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TWEAK Inhibits TRAF2-Mediated CD40 Signaling by Destabilization of CD40 Signaling Complexes

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We found recently that TNF-like weak inducer of apoptosis (TWEAK) and fibroblast growth factor–inducible-14 (Fn14) by virtue of their strong capability to reduce the freely available cytoplasmic pool of TNFR-associated factor (TRAF)2 and cellular inhibitors of apoptosis (cIAPs) antagonize the functions of these molecules in TNFR1 signaling, resulting in sensitization for apoptosis and inhibition of classical NF-κB signaling. In this study, we demonstrate that priming of cells with TWEAK also interferes with activation of the classical NF-κB pathway by CD40. Likewise, there was strong inhibition of CD40 ligand (CD40L)–induced activation of MAPKs in TWEAK-primed cells. FACS analysis and CD40L binding studies revealed unchanged CD40 expression and normal CD40L–CD40 interaction in TWEAK-primed cells. CD40L immunoprecipitates, however, showed severely reduced amounts of CD40 and CD40-associated proteins, indicating impaired formation or reduced stability of CD40L–CD40 signaling complexes. The previously described inhibitory effect of TWEAK on TNFR1 signaling has been traced back to reduced activity of the TNFR1-associated TRAF2–cIAP1/2 ubiquitinase complex and did not affect the stability of the immunoprecipitable TNFR1 receptor complex. Thus, the inhibitory effect of TWEAK on CD40 signaling must be based at least partly on other mechanisms. In line with this, signaling by the CD40-related TRAF2-interacting receptor TNFR2 was also attenuated but still immunoprecipitable in TWEAK-primed cells. Collectively, we show that Fn14 activation by soluble TWEAK impairs CD40L–CD40 signaling complex formation and inhibits CD40 signaling and thus identify the Fn14-TWEAK system as a potential novel regulator of CD40-related cellular functions. The Journal of Immunology, 2013, 191: 2308–2318.

Tumor necrosis factor–like weak inducer of apoptosis (TWEAK) is a typical representative of the TNF ligand family and as such it is initially expressed as a type 2 transmembrane protein (1, 2). Membrane-bound TWEAK (memTWEAK) can be cleaved by furin proteases in the stalk region separating the transmembrane domain from the conserved extracellular C-terminal TNF homology domain, resulting in the release of soluble TWEAK. The only known unquestioned verified signaling-competent receptor of TWEAK is fibroblast growth factor–inducible-14 (Fn14), an unusual small member of the TNF receptor family (1, 2). Fn14 is highly expressed on most tumor cell lines but rarely on leukemia or lymphoma cell lines. Immunohistological studies further suggest that in vivo Fn14 expression is low in healthy homeostatic tissue but induced in scenarios associated with tissue damage such as ischemia, cachexia, surgical intervention, or tumor development (1, 2). Indeed, Fn14 has originally been identified as a growth factor–inducible protein (3). Although expression of TWEAK has been demonstrated at the mRNA level in a variety of tissue and cell lines, relatively little is known about TWEAK expression at the protein level. The full-length membrane-bound form of TWEAK has been detected only in IFN-γ–treated monocytes, macrophages, dendritic cells, and a few breast and hepatocellular cancer cell lines (1, 2, 4, 5). It has also been recognized that membrane-bound TWEAK is efficiently processed by furin proteases, but production of soluble TWEAK has been barely addressed in vitro in quantitative and cell type–related terms. Thus, it is currently unclear whether the lack of detectable expression of membrane TWEAK in cells positive for TWEAK mRNA is related to highly efficient furin-mediated processing of memTWEAK to soluble TWEAK or to inadequate translation of TWEAK mRNA. Similar to Fn14, immunohistological data indicate that TWEAK protein expression is upregulated in damaged tissue and development, but the corresponding studies give typically no information about the cellular source of TWEAK and its degree of processing. Nevertheless, it appears that the TWEAK-Fn14 system is active in processes related to growth and remodeling of tissue and organs during development and tissue repair. In accordance with this, animal studies implicated the TWEAK-Fn14 system in liver progenitor cell proliferation (6, 7), regulation of muscle development and muscle regeneration (8–11), tumor-associated angiogenesis (12), and in various inflammation-related pathologies including graft-versus-host disease, systemic lupus erythematosus–related nephritis (13), 2,4,6-trinitrobenzene sulfonic acid-induced colitis (14), renal and cerebral ischemia (15–18), and collagen-induced arthritis (19, 20).

Similar to most other TNF receptors, activation of Fn14 typically results in stimulation of the classical and/or alternative NF-κB
pathway. Activation of various MAPKs, the PI3K/Akt pathway, and of apoptotic and necrotic cell death programs has also been reported (1, 2). In several examples of Fn14-mediated cell death, the cytotoxic effect has been traced back to the induction of TNF and subsequent activation of TNFR1, which in turn is able to trigger Fas-associated death domain protein– and caspase-8–mediated apoptosis or receptor interacting protein 1–mediated necrosis (21–23). However, in some studies there is also evidence for a TNF-independent mode of TWEAK/Fn14-mediated apoptosis, although no data are yet available concerning the underlying molecular mechanisms (21). Indeed, in general only little is known with respect to the role that the various Fn14-associated signaling pathways play in the physiological and pathophysiologi- cal TWEAK/Fn14-related processes discussed above. Moreover, corresponding studies must be considered in light of recent findings showing that soluble TWEAK only efficiently engages a subset of the Fn14-associated signaling events that are triggered by memTWEAK (24). For example, whereas both soluble TWEAK and memTWEAK are strong stimuli of the alternative NF-κB pathway, only memTWEAK also efficiently triggers the classical NF-κB pathway (24). Several members of the TNFR-associated factor (TRAF) family and Ral1 have been identified as Fn14-interacting molecules, but their relevance for defined aspects of Fn14 signal transduction is poorly understood (25–27). In molecular terms the best elucidated Fn14 signaling event is presumably TRAF2 recruitment and its relevance for alternative NF-κB signaling (28). TRAF2 forms a complex with cIAP1 and cIAP2 and this TRAF2–cIAP1/2 complex has the potential to interact in the cytoplasm with a complex of TRAF3 and NF-κB–inducing kinase (NIK), an MAPK kinase kinase upstream of IκB kinase (IKK)1 (29). As a consequence of the interaction of the two complexes, NIK becomes K48-ubiquitinated in unstimulated cells by the TRAF2-associated cIAPs and undergoes proteasomal degradation. The TWEAK-induced recruitment of the TRAF2–cIAP1/2 complex to Fn14 results then in depletion of the freely available cytosolic pool of TRAF2-containing complexes, NIK accumulation, and eventually to IKK1-mediated triggering of p100 processing to p52 and translocation of p52-containing NF-κB complexes into the nucleus (29). In addition to its inhibitory role in alternative NF-κB signaling, the TRAF2–cIAP1/2 complex has also a stimulating role in the classical NF-κB pathway in response to TNFR1 activation and also antagonizes TNFR1-induced cell death (30, 31). The TWEAK-induced depletion of cytosolic TRAF2–cIAP1/2 complexes can thus shift the quality of TNFR1 signaling from inflammation and survival to cell death (23, 32).

In this study, we show that TWEAK priming and depletion of TRAF2–cIAP1/2 complexes also affects the formation of CD40 signaling complexes, resulting in an inhibition of the inflammatory activities of CD40 ligand (CD40L).

Materials and Methods

Cell lines and reagents

The human osteosarcoma cell line U2OS was cultivated in DMEM whereas HeLa-CD40 or U20S cells were grown on glass coverslips. After treatment with Flag-TWEAK and/or Fc-Flag-CD40L, cells were fixed for 10 min at room temperature with 2% formalin in PBS, permeabilized for 5 min in PBS containing 0.1% Triton X-100, and nonspecific protein binding sites were blocked with a mixture of 10% goat serum and 1% BSA (30 min, room temperature). Samples were incubated overnight at 4°C with a p65 (Santa Cruz Biotechnology)- or p52/p100 (Upstate Biotechnology)-specific Ab (1:100 in PBS), washed three times (5 min) with PBS, and then treated with a Cy3-labeled secondary Ab (Dianova, Hamburg, Germany; 1:600 dilution in PBS, 1 h, room temperature). After rinsing the coverslips again three times in PBS, they were mounted on glass slides using Fluoromount-G (SouthernBiotech, Birmingham, AL) as an anti-fading compound. Coverslips were analyzed by confocal laser microscopy (MRC-1024; Bio-Rad, Munich, Germany) with a Plan APOCHROMAT 40/1.40 objective attached to an Axiovert 135TV microscope (both Carl Zeiss Microimaging, Göttingen, Germany). By using ImageJ software, nuclear and cytoplasmic fluorescence intensities were determined in >30 cells for every condition to calculate the ratios of nuclear to cytoplasmic fluorescence.

NF-κB Gpl reporter assay

HeLa-CD40 cells were electroporated (20 × 106 cells/ml, 4 mm cuvette, 250 V, 1800 μF, maximum resistance, 30 μg DNA) with the pNF-B-Gpl reporter plasmid and recovered overnight. Next day, cells were harvested and seeded in a 96-well plate at a density of 10 × 106 cells per well, and after an additional day cells were used in triplicates in stimulation experiments with Flag-TWEAK and Fc-Flag-CD40L. To finally determine reporter gene production, supernatants were assayed for luciferase activity.

Immunoprecipitation

One confluent 16-cm cell culture petri dish of cells was used per condition. After treatment, cells were washed twice with ice-cold PBS and scraped with a rubber policeman in 1 ml lysis buffer (30 mM Tris-HCl [pH 7.5], 1% Triton X-100, 10% glycerol, 120 mM NaCl) supplemented with a protease inhibitor mixture (Roche Diagnostics, Mannheim, Germany). Cells were lysed for 20 min on ice and then cleared by centrifugation twice (5 min at 5,000 × g) at 20 min at 14,000 × g) and the Fc-sc-Flag-CD40L (or Gpl-Flag- sc-Flag-CD40L)– or sc-TNF(143N/145R)–associated receptor complexes were precipitated from the cleared lysate with protein G-agarose beads (30 μl 50% slurry) at 4°C overnight. Lysates of untreated cells were...
supplemented with 5 ng ligand used for stimulation and served as a negative control. Protein G beads were finally released by resuspension of the beads in Laemmli sample buffer and incubation for 5 min at >80°C. In some experiments where cells were stimulated with a conventional stabilized trimeric CD40L variant (Flag-TNC-CD40L) (34), CD40 and CD40-associated proteins were immunoprecipitated by adding 1 µg/ml anti-CD40 to lysates of untreated and Flag-TNC-CD40L-stimulated cells. Ab–Ag complexes were then immunoprecipitated again with protein G beads and finally analyzed by Western blotting.

In some experiments immunoprecipitates were treated with calf intestinal phosphatase or lysis buffer supplemented with 4 M guanidinium chloride prior to denaturation in Laemmli sample buffer. For phosphatase treatment, beads were resuspended in 50 µl NEB3 buffer (New England Biolabs) after the fifth wash and incubated for 1 h at 37°C with 1 µl calf intestinal phosphatase (10 U/µl). After a last wash with lysis buffer, beads were again transferred to Laemmli sample buffer. As a control, lysates (10 µg protein) of 5 min Fc-CD40L–stimulated cells were diluted in NEB3 buffer, supplemented with phosphatase, and analyzed with respect to dephosphorylation of phospho-IκBα by Western blotting. Beads resuspended in guanidinium chloride buffer were incubated at 80°C for 30 min and subjected to Western blotting after 4-fold dilution in Laemmli sample buffer.

To crosslink proteins prior to immunoprecipitation in some experiments, the standard immunoprecipitation protocol was modified as follows. Activated CD40 complexes were obtained by stimulation of cells with 1 µg/ml Fc-CD40L for 20 min at 37°C. After the first 10 min of ligand stimulation, the crosslinker N-succinimidyl 3-(2-pyridyldithio)propionate (Thermo Scientific) was added from a 20 mM stock solution in DMSO to reach a final concentration of 1 mM. After an additional 10 min, cells were further processed as described above for the conventional immunoprecipitations.

Equilibrium binding studies with GpL-Flag-CD40L

Cells were cultivated in 24-well plates (2 × 10⁵ cells/well). Cells in half of the wells were blocked with 2 µg/ml Fc-Flag-CD40L for 30 min at 37°C to determine nonspecific binding. Samples were then treated pairwise (blocked versus untreated) with increasing concentrations of GpL-Flag-CD40L (1 h at 37°C). Unbound ligand molecules were removed by 10 rapid washes (total time <1 min) with ice-cold PBS, and cells of each well were collected in 50 µl culture medium (0.5% FCS, penicillin/streptomycin) using a rubber policeman. The cell-bound luciferase activity was quantified with the Gaussia luciferase assay kit (New England Biolabs, Frankfurt am Main, Germany). In brief, samples were treated in black 96-well plates with 10 µl substrate-buffer solution and the resulting light emission was recorded for 1 s per sample using a Lucy 2 luminometer (Anthos Labtec Instruments, Krefeld, Germany). Specific binding of GpL-Flag-CD40L was obtained by subtraction of nonspecific binding (samples blocked with Fc-Flag-CD40L) from total binding (samples with no pretreatment).

Results

TWEAK inhibits CD40L-induced activation of the classical NF-κB pathway

We have previously shown that soluble TWEAK trimers, in contrast to oligomerized or hexameric soluble TWEAK and membrane-bound TWEAK, only poorly activate the classical NF-κB pathway. Soluble TWEAK trimers are nevertheless sufficient to elicit efficient depletion of cytosolic TRAF2-containing complexes, resulting in strong activation of the alternative NF-κB pathway and inhibition of TRAF2-cIAP1/2–mediated functions in TNFR1 signaling (24, 32). To clarify the possibility that TWEAK regulates CD40 signaling in a related fashion, we analyzed the latter in HeLa and HT1080 cells stably transfected with CD40 as well as in 786-O and U2OS cells that display endogenous expression of CD40 (Fig. 1A). All cell lines also showed robust expression of Fn14 (Fig. 1A). As expected from our previous studies, Flag-TWEAK, a recombinant trimeric variant of soluble TWEAK, strongly induced p100 processing but failed to induce rapid phosphorylation and degradation of IκBα (Fig. 1B and data not shown). The poor activation of the classical NF-κB signaling pathway was not due to a general inability of the TWEAK receptor Fn14 to stimulate this pathway because Fc-Flag-TWEAK, a hexameric variant of soluble TWEAK mimicking the activity of memTWEAK, not only triggers p100 processing, the aforementioned hallmark of the alternative NF-κB pathway, but also phosphorylation and degradation of IκBα (24). Stimulation of CD40 using a highly active hexameric variant of CD40L also resulted in rapid and strong

![Figure 1](http://www.jimmunol.org/)

**FIGURE 1.** Activation of NF-κB signaling pathways by trimeric and hexameric TWEAK. (A) The indicated cell lines were assessed for cell surface expression of CD40 and Fn14 by flow cytometry using the CD40-specific mAb clone 82111, the Fn14-specific mAb ITEM-4, and corresponding control Abs. (B) HeLa-CD40, HT1080-CD40, U2OS, and 786-O cells were stimulated with Flag-TWEAK or Fc-Flag-TWEAK overnight and total cell lysates were analyzed the next day by Western blotting for the presence of the indicated proteins.
phosphorylation and degradation of IkBα in all four investigated cell lines (Fig. 2A). This CD40 response, however, was strongly diminished when cells were pretreated with Flag-TWEAK (Fig. 2A). To further ensure that the inhibitory effect of Flag-TWEAK on CD40L-induced phosphorylation and degradation of IkBα indeed translates into reduced classical NF-κB activation, nuclear translocation of p65 and production of an NF-κB reporter gene were analyzed. Immunocolocalization experiments revealed strong CD40L-induced nuclear translocation of p65 in otherwise untreated U2OS and HeLa-CD40 cells. In contrast, when these cells were primed by TWEAK there was almost no p65 redistribution upon CD40 activation (Fig. 2B). Likewise, in the cell culture supernatants of HeLa-CD40 cells transiently transfected with an NF-κB-controlled reporter gene encoding secretable GpL, there was a severe reduction of CD40L-induced luciferase activity upon TWEAK priming (Fig. 2C). In good accordance with the idea that the inhibitory effect of TWEAK priming is related to Fn14 depletion of TRAF2–cIAP1/2 complexes, it becomes apparent starting at 1.5–3 h priming time (Fig. 2D) what matches well with the known kinetics of TWEAK-induced p100 processing. Particularly, cell culture supernatants containing soluble TWEAK released from membrane-bound TWEAK also diminished CD40 signaling, indicating that the TWEAK-CD40 crosstalk can also take place with the naturally occurring soluble form of the molecule (Fig. 2E).

**TWEAK inhibits CD40L-induced activation of MAPKs**

Besides NF-κB, CD40 also utilizes various MAPKs for signal transduction. We therefore analyzed next whether TWEAK priming also interferes with these aspects of CD40 signaling. In all four cell lines investigated above with respect to CD40L-induced activation of the classical NF-κB pathway, we also observed strong activation of p38 and JNK and, to a lesser extent, ERK (Fig. 3). Again, all three responses were severely affected in TWEAK-primed cells (Fig. 3). Thus, TWEAK priming affects multiple proinflammatory CD40 signaling cascades.

**CD40L-induced CD40 signaling complex formation is severely affected in TWEAK-primed cells**

To evaluate the effect of TWEAK priming on early events in CD40 signal transduction, we investigated the composition of the CD40 signaling complex by protein G immunoprecipitation of cells stimulated with Fc-CD40L. In 786-O cells challenged with Fc-CD40L for 5 or 15 min, there was efficient coimmunoprecipitation of CD40 with its ligand (Fig. 4A). In contrast, in control cell lysates supplemented with an amount of Fc-CD40L comparable or exceeding to those that remained bound to Fc-CD40L–stimulated cells, there were only traces of CD40 in protein G immunoprecipitates (Fig. 4A). The anti-CD40 Ab–reactive band in the Fc-CD40L immunoprecipitates migrated significantly slower, as did most of the total CD40 contained in the corresponding lysates. The identity of the differently migrating CD40 bands was thus confirmed by a second independent CD40-specific Ab (Fig. 4B). To clarify whether the slower migrating CD40 band in Fc-CD40L immunoprecipitates represents a phosphorylated or not fully unfolded CD40 species, we treated immunoprecipitates with calf intestinal phosphatase or guanidinium chloride prior to Western blot analysis. However, both treatments had no effect on migration of immunoprecipitated CD40 (Fig. 4C). It is therefore unlikely that the slower migration of the immunoprecipitated CD40 species is caused by phosphorylation or incomplete denaturation. Based on the size difference of ~8–10 kDa between cytosolic CD40 and immunoprecipitated liganded CD40, it is tempting to speculate that the reduced mobility of the latter results from monoubiquitination or modification with another ubiquitin-related modifier protein.

Fc-CD40L also coprecipitated TRAF2, cIAP1, and cIAP2 (Fig. 4A). Notably, in TWEAK-primed samples the amount of coimmunoprecipitated CD40 was drastically reduced, and accordingly there were also strongly reduced amounts of TRAF2, cIAP1, and cIAP2 in the immunoprecipitates (Fig. 4A). To rule out that inefficient CD40 signaling complex formation in TWEAK-primed 786-O cells is an unrepresentative cell type–specific phenomenon, we also performed immunoprecipitation experiments with protein G in Fc-CD40L–stimulated U2OS, HT1080-CD40, and HeLa-CD40 cells. In all cases, there was again efficient coprecipitation of CD40 and TRAF2 with Fc-CD40L, indicative of CD40 signaling complex formation (Fig. 4D). As in 786-O cells, CD40L-induced formation of the CD40 signaling complex was severely affected upon TWEAK priming in all of these cell lines (Fig. 4D). Thus, the inhibitory effect of TWEAK on CD40L-induced proinflammatory CD40 signaling generally coincidences with a strong inhibition of ligand-induced CD40 signaling complex formation.

**CD40 cell surface expression and CD40L–CD40 interaction occur unchanged in TWEAK-primed cells**

To evaluate whether the reduced formation of the CD40 signaling complex in TWEAK-primed cells is related to downregulation of cell surface–expressed CD40, we analyzed the latter by FACs. In none of the CD40-expressing cell lines investigated was there a significant difference between untreated and TWEAK-primed cells (Fig. 5A). To confirm this by an independent method and also to gain insight into the stability of the CD40L–CD40 interaction, we also performed ligand binding studies. We recently demonstrated for TWEAK and the related ligands TNF and CD95L that these molecules can be labeled by genetic engineering using GpL as a reporter domain (36, 37). We therefore fused in a similar approach the GpL domain to CD40L and used the resulting GpL-Flag-CD40L fusion protein for cellular binding studies. GpL-Flag-CD40L was comparably as active as Flag-CD40L and displayed, similar to the latter, enhanced CD40 stimulatory activity upon oligomerization with Flag-specific Abs (data not shown). We also generated a GpL variant of the Fc-CD40L fusion protein used in this study for immunoprecipitation and CD40 activation, which is already highly active without further oligomerization. To avoid the consideration of potential avidity effects related to the hexameric organization of Fc-CD40L in the analysis of the binding studies, we used the trimeric GpL-Flag-CD40L molecule for investigation of the CD40L–CD40 interaction. Equilibrium binding studies resulted in $K_D$ values between 2000 and 7000 pM (Fig. 5B). More important, the affinity of CD40L–CD40 interaction remained essentially unaffected in TWEAK-primed cells, and maximum binding of the GpL ligand variant was also hardly affected, indicating again unchanged cell surface expression of CD40 (Fig. 5B). Thus, TWEAK priming showed neither a major effect on CD40 cell surface expression nor on the stability of the CD40L–CD40 complex in intact cells. One or more of the following mechanisms must therefore account for the strong reduction of CD40 in the protein G immunoprecipitates of Fc-CD40L–stimulated TWEAK-primed cells: first, attenuation of the interaction of protein G and CD40L-bound Fc-CD40L and/or weaker stability of the CD40L–CD40 interaction after solubilization; second, redistribution of CD40 into an insoluble compartment; or third, CD40 degradation. To initially address these issues, we performed TWEAK-CD40L crosstalk experiments with the GpL-tagged version of Fc-CD40L, which allowed accurate and quantitative tracing of the ligand during the various steps of an immunoprecipitation experiment. In accordance with the finding that TWEAK priming has no major impact on CD40 cell surface expression, we obtained comparable amounts of cell-associated

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FIGURE 2. TWEAK priming inhibits CD40L-induced activation of the classical NF-κB pathway. (A) Cells were primed for 6 h with Flag-TWEAK (200 ng/ml) or remained untreated. Cells were then challenged with Fc-Flag-CD40L (100 ng/ml) and activation of the classical NF-κB pathway was evaluated by Western blotting for the presence of IκBα and phospho-IκBα. Tubulin was detected as a control for using an equal protein input. (B) HeLa-CD40 and U2OS cells were grown on glass coverslips, primed with Flag-TWEAK for 6 h, stimulated for an additional hour with 100 ng/ml Fc-Flag-CD40L, and were then finally stained with Abs specific for p65. Shown are representative images (original magnification ×400) and the ratio of nuclear to cytoplasmic fluorescence intensity. (C) HeLa-CD40 cells were transiently transfected with a reporter plasmid encoding a NF-κB–regulated GpL gene. On the next day, transfected cells were seeded in a 96-well plate, and after an additional day half of the wells were primed with 200 ng/ml Flag-TWEAK for 6 h. Cells were stimulated with the indicated concentrations of Fc-Flag-CD40L and, finally, supernatants were collected and analyzed for luciferase activity. (D) 786-O cells were primed for the indicated times with Flag-TWEAK and were then stimulated with Fc-Flag-CD40L (100 ng/ml) for 5 and 15 min before total cell lysates were finally analyzed by Western blotting with respect to the presence of IκBα and phospho-IκBα. (E) 786-O, U2OS, and HT1080CD40 cells were primed for 6 h with supernatants (SN) collected from HEK293 cells transiently transfected by electroporation with an expression... (Figure legend continues)
GpL-Fc-CD40L luciferase activity in both groups. Determination of luciferase activity before and after cell lysis further revealed that >90% of the ligand was successfully immunoprecipitated (Fig. 5C, lower panel). Western blot analysis of pre- and post-immunoprecipitation lysates showed only minor changes in the amount of CD40 irrespective of TWEAK priming (Fig. 5C, upper panel). Because the GpL-Fc-CD40L concentration used for cell stimulation ensures high occupancy of cell surface-accessible CD40, and because immunoprecipitation of the ligand was quite effective, this suggests that a significant amount of total CD40 resided in intracellular compartments, for example, in the Golgi. In view of the unchanged efficacy of GpL-Fc-CD40L immunoprecipitation in TWEAK-primed cells, one can rule out that the observed strong reduction of CD40 in the protein G immunoprecipitates of such samples is caused by weakening the protein G interaction with CD40-bound Fc-CD40L or that there is a redistribution of CD40L–CD40 complexes into an insoluble compartment. Thus, it appears likely that TWEAK induces a cellular state in which the stability of CD40 signaling complexes is diminished. Indeed, anti-CD40 immunoprecipitation experiments allowed a similar conclusion. When untreated and TWEAK-primed cells were stimulated with high concentrations of a stabilized trimeric “Fc”-less variant of CD40L, and CD40 was then immunoprecipitated with CD40-specific Abs, CD40 and CD40-associated proteins were detectable in the nonprimed sample whereas only CD40 was present in the immunoprecipitates of TWEAK-primed cells (Fig. 5D). In further accordance with the formation of less stable CD40 receptor signaling complexes in TWEAK-primed cells, we observed increased association of CD40, TRAF2, and IKK2 with Fc-CD40L when cells were immunoprecipitated with the latter in the presence of the crosslinker N-succinimidyl 3-(2-pyridyldithio)propionate (Fig. 5E).

TWEAK priming attenuates activation of proinflammatory pathways by the CD40-related TRAF2-interacting receptor TNFR2

The inhibitory effect of TWEAK priming described in this study resembles our previous finding showing attenuation of TNFR1-induced NF-κB signaling upon TWEAK priming (32). However, there is at least one major difference in the underlying mechanisms. As described above, TWEAK priming results in inhibition of CD40 signaling by destabilization of the CD40L–CD40 complex in a way not allowing ligand coimmunoprecipitation with the receptor and receptor-associated proteins. In contrast, in the case of inhibition of TNFR1 signaling, ligand coimmunoprecipitation of receptor and receptor-associated proteins was largely unchanged in the TWEAK-primed cells and instead was related to impaired recruitment and activity of receptor-associated E3 ligases. Now, TNFR1 belongs to the death receptor subgroup of the TNF receptor family whose members do not directly interact with the TRAF2–cIAP1/2 complex whereas CD40 directly binds the TRAF2 subunit of this complex. We thus wondered whether signaling by another TRAF2-interacting TNF receptor is regulated by TWEAK in a similar fashion to CD40 signaling. In this respect we analyzed TNFR2. To avoid interference with TNFR1 signaling, we used a TNFR2-specific TNF mutant. Because soluble TNF trimers bind to TNFR2 but fail to activate the receptor properly, we further used the TNFR2-specific mutant in the form of a hexameric fusion protein with a Fc domain to overcome this limitation, yielding a protein mimicking the activity of membrane TNF, the natural activator of TNFR2. Stimulation of HeLa cells stably expressing TNFR2 with this construct resulted in activation of the classical NF-κB pathway as well as of the JNK pathway (Fig. 6A). These responses were attenuated upon TWEAK priming, but the inhibitory effect was clearly less pronounced as previously observed in case of the TWEAK-CD40 crosstalk. More importantly, ligand coimmunoprecipitation experiments revealed more or less equal amounts of TNFR2 in the immunoprecipitates, whereas there was reduced recruitment of TRAF2 and cIAP1/2 and also of the IKK complex, which interacts with TRAF2 and signaling intermediates ubiquitinated by the TRAF2–cIAP1/2 complex (Fig. 6B). Thus, attenuation of TNFR2 signaling by TWEAK seems to be based on depletion of E3 ligases required for signaling and therefore resembles the mechanisms of the TWEAK-TNFR1 crosstalk but are partly different from those of the TWEAK-CD40 crosstalk.

Discussion

In view of the involvement of TRAF2 and the TRAF2-interacting E3 ligases cIAP1 and cIAP2 in CD40 signal transduction (38), as well as our recent findings that TWEAK modulates TNFR1 sig-

FIGURE 3. TWEAK priming inhibits CD40L-induced activation of MAPKs. Untreated cells and cells primed for 6 h with 200 ng/ml Flag-TWEAK (200 ng/ml) were challenged for 0, 5, 15, and 60 min with Fc-Flag-CD40L. Activation of JNK, p38, and ERK1/2 was monitored by Western blotting with Abs specifically recognizing the phosphorylated forms of the various kinases. JNK, p38, and ERK1/2 were also detected to check for changes of protein levels. Tubulin served to control protein input.
FIGURE 4. TWEAK priming impairs CD40 signaling complex formation. (A) 786-O cells were primed with Flag-TWEAK (200 ng/ml, 16 h) or remained untreated. Cells were then stimulated for 5 and 15 min with 500 ng/ml Fc-Flag-CD40L or remained untreated. After removal of unbound Fc-Flag-CD40L, cells were lysed in immunoprecipitation lysis buffer. Lysates of cells not treated with Fc-Flag-CD40L were supplemented with 5 ng Fc-Flag-CD40L, and Fc-Flag-CD40L–associated protein complexes were isolated from all samples by immunoprecipitation with protein G-agarose beads. CD40 and components of the CD40-associated signaling complex were finally analyzed by Western blotting for the presence of the indicated proteins. (B) Cells were treated as in (A) and CD40 levels were analyzed by Western blotting using a second unrelated CD40-specific Ab. (C) Immunoprecipitates were again generated as described in (A), subjected to treatment with calf intestinal phosphatase (CIP) or guanidinium chloride buffer (GuCl), and were finally again analyzed by Western blotting. To control the efficacy of the dephosphorylation procedure, lysates containing phosphorylated IκBα were analyzed in parallel. Because high guanidinium chloride concentrations interfere with SDS-PAGE analysis, the corresponding samples were 4-fold diluted prior Western blotting. (D) The indicated cell lines were treated as described in (A), and immunoprecipitates and cell lysates were analyzed with respect to the presence of CD40 and TRAF2.
FIGURE 5. TWEAK priming does not affect cell surface expression of CD40 and CD40L–CD40 interaction. (A) The indicated cell lines were primed with TWEAK or remained untreated and were then analyzed for CD40 cell surface expression by flow cytometry. (B) For equilibrium binding studies with GpL-Flag-CD40L, cells (2 × 10^5 cells/well) were cultivated in 24-well plates and half of the cells were initially primed with Flag-TWEAK (200 ng/ml, 6 h). To determine nonspecific binding of GpL-Flag-CD40L in the case of 786-O and U2OS cells half of the samples of both groups were pre-treated (1 h, 37˚C) with an excess (1.5 μg/ml) of Fc-Flag-CD40L. In the case of HT1080-CD40 and HeLa-CD40 cells, nonspecific binding of GpL-Flag-CD40L was obtained by parallel treatment of the parental cell lines devoid of CD40 expression. All samples were cultivated for an additional 2 h in the presence of the indicated concentrations of GpL-Flag-CD40L. After removal of unbound ligand, cells were collected in 50 μl medium and cell-associated GpL-Flag-CD40L was quantified using a commercially available luciferase assay. Specific binding values (total binding – nonspecific binding) were fitted by nonlinear regression using GraphPad Prism 5 software to obtain maximal binding (Bmax) and Kd values. (C) The CD40 signaling (Figure legend continues)
naling by reducing the activity of the cellular pool of these signaling intermediates (32), in this study we evaluated whether TWEAK and its receptor Fn14 also affect CD40 signaling. Indeed, we observed in all four cell lines investigated that the capability of CD40 to activate the classical NF-κB pathway and MAPKs is strongly inhibited in cells primed with soluble TWEAK. Especially, the time of TWEAK pretreatment required to reach maximal desensitization of cells for CD40 signaling was ~3 h (Fig. 2D) and thus in the known range of the kinetics of p100 processing (e.g., see Ref. 32). Moreover, TWEAK priming also attenuated proinflammatory signaling by the CD40-related receptor TNFR2, although with lower efficacy (Fig. 6A). These observations were in accordance with the initial idea that depletion of cytosolic TRAF2 and cIAP1/2 complexes by triggering Fn14 with soluble TWEAK interferes with the functionality of TRAF2-cIAP1/2-utilizing receptors and in this manner resembles the mechanisms of the previously described TWEAK-TNFR1 crosstalk (32). Surprisingly, however, immunoprecipitates uncovered a more complex picture. As expected and observed before similarly in the TWEAK–TNFR1 crosstalk, there was a reduction of recruitment of TRAF2 and cIAPs as well as IκKs in the TNFR2 signaling complex of TWEAK-primed cells but no significant changes in the amount of immunoprecipitated receptor (Fig. 6B). There was a severe reduction of TRAF2 and cIAPs in CD40L immunoprecipitates, too, in TWEAK-primed cells, but this was also accompanied by strongly diminished presence of the receptor CD40. Because cell surface expression of the latter and CD40L binding were not reduced in TWEAK-primed cells, this implicates that the CD40L–CD40 signaling complex is less stable in TWEAK-primed cells or the subject of proteolytic degradation. Arguing against the latter, shortening the time for immunoprecipitation of Fc-CD40L from the cell lysates, as well as addition of various protease inhibitors, failed to improve the efficacy of CD40 coimmunoprecipitation (data not shown). From the CD95 ligand–CD95 system there is evidence that the cytosolic adapter proteins recruited to supramolecular ligand–receptor complexes contributed to their stability (39, 40). It is therefore tempting to speculate that in the case of CD40, stabilization of supramolecular ligand receptor clusters by associated adapter proteins, such as TRAF2, is more important than in other cases for the overall stability of these complexes.

CD40 is highly expressed on some types of immune cells, especially B cells and dendritic cells, but it is also found on vascular endothelial cells and smooth muscle cells (41). CD40 stimulation in endothelial cells takes place under proinflammatory conditions and contributes to upregulation of cell adhesion molecules such as ICAM-1, VCAM-1, and E-selectin and consequently in recruitment of immune cells and their transmigration across the endothelium (41). In the vessel wall, CD40 further triggers proliferation and activation of smooth muscle cells. In accordance with these vasculature-associated activities of CD40, it is not surprising that this receptor crucially contributes to atherosclerosis (42). Fn14 is not expressed on APCs but again prominently on endothelial cells and smooth muscle cells particular in situations of tissue damage (43). TWEAK enhances proliferation of endothelial cells and induces tissue factor and plasminogen activator inhibitor 1 in atherosclerotic plaques, and, indeed, pharmacological inhibition of TWEAK with TWEAK-specific Abs or Fn14-Fc reduces vascular damage in the ApoE knockout model of atherosclerosis (44–46). Furthermore, concentrations of soluble TWEAK have been found to be reduced in samples of patients suffering arterial hypertension (47), atherosclerosis (48), or abdominal aortic aneurysm (49) and are of poor prognosis in patients with ischemic heart failure (50, 51) and artery disease (52). Collectively, these data give clear evidence that the TWEAK–Fn14 system is of relevance for atherosclerosis and heart failure–associated pathologies.

FIGURE 6. TWEAK priming attenuates TNFR2 signaling but does not affect the overall stability of the TNF/TNFR2 signaling complex. (A) Cells were primed for 6 h with Flag-TWEAK (200 ng/ml) or remained untreated. Cells were then stimulated with Fc-scTNF(143N/145R) (100 ng/ml) for the indicated times. Activation of the classical NF-κB pathway and the JNK pathway were then evaluated by Western blot detection of IκBα, phospho-IκBα, c-Jun and phospho-c-Jun. (B) HeLa-TNFR2 cells were primed with Flag–TWEAK (200 ng/ml, 15 h) or remained untreated. Cells were then stimulated for 10, 20, and 30 min with 1 μg/ml Fc-scTNF(143N/145R) and after removal of unbound ligand cells were lysed in lysis buffer. As a negative control, lysates of cells not stimulated with Fc-scTNF(143N/145R) were supplemented with 25 ng ligand. Fc-scTNF(143N/145R) containing immune complexes were isolated by immunoprecipitation with protein G-agarose beads and the indicated proteins were detected by Western blotting.

complex of 786-O and U2OS cells was isolated from nonprimed and TWEAK-primed cells as described in Fig. 4 using GpL-Fc-Flag-CD40L instead of Fc-Flag-CD40L. Efficacy of the various steps of the immunoprecipitation procedure was determined by tracing the luciferase activity of the GpL ligand fusion protein (lower panels), and immunoprecipitates were finally again analyzed by Western blotting (upper panels). (D) 786-O cells were primed with Flag-TWEAK (200 ng/ml, 6 h) or remained untreated and were then challenged for 5 and 15 min with 500 ng/ml TNC-Flag-CD40L or remained unstimulated. Cells were washed twice and lysed in immunoprecipitation lysis buffer. Lysates were then supplemented with 1 μg anti-CD40 to isolate CD40 and CD40-associated proteins by immunoprecipitation with protein G-agarose beads. Indicated proteins were detected by Western blotting. (E) The CD40 signaling complex of nonprimed HeLa-CD40 cells and cells stimulated for 16 h with Flag–TWEAK were immunoprecipitated with Fc-Flag-CD40L after 20 min of stimulation. Where indicated, cells were treated with the crosslinker N-succinimidyl 3-(2-pyridyldithio)propionate during the last 10 min of Fc-CD40L stimulation.
In view of the cell type–specific effects of TWEAK and the different signaling capabilities of soluble and membrane TWEAK, however, it is as yet practically impossible to reasonably conclude from the available literature on the potential relevance of the TWEAK–CD40 crosstalk for special aspects of atherosclerosis and heart failure. In this context it might be especially important to keep in mind two aspects: 1) that the induction of a CD40L–unresponsive cellular state by TWEAK needs a few hours, and 2) that the inhibitory effect of TWEAK on CD40 signaling investigated in this study was triggered with the soluble form of the molecule, which itself is a poor activator of the classical NF-κB pathway and of the various MAPKs that are engaged by CD40, memTWEAK, however, can robustly activate these proinflammatory pathways in various cell types and is expressed on activated innate immune cells. memTWEAK–induced proinflammation may thus diminish the consequences of concomitantly triggered desen- sitization of CD40 signaling in endothelial cells in direct contact with the adherent immune cells. Accordingly, it is tempting to speculate that the soluble TWEAK–CD40 crosstalk helps to re- strict endothelial cell reactivity spatially and temporally. In initial experiments, we intended to analyze HUVECs and human coro- nary artery endothelial cells with respect to modulation of CD40–mediated effects by TWEAK. We regularly observed significant expression of CD40 in the primary endothelial cells, but stimulation with Fc–CD40L failed to induce significant upregulation of proinflammatory factors such as IL-8 and various cell adhesion molecules (Supplemental Fig. 1). Owing to this unexpected lack/weakness of CD40 activity, it was not straightforwardly possible to evaluate an inhibitory effect of TWEAK and Fn14 despite robust and functional expression of the latter. At first glance, the poor/missing CD40 response in our experiments with endothelial cells is at variance with the literature. However, reports demonstra- ting robust effects of CD40 activation on endothelial cells are often based on in vivo studies. Indeed, studies analyzing endo- thelial CD40 functions in vitro typically report modest responses that only reach a very small percentage of the same response induced by TNF (53–56). Furthermore, quite strong CD40L stimula- tion protocols have been used in these studies (10–20 μg/ml soluble CD40L or membrane CD40L transfectants) to see the comparable weak effects on CD40 activity (53–56). However, in our experiments with primary endothelial cells, we used Fc– CD40L at similar concentrations as in our other experiments where the concentrations of Fc–CD40L were fully sufficient to drive strong responses and which were also sufficient to drive maturation of dendritic cells (data not shown). Of note, primary endothelial cells were often cotreated with other proinflammatory triggers (IL-4, INF-γ, CMV infection) to increase CD40 expres- sion and CD40 responses (53–56). Conventional cultivated pri- mary endothelial cells might thus not represent a suitable model to analyze the relevance of the CD40–Fc40 crosstalk in this type of cells in vitro. Instead, it appears that this rather needs primary endothelial cells in a proinflammatory environment or appropriate in vivo models where CD40 signaling competence is ensured.

Disclosures

The authors have no financial conflicts of interest.

References


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