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Proinflammatory Effects of Tweak/Fn14 Interactions in Glomerular Mesangial Cells

Sean Campbell,* Linda C. Burkly,† Hua-Xin Gao,* Joan W. Berman,** Lihe Su,† Beth Browning,† Timothy Zheng,§ Lena Schiffer,§ Jennifer S. Michaelson,† and Chaim Putterman2*†

TNF-like weak inducer of apoptosis, or TWEAK, is a relatively new member of the TNF-ligand superfamily. Ligation of the TWEAK receptor Fn14 by TWEAK has proinflammatory effects on fibroblasts, synoviocytes, and endothelial cells. Several of the TWEAK-inducible cytokines are important in the pathogenesis of kidney diseases; however, whether TWEAK can induce a proinflammatory effect on kidney cells is not known. We found that murine mesangial cells express cell surface TWEAK receptor. TWEAK stimulation of mesangial cells led to a dose-dependent increase in CCL2/MCP-1, CCL5/RANTES, CXCL10/IFN-γ-induced protein 10 kDa, and CXCL1/KC. The induced levels of chemokines were comparable to those found following mesangial cell exposure to potent proinflammatory stimuli such as TNF-α + IL-1β, CXCL11/interferon-inducible T cell α chemottractant, CXCR5, mucosal addressin cell adhesion molecule-1, and VCAM-1 were up-regulated by TWEAK as well. TWEAK stimulation of mesangial cells resulted in an increase in phosphorylated IκB, while pretreatment with an IκB phosphorylation inhibitor significantly blocked chemokine induction, implicating activation of the NF-κB signaling pathway in TWEAK-induced chemokine secretion. Importantly, the Fn14-mediated proinflammatory effects of TWEAK on kidney cells were confirmed using mesangial cells derived from Fn14-deficient mice and by injection in vivo of TWEAK into wild-type vs Fn14-deficient mice. Finally, TWEAK-induced chemokine secretion was prevented by treatment with novel murine anti-TWEAK Abs. We conclude that TWEAK induces mesangial cells to secrete proinflammatory chemokines, suggesting a prominent role for TWEAK in the pathogenesis of renal injury. Our results support Ab inhibition of TWEAK as a potential new approach for the treatment of chemokine-dependent inflammatory kidney diseases. The Journal of Immunology, 2006, 176: 1889–1898.

Binding of TNF superfamily ligands (e.g., TNF, CD40L/CD154, Fas ligand) to their cognate receptors plays a crucial role in several fundamental biological processes, including apoptosis, cellular differentiation, and inflammation (1). TNF ligands and receptors are pivotal in maintaining normal immune homeostasis, and in the pathogenesis of autoimmune diseases as well. TWEAK-like weak inducer of apoptosis, or TWEAK, is a member of the TNF ligand superfamily originally described in 1997 (2). The TWEAK gene encodes for the synthesis of a type II transmembrane protein that, similar to TNF, is cleaved into a circulating trimeric form that mediates its biologic effects (2–4). The TNFR superfamily member Fn14 (TweakR) was conclusively identified as the receptor for TWEAK in 2001 (5). Fn14 mRNA is widely expressed, and can be found in endothelial and vascular smooth muscle cells, PBMC, kidney, heart, lung, skin, spleen, and brain. Further details regarding this relatively new ligand-receptor pair can be found in recent comprehensive reviews (6–8).

Although initial studies focused on a potential role for TWEAK/Fn14 interactions in apoptosis, more recent evidence demonstrates significant and physiologically relevant effects of TWEAK in angiogenesis and inflammation. In HUVECs, TWEAK (alone or in combination with other growth factors) enhances cell proliferation, migration, and resistance to apoptosis, and induces ICAM-1, E-selectin, CCL2/MCP-1, and IL-8 expression (9–12). Proinflammatory cytokine secretion has also been recently described following TWEAK stimulation of synoviocytes, fibroblasts (13), bronchial epithelial cells (14), and macrophages (15).

Chemokines that are induced in response to TWEAK are pivotal in the pathogenesis of many inflammatory renal diseases. However, while Fn14 mRNA is detected in normal kidney (5), it is not known which cells (if any) actually express the TWEAK receptor, or whether kidney cells are in fact responsive to TWEAK. In this study, we characterize the response of mesangial cells to TWEAK, primarily in induction of proinflammatory chemokine secretion, and identify a possible novel role for TWEAK in the pathogenesis of inflammatory renal diseases.

Materials and Methods

Cells and cell lines

Immortalized mesangial cells from BALB/c mice were derived, as described (16). Immortalized mesangial cells derived from C57BL/6 mice...
were a gift from M. Madaio (University of Pennsylvania School of Medicine, Philadelphia, PA). The B2 cell lines ZA8A3 and 71CF4 derived from C57BL/6 mice were a gift from C. Mohan (Southwestern Medical Center, Dallas, TX). Cell lines were maintained on tissue culture-treated plates in 10% DMEM (DMEM supplemented with 10% FCS, nonessential amino acids, glutamine, pyruvate, HEPES, and penicillin-streptomycin).

Primary mesangial cells were generated as follows: kidneys from 6-wk-old mice were diced in cold PBS, and passed through a series of progressively smaller stainless steel sieves (180, 100, and 71 μm). The resultant suspension was centrifuged at 2300 rpm for 5 min, and the supernatant was discarded. The glomeruli were digested with a 100 μg/ml solution of collagenase IV-S (Sigma-Aldrich) diluted in Leffert’s buffer for 30 min with gentle vortexing every 10 min, and washed twice in 20% DMEM. The enriched glomerular fraction was resuspended in 20% lagenase IV-S (Sigma-Aldrich) diluted in Leffert’s buffer for 30 min with TNF-α, IL-1β, IP-10, and CXCL1/KC in cell supernatants were performed Sigma-Aldrich.

Flow cytometry
Mesangial cells were detached with 2 mM EDTA in PBS, washed, and resuspended in 4 × 10^5 cells/ml in PBS/1% BSA (FACS buffer). A total of 2 × 10^5 cells (50 μl) was aliquoted into tubes, and incubated with 100 ng/ml Fc-TWEAK for 30 min at room temperature. The cells were washed in FACS buffer three times, and incubated with a 1/200 dilution of PE-labeled goat anti-human IgG-Fc (Jackson ImmunoResearch Laboratories) for 30 min at room temperature in the dark. Following three additional washes with FACS buffer, the cells were resuspended in 1% paraformaldehyde in PBS and analyzed by flow cytometry (BD Immunocytometry Systems).

ELISA
ELISA for CCL2/MCP-1, CCL5/RANTES, CXCL10/IP-10 induced protein 10 kDa (IP-10), and CXCL1/JKC in cell supernatants were performed using kits from R&D Systems and the manufacturer’s protocols. Murine TNF-α and IL-1β were from R&D Systems. LPS was purchased from Sigma-Aldrich.

Multiplex bead assay
Cell culture supernatants were analyzed using the Beadlyte Cytokine Profiler system (Upstate Cell Signaling Solutions), according to the following protocol. Samples or standards were incubated with the capture bead sets for the different cytokines in a 96-well filter plate for 2 h at room temperature. The capture beads with bound cytokine analytes were washed in the filter plate, and biotinylated reporter Abs were added for 1.5 h to complete the sandwich immunoassay. Streptavidin-PE was then added to each well and, after 30-min incubation, the beads were washed and resuspended in assay buffer. The median fluorescence intensity of 50 beads per cytokine was read using a Luminex100 instrument (Luminex). Concentrations were interpolated from standard curves, and samples were run in duplicate.

Design of primers, probes, and oligonucleotide standard templates for real-time PCR (TaqMan)
Oligonucleotide primers and TaqMan minor groove binder probes were designed from Affymetrix consensus sequences using Primer Express version 2.0.0 (Applied Biosystems). TaqMan minor groove binder probes were designed with a 5′ covalently linked fluorescent reporter dye and a minor groove binder/nonfluorescent quencher covalently linked to the 3′ end. Oligonucleotide standard templates were designed by the addition of 10 bp of gene-specific sequence to the 5′ and 3′ ends of the ampiclon. Reverse-phase HPLC-purified primers and oligonucleotide standard templates were purchased from Biosearch Technologies. HPLC-purified probes were purchased from Applied Biosystems. Primers and probe for GAPDH, used as an internal control, were purchased from Applied Biosystems.

cDNA synthesis and oligonucleotide standard curves
For preparation of RNA, 5 × 10^6 mesangial cells were detached from tissue culture plates and mixed with 1.5 ml of TRIzol reagent (Invitrogen Life Technologies), according to the manufacturer’s protocol, with an additional phenol:chloroform:isoamyl alcohol (25:24:1 (pH 6.6)) extraction. Purified total RNA was resuspended in diethyl pyrocarbonate H2O (Ambion), and the absorbance at 260 and 280 nm was recorded (Spectra Max Plus; Molecular Devices). Residual DNA was removed from 5 μg of total RNA using 5 U of DNase I amplification grade (Invitrogen Life Technologies) at 20°C for 15 min, followed by enzyme inactivation, according to manufacturer’s protocol. The DNase-treated RNA was used in a cDNA synthesis reaction using a high capacity cDNA archive kit, according to the manufacturer’s protocol (Applied Biosystems). cDNA reactions were stored at −20°C until use. Oligonucleotide templates were pooled and then serially diluted 1/10 eight times in 25 ng/μL yeast RNA (Ambion) to include a final range of 500 fM to 5 μM.

TaqMan thermal cycling
PCR for samples and standards were mixed in a 96-well plate, transferred to a 384-well optical plate (Applied Biosystems), and cycled in a 7900HT (Applied Biosystems) thermal cycler under the following conditions: 50°C for 2 min (uracil N-deglycosylase digest), 95°C for 10 min (activation of Taq thermostable polymerase), and 40 cycles of 95°C for 15 s and 60°C for 1 s. The fluorescence emission was collected every 7 s for the length of the run for each reaction well. Relative transcript quantities were determined for each sample by comparison with an oligonucleotide standard curve using Sequence Detection Software (Applied Biosystems).

Real-time PCR by SYBR green
Primers were designed using the PRIMER3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi), based on published sequence data from the Ensembl database (www.ensembl.org/Mus_musculus/). At least one intron was included to avoid genomic DNA amplification. Amplions ranged from 80 to 120 bp. Total RNA was reverse transcribed, and real-time PCR was performed in triplicate by the SYBR green method and the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems), using the following conditions; 10 min at 95°C, and 45 cycles of 95°C for 10 s, 60°C for 20 s, and 72°C for 30 s.

Signal transduction
A total of 3 × 10^5 mesangial cells was grown in 5 ml of tissue culture-treated plates for 24 h. The medium was removed, and fresh medium alone (standard medium) or medium + 100 ng/ml TWEAK was added to each plate for 0, 5, 20, 40, or 60 min. At the end of each time point, the cells were collected using the protocol provided by Cell Signaling Technology with the following modifications. Five hundred microliters of Laemmli buffer (Bio-Rad) with 50 mM DTT were added to each plate. The cells were collected by scraping, and the cell solution was passed six times through a 21-gauge needle. The lysates were boiled for 5 min, cooled on ice, and then run on a 10–20% Tris-HCl polyacrylamide gel (Bio-Rad). Using standard transfer techniques, the gel was transferred to a polyvinylidene difluoride membrane (Bio-Rad). The membrane was then probed with Abs against unphosphorylated and phosphorylated 14kD-α (Cell Signaling Technology).

Generation of TWEAK-deficient mice and isolation of anti-TWEAK mAbs
To generate TWEAK-deficient mice, a 8.12-kb HindIII subclone containing 3.36 kb of upstream sequence and the first five exons of the TWEAK gene was obtained from a BAC clone of 129 svJ genomic DNA (Genome Systems). The targeting vector was constructed from this subclone using the bacterial recombination method, as described (17). Construction of the targeting vector introduced an in-frame human CD2 CDNA upstream of a floxed deleted neomycin resistance gene in the place of the TWEAK coding sequence, with the translational start site through exon 3, thereby removing the first three TWEAK exons. TWEAK-deficient mice were generated by standard procedures using homologous recombination in embryonic stem cells (18). The neomycin expression cassette was excised from the locus by mating the neomycin-resistance mice with MSK2-Cre recombinase-expressing mice (19). Homozygous TWEAK-deficient, neomycin-deleted mice were generated by breeding heterozygous mice. Absence of TWEAK mRNA expression in knockout mice was confirmed by RT-PCR and Northern blot assays.

Murine Abs to TWEAK were generated by immunizing TWEAK knockout mice with 25 μg of TWEAK in CFA, followed by two to three boosts with 25 μg of murine TWEAK in IFA. A final boost just before sacrifice was given with 25 μg of murine TWEAK in the IFA adjuvant system (Cortiva). Hybridoma supernatants from spleenocyte fusions were screened for binding activity using standard methods of solid-phase ELISA, flow cytometry, and cell-based functional assays.
Generation of TWEAK and Fn14 constructs

Soluble mouse rTWEAK was generated with a 6× His tag attached at the N terminus of TWEAK (aa 106–249) with the N139Q substitution at the N-linked glycosylation site, and was expressed in Pichia, followed by purification with a precharged Ni Sepharose column (below referred to as TWEAK).

The human (h)Fc-TWEAK construct was generated by fusing the Fc portion of human IgG1 (20) to the N terminus of a cDNA fragment encoding soluble human TWEAK (aa 106–249). The construct was transfected into the 293T cell line to generate a stable expression line for protein production and subsequent protein A purification, as described (21). The murine (m)Fc-TWEAK construct (used for in vivo studies) was generated based on this hFc-TWEAK construct. Briefly, a DNA fragment encoding the soluble portion of mouse TWEAK was generated by PCR to replace the human TWEAK portion of the hFc-TWEAK construct, to obtain hFc-mTWEAK. Subsequently, a DNA fragment encoding the Fc portion of mIgG2a was generated by PCR to replace the hFc portion of hFc-mTWEAK, thus obtaining the final mFc-TWEAK construct. A stable Chinese hamster ovary cell line expressing mFc-TWEAK was generated for protein production.

To generate the Fn14-Fc construct, a cDNA fragment encoding the extracellular portion of murine Fn14 (aa 1–75) was ligated to the pEAG293 vector containing mouse IgG2a. The resulting murine Fn14-mFc was subsequently subcloned into the pV90 vector for stable expression in a Chinese hamster ovary cell line, as described (21). Due to high sequence homology (6, 7), the human Fc-TWEAK and murine Fn14-Fc constructs each recognized human and murine TWEAK receptor and ligand, respectively.

Cell proliferation assay

Mesangial cells (10⁴ cells/well) were plated in 96-well plates in quadruplicate and left to attach for 24 h. Cytokines (TWEAK at 100 ng/ml and/or IFN-γ at 80 IU/ml) were added on day 2, and the cells were refed 2 days later. Seventy-two hours after initial addition of the cytokines, 20 μl of the CellTiter 96 Aqueous One reagent (Promega) was added per well. Plates were read at 490 nm after 30–60 min.

TUNEL staining

A total of 5 × 10⁴ mesangial cells was grown in tissue culture-treated plates for 16 h. The medium was removed, and 100 ng/ml TWEAK, 100 IU/ml IFN-γ, or 100 ng/ml TWEAK + 100 IU/ml IFN-γ was added to each well for 48 h. The cells were collected using trypsin/EDTA, and TUNEL was stained using the In Situ Death Detection kit (Roche). TUNEL staining was detected by flow cytometry, and the data were analyzed using the CellQuest program.

Injection of mFc-TWEAK in vivo

Four- to 6-wk-old female C57BL/6-SCID mice were obtained from The Jackson Laboratory. Female Fn14-deficient C57BL/6 mice were generated at Biogen Idec, as described (22), and used at 4–6 wk of age. Mice were injected i.v. with 200 μg/mouse (in 100 μl of PBS) mFc-TWEAK or P1.17 (an irrelevant mouse IgG2a mAb (American Type Culture Collection), which shares the same Fc as mFc-TWEAK), or the same volume of PBS alone. Sixteen hours following the injection, the mice were sacrificed and the kidneys were obtained for RNA isolation and real-time PCR, as described above.

Studies involving animals

Animal studies were approved by the appropriate institutional review committees at the Albert Einstein College of Medicine and Biogen Idec.

Results

Murine mesangial cells express Fn14

Although Fn14 mRNA expression is detected in kidney tissue (5), it is unknown which cells express the receptor and whether actual protein is being displayed. Using real-time PCR, we found that transformed mesangial cell lines from the BALB/c (data not shown) and C57BL/6 mouse strains express Fn14 mRNA, comparable to the positive control murine Eph4 mammary epithelial cells (21). B cells, which do not express Fn14, were negative in this assay (Fig. 1A). We then examined whether there is cell surface expression of the TWEAK receptor on mesangial cells by flow cytometry. We found that there was significant binding to the surface of murine mesangial cells by hFc-TWEAK (Fig. 1B), but not by a CTLA4-Ig control fusion protein (R&D Systems) or a human IgG1 mAb (HP010; Serotec), which share the same IgG1 Fc as hFc-TWEAK (data not shown). To confirm the specificity of the interaction of TWEAK with a mesangial membrane receptor, blocking experiments were performed by preincubating hFc-TWEAK with an excess of Fn14-Fc before incubation with the mesangial cells. Prior incubation with Fn14-Fc eliminated the binding of hFc-TWEAK to mesangial cells (Fig. 1B). Our results indicate that a TWEAK receptor is indeed expressed on mesangial cells, and that the binding of hFc-TWEAK to mesangial cells is not mediated by the Fc portion of the fusion protein. Flow cytometry experiments using an anti-Fn14 mAb confirmed that Fn14 is the TWEAK receptor expressed on murine mesangial cells (data not shown).

TWEAK induces proinflammatory chemokine and cytokine production in mesangial cells via Fn14

TWEAK induces CCL2/MCP-1 in endothelial cells and CCL5/RANTES and CXCL10/IP-10 secretion in fibroblasts and synovocytes (9, 13). To determine whether TWEAK can up-regulate CCL2/MCP-1 and CCL5/RANTES in mesangial cells, we examined mRNA levels by real-time PCR in mesangial cells treated...
with 100 ng/ml TWEAK for 24 h. TWEAK treatment of transformed C57BL/6 mesangial cells up-regulated CCL2/MCP-1 mRNA by almost 5-fold, and CCL5/RANTES mRNA by 2-fold (data not shown). Similar induction of CCL2/MCP-1 and CCL5/RANTES mRNA expression was also seen using primary mesangial cells.

To analyze the effects of TWEAK treatment on chemokine secretion, we measured chemokine levels by sandwich ELISAs in conditioned medium from mesangial cells treated with TWEAK. We used primary and transformed cells from BALB/c and C57BL/6 for our experiments; because similar responses were observed in the various cell lines, only the data from the C57BL/6 mesangial cell line will be presented. TWEAK (1–1000 ng/ml) induces a dose-dependent increase in CCL2/MCP-1, CCL5/RANTES, and CXCL10/IP-10 secretion in murine mesangial cells (Fig. 2). CCL2/MCP-1 secretion was significantly higher than in control-treated cells (medium alone) at 10, 100, and 1000 ng/ml TWEAK ($p < 0.015, 0.004$, and $0.0003$, respectively). Furthermore, increased CCL2/MCP-1 concentrations were observed with escalating doses of TWEAK from 1 to 10 ng/ml ($p < 0.0025$), 10 to 100 ng/ml ($p < 0.0055$), and 100 to 1000 ng/ml ($p < 0.0035$).

Very similar results were seen with CCL5/RANTES and CXCL10/IP-10 induction, with chemokine concentrations increasingly significant vs medium beginning at 10 and 5 ng/ml TWEAK ($p < 0.015$ and $p < 0.003$, respectively), and likewise displaying significant dose-dependent increases between the different TWEAK concentrations tested (1 or 5–10 ng/ml ($p < 0.03$ for CCL5/RANTES and $p < 0.007$ for CXCL10/IP-10), 10–100 ng/ml ($p < 0.0015$ and $p < 0.0045$), and 100–1000 ng/ml ($p < 0.006$ and $p < 0.02$)). In all cases, chemokine secretion was significantly blocked by preincubation of TWEAK with Fn14-Fc (CCL2/MCP-1, $p < 0.0045$; CCL5/RANTES, $p < 0.00005$; and CXCL10/IP-10, $p < 0.01$). The magnitude of the chemokine response induced by higher levels of TWEAK was in a similar range to that induced by LPS or a combination of TNF-α and IL-1β, which are known proinflammatory stimuli for mesangial cells (Fig. 2).

To examine whether additional cytokines and chemokines are induced by TWEAK, we used the multiplexed, bead-based mouse cytokine assay, comparing the cytokine/chemokine profile in supernatant from TWEAK-treated mesangial cells (100 ng/ml for 24 h) with that from untreated cells. We detected a multifold increase in CCL2/MCP-1 and CCL5/RANTES levels (CCL2/MCP-1 and CCL5/RANTES of 3350 ± 115 pg/ml and 9275 ± 29 pg/ml in TWEAK-treated cells as compared with undetectable levels in the case of CCL2/MCP-1 and 1550 ± 115 pg/ml CCL5/RANTES, respectively, in control-treated cells (medium alone)). This increase in chemokine secretion in response to TWEAK was statistically significant ($p < 0.001$ and $p < 0.0002$ for CCL2/MCP-1 and CCL5/RANTES, respectively). Furthermore, the bead assay did confirm the multifold rises in CCL2/MCP-1 and CCL5/RANTES in response to TWEAK measured previously by ELISA. In contrast to the observed increases in CCL2/MCP-1 and CCL5/RANTES, other cytokines inducible in mesangial cells, including TNF-α, IL-4, IL-6, IL-10, IL-12, and GM-CSF, were not detectable in cell supernatants from TWEAK-stimulated mesangial cells. CXCL10/IP-10 was not available for measurement in this assay format. However, TWEAK treatment more than doubled the levels of CXCL1/KC from 1975 ± 29 pg/ml in control-treated cells to 4400 ± 115 pg/ml in TWEAK-treated cell supernatants ($p < 0.002$). A dose-dependent increase in CXCL1/KC in response to TWEAK was subsequently also confirmed by ELISA (data not shown).

We next performed time course analyses to quantify further the induction of chemokines in response to TWEAK treatment of mesangial cells. There was an increase over time in the concentrations of secreted CCL2/MCP-1, CCL5/RANTES, and CXCL1/KC, with the peak chemokine concentrations in this study measured at 24 h (Fig. 3). CCL2/MCP-1 induction relative to medium was significant at 6 h ($p < 0.0004$), with concentrations increasing further between 6 and 12 h ($p < 0.0007$) and between 12 and 24 h ($p < 0.003$). A very similar pattern was seen for CCL5/RANTES and CXCL1/KC in response to TWEAK, with induction relative to medium that was significant at 6 h ($p < 0.0025$ and $p < 0.008$, respectively), and chemokine concentrations increasing further between 6 and 12 h ($p < 0.0045$ and $p < 0.2$, respectively) and between 12 and 24 h ($p < 0.0004$ and $p < 0.004$, respectively). Chemokine induction in response to TWEAK was inhibited by Fn14-Fc, but not by P1.17. Addition of TNF-α to TWEAK-treated cells further increased chemokine levels, at least for CCL5/RANTES at 12 h (Fig. 3). However, chemokine stimulation in response to TWEAK plus TNF-α was usually less than additive, perhaps because a suboptimal concentration of TNF-α would be required to observe such an effect.

FIGURE 2. Chemokine secretion in response to TWEAK stimulation is dose dependent. C57BL/6 mesangial cells were cultured in standard medium (Media), or stimulated for 24 h with 10 ng/ml LPS, a combination of 100 ng/ml TNF-α and 20 ng/ml IL-1β, TWEAK at a concentration of 1 (5 for CXCL10/IP-10), 10, 100, 1000, and 100 ng/ml TWEAK preincubated with 2 μg/ml Fn14-Fc. Chemokine concentrations in conditioned medium were measured by commercial ELISA kits. A, CCL2/MCP-1; B, CCL5/RANTES; C, CXCL10/IP-10. Shown here are the mean ± SD values for duplicate wells; similar results were found in several independent experiments.
To learn whether the observed induction of chemokine production by TWEAK in mesangial cells is mediated via Fn14, we isolated mesangial cells from Fn14 knockout C57BL/6 mice (22). We confirmed that the cells lacked Fn14 by real-time PCR and flow cytometry (data not shown), and stimulated these Fn14-deficient mesangial cells with increasing concentrations of TWEAK. Mesangial cells isolated concurrently from Fn14-sufficient C57BL/6 littermates were used as a control. We found that while mesangial cells from Fn14 wild-type littermates responded to TWEAK with dose-dependent increases in CXCL10/IP-10, as shown above, chemokine secretion was not evident in response to TWEAK (but preserved in response to TNF) in Fn14-deficient mesangial cells (Fig. 4). Similar results were found for CCL2/MCP-1, CCL5/RANTES, and CXCL1/KC (data not shown).

CXCL1/KC was not previously identified as a TWEAK-regulated chemokine. To determine whether there are other novel inflammatory mediators induced by TWEAK that may be relevant in the pathogenesis of glomerular disease, we treated mesangial cells with 100 ng/ml TWEAK for 24 h and looked for mRNA up-regulation of selected chemokine and cytokine genes. By real-time PCR, TWEAK significantly up-regulates the gene expression of CXCL11 (interferon-inducible T cell /chemoattractant), CXCR5, mucosal addressin cell adhesion molecule-1, and VCAM-1 in mesangial cells (Fig. 5).

We demonstrated that TWEAK binding to Fn14 in mesangial cells in vitro induces production of proinflammatory cytokines. To support the in vivo relevance of this observation, we injected mFc-TWEAK i.v. into C57BL/6-SCID mice, and isolated total kidney RNA at 16 h postinjection. Control mice were injected with P1.17 (as an Fc control) or PBS. We found that mice injected with Fc-TWEAK had significantly increased up-regulation of kidney chemokine RNA, including CCL2/MCP-1 ($p < 0.025$ vs P1.17) and...

![Figure 3](http://www.jimmunol.org/content/ji/169/6/1893/F3.large.png)  
**FIGURE 3.** Chemokine secretion in response to TWEAK stimulation increases over time. C57BL/6 mesangial cells were cultured in standard medium (Media), or stimulated for 24 h with 100 ng/ml TNF-$\alpha$, 100 ng/ml TWEAK, a combination of 100 ng/ml TNF-$\alpha$ and 100 ng/ml TWEAK, 100 ng/ml TWEAK preincubated with 2 $\mu$g/ml Fn14-Fc, and 100 ng/ml TWEAK preincubated with 5 $\mu$g/ml P1.17. A, CCL2/MCP-1; B, CCL5/RANTES; C, CXCL1/KC. The effects of TWEAK are blocked by Fn14-Fc, but not by the Fc-matched mAb P1.17. Shown here are the mean ± SD values for duplicate wells; similar results were found in several independent experiments.

![Figure 4](http://www.jimmunol.org/content/ji/169/6/1893/F4.large.png)  
**FIGURE 4.** Fn14 mediates chemokine secretion in response to TWEAK in mesangial cells. C57BL/6 mesangial cells from Fn14-deficient and wild-type mice were incubated with 100 ng/ml TNF-$\alpha$ or 0.1–1000 ng/ml TWEAK for 24 h, and the concentration of CXCL10/IP-10 in the supernatant was measured by ELISA. Shown here are the mean ± SD values for duplicate wells. Similar results were seen for CCL2/MCP-1, CCL5/RANTES, and CXCL1/KC.

![Figure 5](http://www.jimmunol.org/content/ji/169/6/1893/F5.large.png)  
**FIGURE 5.** TWEAK up-regulates additional inflammatory mediators in mesangial cells. C57BL/6 mesangial cells were cultured in standard medium with or without 100 ng/ml TWEAK for 24 h, and the RNA was isolated, and real-time PCR (SYBR green) was performed, as described in Materials and Methods. Each real-time PCR assay was done in triplicate, and the mean of the three repeats was used as the result for that sample. Shown here are the mean ± SD values for fold change when each sample was separately normalized to GAPDH, $\beta$-actin, and $\beta$-2-microglobulin; similar results were found in several independent experiments.
CXCL10/IP-10 (p < 0.0015 vs P1.17) (Fig. 6A). To further confirm that circulating TWEAK up-regulates kidney chemokines and that this effect is mediated by Fn14, we injected mFc-TWEAK into female C57BL/6 Fn14 knockout mice and Fn14-sufficient (wild-type) littermate controls. We found that while Fe-TWEAK had the same effect as PBS on chemokine up-regulation in Fn14-deficient mice, injection of TWEAK in vivo significantly up-regulates kidney CCL2/MCP-1 (p < 0.035) and CXCL10/IP-10 (p < 0.025) gene expression in Fn14-sufficient mice (Fig. 6B).

The proinflammatory effects of TWEAK are not mediated by IL-1β, IL-6, or TNF-α

IL-1β and IL-6 have been shown to induce chemokine secretion in several different cell types, including mesangial cells. Therefore, we tested for induction of these cytokines in the supernatants of treated cells to determine whether TWEAK stimulation of chemokines might be indirect. However, we did not detect any significant levels of IL-1β and/or IL-6 in the supernatants of TWEAK-stimulated mesangial cells in the multiplexed bead assay. To further verify that the stimulatory effect of TWEAK on chemokine secretion by mesangial cells was not due to levels of IL-1β and IL-6 below the detection limit of the bead assay, we repeated the TWEAK stimulation experiments with addition of Abs to IL-1β and IL-6 (R&D Systems). Anti-IL-1β had no effect on the production of CCL2/MCP-1 and CXCL1/KC relative to the isotype-matched control; anti-IL-1β decreased CCL5/RANTES secretion, but significantly less than the isotype-control Ab or soluble Fn14 (p < 0.0002 for each comparison) (Fig. 7). Similarly, the effect of the anti-IL-6 Ab on TWEAK-induced chemokine secretion was equivalent (in the case of CCL2/MCP-1, p = 0.2) or less (p < 0.008 for CCL5/RANTES, and p < 0.02 for CXCL1/KC) than any inhibition seen with the isotype-matched rat Ab. Blocking TNF-α using a rat anti-mouse TNF-α mAb (clone MP6-XT3; eBioscience) also had no effect on TWEAK-induced chemokine production (data not shown). The latter experiment indicates that in contrast to KYM-1 rhabdomyosarcoma cells (23), the effect of TWEAK on mesangial cells is direct and not mediated by endogenous TNF-α.

**FIGURE 6.** Administration of TWEAK in vivo up-regulates kidney CCL2/MCP-1 and CXCL10/IP-10 gene expression. A, Female C57BL/6-SCID mice (4–6 wk old) were injected i.v. with 100 μl of PBS, or 200 μg/mouse (in 100 μl of PBS) mFc-TWEAK or P1.17 (n = 5 in each group). B, Female C57BL/6 Fn14 knockout mice (n = 6) or wild-type littermates (n = 5) were injected i.v. with mFc-TWEAK. Mice injected i.v. with the same volume of PBS (n = 2 in both the Fn14 knockout and wild-type group) were used to determine the baseline for comparison. A and B, 16 h following the injection, the mice were sacrificed and the kidneys were obtained for RNA isolation and real-time PCR, as described in Materials and Methods. Shown here are the mean ± SD of the values for each sample when normalized against GAPDH, except for the PBS-injected mice in B, for which the mean alone is shown. For illustration purposes, the normalized values for CCL2/MCP-1 were multiplied by 10^2, and the values for CXCL10/IP-10 were multiplied by 10^1.

**FIGURE 7.** Abs to IL-1β or IL-6 do not inhibit chemokine secretion in response to TWEAK. C57BL/6 mesangial cells were stimulated for 24 h with and without 100 ng/ml TWEAK and 5 μg/ml monoclonal rat anti-mouse IL-1β and IL-6. Rat IgG is an IgG2b mAb that was the rat isotype-matched control. A, CCL2/MCP-1; B, CCL5/RANTES; C, CXCL1/KC. Shown here are the mean ± SD values for duplicate wells; similar results were found in several independent experiments.
TWEAK treatment of mesangial cells induces phosphorylation of IκB-α.

It has been previously shown that TWEAK activates the NF-κB signaling cascade in fibroblasts (24); to determine whether a similar mechanism may be operative in mesangial cells, we examined IκB-α phosphorylation in TWEAK-treated mesangial cells. Mesangial cells displayed progressive IκB-α phosphorylation, beginning at 5 min after exposure to 100 ng/ml TWEAK and increasing throughout the first 60 min of stimulation (Fig. 8A). The relatively delayed kinetics of TWEAK-induced IκB-α phosphorylation in mesangial cells, as compared with that induced by TNF-α, has been reported also in bronchial epithelial cells (14).

To investigate whether TWEAK-induced chemokine secretion seen in mesangial cells is mediated by NF-κB activation or whether there is a major contribution of other signaling pathways (7), we treated mesangial cells with BAY11-7082 (E-3-(4-methylphenylsulfonyl)-2-propenenitrile; Calbiochem), an IκB-α phosphorylation inhibitor (25), at 5 μM for 30 min before TWEAK stimulation (14). Treatment of mesangial cells with BAY11-7082 significantly reduces chemokine production in response to TWEAK (Fig. 8B), confirming that TWEAK-induced chemokine secretion in these cells is mediated by activation of the NF-κB signaling pathway.

Murine anti-TWEAK mAbs block the proinflammatory effect of TWEAK on mesangial cells.

We developed mouse anti-TWEAK Abs as reagents for blocking the activities of TWEAK. A panel of anti-TWEAK Abs was derived by TWEAK immunization of TWEAK knockout 129/B6 mice, followed by recovery of anti-TWEAK mAbs by standard hybridoma technology. From the series of anti-TWEAK Abs that were generated, two Abs (2.P5.G9 and 2.P2.D10) showed the most effective blocking of TWEAK and were chosen for further study. Treatment of mesangial cells with the anti-TWEAK mAbs 2.P5.G9 and 2.P2.D10 (both of the IgG2a isotype) prevented the proinflammatory effects of TWEAK on these cells (Fig. 9). The 2.P5.G9 and 2.P2.D10 each significantly decreased CCL2/MCP-1 secretion from TWEAK-stimulated mesangial cells (p < 0.002), while the P1.17 isotype-matched control Ab showed no effect (p = 0.7).

Depending on the cell type and physiologic context, TWEAK can promote cell proliferation and/or cell death (3, 26). To determine which effect, if any, is induced in mesangial cells, we examined the effects of TWEAK on cell proliferation. Treatment with TWEAK and IFN-γ each had a small, but significant inhibitory effect on cell proliferation, relative to medium alone (p < 0.05). However, treatment using a combination of TWEAK and IFN-γ was synergistic, and significantly inhibited mesangial cell proliferation relative to TWEAK (Fig. 8B), confirming that TWEAK-induced chemokine secretion in these cells is mediated by activation of the NF-κB signaling pathway.

FIGURE 8. TWEAK induction of chemokines in mesangial cells is mediated by NF-κB. A, TWEAK activation of mesangial cells results in phosphorylation of IκB-α. Cell lysates from TWEAK-treated (100 ng/ml) and untreated C57BL/6 mesangial cells at the designated time points (in minutes) were separated by gel electrophoresis, and blotted with Abs against unphosphorylated (IκB) and phosphorylated IκB-α (P-IκB). B, Treatment of mesangial cells with an inhibitor of IκB-α phosphorylation significantly down-regulates chemokine production in response to TWEAK. BAY11-7082 was added to the culture medium at 5 μM, 30 min before adding TWEAK at a concentration of 100 ng/ml. After 24 h, chemokines were measured in the supernatant by ELISA. Shown here are the results for RANTES and KC; BAY11-7082 treatment significantly reduces MCP-1 levels as well. Higher concentrations of BAY11-7082 resulted in further reductions in secreted chemokine levels. The graph displays the mean ± SD values for duplicate wells; similar results were found in a repeat experiment.

FIGURE 9. Murine anti-TWEAK mAbs prevent the induction of chemokines in response to TWEAK stimulation of mesangial cells. The 2.P5.G9 and 2.P2.D10 are two IgG2a mouse mAbs against murine TWEAK, derived by immunization of TWEAK knockout mice, as described in Materials and Methods. P1.17 is an isotype-matched control Ab. C57BL/6 mesangial cells were left untreated, or treated with TWEAK with and without these mAbs at a concentration of 5 μg/ml for 24 h. Shown here are the mean ± SD values for duplicate wells; similar results were found in several independent experiments.
treatment with either cytokine alone (p < 0.003) (Fig. 10A). To
determine whether TWEAK induces apoptosis, TWEAK-treated
mesangial cells were stained by TUNEL and analyzed by flow
cytometry. Treatment with TWEAK or IFN-γ alone induced apo-
ptosis in a small percentage of mesangial cells as compared with
no treatment. However, in combination with IFN-γ, TWEAK had
a synergistic effect in inducing mesangial cell apoptosis (Fig. 10B).
The proapoptotic effect of TWEAK in combination with IFN-γ
was confirmed using propidium iodide and annexin V staining.
Furthermore, TWEAK-induced apoptosis in mesangial cells could
be blocked using the ITEM-4 anti-Fn14 or the 2.P5.G9 mAb (data
not shown).

**Discussion**

In this study, we found that murine mesangial cells express cell
surface TWEAK receptors. Stimulation of mesangial cells by
TWEAK had a marked and direct proinflammatory effect, with
significantly increased levels of secreted protein levels of CCL2/MCP-1,
CCL5/RANTES, CXCL10/IP-10, and CXCL1/KC. These effects
were mediated via the Fn14 receptor and the NF-κB signaling
pathway. Chemokine induction in response to TWEAK was time
and dose dependent, and was abolished by blocking TWEAK with
soluble Fn14 and by two novel murine IgG2a anti-TWEAK Abs.
Finally, injection of TWEAK in vivo significantly up-regulated the
kidney gene expression of CCL2/MCP-1 and CXCL10/IP-10, an
effect mediated by the Fn14 receptor.

Chemokines are critical for the recruitment of specific leukocyte
subsets to areas of tissue injury and inflammation (27). In the
context of the kidney, chemokines and chemokine receptors are cru-
cially involved in initiation, amplification, progression, and reso-
lution of renal disease from a variety of inflammatory insults, both
immune mediated (such as nephrototoxic serum nephritis, lupus ne-
phritis, immune complex glomerulonephritis, and experimental tu-
balointerstitial disease) and nonimmune mediated (such as renal
ischemia and ureteral obstruction) (27–31).

Among the many chemokines described in association with ex-
perimental kidney disease models and human diseases, CCL2/
MCP-1 and CCL5/RANTES play prominent roles in inflammatory
renal syndromes through their effects in the local recruitment of
monocytes and activated T cells (32). As an example, several lines
of evidence, including temporal expression patterns in the kidney,
support an important role for CCL2/MCP-1 and CCL5/RANTES
in the pathogenesis of lupus nephritis (32–34). Moreover, en-
hanced kidney expression of CCL5/RANTES accelerates renal
disease in the lupus-prone MRL-lpr/lpr mouse strain (35), while
down-regulating the effects of CCL2/MCP-1 using a small mole-
cule inhibitor (36) or by genetic approaches (37, 38) ameliorates
lupus nephritis in this mouse model. We describe in this study two
murine anti-TWEAK Abs, and find that these Abs prevent CCL2/
MCP-1 and CCL5/RANTES secretion in response to TWEAK.

These in vitro results are very promising, and suggest a novel
therapeutic approach to the treatment of renal disease: Ab inhibi-
tion of local and circulating TWEAK, an intervention intended to
block the proinflammatory effects of TWEAK on resident glomer-
ular cells, lower chemokine levels, and prevent leukocyte migra-
tion into the kidney.

We observed as well enhanced CXCL10/IP-10 secretion in re-
response to TWEAK stimulation of mesangial cells. Cells positive
for CXCR3, the receptor for CXCL10/IP-10, are prominent in
interstitial infiltrates in kidney biopsies in several renal diseases,
including IgA nephropathy, lupus nephritis, and membranoprolif-
erative glomerulonephritis. Furthermore, the number of CXCR3-
positive cells shows a positive correlation with decreased renal
function, proteinuria, and glomerulosclerosis, all important predic-
tors of the ultimate renal prognosis (39). Although by no means
definitive, these latter results would suggest that CXCL10/IP-10
binding to CXCR3 is an important determinant in the progressive
loss of renal function that may occur following a variety of in-
flammatory insults.

The chemokine CXCL1/KC induced by TWEAK may be in-
volved in a positive feedback loop that promotes glomerular in-
flammation. CXCL1/KC is a known chemoattractant stimulus for
neutrophils and mesangial cells (40). Furthermore, CXCL1/KC
up-regulates chemokine expression in mesangial cells, and may
also stimulate its own synthesis (41). Therefore, TWEAK stimu-
lation of CXCL1/KC production, as observed in our studies, may
serve to perpetuate cellular recruitment and renal infiltration by
inflammatory cells in different forms of nephritis.

Our in vivo experiments with i.v. administration of Fc-TWEAK
corroborate our studies with mesangial cells in vitro. It is possible
that kidney cells other than mesangial cells also display Fn14 and
may have contributed to the response seen following i.v. administration of TWEAK. In contrast, despite mesangial cells being only a small fraction of the cellular composition of the kidney (which also contains tubular, epithelial, and glomerular endothelial cells), we still were able to demonstrate that TWEAK leads to a significant increase in kidney CCL2/MCP-1 and CXCL10/IP-10 gene expression in vivo. Therefore, we believe that these in vivo studies lend further support to our in vitro findings in mesangial cells and indicate a role for circulating TWEAK as a novel mediator in inflammatory kidney diseases.

Although proinflammatory effects of TWEAK engagement have been described in several cell types, the complete scope of TWEAK effects has yet to be fully appreciated. In human endothelial cells, TWEAK up-regulates surface expression of the adhesion molecules ICAM-1 and E-selectin, and enhances secretion of CCL2/MCP-1 and IL-8 (9). In synoviocytes and fibroblasts, PGE2, MMP-1, IL-8, CCL5/RANTES, and CXCL10/IP-10 induction were seen (13). TWEAK treatment stimulates the secretion of IL-8 and GM-CSF from bronchial epithelial cells (14), and of CCL5/RANTES (but not CCL2/MCP-1 or IL-8) from keratinocytes (4). Finally, proinflammatory effects induced by TWEAK were also reported in astrocytes (42) and macrophages (15). However, kidney cells, and specifically mesangial cells, were not previously recognized as being TWEAK responsive. It is also important to note that while there are some chemokines/cytokines induced in response to TWEAK that are secreted by more than one cell type (e.g., CCL2/MCP-1), there are also important differences in the pattern of chemokine/cytokine induction. Indeed, we found that in addition to CCL2/MCP-1, CCL5/RANTES, and CXCL10/IP-10, TWEAK stimulates the expression of CXCL1/KC, CXCL11/interferon-inducible T cell α chemoattractant, CXC5, mucosal addressin cell adhesion molecule-1, and VCAM-1, the latter all not previously known to be regulated by TWEAK. The proinflammatory effects of TWEAK on kidney and other cell types will most likely depend on the type of cell being stimulated, its state of activation, and which additional stimulatory and inhibitory factors are present. Furthermore, TWEAK does not stimulate mesangial cells indiscriminately, as we found multiple chemokines and cytokines that were unaffected by TWEAK treatment. Rather, the effect of TWEAK on mesangial cells is specific for certain, but not other chemokines.

The effects of TWEAK on cell survival are context dependent. On one hand, TWEAK displays a proapoptotic effect on several human tumor cell lines (26, 43), although the effect is usually weak, as the name of this cytokine suggests. In contrast, in endothelial cells, TWEAK has a proliferative and prosurvival effect, either alone or in combination with other endothelial cell growth factors (11, 12). Moreover, TWEAK was found to play a dual role as an angiogenic regulator, with induction of endothelial cell survival in cooperation with basic fibroblast growth factor, while antagonizing a morphogenic response to vascular endothelial growth factor without affecting vascular endothelial growth factor-induced proliferation (11). We found that in mesangial cells, similar to what is observed in some tumor cell lines, TWEAK can synergize with IFN-γ to induce a measurable and significant increase in apoptosis. It is interesting that glomerular cell apoptosis has been linked to the pathogenesis and progression of lupus nephritis (44, 45), despite the proliferative phenotype observed in lupus renal disease. Nevertheless, the possibility that in certain cellular microenvironments TWEAK will demonstrate a proliferative effect on mesangial cells has not yet been completely excluded.

Polek et al. (46) reported that TWEAK can induce differentiation into osteoclasts of RAW cells that reportedly do not display the Fn14 receptor. As no other TWEAK receptor has been demonstrated to date, this observation may be explained by the recent finding by De Ketelaere et al. (47) that soluble TWEAK can internalize into several cell lines (not via Fn14) and translocate directly to the nucleus, leading to nuclear translocation of GSK-3β and p65 and induction of NF-κB-driven gene expression. Because in our studies TWEAK did not induce any measurable increase in chemokine production in Fn14-deficient mesangial cells, it appears that Fn14 alone mediates the inflammatory effects of TWEAK in these cells.

Fn14, the shortest TNFR superfamily member associated to date, displays a single putative TNFR-associated factor (TRAF) binding site that associates with TRAFs 1, 2, 3, and 5 (48, 49). Association of TRAFs is known to activate NF-κB-associated intracellular signaling pathways. Furthermore, the induction of NF-κB-regulated genes such as IL-6, IL-8, CCL5/RANTES, and ICAM-1 in TWEAK-treated cell lines also points to a role for the NF-κB pathway in transducing TWEAK signaling intracellularly (6), as indeed confirmed by several investigators (7). However, the blocking of chemokine secretion in TWEAK-treated astrocytes by an inhibitor of p38 (42) indicates that additional signaling pathways may also be involved. Nevertheless, while we have not ruled out that other pathways may be operative as well, in mesangial cells we clearly demonstrated that TWEAK-induced chemokine secretion is mediated by NF-κB signaling.

In conclusion, we demonstrate that TWEAK stimulation induces dose- and time-dependent secretion of potent chemoattractant cytokines in mesangial cells. As the chemokines induced by TWEAK are known to have an essential role in the pathogenesis of a variety of renal disorders, our results suggest a possible, previously unappreciated pathogenic role of TWEAK in immunological as well as in nonimmune mediated kidney disease. Although hamster anti-TWEAK Abs have been reported previously (13), Abs of murine origin that are likely to be less immunogenic and have a longer t1/2 in vivo would be very helpful in studying murine models of kidney disease. Thus, studies to support or disprove our hypothesis concerning an essential role for TWEAK in kidney diseases will be greatly facilitated by the availability of the novel blocking anti-TWEAK Abs of murine origin described in this work. Finally, we propose that TWEAK blockade may be a promising new approach for the treatment of chemokine-dependent renal diseases.

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