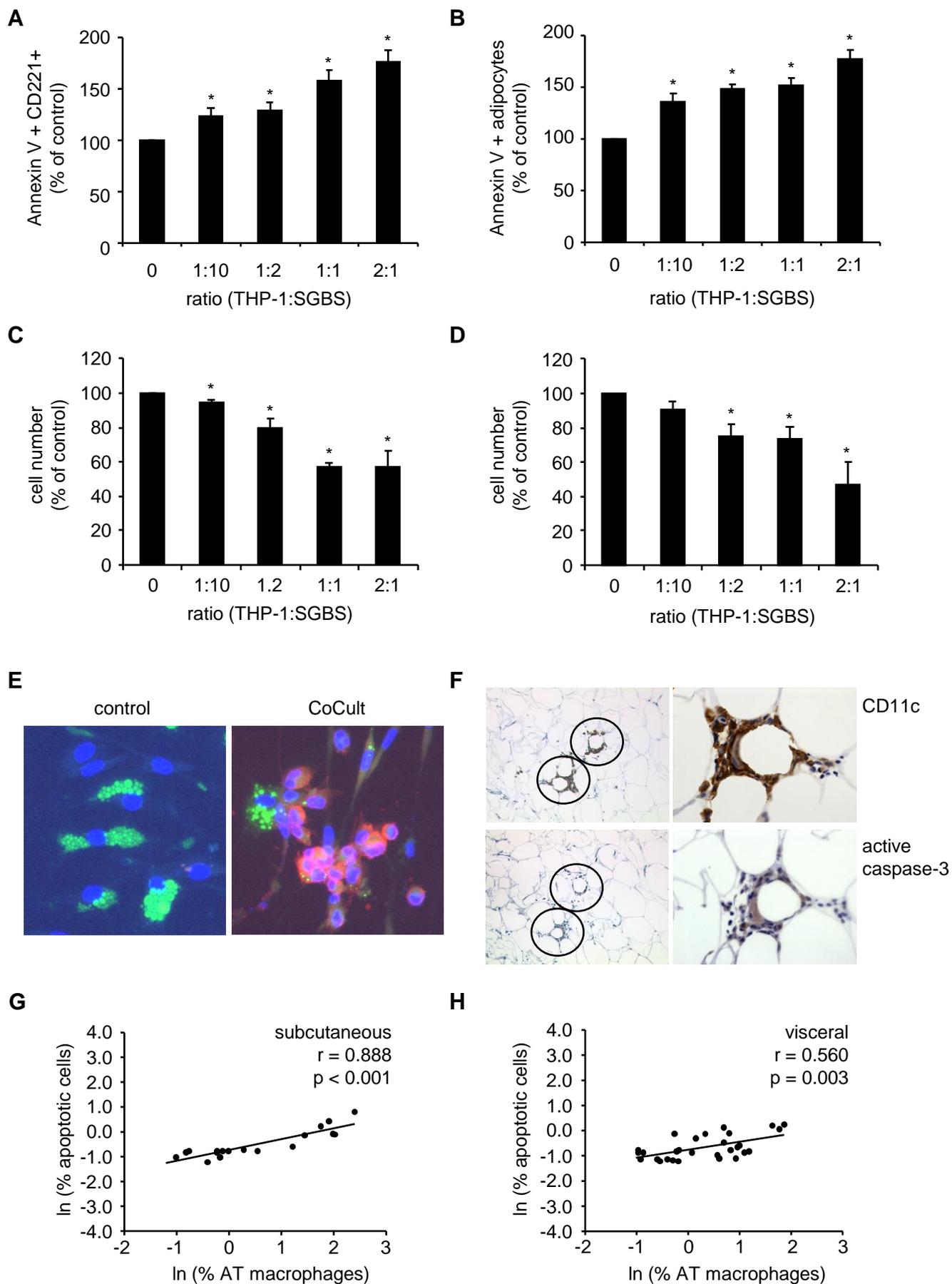


Figure 6

