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Selective deletion of adipocytes, but not preadipocytes, by TNF-α through C/EBP- and PPARγ-mediated suppression of NF-κB

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Tumor necrosis factor-α (TNF-α) is a key regulator of adipose tissue mass, but mechanisms underlying this effect have not been fully elucidated. We found that exposure to TNF-α caused a significant decrease in the number of adipocytes, but not preadipocytes. Subsequent experiments revealed that TNF-α selectively deleted adipocytes through induction of apoptosis. Following exposure to TNF-α, rapid activation of nuclear factor-κB (NF-κB) was observed only in preadipocytes, but not in adipocytes. Inhibition of NF-κB rendered preadipocytes susceptible to TNF-α-induced apoptosis, suggesting that different activity of NF-κB is the determinant for the distinct apoptotic responses. During adipocyte differentiation, expression and activity of peroxisome proliferator-activated receptor-γ (PPARγ) were upregulated. Treatment of preadipocytes with a PPARγ agonist attenuated NF-κB activation and rendered the cells vulnerable to TNF-α-induced apoptosis. Conversely, treatment of adipocytes with a PPARγ antagonist enhanced NF-κB activation and conferred resistance to TNF-α-induced apoptosis, suggesting involvement of PPARγ in the suppression of NF-κB in adipocytes. We also found that, following differentiation, expression and activity of CCAAT/enhancer binding protein (C/EBP), especially C/EBPα and C/EBPβ, were upregulated in adipocytes. Overexpression of individual C/EBPs significantly inhibited activation of NF-κB in preadipocytes. Furthermore, transfection with siRNA for C/EBPα or C/EBPβ enhanced activity of NF-κB in adipocytes, suggesting that C/EBP is also involved in the repression of NF-κB in adipocytes. These results suggested novel mechanisms by which TNF-α selectively deletes adipocytes in the adipose tissue. The C/EBP- and PPARγ-mediated suppression of NF-κB may contribute to TNF-α-related loss of adipose tissue mass under certain pathological situations, such as cachexia.

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Cachexia is caused under several pathological conditions including cancer and infection. It is characterized by a loss of lean body mass, especially skeletal muscles and adipose tissues.[1] The pathogenesis of cachexia is mutlifactorial, but previous reports suggested that inflammatory cytokines, especially tumor necrosis factor-α (TNF-α), have a key role.[2] In cachexia, elevation in plasma levels of TNF-α is often observed. It is believed that the circulating TNF-α causes lipolysis and inhibits lipogenesis, leading to reduction in adipose tissue mass.[2] However, it is well known that TNF-α has the potential to induce apoptosis in various cell types.[3] TNF-α also affects differentiation of several cells including adipocytes.[4] TNF-α-triggered loss of adipose mass may, therefore, be caused not only by reduced store of lipids, but also by inhibition of adipogenesis or deletion of adipocytes through apoptosis. Indeed, previous reports showed that either secreted or transmembrane TNF-α has the potential to inhibit adipocyte differentiation through activation of TNF receptor 1.[5,6] In contrast, information is limited regarding whether and how TNF-α affects the fate of adipose tissue cells, especially preadipocytes. Recently, we found that preadipocytes were resistant to TNF-α-induced cell injury. In contrast, adipocytes were vulnerable to TNF-α-induced cell death. In this report, we investigate molecular mechanisms underlying this observation, especially focusing on differential regulation of nuclear factor-κB (NF-κB) by peroxisome

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proliferator-activated receptor-γ (PPARγ) and CCAAT/enhancer binding protein (C/EBP). The results of our current study suggest that, in the adipose tissue, TNF-α selectively deletes mature adipocytes in which activation of NF-κB is blunted by constitutively expressed PPARγ and C/EBP.

MATERIALS AND METHODS

Reagents

Insulin, 3-isobutyl-1-methylxanthine (IBMX), dexamethasone, oil red O, GW9662 and Hoechst 33258 were purchased from Sigma-Aldrich Japan (Tokyo, Japan). SC-514 was from Biomol International (Plymouth Meeting, PA, USA), troglitazone was from Cayman Chemical (Chicago, IL, USA) and MG132 was from Peptide Institute (Osaka Japan). Human recombinant TNF-α was purchased from Genzyme (Cambridge, MA, USA) and used at 10 ng/ml. Experiments were performed in the presence of 10% fetal bovine serum (FBS).

Induction of Adipocyte Differentiation

Murine 3T3-L1 preadipocytes were purchased from Health Science Research Resources Bank (Osaka, Japan) and maintained in Dulbecco's modified Eagle's medium/F-12 (Wako Pure Chemical) supplemented with 10% FBS (basal medium). For the induction of adipocyte differentiation, cells were (1) precultured in basal medium for 2 days, (2) treated with differentiation medium containing 10 μg/ml insulin, 0.25 μM dexamethasone and 500 μM IBMX (IDI medium) for 2 days (D2 adipocyte) and (3) incubated in basal medium supplemented with insulin alone for 2 days (D4 adipocyte), as we have described previously.[7] The cells were further incubated in basal medium for additional 2 days (D6 adipocyte) and subjected to analyses.

Oil Red O Staining

To quantify lipid accumulation, we fixed cells with 10% formalin in PBS for 10 min, rinsed with 60% isopropanol and stained by oil red O in 60% isopropanol for 20 min. After the staining, cells were rinsed several times with 60% isopropanol and subjected to microscopic analysis.[7] To evaluate the amount of lipid quantitatively, we added cells with isopropanol containing 4% Nonidet P-40, lysed by agitation for 5 min and subjected to spectrophotometric analysis (absorbance: 520 nm wavelength).

Hoechst Staining

Cells fixed in 4% formaldehyde were stained by Hoechst 33258 (10 μg/ml) for 2 h. Apoptosis was identified using morphological criteria including shrinkage of the cytoplasm and nuclear condensation, as described before.[8] Fluorescence microscopic analysis was performed using IX71 microscope (Olympus, Tokyo, Japan).

Single-Cell Chasing

Preadipocytes and adipocytes were treated with trypsin or type I/IV collagenase cocktail (0.5 mg/ml type I collagenase and 0.5 mg/ml type IV collagenase), dispersed and seeded into 35 mm tissue culture plates at a low density. After incubation for 24 h, completely isolated single cell was individually marked with a circle using a marker pen and treated with or without TNF-α for up to 6 days. Microscopic analysis was performed every 2 days to determine the number of survivor cells or the number of survivor (+) circles.

Northern Blot Analysis

Northern blot analysis was performed as described before.[7,9] cDNAs for adiponectin,[10] PPARγ (purchased from Addgene, Cambridge, MA, USA),[11] C/EBPα, C/EBPβ and C/EBPδ[12] (provided by Dr Ez-Zoubir Amri, CNRS, Nice, France) were used for preparation of radiolabeled probes. The level of 28S rRNA was used as a loading control.

Western Blot Analysis

Western blot analysis was performed as described before.[13] Primary antibodies used were anti-IκBα antibody (1:200 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-IκBβ antibody (1:200 dilution; Santa Cruz Biotechnology) and anti-caspase-3 antibody (1:1000 dilution; Cell Signaling Technology, Beverly, MA, USA). As a loading control, levels of β-actin were evaluated using anti-β-actin antibody (1:6000 dilution; Sigma-Aldrich Japan).

Transient Transfection and Reporter Assays

Using GeneJuice Transfection Reagent (Novagen, Madison, WI, USA), preadipocytes were transiently transfected with pNF-κB-Luc (Panomics, Fremont, CA, USA), pPPRE(A)-Luc (provided by Dr Shunji Ishihara, Shimane University School of Medicine, Shimane, Japan)[14] or pC/EBP-Luc (provided by Dr Yoshihiko Nishio, Shiga University of Medical Science, Shiga, Japan)[15] that introduces a luciferase gene under the control of the κB site, the peroxisome proliferator responsive element (PPRE), or the C/EBP binding site. The cells were then treated with TNF-α or IDI for 8–12 h or 48 h. In some experiments, cells were transiently transfected with the reporter plasmids together with pCMV-C/EBPα, pCMV-C/EBPβ, pCMV-C/EBPδ,[12] C/EBPα siRNA (pBSKU6-C/EBPα-siRNA-2; provided by Dr Jianhua Shao, University of Kentucky, Kentucky)[16] or C/EBPβ siRNA (pRNA-U6.1-siC/EBPβ-1).[17] After the stimulation, cells were subjected to luciferase assay using Luciferase Assay System (Promega, Madison, WI, USA).

Statistical Analysis

Reporter assays and quantitative analysis of lipid content were performed in quadruplicate. Data were expressed as mean ± s.e. Statistical analysis was performed using the non-parametric Mann–Whitney *U*-test to compare data in different groups. *P*-value <0.05 was considered to indicate a statistically significant difference.

RESULTS

Reduction in the Number of Adipocytes, but Not Preadipocytes, by TNF-α through Induction of Apoptosis

TNF-α is considered as a key regulator of adipose tissue mass. We examined whether TNF-α affects generation and maintenance of adipocytes, using an *in vitro* model of adipogenesis. When 3T3-L1 preadipocytes were treated with IDI medium, lipid-laden adipocytes appeared at day 4, and mature adipocytes were generated within 10 days (Figure 1a). However, when the differentiation was induced in the presence of TNF-α during the initial 4 days, the number of adipocytes decreased. This effect was dependent on the concentration of TNF-α, and dramatic reduction in the number of adipocytes was observed at 10 ng/ml (Figure 1b). Oil red O staining confirmed absence of lipid-laden adipocytes by the treatment with TNF-α (Figures 1c and d). Because morphological feature of preadipocytes was not affected by TNF-α (Figure 1b), the suppressive effect was not due to cytotoxicity of TNF-α on preadipocytes.

There are several possibilities that explain the lack of lipid-laden adipocytes in TNF-α-treated cultures. Those are (1) enhanced lipolysis and/or reduced lipogenesis, (2) suppression of adipocyte differentiation and (3) selective deletion of adipocytes after differentiation. Indeed, previous reports showed that TNF-α has the potential to modulate lipogenesis and lipolysis in adipocytes.[2] It is also known that TNF-α inhibits adipogenesis at picomolar concentrations.[5,6] Previous studies showed induction of apoptosis by TNF-α in human and rat adipocytes in primary cultures.[18,19] To examine the proapoptotic potential of TNF-α in 3T3-L1 adipocytes, we treated differentiated adipocytes (D4 adipocytes) with TNF-α for 48 h and subjected to phase-contrast microscopy and oil red O staining. Even after completion of the differentiation process, treatment with TNF-α markedly reduced the number of lipid-laden, mature adipocytes (Figure 1e). In contrast, preadipocytes without lipids rather increased in the presence of TNF-α. This result suggested selective deletion of adipocytes by TNF-α. To confirm this possibility, we treated preadipocytes and adipocytes (D4 adipocytes) with TNF-α for 48 h, and performed Hoechst staining to evaluate apoptotic cell death. Phase-contrast microscopy showed that, when preadipocytes were treated with TNF-α, morphological change of the cells was not detectable (Figure 1f, top row). Hoechst staining showed that nuclei of the cells were preserved without condensation (Figure 1f, bottom row). In contrast, when adipocytes were treated with TNF-α, substantial cellular damage was observed (Figure 1g, top row). Scattering of lipid droplets outside adipocytes was occasionally observed (Supplementary Figure 1). Hoechst staining showed that a number of cells showed nuclear condensation typical of apoptosis (Figure 1g, bottom row). Consistent with this result, western blot analysis revealed that TNF-α caused substantial activation of caspase-3 in adipocytes, but not in preadipocytes (Figure 1h).

To further confirm that adipocytes, but not preadipocytes, are susceptible to TNF-α-induced apoptosis, we performed 'single-cell chasing'. For this purpose, D4 adipocytes (Figure 2a) and D12 adipocytes (Figure 2b) were dispersed and seeded at a low density. After attachment of the cells, isolated single cells were individually marked by circles (Supplementary Figure 2a) and treated with or without TNF-α for up to 6 days. Microscopic analysis revealed that in the absence of TNF-α, the majority of adipocytes survived and remained on the plate with increasing lipid content (Figures 2a and b, top). Proliferation and migration of adipocytes were not observed. Percentages of survivors were 67% in D4 adipocytes and 86% in D12 adipocytes (Figures 2a and b, graphs). In contrast, in the presence of TNF-α, the number of survivor cells (remaining cells on the plate) decreased during the course of culture (Figures 2a and b, bottom). Apoptotic cells with shrinkage of the cytoplasm were often observed. After 6 days, all adipocytes disappeared from the plate (Figures 2a and b, graphs).

We performed the similar experiment using preadipocytes. In contrast to adipocytes, the number of preadipocytes (+) circles did not decrease during incubation even in the presence of TNF-α (Figure 2c, graph). The cells proliferated actively, and the number of preadipocytes within individual circles rather increased even in the presence of TNF-α (Figure 2c, photograph; Supplementary Figure 2b). These results provided additional evidence that adipocytes, but not preadipocytes, are selectively deleted by TNF-α.

Blunted Activation of NF-κB and Consequent Apoptosis in TNF-α-Exposed Adipocytes

The sensitivity of mammalian cells to TNF-α-induced apoptosis varies from cell type to cell type, but most cells become sensitive to TNF-α on suppression of the NF-κB pathway. In this concept, the death-inducing capability of TNF-α is masked by concomitant activation of NF-κB.[20] We speculated that activity of NF-κB could be different between preadipocytes and adipocytes under TNF-α-stimulated conditions. IκBα and IκBβ are degraded on stimulation with TNF-α and regarded as endogenous indicators for NF-κB activation. We examined kinetics of the levels of IκBα and IκBβ proteins in TNF-α-stimulated preadipocytes and adipocytes. As shown in Figure 3a, rapid degradation of IκBs was observed in preadipocytes following stimulation with TNF-α (left). The degradation was observed within 0.5 h and partially recovered after 1 h in IκBα. In contrast, in TNF-α-stimulated adipocytes, degradation of IκBα was only modest, and the level of IκBβ was not affected by TNF-α (right). These results suggested that activation of NF-κB by TNF-α was blunted in adipocytes, but not in preadipocytes.

To confirm that the different activity of NF-κB is the determinant for the different susceptibility to apoptosis, we stimulated preadipocytes with TNF-α in the absence or presence of SC-514, a selective inhibitor of IκB kinase-2/NF-κB,[21] and performed microscopic analyses. Like adipocytes, TNF-α-treated preadipocytes showed dramatic cell damage only under the suppression of NF-κB (Figure 3b, left). The

cells showed shrinkage of the cytoplasm, membrane blebbing (left, high-power field (HPF)) and nuclear condensation (right, HPF) typical of apoptosis. Quantitative analysis showed that TNF-α induced apoptosis of preadipocytes only in the presence of SC-514 (45.7 ± 3.1 *vs* $1.8 \pm 1.0\%$ in SC-514-untreated cells; Figure 3c). Of note, SC-514 alone did

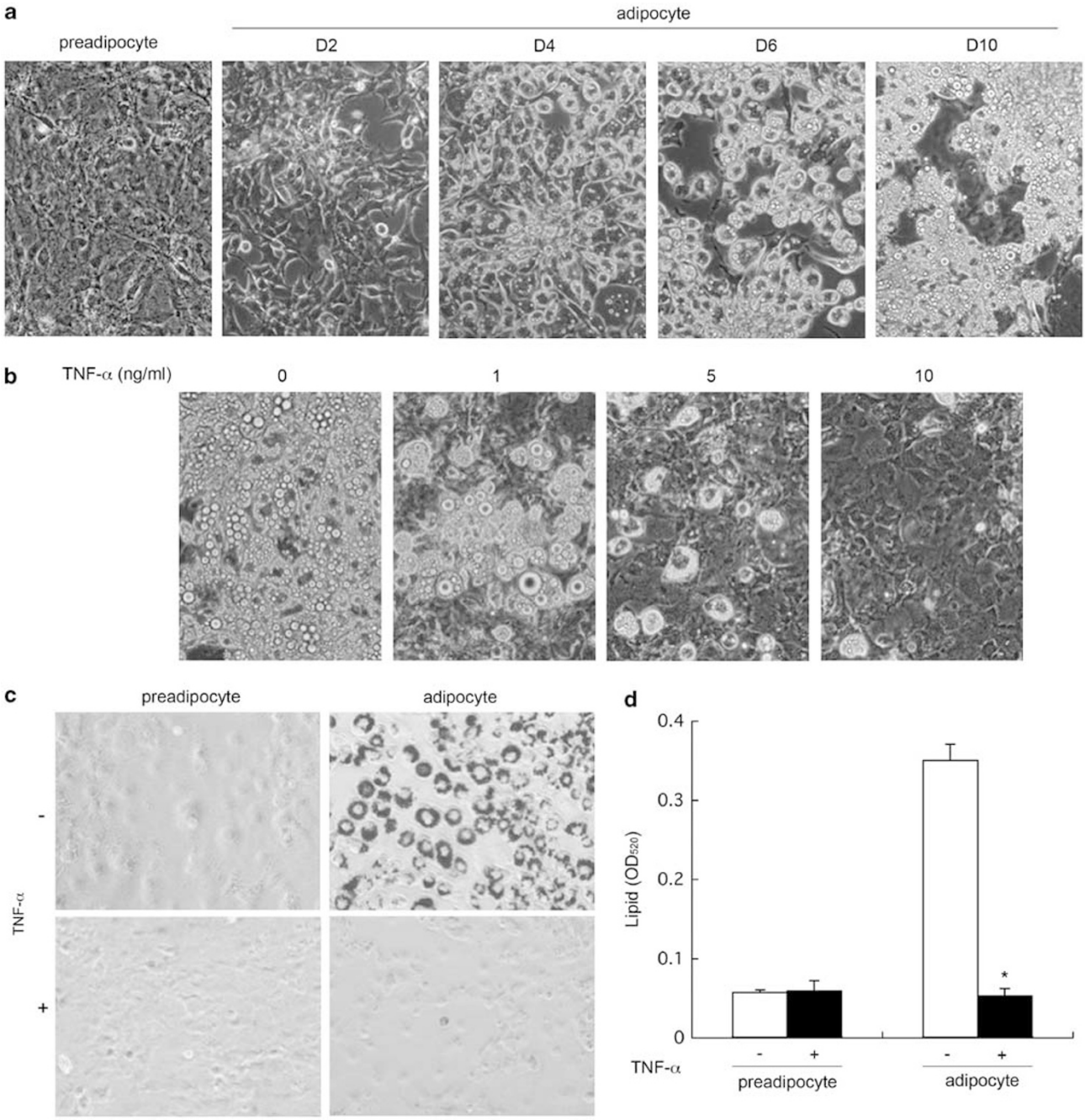

Figure 1 Reduction in the number of adipocytes under TNF-α-stimulated conditions. (**a**) 3T3-L1 preadipocytes were differentiated into adipocytes by IDI (insulin, dexamethasone and IBMX) and microscopic analysis was performed. D2, D4, D6 and D10 indicate adipocytes 2, 4, 6 and 10 days after the initiation of IDI treatment. (**b**) Preadipocytes were exposed to serial concentrations of TNF-α during differentiation in IDI medium (2 days) and insulin medium (2 days). Microscopic analysis was performed after additional incubation in basal medium for 5 days (D9). (**c**, **d**) Preadipocytes were treated without (preadipocyte) or with IDI (adipocyte) in the absence (−) or presence (+) of 10 ng/ml TNF-α for 2 days, and oil red O staining was performed after 5 days. (**c**) Light microscopy; (**d**) quantitative analysis of lipid content. In (**d**), assays were performed in quadruplicate, and data are presented as mean ± s.e. An asterisk indicates a statistically significant difference ($P < 0.05$). (**e**) D4 adipocytes were treated with or without TNF-α for 2 days and subjected to phase-contrast microscopy (top) and oil red O staining (bottom). (**f**, **g**) Preadipocytes (**f**) and D4 adipocytes (**g**) were treated with or without TNF-α for 2 days and subjected to phase-contrast microscopy (top) and Hoechst staining (bottom). Arrows point to adipocytes with nuclear condensation. (**h**) Preadipocytes and D6 adipocytes were treated with or without TNF-α for 24 h and subjected to western blot analysis of caspase-3. The level of β-actin is shown at the bottom as a loading control.

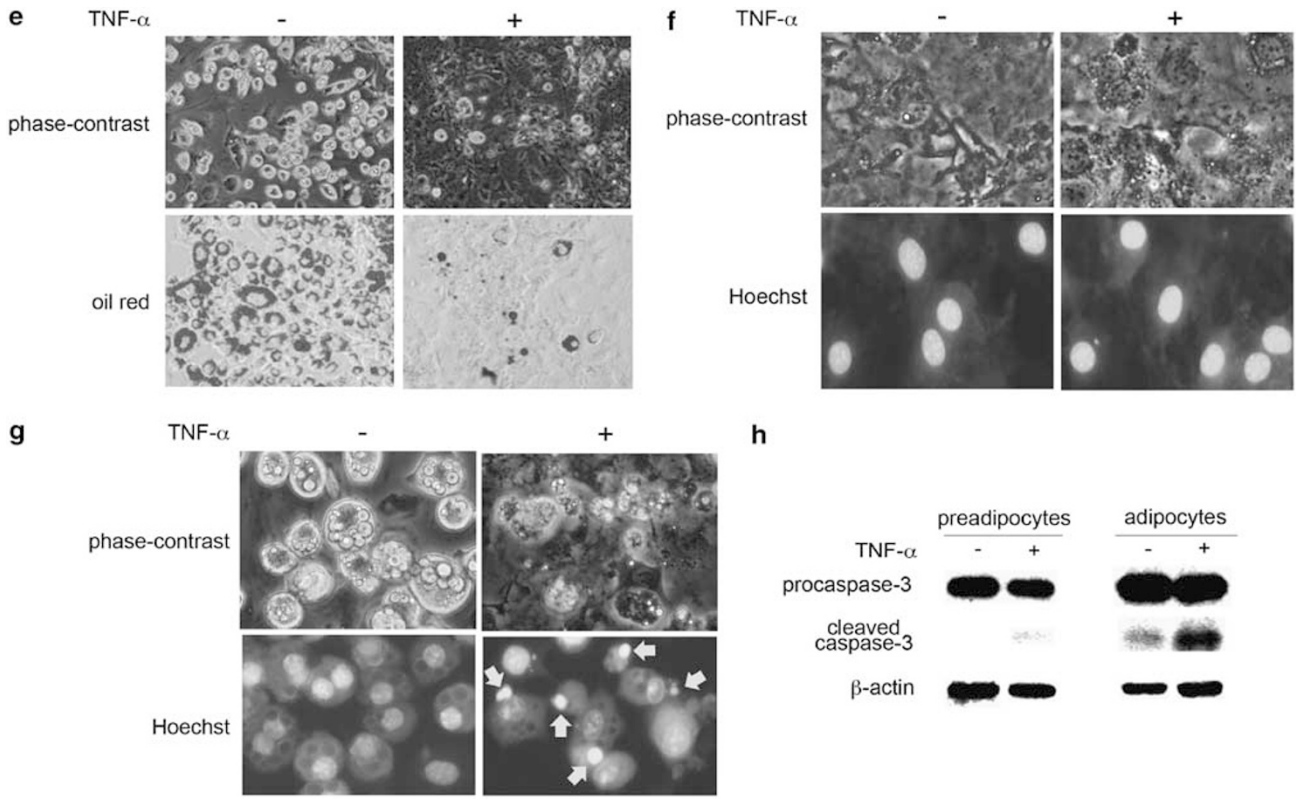

Figure 1 Continued.

not induce apoptosis. Similar results were also obtained in the presence of another inhibitor of NF-κB, MG132 (Supplementary Figure 3). These results confirmed that adipocytes, but not preadipocytes, are susceptible to TNF-α-induced apoptosis because of blunted activation of NF-κB.

Involvement of PPARγ in the Suppression of NF-κB

It is known that, during differentiation of preadipocytes, several adipocyte-specific genes are induced. Those include adiponectin and PPARγ.[22,23] A previous study indicated that, in activated macrophages, NF-κB bound to PPARγ and formed transcriptionally inactive complexes, leading to inhibition of NF-κB-promoted gene expression.[24] To examine involvement of PPARγ in the suppression of NF-κB in adipocytes, we first confirmed induction of *PPARγ* in 3T3-L1 cells during differentiation. Northern blot analysis revealed that, like adiponectin, expression of *PPARγ* was low in preadipocytes but was substantially induced in adipocytes (Figure 4a). The induced PPARγ was functional, because reporter assay showed that induction of differentiation by IDI significantly induced PPRE-mediated transcription (Figure 4b). To examine whether induction of PPARγ is causative of suppression of NF-κB in adipocytes, we transiently transfected preadipocytes with pNF-κB-Luc and induced differentiation by IDI (2 days). The adipocytes were then treated with or without TNF-α in the presence of troglitazone (PPARγ agonist) or GW9662 (PPARγ antagonist) and subjected to luciferase assay. In the absence of TNF-α, both troglitazone and GW9662 did not significantly affect NF-κB activity (Figure 4c, left). However, under the stimulation with TNF-α, treatment with troglitazone attenuated NF-κB activation, and treatment with GW9662 enhanced activation of NF-κB (Figure 4c, right).

To examine whether activation of PPARγ is involved in the vulnerability of adipocytes to TNF-α-induced apoptosis, we performed single-cell chasing in the presence of GW9662. When D6 adipocytes were treated with TNF-α, the number of survivor cells decreased in a time-dependent manner. However, in the presence of GW9662, the proapoptotic effect of TNF-α was markedly diminished (Figure 4d). Consistent with this result, when preadipocytes were stimulated with TNF-α in the presence of troglitazone, the cells became susceptible to TNF-α-induced apoptosis (Figure 4e). Treatment with troglitazone alone did not decrease the number of survivor cells. These results suggested that activation of PPARγ is responsible for the sensitiveness of adipocytes to TNF-α-induced apoptosis through suppression of NF-κB.

Involvement of C/EBP in the Suppression of NF-κB

As described, several adipocyte-specific genes are induced during differentiation of preadipocytes. In addition to PPARγ, C/EBPα is also induced during adipogenesis.[25] A previous report indicated that C/EBPβ may inhibit activation of NF-κB in monocytic cells.[26] C/EBP family members

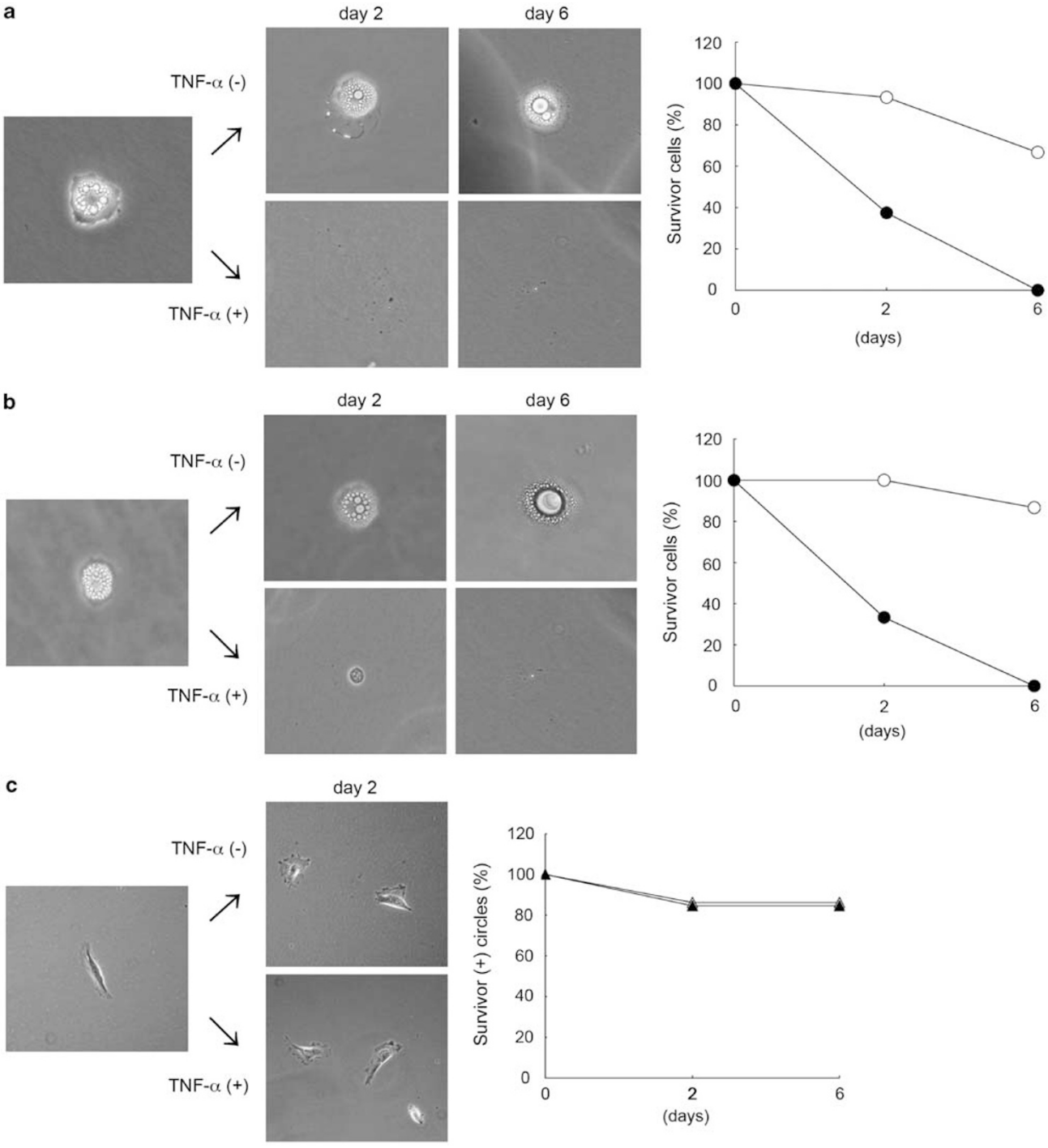

Figure 2 Selective deletion of adipocytes by TNF-α: single-cell chasing. D4 adipocytes (**a**), D12 adipocytes (**b**) and preadipocytes (**c**) were dispersed and seeded at a low density. After attachment of the cells on plastic plates, isolated single cells were individually marked with circles. The plates were then divided into two groups, either untreated or treated with TNF-α for up to 6 days and subjected to phase-contrast microscopy (left). Kinetics of percentages of survivor cells (or survivor (+) circle) is shown in the right graphs. Total 15–20 cells (circles) were examined for individual groups. Open circle/triangle, TNF-α (−); closed circle/triangle, TNF-α (+).

share substantial sequence identity in the basic region and the leucine zipper domain, form heterodimers and bind to the C/EBP recognition sequence in the promoter regions of target genes.[27] We speculated that induction of C/EBP may cause suppression of NF-κB in adipocytes. To examine this possibility, we first investigated induction of C/EBP family members, *C/EBPα*, *C/EBPβ* and *C/EBPδ*, in IDI-treated 3T3-L1 cells. As shown in Figure 5a, expression of *C/EBPα*

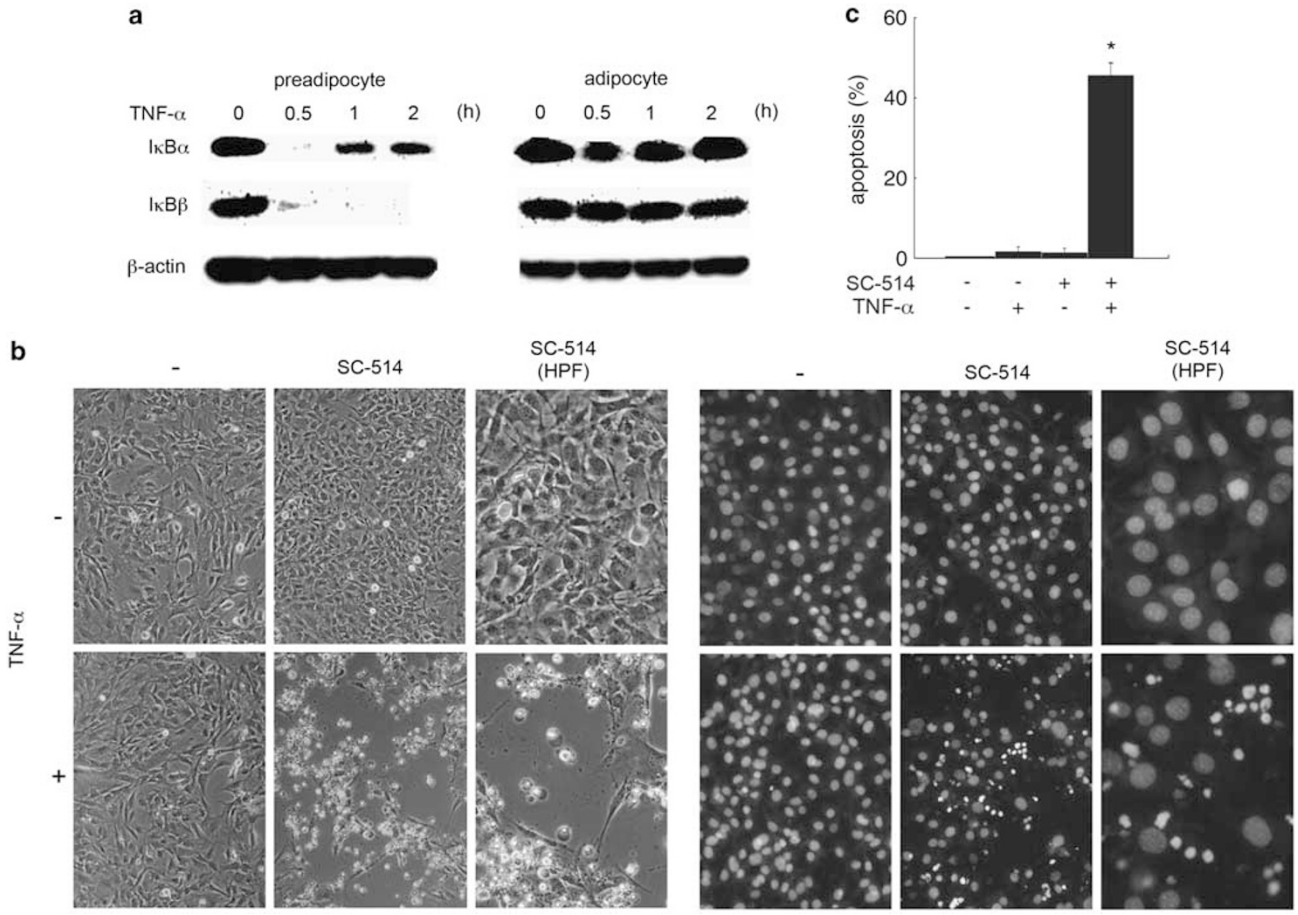

Figure 3 Blunted activation of NF-κB and consequent apoptosis in TNF-α-exposed adipocytes. (**a**) Preadipocytes and D6 adipocytes were treated with TNF-α for up to 2 h and subjected to western blot analysis of IκBα and IκBβ. The level of β-actin is shown at the bottom as a loading control. (**b**) Preadipocytes were treated with TNF-α in the absence or presence of 100 μM SC-514 and subjected to phase-contrast microscopy (left) and Hoechst 33258 staining (right). HPF, high-power field. (**c**) Quantitative analysis of the result shown in (**b**). Percentages of apoptotic cells with condensed nuclei are shown. Assays were performed in quadruplicate, and data are presented as mean ± s.e. An asterisk indicates a statistically significant difference ($P<0.05$).

was absent in preadipocytes, but basal expression of *C/EBPβ* was detectable. Basal expression of *C/EBPδ* was very low in preadipocytes. When adipogenesis was induced by IDI, expression of *C/EBPα* and *C/EBPβ*, but not *C/EBPδ*, was substantially induced. The induced C/EBPs were functional, because reporter assay showed that induction of C/EBP by IDI was associated with significant upregulation of C/EBP-mediated transcription (Figure 5b).

To examine involvement of C/EBPα and C/EBPβ in the suppression of NF-κB, we co-transfected preadipocytes with *C/EBPα* or *C/EBPβ* together with pNF-κB-Luc. After 48 h, the cells were treated with or without TNF-α and subjected to luciferase assay. As shown in Figure 5c, overexpression of C/EBPα or C/EBPβ significantly reduced activity of NF-κB in preadipocytes under basal (left) and TNF-α-stimulated (right) conditions. Similar results were also obtained in preadipocytes transfected with *C/EBPδ* (Supplementary Figure 4). These results suggested the potential of C/EBP, especially C/EBPα and C/EBPβ, to lower NF-κB activity in adipocytes. To further confirm this result, we performed loss-of-function studies using RNA interference. First, preadipocytes were transiently transfected with pC/EBPα or pC/EBPβ together with siC/EBPα or siC/EBPβ and subjected to northern blot analysis of *C/EBP* and *C/EBPβ*. As shown in Figure 5d, expression levels of *C/EBPα* and *C/EBPβ* were substantially lowered by transfection with individual siRNAs. Using these expression constructs, we further investigated the roles of C/EBPs in the regulation of NF-κB. Preadipocytes were transiently co-transfected with siC/EBPα or siC/EBPβ together with pNF-κB-Luc, treated with IDI to induce adipocyte differentiation and subjected to luciferase assay to evaluate NF-κB activity. The results showed that knockdown of C/EBPα or C/EBPβ significantly increased activity of NF-κB in adipocytes (Figure 5e, left). This suppressive effect was similarly observed under the stimulation with TNF-α (Figure 5e, right). These results suggested that C/EBPα and C/EBPβ are involved in the blunted activation of NF-κB in adipocytes.

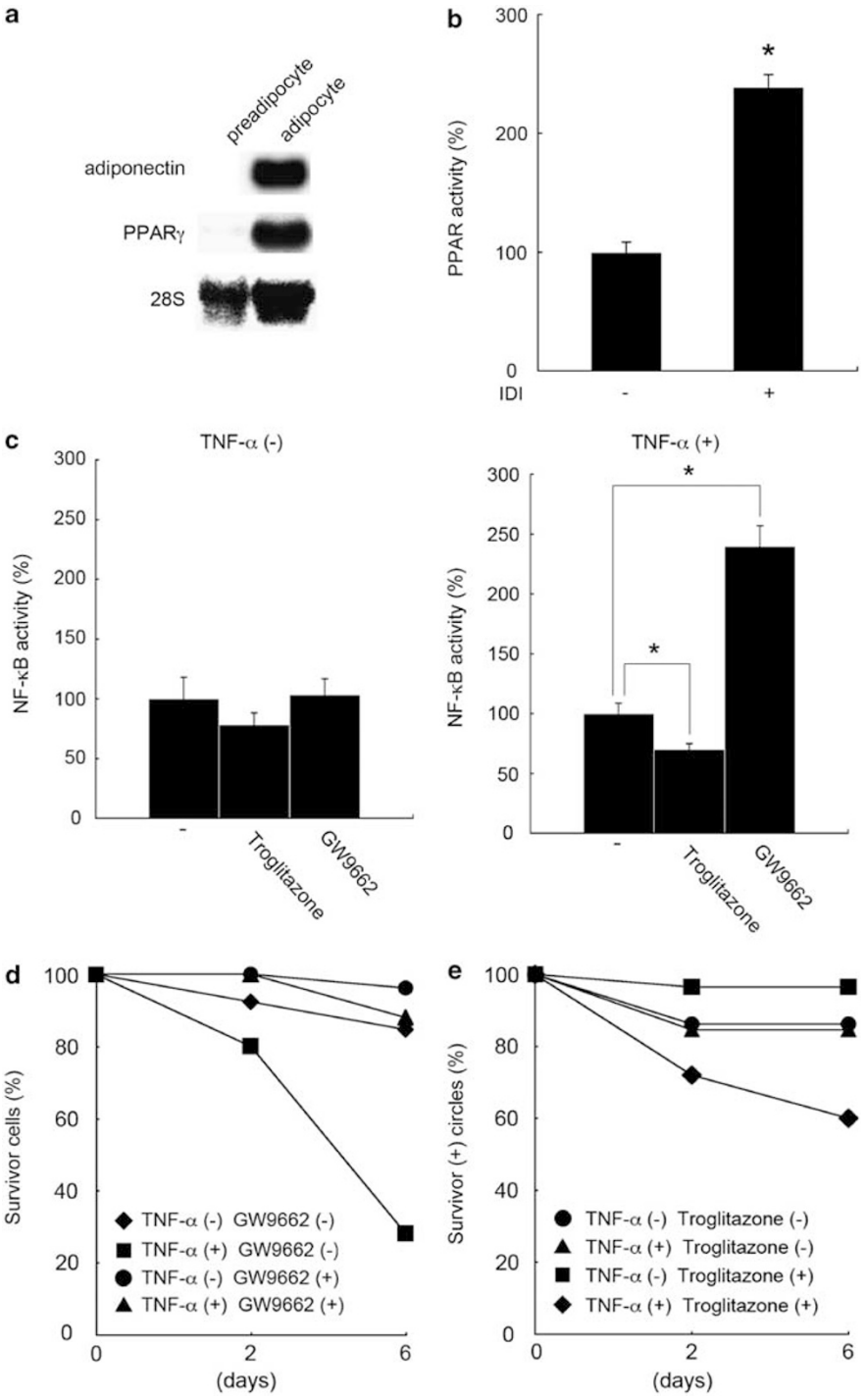

Figure 4 Involvement of PPARγ in the suppression of NF-κB in adipocytes. (**a**) Preadipocytes and D6 adipocytes were subjected to northern blot analysis of adiponectin and *PPARγ*. Expression of 28S rRNA is shown at the bottom as a loading control. (**b**) Preadipocytes were transiently transfected with pPPRE(A)-Luc, treated with or without IDI for 2 days and subjected to luciferase assay to evaluate activity of PPARγ. (**c**) Preadipocytes were transiently transfected with pNF-κB-Luc, and after 24 h, differentiation was induced by the treatment with IDI for 2 days. The adipocytes were then treated with (right) or without (left) TNF-α for 8 h in the presence of 30 μM troglitazone or 30 μM GW9662 and subjected to luciferase assay. In (**b**) and (**c**), data are presented as mean ± s.e. Asterisks indicate statistically significant differences ($P<0.05$). Assays were performed in quadruplicate. (**d**) D7 adipocytes were treated with or without TNF-α in the presence or absence of GW9662 for up to 6 days, and percentages of survivor cells were evaluated by single cell chasing. Total 25–27 cells in individual groups were examined. (**e**) Preadipocytes were treated with or without TNF-α in the presence or absence of troglitazone for up to 6 days, and percentages of survivor (+) circles were evaluated by single cell chasing. Total 25–29 circles in individual groups were examined.

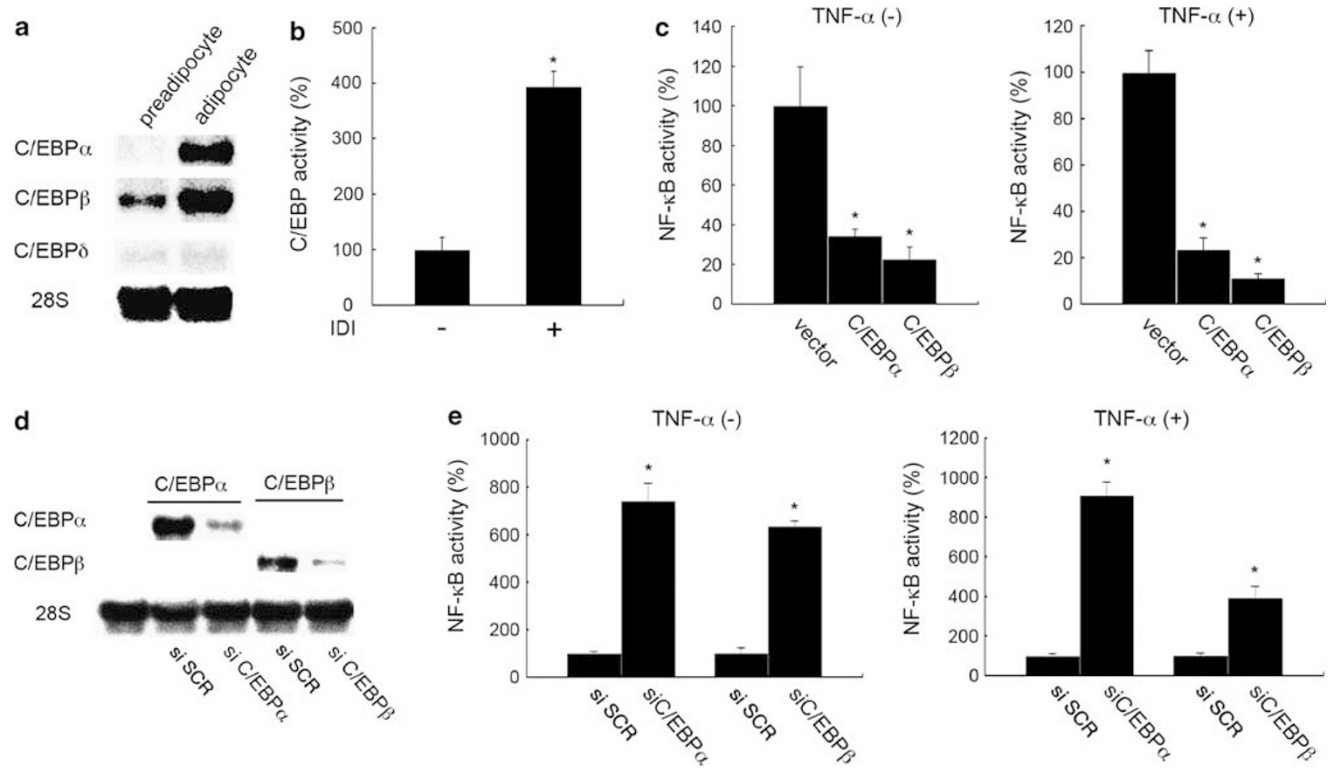

Figure 5 Involvement of C/EBP in the suppression of NF-κB. (**a**) Preadipocytes and D6 adipocytes were subjected to northern blot analysis of *C/EBPα*, *C/EBPβ* and *C/EBPδ*. (**b**) Preadipocytes were transiently transfected with pC/EBP-Luc, treated with or without IDI for 2 days and subjected to luciferase assay to evaluate activity of C/EBP. (**c**) Preadipocytes were transiently co-transfected with pNF-κB-Luc together with empty vector, pC/EBPα or pC/EBPβ. After 2 days, the cells were treated with (right) or without (left) TNF-α for 8 h and subjected to luciferase assay to evaluate activity of NF-κB. (**d**) Preadipocytes were transiently transfected with pC/EBPα or pC/EBPβ together with scrambled siRNA (siSCR), C/EBPα siRNA (siC/EBPα) or C/EBPβ siRNA (siC/EBPβ). After 24 h, the cells were collected and subjected to northern blot analysis of *C/EBPα* and *C/EBPβ*. (**e**) Preadipocytes were transiently co-transfected with pNF-κB-Luc together with siSCR, siC/EBPα or siC/EBPβ. After the treatment with IDI for 2 days, the adipocytes were treated with (right) or without (left) TNF-α for 8 h and subjected to luciferase assay. In (**b**, **c**, **e**), data are presented as mean ± s.e. Assays were performed in quadruplicate. Asterisks indicate statistically significant differences ($P<0.05$).

DISCUSSION

TNF-α is a pleiotropic cytokine that regulates a number of biological processes including immune/inflammatory function, cell differentiation, proliferation, apoptosis and energy metabolism. Previous reports indicated that high levels of circulating TNF-α are observed in cachectic conditions characterized by reduction in adipose tissue mass.[28] TNF-α affects several functions of adipocytes including glucose homeostasis, lipolysis and lipogenesis. TNF-α causes depletion of insulin-responsive glucose transporter GLUT4, suppresses lipogenesis through inhibition of lipoprotein lipase and stimulates lipolysis in cultured adipocytes.[29,30] Some reports also suggested that TNF-α inhibits adipocyte differentiation through activation of TNF receptor 1 and consequent suppression of adipogenesis-committed genes including PPARγ and C/EBPα.[5,6] These biological effects may contribute to reduction of adipose tissue mass by TNF-α. However, TNF-α-triggered loss of adipose mass may be caused not only by reduced store of lipids and inhibition of adipogenesis, but also by deletion of adipose tissue cells through apoptosis. In this report, we elucidated that TNF-α preferentially deletes adipocytes, but not preadipocytes. This selective ablation was due to blunted activation of NF-κB in adipocytes. We further disclosed that constitutively expressed PPARγ and C/EBP are responsible for inhibition of NF-κB in adipocytes. These data suggested that PPARγ and C/EBP not only control adipogenesis but also regulate the fate of adipocytes.

Prins *et al*[18] previously reported that both preadipocytes and mature adipocytes derived from human adipose tissues were susceptible to TNF-α-induced apoptosis. In contrast, in this report, we showed that murine preadipocytes, but not adipocytes, were resistant to TNF-α-induced injury. The reason for the discrepancy is currently unclear. One possibility is that 3T3-L1 preadipocytes (cell line) might have different characteristics from those of preadipocytes in primary cultures; eg, the stage of differentiation could be different between 3T3-L1 preadipocytes and preadipocytes in primary cultures. However, using rat preadipocytes in primary cultures, Qian *et al*[19] also showed that preadipocytes, but not adipocytes, were resistant to TNF-α-triggered apoptosis. We found that individual reports used different culture conditions, especially serum concentrations. Our group and Qian *et al* treated preadipocytes with TNF-α in the presence of 10% FBS, whereas Prins *et al* treated the cells under a serum-free condition.[18,19] Of note, Prins *et al*.[18] also

showed that TNF-α did not induce apoptosis of human preadipocytes under a serum-containing condition. Some survival factors in serum, therefore, may confer resistance to TNF-α on preadipocytes, and responses to such survival factors might be different between preadipocytes and adipocytes.

PPARs are ligand-activated transcription factors that belong to the nuclear receptor superfamily. PPARα is ubiquitously expressed in the liver, skeletal muscle, kidney, heart and the vascular wall. In contrast, PPARγ is predominantly expressed in the adipose tissue and macrophages. Previous reports suggested that PPARα has the potential to modulate inflammatory responses. For example, PPARα may negatively regulates expression of inflammation-associated genes by antagonizing activator protein 1, NF-κB and nuclear factor of activated T cells, as reviewed by Delerive *et al.*[31] Among members of the PPAR family, the anti-inflammatory action has been well documented in PPARα.[32,33] PPARα may physically interact with p65 through its Rel homology domain that mediates dimerization and interaction with IκB.[33] PPARα may also increase the level of nuclear IκBα, leading to reduction in NF-κB DNA-binding activity.[34] In contrast to the abundant information on the suppressive effect of PPARα on NF-κB, little is known about anti-inflammatory effects of other PPARs. In vascular smooth muscle cells and endothelial cells, previous reports showed that, in contrast to PPARα agonists, PPARγ agonists did not influence cytokine-induced activation of NF-κB.[35,36] In contrast, our present report showed the potential of PPARγ to suppress NF-κB in adipocytes. The reason for the lack of NF-κB suppression by PPARγ agonists in vascular cells is currently unclear, but may simply be due to the fact that the expression level of PPARγ is low in these cell types. Indeed, a previous study suggested that PPARγ bound NF-κB and formed transcriptionally inactive complex, leading to inhibition of NF-κB-mediated transcription in activated macrophages, another cell type that abundantly expresses PPARγ.[24] Molecular mechanisms involved in the suppression of NF-κB by PPARγ are not fully elucidated to date, but like PPARα, physical interactions of PPARγ with NF-κB subunits might be important.[24]

Like PPAR, C/EBP is a family of leucine zipper transcription factors involved in the regulation of various cell function. Previous investigations elucidated that C/EBP has the potential to interact physically or functionally with a number of different transcription factors including NF-κB.[37] All members of the NF-κB family have the Rel homology domain that mediates DNA binding, dimerization and nuclear targeting. C/EBP contains the bZIP region composed of a basic region involved in DNA binding and a leucine zipper motif involved in dimerization. Stein *et al*[37] reported that the C/EBP family members cross-coupled with the members of NF-κB family through physical association between the Rel homology domain and the bZIP region. This cross-coupling may affect activity of the κB sites and the C/EBP-binding sites.[37] A previous report indicated that C/EBPβ may inhibit activation of NF-κB in monocytic cells.[26] However, information is still limited regarding regulation of NF-κB by C/EBP members. In particular, regulation of NF-κB by C/EBPs other than C/EBPβ has not been elucidated. In 3T3-L1 cells, C/EBPα and C/EBPβ were preferentially induced during adipogenesis. This result led us to examine the role of C/EBPs in the regulation of NF-κB and found that C/EBPα as well as C/EBPβ is crucial in the suppression of NF-κB in adipocytes. Molecular mechanisms underlying this phenomenon are currently unclear, but like C/EBPβ in macrophages, direct, physical interactions of C/EBPs with NF-κB subunits may be involved in their inhibitory actions.[26]

In this report, we show both PPARγ and C/EBPs control the fate of adipocytes by suppression of NF-κB. Currently, it is undetermined whether both factors regulate activity of NF-κB independently or cooperate in a single pathway. It is known that, during adipogenesis, C/EBP functions as an inducer of PPARγ.[38] Rosen and colleagues[39,40] reported that PPARγ was able to promote adipogenesis even in the absence of C/EBPα, whereas C/EBPα could not promote adipogenesis in the absence of PPARγ. These results suggest that C/EBP and PPARγ participate in a single pathway of adipogenesis with PPARγ being the proximal effector. On the basis of current knowledge, it is reasonable to speculate that induction of PPARγ is an event downstream of C/EBP, and the C/EBP–PPARγ pathway possibly contributes to the suppression of NF-κB in adipocytes.

In this study, we elucidated the potential of TNF-α for selective deletion of adipocytes in the adipose tissue. The results of our current study, together with previous reports,[2,5,6] show that TNF-α reduces adipose tissue mass through multiple mechanisms including inhibition of lipogenesis, facilitation of lipolysis, suppression of adipogenesis and selective induction of adipocyte apoptosis.

Supplementary Information accompanies the paper on the Laboratory Investigation website (http://www.laboratoryinvestigation.org)

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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