Adipocytes as Immune Cells: Differential Expression of TWEAK, BAFF, and APRIL and Their Receptors (Fn14, BAFF-R, TACI, and BCMA) at Different Stages of Normal and Pathological Adipose Tissue Development

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Adipocytes as Immune Cells: Differential Expression of TWEAK, BAFF, and APRIL and Their Receptors (Fn14, BAFF-R, TACI, and BCMA) at Different Stages of Normal and Pathological Adipose Tissue Development

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Adipose tissue represents a rich source of multipotent stem cells. Mesenchymal cells, isolated from this source, can differentiate to other cell types in vitro and therefore can be used for a number of regenerative therapies. Our view of adipose tissue has recently changed, establishing adipocytes as new members of the immune system, as they produce a number of proinflammatory cytokines (such as IL-6 and TNF-α) and chemokines, in addition to adipokines (leptin, adiponectin, resistin) and molecules associated with the innate immune system. In this paper, we report the differential expression of TNF-superfamily members B cell activating factor of the TNF Family (BAFF), a proliferation inducing ligand (APRIL), and TNF-like weak inducer of apoptosis (TWEAK) in immature-appearing and mature adipocytes and in benign and malignant adipose tissue-derived tumors. These ligands act through their cognate receptors, BAFF receptor, transmembrane activator and calcium signal-modulating cyclophilic ligand (TACI), B cell maturation Ag (BCMA), and fibroblast growth factor-inducible 14 (Fn14), which are also expressed in these cells. We further report the existence of functional BCMA, TACI, and Fn14 receptors and their ligands BAFF, APRIL, and TWEAK on adipose tissue-derived mesenchymal cells, their interaction modifying the rate of adipogenesis. Our data integrate BAFF, APRIL, and TWEAK and their receptors BCMA, TACI, and Fn14 as novel potential mediators of adipogenesis, in addition to their specific role in immunity, and define immature and mature adipocytes as source of immune mediators. The Journal of Immunology, 2009, 183: 5948–5956.

Although adipose tissue has long been regarded as an almost inert tissue, dedicated solely to energy storage and release, recent data dramatically changed our opinion. Indeed, recent insights into the metabolic and immunological functions of preadipocytes and adipocytes revealed that they are potent producers of proinflammatory cytokines (such as IL-6 and TNF-α) and chemokines, regulating monocyte/macrophage function (1) and molecules associated with the innate immune system, such as the C1q/TNF-related superfamily. Finally, preadipocytes and adipocytes express a broad spectrum of functional Toll-like receptors and can convert into macrophage-like cells (reviewed in Ref. 2). These data clearly establish adipose tissue as a new player in immune reactions.

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The TNF superfamily is a group of receptors and ligands consisting of 19 ligands and 29 receptors. They orchestrate a wide range of biological functions, from the regulation of activation and cell death in the immune system to tissue homeostasis and cancer cell modulation (3). Among the different members of this family, an increased interest has been shown recently for the subset of B cell activating factor of the TNF Family (BAFF), a proliferation inducing ligand (APRIL), and TNF-like weak inducer of apoptosis (TWEAK). APRIL (CD256, TNF-SF13) (4–6) and BAFF (CD257, TNF-SF13B) (6–9) have been identified as trophic factors in lymphocyte malignancies and immune-related disorders (10). APRIL and BAFF have also been identified in a number of immune-related and immune-independent tissues (spleen, liver, lung, heart, intestine, kidney, and thymus) (11). They both bind to two TNF-R family members, transmembrane activator and calcium signal-modulating cyclophilic ligand (TACI) (TNFRSF13B) and B cell maturation Ag (BCMA) (TNFRSF17) (12, reviewed in Ref. 13). APRIL binds also syndecan-1 (CD138), a heparan sulfate proteoglycan present on the surface of many cell types (14, 15), allowing ligand polymerization and cross-linking (14–17). BAFF

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specifically binds to another TNF-R family member, BAFF-receptor (BAFF-R) (TNFRSF13C) (18). Binding to these receptors triggers diverse signaling pathways, including translocation of NFκB or activation of mitogen-activated kinases (19, reviewed in Ref. 20).

TWEAK/Fn14 represents another ligand/receptor couple, also mediating pluripotent responses. First described as a TNF-like weak inducer of apoptosis, TWEAK (TNF-SF12) has since emerged as a cytokine, regulating multiple cellular responses, including proinflammatory activity, angiogenesis, cellular proliferation, differentiation, and migratory capacity, suggesting a potential role in inflammation and cancer (reviewed in Ref. 21). TWEAK is highly expressed by inflammatory cells, expressed as a type II-transmembrane protein that can be cleaved to function as a soluble cytokine (22). It acts through its cognate receptor, fibroblast growth factor-inducible 14 (Fn14) (TNFRSF12A), identified also on nonlymphoid cell types and triggers multiple, often conflicting, cellular responses ranging from proliferation to cell death. TWEAK, and in particular Fn14, was relatively low in normal tissues, but TWEAK undergoes dramatic up-regulation in tissue injury and diseases, whereas Fn14 is expressed by many tissue-resident progenitor cells (23, 24). More recently, the concept that inflammatory cells regulate tissue regeneration via their effects on tissue progenitor cells has also been advanced (25). In this context, it is now evident that TWEAK is a multifunctional cytokine, similar in this regard to its sibling TNF-α.

Until recently, the presence of BAFF and APRIL or their receptors in adipose tissue was not evidenced. However, in a recent work, we have reported the expression of BAFF, but not APRIL, in mature breast adipocytes (26). In this study, we performed a thorough investigation of BAFF, APRIL, and TWEAK expression in normal, mature, and immature-appearing adipose tissue cells from different origins (s.c., visceral, and perirenal fat tissue). We report the expression of these ligands, as well as their respective receptors (BAFF-R, BCMA, TACI, and Fn14) at different stages of human adipose tissue maturation, establishing this tissue as a novel source of TFN members and strengthening the link between adipose tissue and the immune system. This distribution is further contributing to understanding the mechanisms by which adipose tissue and the immune system interact. This distribution is further contributing to understanding the mechanisms by which adipose tissue and the immune system interact. Additionally, it is now evident that TWEAK is a multifunctional cytokine, similar in its function to its sibling TNF-α.

Materials and Methods

Tissue selection

Specimens of human skin or breast or renal peritumoral fat (n = 20, 52, and 32, respectively) were obtained from the University of Crete, School of Medicine, Department of Pathology (Crete, Greece) tissue bank, after permission of the University Hospital Research and Ethics Committee. The demographic characteristics of the patients are presented in supplemental Table 1. Furthermore, one hibernoma, two cases of lipomas, and two liposarcomas were also examined. Eight tissue slides (3 μm) were cut serially, one for H&E staining and seven for specific immunostaining for BAFF, APRIL, TWEAK, BAFF-R, BCMA, TACI, and Fn14. The slides were reviewed by two investigators independently and blindly and the final conclusion was reached in consensus. In the few cases with discrepancy between the two observers, the immuno-stained slides were reviewed in a double viewing microscope, so that the discrepancy was settled.

Immuno/histochemistry

After deparaffinization and hydration, slides were subjected to three 5-min cycles of citrate buffer (0.01M, pH 6.0) incubation in a 500-W microwave oven and treated with 3% hydrogen peroxide for 15 min, as appropriate. They were incubated for 30 min with primary Abs to APRIL (hApril-8 monoclonal Ab, 1/100 dilution, Alexis Biochemicals); BAFF (ALX-804–131 mAb/Buffy-2 clone, dilution 1/100, Alexis Biochemicals), TWEAK (sc-12405, 1/100 dilution, Santa Cruz Biotechnology), BCMA (Vicky-1 rat mAb, 1/100 dilution, ALX-804–151, Alexis Biochemicals), TACI (IMG-249 rabbit polyclonal Ab, 1/150 dilution, Imgenex), BAFF-R (goat polyclonal, AFI1162, dilution 1/100, R&D Systems), or Fn14 (mouse monoclonal, sc-56250, dilution 1/50, Santa Cruz Biotechnology). The UltraVision LP Detection System (TL-060-AL, Lab Vision, Fremont, CA) was used. The HRP detection kit (BD Biotechnologies) or the K0689 LSAB kits (DakoCytomation) were used for immunodetection, as appropriate. Counterstaining was performed using Mayer’s hematoxylin. Known positive and negative controls (omission of the primary antibody) were used in every run (supplemental Fig. 1).

Isolation, culture, and differentiation of mesenchymal cells

Liposarpirates were obtained by liposuction of subcutaneous adipose tissue of abdomen or hips of healthy donors (n = 6), aged 42 ± 5 years old (mean ± SD) after informed consent. Human ADMC were isolated as previously described (27). Briefly, liposarpirates were washed with PBS, incubated with 1 mg/ml collagenase type I (Sigma-Aldrich) for 45 min at 37°C, and tissue was centrifuged at 1200 rpm for 10 min. Erthrocytes were removed by a 10-min incubation at room temperature in an erythrocyte lysis buffer (150 mM NH4Cl, 10 mM NaHCO3, 1 mM EDTA, pH 7.5). Cells were cultured in low glucose (1g/L) DMEM, supplemented with 10% FBS and penicillin/streptomycin at 37°C and 5% CO2 in a humidified atmosphere (28). Two days after seeding, floating cells were removed and thereafter medium was changed every 3 days. Cells were passaged by trypsinization after reaching 70–90% confluence.

For adipogenic induction, human ADMC were cultured in adipogenic medium consisting of basal medium supplemented with 10 μg/ml insulin, 1 μM dexamethasone, 0.5 mM methylxanthine, and 200 μM indomethacin. Medium was changed every 2 days and 2 weeks of culture, adipogenesis was evaluated by Oil Red O staining. For the latter, cells were fixed with 4% formaldehyde for 12 h, stained for 45 min with 2 volumes of 60% Oil Red O (diluted in isopropanol)/1 volume PBS and thoroughly washed with PBS. Pictures were taken with an inverted Leica microscope. For quantification of staining, the dye was solubilized with isopropanol and red color intensity (proportional to the lipid content of cells) was measured in a plate photometer at 600 nm. Results were normalized per mg of total protein.

Direct immunofluorescence

ADMC (passage-2) were characterized by flow cytometry using the following Abs conjugated to fluorochromes: anti-CD2HFTC, anti-CD3FTC, anti-CD4FTC, anti-CD8FTC, anti-CD19PE, anti-CD31PE, anti-CD44FITC, anti-CD54PE, anti-CD73PE, anti-CD138FITC, anti-CD90FITC (all from BD Pharmingen); anti-CD3 (H-271, 1/50) (Santa Cruz Biotechnology); and TNFR1 (H-271, 1/50), and TNFR2 (PA1–21148, 1/50; Thermo Fisher Scientific) have been used. Briefly, cells were trypsinized after reaching 70–90% confluence.

Indirect immunofluorescence

For identification of TNFR-Superfamily (TNFR-SF) members, primary Abs against BAFF-R (goat polyclonal, AFI1162, dilution 1/100; R&D Systems); BCMA (Vicky-1 rat mAb, 1/100 dilution, ALX-804–151; Alexis Biochemicals); TACI (IMG-249 rabbit polyclonal Ab, 1/150 dilution; Imgenex); Fn14 (mouse monoclonal, sc-56250, dilution 1/50), TNF-α (2C8, 1/50), and TNFR1 (H-271, 1/50) (Santa Cruz Biotechnology); and TNFR2 (PA1–21148, 1/50; Thermo Fisher Scientific) have been used. Briefly, cells were detached by gentle scraping, washed in PBS/1% BSA, and incubated with Abs, according to the manufacturer’s instructions. Fluorochrome-conjugated F(ab)2 or IgG2a were used as negative controls. Staining was quantified by flow cytometry using a FACScan operator (BD Biosciences) and results were analyzed with the FACScan and Cell Quest softwares (BD Biosciences).
Cells were again washed and resuspended in PBS/3%BSA/0.2% NaN₃. For fluorescent staining against BCMA, we used a biotinylated goat anti-rat secondary Ab (used at 1/100, Sigma-Aldrich), and FITC-conjugated avidin (1/200; BD Biosciences). Staining was quantified by flow cytometry.

Detection of functional TNFR-SF members on cells

Cells were harvested in cold 1 mM EDTA in PBS and incubated for 20 min in a buffer containing 10% human serum, 1 mM EDTA, 1% BSA, and 0.2% NaN₃ in PBS. FLAG-BAFF, FLAG-APRIL, or FLAG-TWEAK were added to a concentration of 100 ng/ml. Incubation buffer was used as a mock control. Then monoclonal mouse (M2; Sigma-Aldrich), anti-FLAG (8 μg/ml), and FITC-conjugated goat anti-mouse IgG (1:50) were added sequentially. Staining was quantified by flow cytometry using the aforementioned operator.

Quantitative RT-PCR (qRT-PCR)

Total RNA was isolated from cell cultures with the Miniprep RNA isolation kit (Macherey-Nagel) and from harvested (adipose) tissue with Trizol (Life Technologies). RNA samples treated independently. ADMC mRNA expression was used as control.

Analysis of published gene-array data

Published gene array data that included expression analysis of mesenchymal stem cells (MSCs) that were driven to adipocytes in vitro were used. In the Gene Expression Omnibus database, we identified dataset GSE9451 (K. Shimizu et al., Hiroshima University, Hiroshima, Japan) that included gene array data from MSCs that were in vitro differentiated to either fibroblast, osteoblast, chondrocyte, or adipocyte in triplicates. The control (MSC) and adipocyte gene-array data were extracted and loaded to Gene-spring GX V10.0.1 (Agilent Technologies) in order to calculate fold changes in gene expression (adipocytes/MSCs), and to identify statistically significant changes (unpaired t test) in the expression of members of the TNF superfamily BAFF, APRIL, TACI, BCMA, BAFF-R, TWEAK, and FN14 between adipocytes and MSCs.

Assay of secreted APRIL, BAFF, and TWEAK

Assays of secreted TNF-SF ligands in the culture medium were performed on supernatant medium after 24 h incubation with cells. The ELISA for these ligands were provided by Bender MedSystems (catalogue numbers: human BAFF BMS2007INST, human APRIL BMS2008, human TWEAK BMS2006INST). The sensitivity of the assays was 130, 400, and 9.8 pg/ml for BAFF, APRIL, and TWEAK, respectively, whereas the corresponding intra-assay coefficient of variation were 6.7%, 7.3% and 7.9%, respectively. Due to the assay characteristics, production of BAFF, APRIL, and TWEAK content was assayed in 5× concentrated supernatant medium.

Statistical analysis

Statistical analysis was performed with the Statistical Package for Social Sciences, v 16 (SPSS) using the appropriate parametric tests. Statistical significance was set to p < 0.05.

Results

Expression of BAFF, APRIL, TWEAK, and their receptors in adipose tissue sections

Using immunological staining, we have tested for the presence of BAFF, APRIL, TWEAK, and their receptors in adipose tissue sections (Fig. 1). APRIL was constantly expressed in visceral adipose tissue (perinephric fat), in which adipocytes appeared immature with multilocular cytoplasm (herein described as immature-appearing adipocytes). Staining was restricted in well described intracellular dots in specific cytoplasmic areas in relation to the mode of APRIL synthesis and secretion through secretory vesicles. In contrast, in peripheral adipocytes (s.c. fat, with mature-appearing adipocytes), staining was observed in the cell periphery was more heavily stained. In some cases, an additional perinuclear staining was also observed. BAFF, but not APRIL, was also expressed in the vascular endothelium (Fig. 1, arrowheads).
The finding that BAFF and APRIL are expressed in normal adipose tissue suggests a possible local action. Therefore, we have assayed whether their receptors BCMA, TACI, and BAFF-R are also present. BCMA and TACI were equally present in immature-appearing adipocytes, but not in mature adipose tissue. It is notable that vessels (Fig. 1, arrowheads) were negative for BCMA but moderately positive for TACI staining. In contrast, BAFF-R was constantly present in immature-appearing and mature adipocytes, but negative in endothelial cells. These data suggest that in mature adipose tissue, BAFF might exert its action through the equally expressed BAFF-R, while in immature-appearing adipose tissue both BAFF and APRIL, as well as their three receptors (BCMA, TACI, and BAFF-R) are expressed, suggesting more complex interactions.

TWEAK was heterogeneously present in immature-appearing adipose tissue, in which negative and positive areas were found, while no TWEAK expression has been evidenced in mature adipose tissue. In contrast, Fn14 was always present as discrete membrane dots, both in immature-appearing and mature adipose tissue, although mature adipocytes exhibited an apparent lower staining of Fn14 compared with that of immature tissue. In addition, blood vessels (Fig. 1, arrowheads) were constantly negative for TWEAK but positive for Fn14 staining. A comparison of staining intensities of TNF-SF ligands and receptors is presented in Table I.

The above data indicate that TNF-SF members APRIL, BAFF, and TWEAK are differentially expressed in the adipose tissue. TNF-α has been reported to be produced by adipocytes, preadipocytes, and infiltrating macrophages in the adipose tissue of (predominantly obese) humans, exerting auto- or paracrine effects (29).

In this paper, we report that TNF-α and its receptors (TNFR1 and TNFR2) were also expressed differentially in adipose tissue (supplemental Fig. 2). TNF-α was preferentially expressed in immature-appearing adipocytes and endothelial cells. The same pattern was found for TNFR2. In contrast, TNFR1 present in mature-appearing adipocytes with a greater intensity. Endothelial cells were equally stained for TNFR1 and TNFR2.

Expression of BAFF, APRIL, TWEAK, and their receptors in adipose tissue-derived tumors

The above data indicate a differential expression of BAFF, APRIL, TWEAK, and their receptors in nonneoplastic, immature-, and mature-appearing adipose tissue in a different pattern than TNF-α and its receptors. This result suggests a possible differential role of these ligands in adipogenesis. We have assayed whether the expression of these molecules varies in adipose tissue tumors, in which proliferation and differentiation of adipocytes display enormous variability. We have examined three cases of lipomas (benign tumors consisting of nonmalignant normal-appearing adipocytes), one case of hibernoma, a rare benign brown fat tumor resembling immature-appearing adipose tissue (30), and two cases of malignant liposarcomas (Fig. 2).

Lipomas showed staining for BAFF and BAFF-R; Fn14 staining was also present, but at lower intensity than in normal mature adipose tissue-derived tumors (Table I). APRIL and its receptors BCMA and BAFF-R were also present. BCMA and TACI were equally present in immature-appearing and mature adipose tissue. In contrast, Fn14 was always present as discrete membrane dots, both in immature-appearing and mature adipose tissue.

In the hibernoma, BCMA and TACI staining was negative, while BAFF was heterogeneously present, with areas of positive staining. TWEAK expression was negative in hibernoma, while BAFF-R was constantly present in immature-appearing and mature adipose tissue.

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adipocytes, and TWEAK staining was absent. In this respect, expression of these TNF-SF members in lipoma resembles the expression observed in mature-appearing adipose tissue. However, an additional low APRIL and TACI staining was evidenced. Hibernoma was also heavily stained for BAFF, BAFF-R, and Fn14, whereas APRIL, TACI, BCMA, and TWEAK staining was also evident in a pattern similar to that observed in immature-appearing adipose tissue. It is interesting that this staining was restricted to immature-appearing adipocytes, whereas, in mature appearing adipocytes, the latter molecules were absent. Finally, in liposarcomas, a heterogeneous APRIL, BAFF, BAFF-R, BCMA, TWEAK, and Fn14 staining was observed, whereas TACI presented a homogeneous staining, suggesting a potential role of this receptor in the development of sarcoma tumors (Table I). The atypical neoplastic adipocytes show staining patterns similar to that of hibernomas.

**TNF-SF ligands and receptors immunocytochemistry in ADMC and in in vitro differentiated adipocytes**

Our results indicate that normal, immature-appearing, and mature adipose tissue and adipose tissue-derived tumors express differentially BAFF, APRIL, TWEAK and their receptors. To further explore this differential expression, we isolated ADMC and followed the expression of these molecules during adipogenesis. These cells were found to present striking similarities with bone-marrow-derived mesenchymal cells, presenting also an additional partial staining for endothelial markers (supplemental Table III).

We investigated the presence of TNF-SF ligands (BAFF, APRIL, TWEAK) and their receptors (BAFF-R, TACI, BCMA, Fn14) both in ADMC and in vitro differentiated adipocytes (Fig. 3). APRIL presented a weak staining in ADMC, found as discrete intracellular dots, present also in differentiated cells. ADMC and differentiated adipocytes expressed both BAFF and TWEAK. Differentiated cells were more heavily stained than their precursors. The cognate receptors of these TNF-SF members were also evidenced in ADMC and in vitro differentiated adipocytes. BAFF-R, BCMA, and TACI, receptors for BAFF and APRIL, were present in ADMC. After adipogenesis, BCMA staining intensity was increased, TACI staining was decreased, while the presence of BAFF-R was maintained at low levels. In contrast, an intense membrane staining for Fn14 was found in ADMC; this staining was decreased after adipocyte differentiation and was mainly found intracellularly (Table I).

ADMC cells, either nondifferentiated or differentiated toward adipocytes, do not express (<1%) CD2, CD3, CD4, CD8, CD15, CD19, HLA-DR, and CD45, indicative of the nonexistence of hemopoietic cells (supplemental Table IV). In this respect, our data indicate that TNF-SF ligands and receptors are expressed by adipocytes themselves and that ADMC do not differentiate to blood cells (lymphocytes, macrophages/monocytes, granulocytes) that may also secrete these molecules. These results are consistent with immunostaining data presented in Figs. 1–3.

The above data in isolated cells, cultured on plastic, present some discrepancies from those presented in tissue sections. Indeed, in fully differentiated adipose tissue, neither APRIL, TWEAK, TACI, nor BCMA are expressed, although they continue to be expressed in isolated differentiated adipocytes. This result provides evidence about a more complex regulation of the TNF-SF system in the adipose tissue, suggesting that potential interactions between immature-appearing adipocytes and humoral or tissue factors might regulate their expression.

The expression of the above TNF-SF members and receptors was further verified by qRT-PCR on total RNA isolated from ADMC, undifferentiated or differentiated in vitro toward adipocytes (Fig. 4A). Adipocyte differentiation was verified both phenotypically (Oil Red O staining; Fig. 3) and with qRT-PCR of lipoprotein lipase (LPL) and peroxisome proliferator activated receptor γ (Fig. 4B). After 2 weeks of differentiation, results of BAFF and BAFF-R transcripts were comparable with their immunostaining on ADMC and differentiated cells. APRIL, as well as TACI and BCMA, exhibited a significant increase of their expression in differentiated cells as compared with that of ADMC, paralleling the increased staining found in differentiated adipocytes. It is interesting that Fn14 expression decreased dramatically in differentiated cells, paralleling the decreased staining observed in differentiated cells, whereas TWEAK mRNA expression showed a slight increase in differentiated adipocytes. In addition, the changes

**FIGURE 3.** Expression of BAFF, APRIL, TWEAK, and their receptors (BCMA, TACI, BAFF-R, and Fn14) in ADMC and differentiated adipocytes. Bottom left, Phase-contrast microphotograph of ADMC and the same culture after adipocyte differentiation, both in phase contrast and after Oil Red O staining. Bar = 50 μm.
in the expression of these TNF-SF ligands and their receptors follow in a parallel way the gradual differentiation of ADMC toward adipocytes, which is evidenced by a gradual increase of adipogenic markers (LPL, PPARγ) in ADMC and the same cells after 1 or 2 wk of adipocyte differentiation. Mean ± SEM of five different cases. B, Expression of LPL and PPARγ mRNA in ADMC undifferentiated and differentiated for 1 and 2 wk toward adipocytes. C, Analysis of the NCBI GSE9451 gene-array dataset for the expression of TNF-SF ligands and receptors, as in A.

FIGURE 4. A, Expression of BAFF, APRIL, TWEAK, and their receptors (BCMA, TACI, BAFF-R, and Fn14) mRNA in ADMC and the same cells after 1 or 2 wk of adipocyte differentiation. Mean ± SEM of five different cases. B, Expression of LPL and PPARγ mRNA in ADMC undifferentiated and differentiated for 1 and 2 wk toward adipocytes. C, Analysis of the NCBI GSE9451 gene-array dataset for the expression of TNF-SF ligands and receptors, as in A.

Effect of BAFF, APRIL, and TWEAK on adipogenesis

As presented above, isolated ADMC express BAFF, APRIL, and TWEAK, as well as BCMA, TACI, and Fn14, whereas BAFF-R is expressed at low quantities. The existence of both ligands and receptors on the same cell population is indicative of a possible autocrine/paracrine action and suggests a possible role of these factors in adipogenesis. Indeed, we found that FLAG-tagged recombinant BAFF, APRIL, and TWEAK can bind on ADMC, evidencing the existence of functional receptors on the cell surface (Fig. 5A). The presence of BCMA, TACI, and Fn14 molecules on ADMC surface was also confirmed by flow cytometry (Fig. 5B), although we have not observed the presence of BAFF-R. These results suggest that APRIL, BAFF, and TWEAK possibly act on adipocytes precursors through these three receptors. It is notable that the observed BCMA membrane expression is rare, because this receptor is usually identified intracellularly (12).

APRIL has been reported to bind also to syndecan-1 (a membrane heparan sulfate proteoglycan) (31, 32) through its N-terminal domain, inducing its oligomerization, TACI- and/or BCMA-mediated activation, and resulting in the generation of migration or survival signals (14, 15). However, we have not detected the presence of syndecan-1 (CD138) on adipocyte-derived mesenchymal cells (not shown). Furthermore, FLAG-tagged APRIL binding on ADMC was not decreased in presence of a 250-fold molar concentration of heparin, suggesting that membrane proteoglycans might not be implicated in the binding of APRIL and therefore in its potential action in ADMC (supplemental Fig. 3). In conclusion, BAFF, APRIL, and TWEAK could exert their effects through functional TACI, BCMA, and Fn14 receptors expressed on ADMC.

We further investigated whether the addition of human recombinant BAFF, APRIL, and TWEAK can modify adipogenesis. However, as these ligands have, in other systems, notable effects on cell proliferation and survival, we have tested whether this might be the case in ADMC differentiated toward adipocytes. As presented in supplemental Fig. 4, neither ligand (100 ng/ml) had an effect on cell survival after a short or a long incubation time period (4 and 7 days, respectively). Conversely, at the same concentrations, we observed an increased differentiation of ADMC toward adipocytes in the presence of BAFF, whereas addition of APRIL and especially of TWEAK significantly decreased the adipogenic differentiation. Addition of APRIL resulted in a dose-dependent decrease of adipogenesis after 14 days, whereas the inhibitory effect of TWEAK was evident as early as day 5 (not shown) and maximal at concentrations as low as 25 ng/ml (Fig. 5C). This result further suggests the involvement of BAFF as a trophic factor of the mature adipose tissue with a proadipogenic potential and provides a hint about the absence of the antiadipogenic APRIL, BCMA, and TACI system in mature adipose tissue. However, the absence on cell membranes of mature adipocytes of Fn14 suggests an additional role of TWEAK/Fn14.

As presented above, Fn14 is constantly present in endothelial cells. Therefore, the system TWEAK/Fn14 could play a role in the angiogenesis of the adipose tissue. To investigate a possible role of TWEAK/Fn14 in angiogenesis and as ADMC contain endothelial precursor cells (33, 34), we have investigated the expression of the endothelial marker ICAM-1 (CD54). ICAM-1 is an intercellular adhesion factor, constantly present in leukocytes and endothelial cells (35) that is greatly enhanced upon cytokine stimulation (IL-1 and TNF-α). Incubation of ADMC with TWEAK results in an enhanced expression of CD54 (Fig. 5D), a result not observed in the case of BAFF or APRIL (not shown).

Discussion

After their initial characterization, MSCs were found within the connective tissue of many organs (36), although their proliferation and differentiation characteristics vary. Similarities in surface marker profiles, morphology, and differentiation potentials assayed in vitro led to suggestions that each of these multipotent cell types arise from a common adult progenitor cell (37) and then may adopt tissue-specific attributes according to its particular niche (38). Adipose tissue represents a rich source of such cells. Indeed, mesenchymal cells, isolated from this easy source (28), can differentiate to other cell types in vitro (27) and therefore have been used for a number of regenerative therapies currently under investigation. However, recent data indicate that adult mesenchymal cells can
also be transformed in cancer-associated fibroblasts, a necessary stroma element promoting the growth of epithelial solid tumors (39, 40). In this respect, a detailed analysis of the differentiation potential and the involved mechanisms are of primary importance. The results of the present study integrate this effort by reporting that the subset of the TNF-SF ligands APRIL, BAFF, and TWEAK and their receptors BCMA, TACI, BAFF-R, and Fn14 are involved in the differentiation process of ADMC toward adipocytes and (possibly) endothelial cells. In this respect, they may provide an additional spectrum of agents for use in therapeutic interventions.

FIGURE 5.  A, Binding of FLAG-tagged APRIL, BAFF, and TWEAK on ADMC assayed by flow cytometry. Black hollow curves represent the omission of the corresponding ligand, while grey curves represent binding of FLAG-tagged APRIL, BAFF, and TWEAK.  B, Binding of anti-BAFF-R, anti-BCMA, anti-TACI, and anti-Fn14 Abs on ADMC by flow cytometry. Hollow curves show isotype matching controls (replacement of Abs by corresponding pre-immune Igs), while grey curves show binding of anti-BAFF-R, anti-TACI, anti-BCMA, and anti-Fn14.  C, Effect of BAFF, APRIL, and TWEAK on adipocyte differentiation of ADMC. Mean ± SEM of two different cases, assayed in triplicate.  D, Effect of APRIL, BAFF, and TWEAK on the ICAM-1 (CD54) expression in ADMC.

TNF was the first proinflammatory mediator shown to be secreted by adipocytes (Ref. 41 and supplemental Fig. 2). Additionally, recent data identified a whole new family of C1q-TNF members, synthesized and secreted by the adipose tissue (42, 43), including adiponectin, C1q, and CORS-26 (collagenous repeat containing sequence of 26 kDa protein, also known as CTRP-3). They are highly expressed and secreted by adipose tissue (reviewed in Ref. 2) and linked to a number of processes, such as inflammation, apoptosis, autoimmunity, host defense, hibernation, organogenesis, cell differentiation, metabolism, and insulin signaling. In this paper, we report that other members of the TNF-SF (BAFF, APRIL, and TWEAK) are also synthesized and secreted from adipocytes and ADMC, which can act in an auto/paracrine way, regulating the differentiation potential of these cells. We also show that this expression is modulated along the differentiation of ADMC to mature adipocytes. BAFF, APRIL, and TWEAK and their receptors were, for a long time, regarded as elements of the immune system, regulating the differentiation and fate of B lymphocytes (reviewed in Ref. 44). However, in recent years these molecules have been identified in a number of normal and pathological tissues (11, 21, 45–47). In this paper, we report, for the first time, that adipose tissue-resident adult mesenchymal cells express and synthesize these ligands. In addition, the same population of mesenchymal cells expresses their cognate receptors BAFF-R, TACI, BCMA, and Fn14 on the surface of the cells. An interesting result is the rare localization of BCMA on the cell membrane of ADMC. Indeed, usually, staining for BCMA is found intracellularly, located in the Golgi apparatus (12). In addition, Fn14 (the specific TWEAK receptor) is predominantly present at the membrane level in ADMC, whereas it translocates intracellularly in in vitro maturing adipocytes. These receptors are functional as they can bind in vitro their corresponding ligands.
The expression of ligands and receptors on ADMC indicate a possible role in their differentiation, expanding their potential targets beyond the immune system. We report that BAFF enhances adipogenesis, while APRIL and especially TWEAK inhibit it. APRIL, in contrast to many other TNF-related ligands, is a factor promoting proliferation (4, 48) of cells in tissue culture. Addition of recombinant APRIL or transfection of tumor lines with APRIL provided a significant growth advantage to lymphoid and nonlymphoid cells (49). Our data show that APRIL decreases adipocyte differentiation, whereas no effect of APRIL on ADMC proliferation was evident (not shown), suggesting other roles of this ligand in ADMC that have yet to be determined. It is interesting to note that in vitro differentiated adipocytes continue to express all ligands and receptors, suggesting that the final differentiation process is the result of a fine interplay between elements of this system. In addition, Fn14 internalizes in differentiated adipocytes. However, TWEAK has been recognized in many tissue-resident progenitor cells (23, 24) and a potential role in tissue regeneration has been proposed (25). In addition, TWEAK enhances endothelial cell maturation of ADMC, a result supporting the recent finding of the perivascular residence of ADMC (50) and providing a biological support of the reported role of the system (TWEAK/Fn14) in the pathogenesis of atherosclerosis (51).

The role and regulation of this TNF-SF system appears to be more complicated at the organism level. Indeed, staining of tissue sections revealed some discrepancies between tissue expression and in vitro differentiated adipocytes. In fully differentiated adipose tissue, neither APRIL, TWEAK, TACI, nor BCMA are expressed, whereas they are expressed in fully isolated differentiated adipocytes. These results suggest that there are potential interactions between adipocytes and humoral or tissue factors that might regulate the expression of these ligands and receptors. These data further indicate that dedifferentiation of cells results in the acquisition of a stem cell-like phenotype and that the TNF-SF members APRIL and TWEAK could possibly be used for the therapeutic manipulation of these tumors, promoting the possible reprogramming of tumor cells.

Adipose tissue was long regarded as a silent and passive organ, storing excess energy as triglycerides and releasing energy as fatty acids. However, adipose tissue is now recognized as an endocrine and immune organ, secreting a wide variety of hormones, cytokines, chemokines, and growth factors that influences metabolism, vascular and endothelial function, appetite and satiety, immunity, fertility, inflammation, tumor growth, and many other processes (for recent reviews, see Refs. 2, 52). In this respect, a thorough investigation of this tissue/organ might provide new insights for a wide spectrum of diseases, from the metabolic syndrome to specific immunological and inflammatory conditions. Results presented in this paper integrate BAFF, APRIL, and TWEAK as novel potential mediators of additional immune actions, taking into consideration their direct implications in the development and maturation of the immune system and autoimmunity (44), and provide further evidence of the immune role of the adipose tissue (2). Moreover, as shown, these ligands and cognitive receptors might be factors influencing the genetic programming of mesenchymal cells toward adipogenesis, providing further elements for the manipulation and/or treatment of adipose tissue-derived benign and malignant tumors.

Acknowledgments

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Disclosures

The authors have no financial conflict of interest.


SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure 1
Detection of APRIL, BAFF, TWEAK, BAFF-R, TACI, BCMA and Fn14, in sections of human tonsil. Representative sections of a germinal center are shown. Bar= 100 μm.

Supplemental Figure 2
TNFα and TNFR1 & 2 in mature- (upper lane) and immature-appearing adipocytes (lower lane). Bar= 50 μm.

Supplemental Figure 3
Addition of heparin does not inhibit APRIL binding on ADMC
Flow-cytometry results show binding of FLAG-APRIL(100 ng/mL) on ADMC, in presence of a 250-fold molecular excess of heparin (blue) and in absence of heparin (red). Binding of mock control (black).

Supplemental Figure 4
ADMC viability after treatment with APRIL, BAFF and TWEAK.
Cells were treated with APRIL, BAFF or TWEAK (100 ng/ml), with medium renewal every 2 days and cell viability was measured by the MTT assay.
## Supplemental Table I

Demographic characteristics of patients.

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<td>52</td>
<td>32</td>
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### Supplemental Table II

Primers used in qRT-PCR assays

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<td>Fn14</td>
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Membrane antigen molecules detected in ADMC and differentiated adipocytes (Diff. ADMC); comparison with bone marrow-derived mesenchymal cells (BMMCs). These progenitor cells, isolated in large quantities from adipose tissue, may express different phenotypes in vitro (adipocytes, chondrocytes, osteocytes, neural cells), under appropriate and routinely-controlled culture conditions, as reported previously (1, 2). We have confirmed that they present striking phenotypic similarities with BMMC (Supplementary Table 1), being highly positive for stem cell precursor markers, such as CD13, CD54, CD44 and CD73 and CD90 (88%, 81%, 80%, 73% and 99%, respectively). Furthermore, they exhibit partial positivity for CD34 (14%), as they also contain endothelial precursor cells (3, 4) and an increasing expression of CD105, increasing with cell passage. On the contrary, they are negative (<1%) for markers of hematopoietic lineages, such as CD14, CD31, CD45, CD133 and CD144. These observations, in addition to their similar fibroblastic morphology, indicate great phenotypical similarity with BMMC, as previously demonstrated (5-9). After their in vitro differentiation towards adipocytes, revealed by morphological changes, lipid droplet accumulation and Oil Red O staining (Figure 3), or LPL and PPARγ expression (Figure 4B), they exhibit decreased expression of CD proteins indicative for “stemness”, like CD13, CD44, CD73 or CD90. We have also found that CD54 (ICAM-1) expression is dramatically reduced after adipogenesis (80.9% in progenitors versus 3.2% in differentiated adipocytes).

<table>
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<th>ADMC</th>
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<tr>
<td></td>
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<tr>
<td>CD13</td>
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<td>CD144</td>
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* Data obtained from the indicated references

Supplementary Bibliography

Supplemental Table IV

Expression of membrane surface markers of myeloid and lymphoid differentiation in ADSC and mature adipocytes. Cells were stained with the specific antibodies before (ADSC) and after two weeks of adipocyte differentiation in an adipocyte medium, as described in Material and Methods. Table represents mean staining intensity of three different experiments with adipocytes isolated from different individuals.

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