FEBS 25286 FEBS Letters 506 (2001) 97–102

Signaling pathways utilized by tumor necrosis factor receptor 1 in adipocytes to suppress differentiation

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Received 27 August 2001; revised 5 September 2001; accepted 5 September 2001

First published online 14 September 2001

Edited by Guido Tettamanti

Abstract Tumor necrosis factor-α (TNFα) has profound effects on cultured adipocytes, one of which is the inhibition of terminal differentiation. Previous studies in TNF receptor (TNFR)-deficient preadipocytes have demonstrated that the anti-adipogenic effect of both secreted and transmembrane TNFα is mediated solely by TNFR1. In this study, we performed a structure–function analysis of the intracellular domains of TNFR1 and investigated the signaling pathway(s) involved in TNFR1-mediated inhibition of adipocyte differentiation. Our results show that repression of adipogenesis required the juxtamembrane and death domains and was independent of the pathways involving nuclear factor κB and neutral sphingomyelinase. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Adipocyte differentiation; Obesity; Signal transduction; Gene expression

1. Introduction

The multiple biological functions of tumor necrosis factor-α (TNFα) are mediated by two distinct TNF receptors, TNFR1 and TNFR2. These two receptors have no homology in the intracellular domains but exhibit a low degree of similarity in the ligand binding regions located in the extracellular domains, indicating that they are capable of inducing distinct cellular responses [1]. So far, the majority of TNFα activities have been attributed to TNFR1 though it has been reported that TNFR2 can also mediate many cellular responses [1,2]. In adipocytes both TNF receptors are active and signal for a variety of functions but TNFα-induced inhibition of adipogenesis is mediated only by TNFR1. However, no structure–function analysis of this receptor has been reported in adipocytes.

Signaling pathways utilized by each TNFR have been extensively studied in other cell types. No intrinsic catalytic activity has been observed for either TNFR and they signal through recruiting cytosolic factors upon ligand-induced oligomerization [3,4]. Several functional domains have been revealed in TNFR1 through structure–function analyses. The death domain, a 80 amino acid region located to the C-terminus of TNFR1, signals apoptosis, activation of nuclear factor (NF)-κB and c-Jun N-terminal kinase. All the above functions are fulfilled through the interaction between the death domain and TRADD (TNFR1-associated death domain protein) [5]. Several downstream effectors of TRADD have been identified, including FADD (Fas-associated death domain protein), TRAF2 (TNFR-associated factor 2) and RIP (receptor interaction protein). The direct interaction between TRADD and FADD [4,6] is responsible for signaling apoptosis by sequentially activating caspase 8 (Flice/MACH/Mch5) [7] and caspase 10 (Flice2/Mch4). The interactions between TRADD and TRAF2 as well as RIP are essential in TNFα-mediated NF-κB activation [4,8]. Recently, a regulatory factor of death domain-containing receptors was identified and named silencer of death domains (SODD) [9]. This provides a negative feedback mechanism of controlling death domain protein signaling.

In addition to the death domain, two other regions of TNFR1 have also been found to physically recruit other factors: the NSD motif (neutral sphingomyelinase (N-SMase) activating domain) and the juxtamembrane domain (JM). The NSD motif is a region containing nine amino acid residues located immediately proximal to the death domain. It interacts with a WD-repeat protein, FAN (factor associated with N-SMase activation) [10], and activates membrane N-Smase, which produces the lipid signaling molecule ceramide through hydrolyzing plasma membrane sphingomyelin. The juxtamembrane region of TNFR1 contains about 100 amino acids and interacts with an isoform of phosphatidylinositol-4-phosphate-5-kinase (PIP_5K), which produces phosphatidylinositol 4,5-bisphosphate [11], a precursor of the important second messengers phosphatidyl 1,4,5-triphosphate and 1,2-diacylglycerol.

The intracellular domain (ID) of TNFR1 has also been found to interact with a number of other molecules, such as Grb2, TNFR-associated kinase, mitogen-activated protein (MAP) kinase activating death domain protein and TNFR-associated protein [12–15], which adds more information to a comprehensive understanding of TNFR1 signaling pathways.

Here, we examine the TNFR1 functional domains that are responsible for mediating the anti-adipogenic effects of transmembrane TNFα (mTNF) and demonstrate that several pathways are involved in this TNFα-mediated cellular response.

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Abbreviations: TNFR, tumor necrosis factor receptor; ID, intracellular domain; JM, juxtamembrane domain; NSM, neutral sphingomyelinase domain; ED, extracellular domain; TM, transmembrane domain; mTNF, transmembrane TNFα; sTNF, soluble TNFα; PIP_5K, phosphatidylinositol-4-phosphate-5-kinase; TRADD, TNFR1-associated death domain protein; FADD, Fas-associated death domain protein; TRAF2, TNFR-associated factor 2; RIP, receptor interaction protein

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PII: S0014-5793(01)02889-7

2. Materials and methods

2.1. Cells and reagents

TNFR1$^{-/-}$R2$^{-/-}$ cell lines were established as described previously [16] and tested for their capacity to differentiate into adipocytes. The best differentiating line only yielded $<80\%$ adipocytes and was further improved by subcloning. Among the 50 clones tested for adipogenic potential, a clone which yielded almost 100% adipocytes was identified and used for all the experiments. The absence of endogenous TNFRs was confirmed by genotyping and Northern blot analysis. Recombinant murine soluble TNFα (sTNF) was purchased from Genzyme (Cambridge, MA, USA). Hygromycin B and puromycin were from Sigma (St. Louis, MO, USA).

2.2. Vector construction

Cloning of the mTNFΔ1–9K11E and TNFR1 cDNAs into retroviral vectors containing hygromycin B and puromycin resistance genes, respectively, has been described in our previous study [17]. Mutagenesis of TNFR1 cDNA was performed in pBluescript vector. Two of the deletion mutants, TNFR1ΔID and TNFR1ΔDD, were produced by introducing stop codons at residues 234 (ATG to TAG) and 354 (GCA to TGA), respectively, using the Chameleon double-stranded, site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). The primers used were 5′-CCGGGGATATCGGCACTA-TAAACTGATGAAG and 5′-CAGAATCGCAAGGTCTCAATTG-TCAGGACGTTGC. The other three deletion mutants, TNFR1ΔJM, TNFR1ΔNSM and TNFR1ΔNSMΔDD, and the hybrid mutant TNFR2/R1 were generated through ligation of PCR fragments. To delete the JM, the extracellular (ED) and transmembrane portions (TM) of TNFR1 were amplified by PCR (5′ primer CCCGGGATGGGTCTCCCCACCGTGCCTGGC and 3′ primer GGTACCTAAACTGATGAAGATAAAGGATAGAAGGC) and ligated to the rest of the intracellular domain (without JM, amplified by 5′ primer GGTACCACCTCTGTTCAGAAATGGGAAGACTCC-GCC and 3′ primer TTATCGCGGGAGGCGGGTCGTGGAC), which introduced a *Kpn*I site. To delete the NSM, the upstream region of NSM was amplified by PCR (5′ primer CCCGGGATGGGTCT-CCCCACCGTGCCTGGC and 3′ primer GGTACCTGGCACG-GAGCAGAGTGATTCG) and ligated to the death domain (amplified by 5′ primer GGTACCGACAATGCAGACCTTGCG and 3′ primer TTATCGCGGGAGGCGGGTCGTGGAC), which also introduced a *Kpn*I site. The TNFR1ΔNSMΔDD mutant was created by amplifying the cDNA encoding ED+TM+JM, with a stop codon introduced at the end of JM (5′ primer GGTACCGACAATGCA-GACCTTGCG and 3′ primer GGGTTATGGCACGGAGCA-GAGTGATTCG). Finally, the hybrid mutant TNFR2/R1 was created by ligating the PCR fragments of the TNFR2 extracellular domain (amplified by the use of 5′ primer CCCGGGATGGC-GCCCGCCGCCCTCTGGGTC and 3′ primer GGTACCTTGTT-CAATAATGGGGGTTGAACCCAACG) to TNFR1 TM and ID (amplified by 5′ primer GGTACCGCGGTGCTGTTGCCCCTGGT-TATCTTG and 3′ primer TTATCGCGGGAGGCGGGTCGTG-GAC), which also introduced a *Kpn*I site. All PCR products were confirmed by sequencing and the final TNFR1 mutants were cloned into a retroviral vector containing the puromycin resistance gene for expression in the pre-adipocytes.

2.3. Transfection and infection

TNFR1 mutants were first transiently transfected into Bosc 23 cells with the Cell Phect calcium phosphate coprecipitation kit (Amersham Pharmacia Biotech) to obtain infectious viral particles [18]. Forty-eight hours after transfection, supernatants containing viral particles were collected, filtered and incubated with recipient cells, as described previously. In this study, mTNFΔ1–9K11E expressing TNFR1$^{-/-}$R2$^{-/-}$ cells were used as recipient cells to achieve coexpression of mTNF and TNFR1 or one of the TNFR1 mutants. Puromycin was used at a concentration of 5 μg/ml for initial 7-day selection and then stable cell lines were maintained in the presence of both puromycin (4 μg/ml) and hygromycin B (200 μg/ml) for all the experiments.

2.4. Total RNA preparation and Northern blotting

The guanidinium thiocyanate method was used to extract all RNA samples [19]. Denatured RNAs were loaded on 1% agarose gel containing 3% formaldehyde. Biotran membrane (ICN) was used for transferring RNA after electrophoresis. After UV cross-linking and baking membranes at 80°C for 1 h, [α-^{32}P]dCTP- (NEN Life Science Products, Boston, MA, USA) labeled cDNA probes were added to hybridization buffer for overnight incubation. After proper washings, blots were exposed to X-omat films and signals were quantitated by the use of NIH image software with 18S rRNA as loading adjustment.

2.5. NF-κB activation

TNFR1$^{-/-}$R2$^{-/-}$ cells expressing TNFR1 mutants or control vectors for TNFRs or mTNF were cotransfected with a NF-κB promoter-driven luciferase reporter gene (provided by Dr. Christopher K. Glass, University of California, San Diego, CA, USA) using the lipofectamine-plus kit (Gibco-BRL Life Science, Grand Island, NY, USA). sTNF was added to the medium at a concentration of 10 ng/ml for 3 or 10 h before harvest which was done 48 h after transfection. The luciferase activity was determined by a luminometer and corrected for transfection efficiency as assessed by β-galactosidase assays.

2.6. Cell differentiation analysis

TNFR1$^{-/-}$R2$^{-/-}$ preadipocytes were grown in Dulbecco's modified Eagle's medium (DMEM, Life Technologies) supplemented with 10% bovine calf serum (Hyclone, Logan, UT, USA). Transfected cells were grown in the same medium in the presence of 200 μg/ml hygromycin B (Sigma) and 4 μg/ml puromycin (Sigma). For differentiation, cells were seeded at 1.0×10^6 on 10-cm petri dishes, grown to 80% confluence in DMEM supplemented with 10% cosmic calf serum (Hyclone) and induced by adipogenic reagents for 4 days (5 μg/ml insulin, 1 μM dexamethasone, 0.5 mM isobutylmethylxanthine and 1 μM BRL49653). Then, cells were kept in medium containing insulin until the vector-infected controls fully differentiated. Adipocyte differentiation was evaluated by oil red O staining, microscopy and mRNA expression of adipogenic markers.

3. Results

3.1. Expression of TNFR1 mutants in TNFR1$^{-/-}$R2$^{-/-}$ preadipocyte cell lines and NF-κB activation

Our previous studies have demonstrated that both sTNF and mTNF could inhibit adipocyte differentiation. TNFR1 alone is sufficient to mediate this anti-adipogenic effect and no potentiation by TNFR2 was observed [17]. To further dissect the signaling pathway(s) utilized by TNFR1 in this action, TNFR1 mutants lacking several functional domains were generated (Fig. 1A). These included the JM, the DD and the NSM. A deletion mutant without the ID was used as a positive control for differentiation analysis. To determine whether the use of TNFR1 versus TNFR2 is due to selective binding of TNFα to TNFR1 or specific to TNFR1 signaling, a hybrid mutant with the TNFR2 extracellular domain and TNFR1 transmembrane as well as intracellular domains was created. All the above mutants were expressed in TNFR1$^{-/-}$R2$^{-/-}$ cells using retroviral vectors, which was confirmed by examining mRNA (Fig. 1B) and protein levels (Fig. 1C). We noticed that mutants without the DD tended to have significantly higher levels of expression with the exception of TNFR1ΔNSM (Fig. 1B, lanes 2, 5 and 7). The membrane-bound form of TNFα was stably expressed in these

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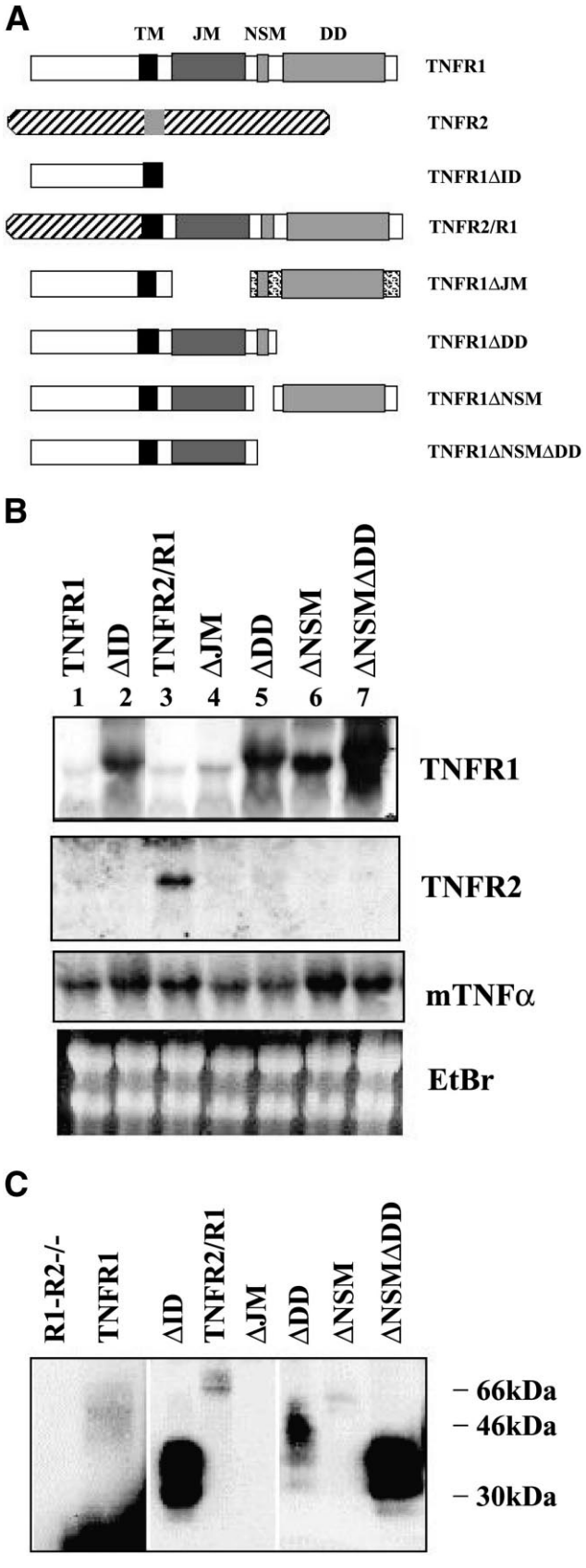
Fig. 1. Mutagenesis of TNFR1. A: Schematic design of TNFR1 mutants. B: mRNA levels of TNFR1 mutants. Top panel is probed with the full-length cDNA for mouse TNFR1. Middle panel is probed with the cDNA of mouse TNFR2 extracellular domain. The expression of membrane-bound TNF is also shown (mTNF). C: Protein levels of TNFR1 mutants. Protein levels are determined by immunoblotting cellular extracts with a monoclonal antibody against mouse TNFα.

cells lines and did not exhibit significant differences in its expression levels.

NF-κB activation was examined to confirm that mutants with the DD are functional despite being expressed at relatively low levels by determining the activity of a NF-κB promoter-driven luciferase reporter gene construct. As shown in Fig. 2, the luciferase reporter gene was activated in TNFR1$^{-/-}$R2$^{-/-}$ cells expressing wild-type TNFR1, TNFR1ΔJM and TNFR1ΔNSM upon TNFα stimulation. The TNFR2/R1 hybrid did not show any function in NF-κB activation though it contains the DD. This is likely to be related to the fact that TNFR2 only activates NF-κB in certain cell types.



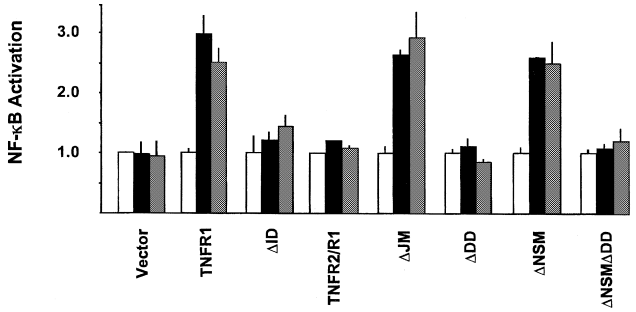


Fig. 2. NF-κB activation by TNFR1 mutants. Open bars, no TNFα treatment; filled bars, treatment with 10 ng/ml recombinant TNFα for 3 h; hatched bars, treatment with 10 ng/ml soluble TNFα for 10 h. Vec, vector-transfected control cells. The results represent mean ± S.E.M. of data obtained from three independent experiments.

3.2. *Differentiation analysis of TNFR1 mutant-expressing TNFR1$^{-/-}$R2$^{-/-}$ cell*

TNFR1$^{-/-}$R2$^{-/-}$ cells simultaneously expressing each of the above mutants and mTNFΔ1–9K11E were induced for differentiation to determine the domains responsible for the TNFR1-mediated anti-adipogenic effect. A mixture of 5 μg/ml insulin, 1 μM dexamethasone, 0.5 mM isobutylmethylxanthine and 1 μM BRL49653 was used for a 4-day induction period. Cells were then maintained in medium containing only insulin until vector-infected cells were fully differentiated. Experiments were then terminated for morphological and molecular comparison of vector-infected control cells with those expressing TNFR1 mutants. As shown in Fig. 3, vector-infected control cells fully differentiated into lipid-laden adipocytes that can be readily identified by oil red O staining. In contrast, cells transfected with wild-type TNFR1 differentiated poorly, indicating inhibition of adipogenesis. Cells expressing TNFR1ΔID differentiated to a similar extent as control cells, suggesting that TNFR1 signaling pathway(s) is necessary for this repression. The fact that the hybrid mutant TNFR2/R1 could also inhibit differentiation further confirmed the necessity of TNFR1 intracellular signaling rather than selective ligand binding. TNFR1 mutants lacking either JM or DD lost their ability to repress adipocyte differentiation, indicating the involvement of both pathways. Cells expressing TNFR1ΔNSM inhibited differentiation to the same extent as wild-type TNFR1 did, which excluded the involvement of NSD. The TNFR1 mutant lacking both NSD and DD behaved similarly to that lacking only DD.

In order to evaluate the effects of TNFR1 mutants on adipocyte differentiation at the molecular level, we next examined expression levels of four adipogenic markers: Glut4, peroxisome proliferator-activated receptor-γ (PPARγ), adipsin and

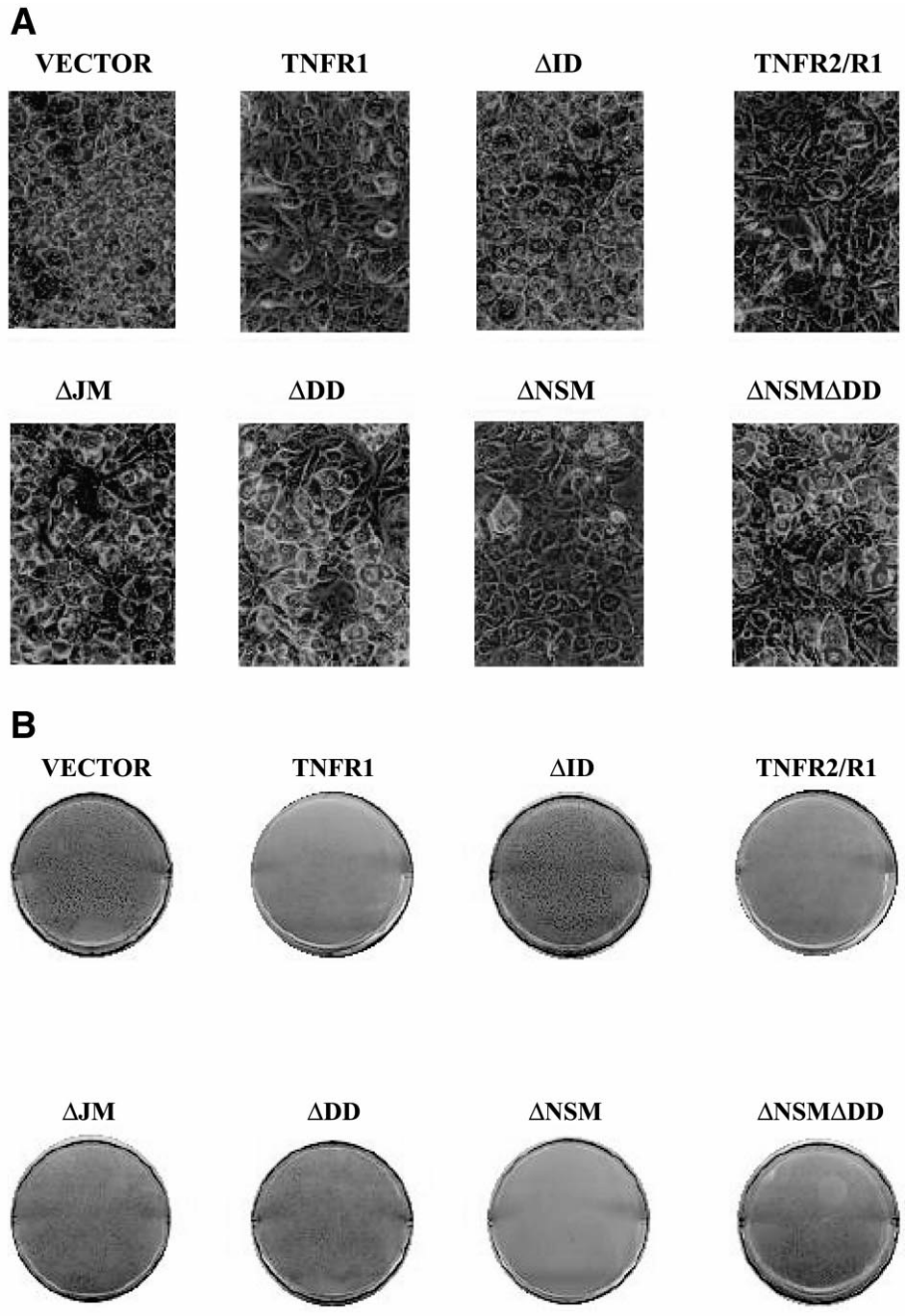


Fig. 3. Differentiation analysis of TNFR1$^{-/-}$R2$^{-/-}$ cells simultaneously expressing mTNFΔ1–9K11E and TNFR1 mutants. A: Microscopy of these cells stained with oil red O after completion of the differentiation procedure. B: An overall view of the same cells on whole plates stained with oil red O.

aP2. The expression levels of all these genes were significantly decreased in cells expressing wild-type TNFR1, the hybrid mutant TNFR2/R1 and TNFR1ΔNSM compared to control cells expressing TNFR1ΔID (Fig. 4A, lanes 1, 3, 6 and 2). Expression of all adipocyte genes was significantly repressed in TNFR1$^{-/-}$R2$^{-/-}$ cells expressing TNFR1ΔJM, TNFR1ΔDD and TNFR1ΔNSMΔDD (Fig. 4A, lanes 4, 5 and 7). Quantitation of three independent experiments is shown in Fig. 4B. These results are consistent with the observed morphological changes, confirming that the anti-adipogenic effect of TNFα is mediated by more than one signaling pathway after aggregation of TNFR1.

4. Discussion

TNFα has profound effects on adipocytes, which include suppression of differentiation, inhibition of insulin-stimulated glucose and free fatty acid uptake and lipogenesis, stimulation of lipolysis and regulation of gene expression. Extensive analyses have primarily focused on the actions of sTNF on adipocyte biology. Our previous studies have demonstrated that mTNF is also biologically active in cultured adipocytes [17]. Interestingly, when expressed in adipocytes, TNFα primarily resides on the cell surface and is processed quite inefficiently to produce secreted protein (Xu and Hotamisligil, unpub-

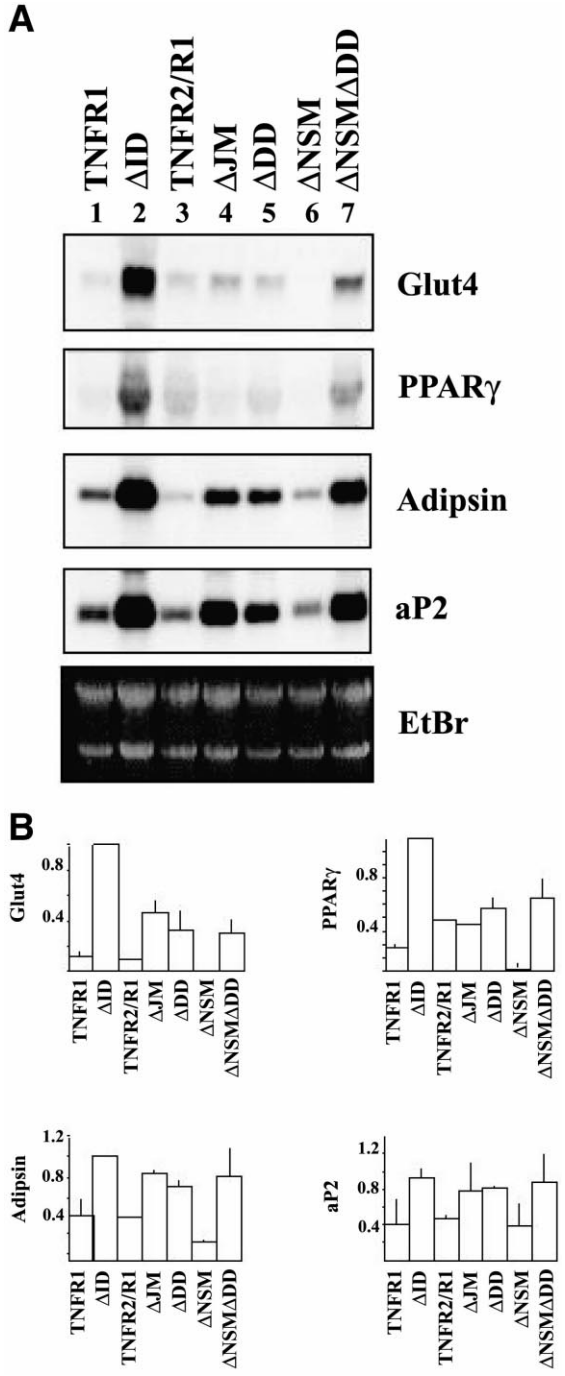


Fig. 4. Expression of adipogenic markers in TNFR1$^{-/-}$R2$^{-/-}$ cells simultaneously expressing mTNFΔ1–9K11E and TNFR1 mutants after differentiation. A: mRNA levels of Glut4, PPARγ, adipsin and aP2 in cells expressing TNFR1 mutants. B: Quantitation of mRNA levels of the genes shown in A relative to control cells, which express TNFR1ΔID in the presence of mTNFΔ1–9K11E. The results represent mean ± S.E.M. of data obtained from three independent experiments.

lished data). In addition, although mTNF has been proposed to be a prime ligand for TNFR2 [20], in adipocytes it acts through TNFR1, alone, to mediate the anti-adipogenic effect [17]. In this study, we investigated the potential functional domains of TNFR1 involved in this effect by establishing a series of TNFR1 mutants and performing a structure–function analysis. By coexpressing these TNFR1 mutants and a membrane-bound TNFα variant (mTNFΔ1–9K11E) in TNFR1$^{-/-}$R2$^{-/-}$ preadipocytes, we were able to demonstrate that both JM and DD of TNFR1 are critical in signaling for the TNF-mediated suppression of adipogenesis.

Our data reveal several critical aspects of TNFR1 signaling as it relates to TNFα actions in adipocytes. First, it appears that activation of NF-κB, alone, is neither necessary nor sufficient for the TNFα-mediated inhibition of adipocyte differentiation. The ΔJM mutant fully activates NF-κB but has no effect on differentiation. Conversely, the TNFR2/R1 chimera blocks differentiation without the activation of NF-κB. Second, the NSD is dispensable for both NF-κB activation and inhibition of adipogenesis, whereas the JM is necessary for the effects on differentiation but not NF-κB activity. Finally, the DD is critically involved in signaling for both of these activities.

There are several potential downstream effectors that might require these functional domains. For example, the JM of TNFR1 interacts with PIP_5K. It has been reported that PIP_5K activity is linked to cell proliferation as well as malignancy [22] and blockage of PIP_5K function via the use of monoclonal antibodies can block the proliferatory effect in response to plasma-derived growth factor (PDGF) and bombesin [23]. PDGF and several other mitogenic factors such as epidermal and fibroblast growth factors and TNFα can also regulate PPARγ activity through activation of the MAP kinase pathway [21]. Since PPARγ is a critical regulator of adipocyte growth and differentiation, one potential mechanism for the requirement of an intact JM domain involves PIP_5K-mediated regulation of growth and differentiation signals for adipogenesis. On the other hand, the DD has also been shown to be critical for several different pathways impacting similar cellular processes, including acidic SMase-mediated ceramide signaling. As a lipid second messenger, ceramide has been demonstrated to activate MAP kinase cascades through stimulating a Ser/Thr ceramide-activated protein kinase [24], which can be a potential mechanism to explain the involvement of the DD in adipogenesis. Additional studies should further dissect these pathways.

Acknowledgements: This work was supported by grants from the American Diabetes Association and National Institutes of Health (to G.S.H.).

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