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TWEAK Is a Novel Arthritogenic Mediator

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TNF-like weak inducer of apoptosis (TWEAK) is a TNF family member with pleiotropic effects on a variety of cell types, one of which is the induction of proinflammatory cytokines by synovial fibroblasts derived from rheumatoid arthritis (RA) patients. In this study, we report that the serum TWEAK level was dramatically elevated during mouse collagen-induced arthritis (CIA) and blocking TWEAK by a neutralizing mAb significantly reduced the clinical severity of CIA. Histological analyses also revealed that TWEAK inhibition diminished joint inflammation, synovial angiogenesis, as well as cartilage and bone erosion. Anti-TWEAK treatment proved efficacious when administered just before the disease onset but not during the priming phase of CIA. Consistent with this, TWEAK inhibition did not affect either cellular or humoral responses to collagen. In contrast, TWEAK inhibition significantly reduced serum levels of a panel of arthritogenic mediators, including chemokines such as MIP-1β (CCL-4), lymphotactin (XCL-1), IFN-γ-inducible protein 10 (IP-10) (CXCL-10), MCP-1 (CCL-2), and RANTES (CCL-5), as well as the matrix metalloprotease-9. Exploring the possible role of the TWEAK/Fn14 pathway in human RA pathogenesis, we showed that TWEAK can target human primary chondrocytes and osteoblast-like cells, in addition to synovial fibroblasts. We further demonstrated that TWEAK induced the production of matrix metalloproteases in human chondrocytes and potently inhibited chondrogenesis and osteogenesis using in vitro models. These results provide evidence for a novel cytokine pathway that contributes to joint tissue inflammation, angiogenesis, and damage, as well as may inhibit endogenous repair, suggesting that TWEAK may be a new therapeutic target for human RA. The Journal of Immunology, 2006, 177: 2610–2620.

Tumor necrosis factor-like weak inducer of apoptosis (TWEAK) is a member of the TNF superfamily originally identified as a weak inducer of apoptosis in certain tumor cell lines (1). As with other members of the TNF superfamily, TWEAK has pleiotropic effects including proangiogenic effects on vascular endothelial cells (2, 3), proinflammatory activities on epithelial and endothelial cells (1, 4–6), as well as proliferation-enhancing effects on endothelial cells and astrocytes (2, 4, 7). The receptor for TWEAK, fibroblast growth factor-inducible gene 14 (Fn14/TWEAK-R), is widely expressed on a variety of tissues (8) and highly up-regulated in the context of tissue injury, regeneration (9–11), and inflammatory responses (10, 12).

Chicheportiche et al. (13) demonstrated that TWEAK can induce the production of proinflammatory cytokines and chemokines by normal human dermal fibroblasts and synovocytes obtained from rheumatoid arthritis (RA) and advanced osteoarthritis patient tissues. In the same series of experiments, TWEAK potentiated proinflammatory responses to TNF and IL-1β in normal human fibroblasts. Additionally, TWEAK had been shown (14) to induce the in vitro differentiation of a monocyte/macrophage cell line into functional osteoclasts. These in vitro activities, along with its ability to promote angiogenesis, suggest that TWEAK may play a role in both joint inflammation and tissue damage in the context of RA and osteoarthritis.

To investigate the possible involvement of the TWEAK/Fn14 pathway in arthropathy in vivo, we evaluated the efficacy profile of an anti-TWEAK-neutralizing mAb in a well-established mouse model for RA collagen-induced arthritis (CIA). By comparing various treatment regimens, we found that TWEAK blockade achieved its maximal clinical efficacy when treatment was initiated just before the collagen II boost and disease onset, and no efficacy was observed with treatment solely during the immunization phase. Blocking TWEAK affected neither the humoral nor cellular response against the immunogen collagen II. In contrast, TWEAK antagonism reduced the serum levels of a panel of known arthritogenic mediators, as well as synovial angiogenesis in the joints of arthritic mice. We further demonstrated expression of the TWEAK receptor Fn14 on multiple human joint cell types and that TWEAK stimulates the production of arthritogenic mediators from human synovocytes and chondrocytes. Interestingly, TWEAK also potently inhibited chondrogenesis and osteoblastogenesis in vitro, suggesting that it might impede endogenous joint repair mechanisms. Collectively, these results indicate that the TWEAK/Fn14 pathway is a novel arthritogenic mediator that contributes to joint tissue pathology through multiple mechanisms and may be a new therapeutic target in human RA.

Materials and Methods

Mice

Male DBA/1 (H-2b) mice were purchased from The Jackson Laboratory and were used at 6–10 wk of age. All experiments were performed according to the guidelines of the institutional animal care and use committee established at Biogen Idec, Inc.
Proteins, mAbs, and primary human cells

Two soluble forms of human TWEAK (recombinant soluble human TWEAK andFc-TWEAK) and the TWEAK-neutralizing hamster anti-TWEAK mAbs AB.G11 and BC.B10 were generated as previously described (2, 15). Mouse anti-TWEAK mAbs were generated by immunizing mice with chicken collagen type II. Lyophilized collagen II (Chondrex) was dissolved (2 mg/ml) in 0.05 N acetic acid and an emulsion was prepared (Ultra Turrax homogenizer, IKA Works) using equal volumes of tetramethylpentadecane; Sigma-Aldrich) by i.p. injection 3 wk before immunization with chicken collagen type II. For immunization, 100 mg of collagen II solution. For immunization, 100 mg of collagen II (mfgGa2a), which is a neutralizing Ab against both murine and human TWEAK, was used exclusively in the CIA studies and contained 0.06 endotoxin units/mg protein. Murine IgG2a control mAb was derived from the murine hybridoma P1.17 (American Type Culture Collection) and contained 7.1 endotoxin units/mg protein. rTNF and IL-1β were purchased from R&D Systems. The anti-human Fn14 mAb ITEM-4 was purchased from E Bioscience.

Human primary synovial fibroblasts were purchased from Cell Applications; and human primary chondrocytes, osteoblasts, and mesenchymal stem cells were purchased from Cambrex. All tissue culture and in vitro differentiation experiments were performed using reagents and protocols provided by the manufacturers. For some experiments, normal human osteoblasts were also derived from trabecular bone and cultured as previously described (16).

Induction of CIA

Male DBA/1 mice were sensitized with 400 μl of pristane (2,6,10,14-tetramethylpentadecane; Sigma-Aldrich) by i.p. injection 3 wk before immunization. Chicken collagen type II (Chondrex) was dissolved (2 mg/ml) in 0.05 N acetic acid and an emulsion was prepared (Ultra Turrax homogenizer, IKA Works) using equal volumes of CFA (2 mg/ml Mycobacterium tuberculosis, strain H37Ra; Chondrex) and collagen II solution. For immunization, 100 μl of the collagen II/CFA emulsion was injected intradermally, distributed over pinnae and one site on the back (100 μg collagen II plus 0.2 mg Mycobacterium tuberculosis H37Rv per mouse). On day 21, mice received an i.p. booster immunization (100 μg) with soluble collagen II diluted in PBS.

Treatments

Unless otherwise stated, P1.17 mlgGa2a (isotype control) and P5G9 anti-TWEAK mAb treatments were administered at 10 mg/kg by i.p. injection on days 20, 23, 27, 30, and 34 after collagen II/CFA immunization. We have previously conducted independent experiments in which both PBS and mlgGa2a control P1.17 Ab were directly compared and did not observe any significant difference between PBS and P1.17 treatment groups. Thus, the effect of anti-TWEAK was directly compared with P1.17 control Ig (same batch as previously used).

Clinical assessment of arthritis

Clinical development of CIA was monitored daily using a previously described (17) scoring system. Briefly, CIA severity was graded by overall assessment of inflammation on all four paws, apportioning a scale ranging from 0 to 4. Each paw was graded according to the following system: 0, normal; 1, mild but definite redness and swelling of the ankle or wrist, or redness and swelling of any severity for 1 or 2 digits; 2, moderate to severe redness and swelling of the ankle or wrist, or more than two digits; 3, redness and swelling (pronounced edema) of the entire paw; and 4, maximally inflamed limb with involvement of multiple joints. The sum of four individual scores was represented as the arthritis index, with the maximal possible score of 16 for each individual mouse. The average arthritis index was calculated on the basis of the number of experimental mice in each group.

Histology

Histological quantification of limbs was performed 38 or 40 days after collagen II/CFA immunization (Novo Pathology). For this, all four limbs from all animals in each group were collected and fixed in 10% neutral-buffered Formalin for 48 h, rinsed in running water, and transferred to a decalcifying solution consisting of 20% formic acid buffered with 10% sodium citrate for 5 days. Samples were then rinsed in running water, transferred to 70% ethanol, and processed into paraffin blocks using an automated tissue processor (ThermoShandon Pathcentre). Tissue sections were stained with trichrome, toluidine blue, and H&E. Histological evaluation of sagittal sections of both tibiotarsal and carpal/metacarpal joints included the following: inflammation, characterized by edema, vascular dilation, presence of fibroblasts, and cellular infiltrates including neutrophils and macrophages; bone erosion/resorption, depressions in the surfaces of cortical bone usually containing osteoclasts; and cartilage loss, decreased staining by toluidine blue, which binds to the basophilic proteoglycans in the cartilage. The levels of inflammation and bone resorption were quantified by scoring the magnitude of each finding on a scale of 0–4 for individual paws, corresponding to normal and severity grades of minimal, mild, moderate, and marked, respectively. For overall inflammation, inflammation of the joint capsule, intra-articular space, s.c. area, and bone were scored separately for each paw and totaled to obtain the overall inflammation score for each animal, with a maximum possible score of 16 for each paw and 64 for each animal. For bone resorption, paw scores were totaled to obtain the final score for each animal with a maximum possible score of 16 per mouse. For cartilage loss, toluidine blue staining was scored as normal, minimally decreased, mildly decreased, moderately decreased, markedly decreased, or severely decreased, and assigned scores of 0–5, respectively, for each paw, and totaled to a maximum possible score of 20 for each animal. For bone erosion, paw scores were totaled to a maximum possible score of 16 for each animal. The final scores shown for each category are averages for all 10 experimental animals in each group. Statistical significance was calculated using the Mann-Whitney U test. All scoring was performed by a pathologist who was blinded to the treatment groups.

Assessment of synovial angiogenesis

Synovial angiogenesis in the tibiotarsal joints of arthritic mice were analyzed by staining with anti-CD31 (Serotec), a standard marker for endothelium (18). For quantification, five randomly selected areas of the synovium from each rear paw were digitally captured at ×10 magnification, and the percentage of CD31 staining area was electronically quantified using a MetaMorph Imaging System (Molecular Devices) and averaged to obtain the percentage of the area staining for CD31 for each paw. The average percentages of the CD31 positive staining area shown were calculated from 17 and 16 rear paws from mlgGa2a and anti-TWEAK treatment groups, respectively. In paws with low clinical scores where there was less synovial vascularity, some adjacent nonsynovial tissues such as the cartilage and bone were also captured in the image. For analysis of the subgroup of paws that achieved the maximal clinical score of 4, regardless of treatment, the final average percentages of positive CD31 staining were obtained. Each 10 paws in each treated group and 4 TWEAK-treated group. A two-tailed t test was used to calculate the p values. To further ensure the specificity of vessel staining, tibiotarsal joint tissue sections were also stained with a polyclonal rabbit anti-human Von Willebrand factor (VWF; DakoCytomation) at 1/2500 dilution. Synovial vessel density based on VWF staining was quantified by counting of the number of well-defined luminal structures in the synovium in five randomly selected fields per joint section and was averaged across sections. VWF staining produced a level of nonspecific staining which precluded quantification by morphometrical analysis. The number of vessels per field was averaged for each rear paw, and average vessels/field were then calculated from 17 and 16 rear paws from the mlgGa2a and anti-TWEAK treatment groups, respectively.

Sera cytokine/chemokine analysis

Mice were bled by retro-orbital sinus under isoflurane anesthesia at various times. Blood was collected into microtainer serum separator tubes (BD Biosciences), allowed to clot, and centrifuged. Serum was decanted and stored at −20°C. All samples from normal and arthritic mice were assayed by either ELISA for TWEAK (see below) or MultiAnalyte Profile testing for a panel of cytokines/chemokines (Rules-Based Medicine). For cytokine/chemokine analysis, serum samples from mice that had been immunized with either mlgGa2a or P5G9 were pooled to develop the CIA scoring system. Serum from anti-TWEAK mAb P5G9 were obtained on days 23, 30, and 40 after CIA collagen II immunization. Analysis of individual mice was performed on day 40 samples, whereas day 23 and 30 samples were pools (two pools of five mice each per treatment group, mlgGa2a or P5G9). The least detectable dose (LDD) is defined as mean + 3 SDs of 20 blank samples and results below LDD are therefore considered less reliable, but not below level of quantification (BLQ).

CD4 T cell proliferation

Spleen cells were derived from individual mice on day 38 and CD4 T cells were enriched by magnetic cell separation using a CD4 T Cell Isolation kit (Miltenyi Biotec). Ninety-five percent CD4 T cells were incubated with 1 × 10^6 irradiated (2500 rad) syngeneic splenocytes as APCs in triplicate flat-bottom 96-well microtiter plates (Costar) for 72 h at 37°C in 5% CO, in the presence of denatured collagen II (30 min, 60°C, 100, 30, 10, 3, 1, 0.3, and 0.1 μg/ml) in DMEM medium plus 10% FCS. Supernatants were collected for IFN-γ production after 72 h from cultures stimulated with 100 μg/ml collagen II. To measure proliferation, 0.2 μCi
levels of collagen II-specific IgG2a and IgG1 were determined by ELISA and results were expressed as arbitrary units per milliliter as previously described (17). Briefly, ELISA grade collagen II (Chondrex) was coated onto 96-well flat-bottom microtiter plates (Nunc MaxiSorb) at 10 μg/ml overnight at 4°C. Following blocking with 2% skimmed milk solution, sera samples were titrated in PBS/0.1% Tween 20 and incubated for 30 min at 37°C. Following three washes, biotin-conjugated goat anti-mouse isotype-specific secondary mAbs (BD Pharmingen) were added and incubated for another 30 min at 37°C, followed by additional washes and the addition of streptavidin-HRP (BD Biosciences) and incubated at room temperature for 1 h. Tetramethylbenzidine substrate solution (BD Biosciences) was added to plates and incubated in the dark at room temperature for up to 1 h. Plates were read at 405 nm. Supernatants from ex vivo CD4 cell proliferation were assayed for IFN-γ by ELISA (R&D Systems).

**Matrix metalloprotease (MMP) production from redifferentiated primary chondrocytes**

The effect of TWEAK on chondrocytes was examined with redifferentiated cultured primary chondrocytes (Cambrex) according to the manufacturer’s protocol. Briefly, cultured human primary chondrocytes at 90% confluence were harvested, washed once in 155 mM NaCl2, and resuspended in 1.2% sodium alginate at 4 × 105 cells/ml. The cell/alginate suspension was then passed through a 22-gauge needle attached to a syringe dropwise into 4–5 volumes of 155 mM NaCl2 to form beads instantly. The formed beads were then allowed to further polymerize for 10 min followed by extensive washing in ~5 volumes of 155 mM NaCl2 five times and one wash in 2 volumes of chondrocyte differentiation medium (CDM). To induce MMP production, equal volume of beads were placed in wells of 12-well plates in 2 ml of regular CDM or CDM supplemented with either 30 ng/ml IL-1β, 20 ng/ml TNF, or 100 ng/ml recombinant soluble human TWEAK. Media were changed three times per week and supernatants were harvested after 4 wk and assayed using MMP activity assay kits (Amersham Biosciences).

**In vitro chondrogenesis and osteogenesis**

To induce chondrocyte differentiation from human mesenchymal stem cells, we followed the protocol provided by Cambrex. Briefly, human mesenchymal stem cells were washed with incomplete chondrogenic medium (differentiation basal medium and differentiation singlequots) and resuspended at a concentration of 5.0 × 106/ml in complete chondrogenic medium (differentiation basal medium and differentiation singlequots and 50 ng/ml TGF-β3). To generate individual chondrogenic pellets, 2.5 × 105 cells/ml suspension was aliquoted into 1.5 ml conical tubes and cultured with or without the addition of 10 ng/ml recombinant soluble human TWEAK or 500 ng/ml heat-denatured TWEAK. Cells were then pelleted at 150 × g for 5 min at room temperature and incubated at 37°C without disturbance for 24 h with cap loafened to allow gas exchange. Cell pellets were fed three times per week with a daily addition of complete chondrogenesis medium with or without TWEAK or heat-denatured preparation of TWEAK by 10 min of boiling. Pellets were harvested 24 days later and fixed in Formalin for either histological staining with Safranin O for glycosaminoglycans, or immunohistochemistry for type II collagen.

Terminal differentiation of human primary osteoblast precursors was also conducted using protocol provided by Cambrex (19). Briefly, 1 × 105 primary normal osteoblast cells (Cambrex) were seeded into 6-well plates and cultured in differentiation-inducing medium containing hydroxycitrate-21-hemisuccinate (200 mM) and β-glycerophosphate (7.5 mM). Cells were cultured for 2–3 wk (media were changed every 3–4 days) before staining with Alizarin Red for calcium deposition. To examine the effect of TWEAK on osteoblastogenesis, Fc-TWEAK (100 ng/ml) with or without the anti-TWEAK mAb ABG.11 (10 μg/ml) was added into the culture medium and replenished every 2 days.

To examine the effect of TWEAK on osteoblast proliferation, trabecular bone-derived osteoblasts were labeled with the fluorescent dye CFSE as described previously (16). Briefly, suspensions of cells were labeled with CFSE (Molecular Probes) at 10 μM in PBS containing 0.1% BSA (Sigma-Aldrich), for 10 min at 37°C. The cells were then washed three times in α-MEM medium containing 10% FBS and cultured for 6 days in the presence or absence of Fc-TWEAK (100 ng/ml), which was replenished after 3 days. Control cultures were treated with colchicine (100 ng/ml; Sigma-Aldrich) to inhibit cell division and provide an input labeling index for the parental population (0 cell divisions). Cells were removed by collagenase digestion and assayed for florescence by flow cytometry (Corixa). List mode data were reanalyzed using Modfit LT software (Verity Software House) to calculate the percentage of cells that had undergone successive cell divisions.

**Real-time RT-PCR analysis**

RNA was prepared from cells using TRIzol reagent (Invitrogen Life Technologies) as per the manufacturer’s protocol. Reverse transcription of 1 μg total RNA per sample was performed using Superscript III (Promega Group), as per the manufacturer’s protocol. Real-time PCR was performed using SYBR Green incorporation by our previously published method (20) for osteocalcin and GAPDH. The primers used for osteocalcin were: forward primer, 5′-ATGAGACCTCACCACCTTCGG-3′; reverse primer, 5′-GCTAGGCAACTGCTGACGTC-3′, which amplify a 257-bp product, and for GAPDH were: forward primer, 5′-ACCAGAGAAGTTTGGATGG3′; reverse primer, 5′-CAGTGAGACACTCCCTCAG-3′, which amplify a 142-bp product. PCR was conducted by a standard protocol for 35 cycles at an annealing temperature of 60°C on a Rotor-Gene thermocycler (Corbett Research).

**Apoptosis assay**

The cell death of cultured human mesenchymal stem cells cultured in the presence or absence of TWEAK was conducted using a modified annexin V7-aminomucinominycin D (7-AAD) staining method that allows detection using fixed cells for biohazard consideration. Briefly, after conventional staining with annexin V-PE and 7-AAD (BD Biosciences), cells were incubated with 20 μg/ml actinomycin D in the presence of 1% paraformaldehyde (in binding buffer) before FACS analysis.

**FACS analysis**

For surface expression of Fn14, cultured human primary synovial fibroblasts (two donors), chondrocytes (one donor), and osteoblasts (one donor) were collected by incubating in PBS containing 5 mM EDTA for 10 min. Cells were then suspended in FACS buffer (PBS with 1% FBS) and stained with the anti-Fn14 mAb ITEM-4 (21) for 1 h followed by a PE-conjugated secondary goat anti-mouse Fc (Jackson ImmunolnResearch Laboratories). The cells were analyzed on a FACS Calibur (BD Biosciences).

**Statistical analysis**

For differences in arthritis severity scores, day of disease onset and day of peak disease a two-tailed Mann-Whitney U test was used. For historological analysis, a one tailed Mann-Whitney U nonparametric test was used. A one-way ANOVA was used to analyze serum TWEAK levels and Student’s t test was used to analyze differences in stimulation indices and Rules-Based Medicine serum analysis, CD31 and VWF staining, as well as real-time RT-PCR analysis. A p value ≤0.05 was considered to be significant.

**Results**

Serum TWEAK level is elevated during CIA

Sera collected on days 23, 28, 30, and 38 after collagen II/CFA immunization were assayed for TWEAK levels and compared with the levels found in normal DBA/1 mice. As shown in Fig. 1A, TWEAK levels were significantly elevated during the course of CIA and peaked at a time when the majority of the animals developed disease (mean values ± SD of 48.3 ± 1.8 ng/ml on day 30 vs 12.0 ± 2.6 ng/ml in normal DBA/1 mice; p < 0.05), suggesting a role of TWEAK in CIA.
Initiation of treatment with anti-TWEAK mAb before collagen II boost significantly reduces disease severity

To assess the effect of TWEAK blockade in CIA, we administered anti-TWEAK or an isotype control Ab (mIgG2a) on day 20, one day before collagen II boost on day 21, followed by four additional mAb treatments on days 23, 27, 30, and 34. As shown in Fig. 1B, anti-TWEAK treatment significantly reduced the overall clinical severity of CIA, as well as the cumulative disease scores and average maximum disease score (Fig. 1C). However, the average day of disease onset and disease incidence rates were not significantly different from the isotype control-treated group (Fig. 1C).

The disease ameliorating effect of anti-TWEAK mAb treatment is mediated during the effector phase rather than the priming phase of CIA

To determine any potential involvement of the TWEAK/Fn14 pathway in the early phase of CIA during autoimmunogenic priming with collagen II, we compared several treatment regimens of mAb administration. In one group we administered prophylactically anti-TWEAK mAb only during the priming phase of the immune response (days −1, 1, 3, and 5 relative to collagen II/CFA immunization) and effector phase, n = 10. *, p < 0.05 (Mann-Whitney U two-tailed test).

Anti-TWEAK mAb treatment does not affect cellular and humoral immune responses to collagen II

Previous studies (22, 23) have demonstrated that the initiation of CIA depends on the anti-collagen II immune response as inhibition of either the cellular or the humoral arms of the adaptive immunity proved efficacious. We, therefore, examined whether TWEAK blockade altered the development and maintenance of anti-collagen II–specific Ab responses. Sera from mice treated with anti-TWEAK mAb according to different regimens were obtained 30 days after collagen II/CFA immunization and assayed for collagen II–specific IgG1 and IgG2a Ab levels. No difference in IgG1 or IgG2a anti-collagen II Ab levels was seen in any of the anti-TWEAK mAb-treated groups as compared with the isotype control-treated group (Fig. 3A). Similarly, we examined the ex vivo T cell proliferation to collagen II on day 38 following anti-TWEAK mAb treatment on days 20, 23, 27, 30, 34, and 38 days after collagen II/CFA immunization. We found that CD4 T cell recall responses to collagen were generally weak, as previously reported (24). Acknowledging that limitation, Fig. 3B nonetheless shows
that the CD4 T cell proliferative response to collagen II was unaffected by the in vivo administration of anti-TWEAK mAb and that IFN-γ production in response to collagen II was also unchanged (Fig. 3B).
controls based on analysis of day 40 samples from individual animals (Fig. 4). Arthritic mice treated with the anti-TWEAK mAb showed significantly \( p < 0.03 \) decreased serum levels of MMP-9, IP-10, lymphotactin, RANTES, and MIP-1\( \beta \) compared with the mIgG2a-treated control group (Fig. 4). Although there was no significant difference at day 40, MCP-1 levels appeared to be lower in the anti-TWEAK treated as compared with the control group on days 23 and 30, as was the case for TNF. Serum IL-6 did not show a relative change in serum level associated with CIA, yet IL-6 levels also appeared to be reduced on days 23 and 30 in the anti-TWEAK mAb group. Although the values for RANTES and TNF are generally lower in anti-TWEAK as compared with the mIgG2a-treated group, the reported levels are below the LDDs in both treatment groups and therefore considered less reliable. We did not see changes associated with anti-TWEAK treatment in other cytokines/chemokines included in the panel, such as LIF, M-CSF, GM-CSF, IL-1, IL-4, IL-10, IL-12, IL-17, IL-18, and IFN-\( \gamma \), among others.

Anti-TWEAK mAb treatment reduced synovial angiogenesis associated with CIA

Given TWEAK’s ability to promote angiogenesis, we also assessed the potential effect of TWEAK antagonism on synovial angiogenesis, a critical process contributing to joint inflammation and pathology associated with RA (25). Joint tissues taken 40 days after collagen II/CFA immunization of mice treated with either control mIgG2a or the anti-TWEAK mAb were stained with an anti-mCD31 Ab. As shown in Fig. 5A, pronounced CD31 staining can be seen in the inflammatory synovial pannus of paws from CIA mice treated with the control mIgG2a, whereas significantly reduced CD31 staining with less intensity was found in anti-TWEAK-treated mice (Fig. 5B). Based on morphometric quantification, there was a statistically significant reduction of synovial angiogenesis following anti-TWEAK treatment (Fig. 5C).

Inflammation often induces angiogenesis and there was marked inhibition of joint inflammation with anti-TWEAK treatment. To discern whether reduced synovial angiogenesis seen in anti-TWEAK-treated mice could be a direct effect on angiogenesis, not merely a secondary effect due to less inflammation, we quantified the CD31 staining in a subgroup of paws in which the maximal clinical score of 4 was reached with or without anti-TWEAK treatment. Our analysis revealed that even among paws with the same maximal clinical score, the average percentage of CD31-positive area in anti-TWEAK-treated paws \( (n = 4) \) was still significantly less than in control mAb-treated paws \( (n = 10) \), suggesting that TWEAK blockade had a direct effect in reducing angiogenesis in the synovium (Fig. 5D). The analysis of this subgroup of severely inflamed paws also allowed us to avoid the potential bias in our quantification (Fig. 5C) due to capturing adjacent nonsynovial tissue in less inflamed paw sections where there was much less synovial cellularity. Because CD31 can potentially also be found on inflammatory cells, we further performed staining of VWF with these joint sections. Consistent with the reduced angiogenesis indicated by CD31 staining, the number of well-defined vessels in the synovium as revealed by VWF staining was also significantly reduced.
reduced with anti-TWEAK treatment (Fig. 5, E–G), indicating inhibition of the TWEAK pathway reduced neovascularization of the synovium in the CIA model.

**TWEAK targets multiple human joint cell types**

To better establish the relevance of the TWEAK/Fn14 pathway in human RA, we surveyed resident cell types of the joint tissue for their surface expression of the TWEAK receptor Fn14. We found that synovial fibroblasts express Fn14, as expected based on a previous report by Chicheportiche et al. (13) demonstrating that they are TWEAK responsive. We further confirmed their observation that, similar to TNF, TWEAK induced the production of proinflammatory cytokines and chemokines such as IL-1, IL-8, and RANTES in synovial fibroblasts (data not shown). In addition, we also identified human primary chondrocyte and osteoblast-like cells as novel target cell types of TWEAK expressing Fn14 (Fig. 6A) and demonstrated that TWEAK induced the production of MMPs by chondrocytes (Fig. 6B). These results suggest that TWEAK may directly contribute to both joint tissue inflammation as well as cartilage and bone damage.

**TWEAK inhibits chondrogenesis and osteogenesis in vitro**

Because the TWEAK receptor Fn14 is expressed on progenitor cells of the mesenchymal lineage (T. S. Zheng, unpublished data), we examined whether the TWEAK/Fn14 pathway might regulate repair mechanisms responsible for replacing damaged bone and cartilage. Using a well-established in vitro system of chondrogenesis from human mesenchymal stem cells (19), we found that TWEAK, but not a heat-inactivated preparation of TWEAK, potently inhibited chondrocyte differentiation, as measured by collagen II deposition (Fig. 7A). Similarly, we found that TWEAK could also block the terminal differentiation of human primary osteoblast precursor cells as measured by calcium deposition, which could be reversed by the neutralizing anti-TWEAK mAb ABG11 (Fig. 7B). The inhibition of osteoblastogenesis by TWEAK was further demonstrated by the dose-dependent inhibition of osteocalcin mRNA expression (Fig. 7C). Because TWEAK was originally identified for its ability to weakly induce apoptosis in certain epithelial tumor lines, we investigated whether TWEAK prevented the terminal differentiation of chondrocytes and osteoblasts.
simply by inducing cell death. As shown in Fig. 7D, incubation of up to 500 ng/ml recombinant human TWEAK for 6 days did not increase cell death as compared with untreated mesenchymal stem cells cultured under the same conditions, as indicated by the percentage of annexin V-positive cells. Similarly, TWEAK also did not induce cell death of cultured osteoblast cells. Instead, TWEAK induced primary osteoblast proliferation, as shown using the CFSE labeling approach (Fig. 7E). These findings suggest the possibility that TWEAK specifically blocks chondrogenesis and osteoblastogenesis and may hinder endogenous repair of damaged cartilage and bone associated with RA.

Discussion

In the present study, we provided both in vitro and in vivo evidence supporting the involvement of a novel TNF family cytokine, TWEAK, known to be expressed by activated macrophages (1), in driving joint tissue inflammation and destruction. Our in vivo animal studies revealed a novel role of this cytokine pathway in the pathogenesis of CIA. TWEAK blockade does not appear to involve direct inhibition of collagen-specific immune responses, consistent with the fact that the TWEAK receptor Fn14 is not expressed on T and B cells (A. Jakubowski, unpublished results). Instead, our studies revealed that inhibition of the TWEAK pathway led to a significant decrease in joint inflammation and synovial angiogenesis. Based on the serum cytokine and chemokine panel analysis, we hypothesize that the most likely mechanism responsible for reduced joint inflammation involves decreased production of an array of arthritogenic mediators as a result of TWEAK inhibition, including MMP-9, RANTES, IP-10, MIP-1β, and IL-6 (26–28). Importantly, the production of these arthritogenic mediators is known (13) to be highly induced in human synovial fibroblasts upon TWEAK stimulation. This suggests that the effect of TWEAK blockade likely takes place in the joints although arthritogenic gene expression within the joints was not directly measured in our study. This notion is also supported by the observation that TWEAK serum level peaks around day 30 when joint manifestations of the disease occur. It is also worth noting that we cannot rule out the possibility that the effect of anti-TWEAK on cytokine/chemokine production could be more profound (both in magnitude and the nature of cytokines/chemokines affected) in the joints than what we were able to detect in the sera.

In addition to contributing to joint inflammation and angiogenesis, we also presented in vitro studies indicating that TWEAK may contribute to joint tissue degeneration by two additional mechanisms. First, TWEAK’s ability to induce MMP production by human chondrocytes indicates that the TWEAK/Fn14 pathway may directly promote bone and cartilage damage. Second, TWEAK may directly suppress joint tissue repair by inhibiting chondrogenesis and osteoblastogenesis from progenitor cells.

The pathogenesis of RA involves components of both adaptive and innate immunity (29). Although targeting either the humoral or cellular arms of the adaptive immune system provides clinical benefits as evidenced by recent positive clinical trial results (30) with Rituxan (anti-CD20, B cell depleting) and CTLA4-Ig, the precise etiology and nature of the pathogenic autoimmune response underlying RA remains obscure. In contrast, a large body of work in the past decade has provided a relatively clear framework of how cells of the innate immune system such as macrophages contribute to arthropathy. It is now recognized that chronic joint inflammation in established RA patients is likely to be self-perpetuated by synovial macrophages and fibroblasts through an elaborate milieu of cytokine mediators including TNF, IL-1, IL-6, IL-15, IL-17, and others. Within this intricate network of cytokines, the macrophage-derived TNF is considered a more upstream player due to its ability to
induce the production of other cytokines in synovial fibroblasts, underlying the effectiveness of TNF blockers in clinical studies. How TWEAK relates to other cytokines in contributing to joint inflammation remains to be better characterized; however, its ability to induce IL-6, IL-1, IL-8, IL-15, and IL-17 from human synovial fibroblasts based on transcription profiling studies (T. S. Zheng, unpublished results) suggests that TWEAK may be another upstream instigator of joint inflammation and tissue degenerative processes in a fashion similar to TNF. Given the rather remarkably similar activities exhibited by TWEAK and TNF on synovial fibroblasts and chondrocytes, one therefore wonders what their relative contributions are in vivo and how these two pathways may interact with each other. For example, these two pathways may be somewhat redundant because blocking either TNF or TWEAK only ameliorates, but does not abrogate clinical manifestations of CIA (31). Alternatively, TWEAK and TNF may synergize with each other in driving disease progression as suggested by their ability to synergistically induce production of several chemokines in human primary dermal fibroblasts (13). A thorough understanding of the interplay between TWEAK and TNF pathways in the context of joint inflammation and tissue damage will be critical to establishing the hierarchical relationship of TWEAK to TNF and other arthritogenic cytokines, and may also provide clues as to

FIGURE 7. TWEAK inhibits chondrogenesis and osteogenesis. A, Chondrogenesis from human mesenchymal stem cells was induced using a pellet-based method and assayed using either collagen II or safranin O staining. Cultures were treated with either TWEAK or heat-denatured TWEAK as indicated. B, Human primary osteoblast cells were cultured in either growth medium, or differentiation medium (DM) without or with Fc-Tweak and the anti-TWEAK-neutralizing mAb ABG.11. Differentiation was measured using Alizarin Red staining for calcium deposition. A and B, Representative figures are shown for results obtained in three independent experiments. C, Human primary osteoblasts cultured in the presence of a concentration range of recombinant TWEAK exhibited dose-dependent down-regulation of osteocalcin expression relative to GAPDH mRNA, as determined by real-time RT-PCR. Data are means of triplicate reactions ± SD. Significant difference to untreated (0 TWEAK) as determined by Student’s t test is indicated by asterisks (p < 0.01). D, Annexin V/7-AAD staining profiles for human mesenchymal stem cells cultured under subconfluent conditions in the presence or absence of human recombinant soluble TWEAK (500 ng/ml) for indicated times. E, Human primary osteoblasts were labeled with CFSE and cultured for 6 days in the presence or absence of TWEAK (100 ng/ml). Cell division was determined by flow cytometry and analysis using ModFit LT software. The percentage of cells having undergone zero to six cell divisions are indicated. Representative results are shown from experiments performed using three independent donor cells.
whether TWEAK might be a critical culprit underlying TNF blockade failure in certain patient populations.

The much reduced synovial angiogenesis in arthritic mice following anti-TWEAK treatment is a significant finding and may have important implications for TWEAK blockade as a potential human RA therapy. Synovial angiogenesis is now thought to be a critical component in RA pathogenesis, contributing to pannus proliferation, infiltration of inflammatory leukocytes, as well as osteophyte formation (25, 32). This notion is supported experimentally by increased angiogenesis found in the synovium biopsy samples from RA patients (33), and by demonstrations that targeting synovial neovascular formation by either inhibiting angiogenesis or inducing endothelial apoptosis could suppress mouse CIA disease progression in vivo (18, 34, 35). In this study, we showed that anti-TWEAK treatment resulted in reduced vessel formation in the synovium of mice with collagen II-induced arthritis based on both CD31 and VWF staining. In addition, we were able to demonstrate that even for a small subset of anti-TWEAK-treated paws that exhibited the maximal clinical score of 4, there was still a statistically significant reduction in CD31 staining as compared with paws from control IgG2a-treated mice with the maximum clinical score, thereby, dissociating the antiangiogenic effect of TWEAK blockade from its anti-inflammatory activity. These results support a direct role of TWEAK in promoting neovascularization formation in the synovium in addition to its proinflammatory effect and are consistent with previous studies (2, 3) indicating that TWEAK is a proangiogenic factor.

Our demonstration that TWEAK inhibited both chondrogenesis from mesenchymal stem cells and osteoblastogenesis from osteoblast precursors in vitro is intriguing. Although the in vivo relevance of these in vitro effects on cartilage and bone formation remains to be established, they may have significant implications. Although the role of inflammatory cytokines in driving tissue damage has been well established, their ability to directly inhibit the differentiation of tissue resident progenitor cells has just begun to emerge (36). The potent inhibitory effect of TWEAK on the terminal differentiation of both chondrocyte and osteoblast lineage cells in vitro suggests that, in addition to promoting joint tissue damage through induction of MMPs and other mediators of tissue damage, TWEAK may also directly impede endogenous mechanisms for bone and cartilage repair mediated by progenitor cells of the mesenchymal lineage (37). This notion is also consistent with the observation (38, 39) that despite the increased frequency of mesenchymal stem cells and osteoblast precursors found in the arthritic joint of human RA patients, effective bone or cartilage repair does not occur in these affected joints. At the present time, we do not know the precise mechanism by which TWEAK inhibited both chondrogenesis and osteoblastogenesis in vitro. Although TWEAK has been shown to induce cell death in several tumor cell lines and macrophages (1), we did not observe accelerated cell death of either mesenchymal stem cells or osteoblast precursor cells with TWEAK treatment. Rather, TWEAK augmented osteoblast proliferation and inhibited the expression of osteocalcin, a gene associated with osteogenesis, suggesting that TWEAK promotes an immature osteoblast phenotype. It has been well established (40) that TNF can inhibit the terminal differentiation of mesenchymal lineage progenitor cells such as myoblasts via an NF-κB-dependent mechanism. Whether or not the inhibition of chondrogenesis and osteoblastogenesis by TWEAK also operates through a similar mechanism remains to be investigated.

With our increased understanding of how multiple cytokines contribute to joint tissue inflammation and damage in human RA patients (41), significantly improved treatment outcomes have been achieved with new biological agents targeting cytokines like TNF, IL-1, and IL-6. Despite considerable progress, a substantial portion of RA patients remain inadequate responders to the current standards of care, indicating the heterogeneous nature of RA pathogenesis and likely the presence of additional arthritogenic mediators. Although we have not directly tested the effect of blocking TWEAK in CIA mice with established arthritis, historical studies (42–44) with TNF and IL-1 inhibition suggest that therapeutic treatment results in CIA mice do not necessarily correlate with clinical experience in RA patients. The results presented from both in vivo studies in CIA and in vitro studies with human joint cell types are consistent with the notion that TWEAK blockade may represent a novel therapeutic opportunity for the treatment of RA.

Based on this study and previously published reports (13), we propose that TWEAK is a novel arthritogenic mediator that may contribute to RA pathogenesis by multiple mechanisms. First, the production of TWEAK by infiltrating or synovium-resident macrophages (although we cannot rule out other sources of TWEAK, such as joint tissue stromal components) promotes joint inflammation by stimulating synovial fibroblasts to produce other inflammatory cytokines and chemokines, including IL-1, IL-6, IL-8, IP-10, RANTES, IL-15, and IL-17 (Ref. 13 and T. S. Zheng, unpublished results). Second, TWEAK directly triggers damage to the cartilage and bone by driving the production of a number of metalloproteases in chondrocytes and promoting osteoclastogenesis (14). Third, TWEAK contributes to joint tissue pathology through directly promoting synovial angiogenesis. And, finally, TWEAK can potentially impede the endogenous repair mechanism by blocking differentiation of precursor cells of the osteoblast and chordrocyte lineage. The potentially multifaceted contribution of TWEAK in RA disease progression suggests that targeting TWEAK may not only alleviate clinical symptoms associated with inflammation, but also promote joint repair by reversing TWEAK-mediated blockade on osteoblast and chondrocyte differentiation from progenitor cells, and therefore be beneficial for the treatment of RA.

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