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TWEAK/Fn14 Interactions Are Instrumental in the Pathogenesis of Nephritis in the Chronic Graft-versus-Host Model of Systemic Lupus erythematosus

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TNF-like weak inducer of apoptosis (TWEAK), a member of the TNF superfamily, is a prominent inducer of proinflammatory cytokines in vitro and in vivo. We previously found that kidney cells display the TWEAK receptor Fn14, and that TWEAK stimulation of mesangial cells and podocytes induces a potent proinflammatory response. Several of the cytokines up-regulated in the kidney in response to TWEAK are instrumental in Lupus nephritis; we therefore hypothesized that TWEAK/Fn14 interactions may be important in the cascade(s) leading to renal damage in systemic Lupus erythematosus. In this study, we analyzed the effects of Fn14 deficiency in the chronic graft-vs-host model of SLE, and the benefits of treatment with an anti-TWEAK mAb in this mouse model. We found that anti-nuclear Ab titers were no different between C57BL/6 Fn14 wild-type and deficient mice injected with allogeneic bm12 splenocytes. However, kidney disease was significantly less severe in Fn14 knockout mice. Furthermore, kidney IgG deposition, IL-6, MCP-1, RANTES, and IP-10, as well as macrophage infiltration, were significantly decreased in Fn14-deficient mice with induced lupus. Similarly, mice with induced Lupus treated with an anti-TWEAK neutralizing mAb had significantly diminished kidney expression of IL-6, MCP-1, IL-10, as well as proteinuria, but similar autoantibody titers, as compared with control-treated mice. We conclude that TWEAK is an important mediator of kidney damage that acts by promoting local inflammatory events, but without impacting adaptive immunity in this experimental LN model. Thus, TWEAK blockade may be a novel therapeutic approach to reduce renal damage in SLE. The Journal of Immunology, 2007, 179: 7949–7958.

E ngagement of TNF receptor superfamily (TNFRSF) members by their cognate ligands (TNF ligand superfamily) is important in the normal function of the immune system and in various autoimmune diseases. Specifically in systemic lupus erythematosus (SLE), abnormal TNF ligand superfamily/TNFRSF interactions are believed to play a central role in disease pathogenesis. Indeed, inhibition of several of these ligand/receptor pairs (e.g., CD40/CD40L, BAFF/BAFF-R) has dramatic therapeutic effects on murine lupus (1–3). Moreover, inhibiting interactions of TNF superfamily ligands and their receptors has also shown initial promise in human SLE (4, 5).

TNF-like weak inducer of apoptosis (TWEAK) is a member of the TNF ligand superfamily (6). Similar to TNF, TWEAK is cleaved into a circulating trimeric form that is thought to mediate its biologic effects. The TNFRSF member Fn14 was conclusively identified as a receptor for TWEAK in 2001 (7); in contrast to the promiscuity seen in some other TNF ligand-receptor pairs, TWEAK and Fn14 are evidently a monogamous ligand-receptor pair. Fn14 is widely expressed at the RNA level in solid organs (kidney, heart, spleen, and brain), and has been reported in several cell types including endothelial and vascular smooth muscle cells, epithelial cells, monocytes/macrophages, fibroblasts, and synoviocytes. Although the expression level is relatively low in normal tissue, Fn14 is highly up-regulated in the contexts of tissue injury and disease (7–11). Binding of TWEAK to the Fn14 receptor induces a strong proinflammatory response in various cell types, including macrophages, fibroblasts, and synoviocytes. Interestingly, several of the cytokines/chemokines induced by TWEAK are major contributors to the pathogenesis of lupus nephritis (LN) (12).

Recently, we have shown that Fn14 is expressed by murine kidney mesangial cells and podocytes, and that TWEAK stimulates mesangial cells to secrete high levels of potent proinflammatory chemokines including MCP-1, RANTES, IP-10, and KC (13). Justo et al. (14) reported that renal tubular cells display Fn14 as well. Furthermore, we found that SLE patients with active kidney disease (nephritis) have higher urinary TWEAK levels than lupus patients with inactive nephritis or with no history of renal involvement. Moreover, disease activity scores of lupus patients with nephritis showed a significant positive correlation with urinary
TWEAK levels (15). Finally, we also found that Fn14 is highly expressed in kidney biopsies of LN patients.

Based on these recent studies, we hypothesized that TWEAK/Fn14 interactions are pivotal in the inflammatory cascade in LN. Thus, blockade of TWEAK/Fn14 interactions may be a unique therapeutic approach that can improve inflammation in a major lupus target organ, and perhaps in other inflammatory glomerulopathies as well. In this study, we tested this hypothesis in the chronic graft-vs-host (cGVH) SLE mouse model.

The classic form of the cGVH lupus model, still in use today, is induced by the transfer of parent T cells into F1 mice (16, 17). In a more recent version of this model described by Eisenberg and colleagues (18, 19), cGVH is induced by the transplant of spleen cells from B6.C-H2<sup>fl/fl</sup>/KHeG (bm12) mice to cisogenic C57BL/6 (B6) recipients (bm12→B6). Donor (bm12) and recipient (B6) cells differ only by three amino acids in their I-A<sub>d</sub>-chain (MHC class II) that is sufficient to induce strong alloreactivity between B6 and bm12. Donor CD4<sup>+</sup> T cells react against incompatible host MHC class II molecules in the recipient, and become activated. These activated donor T cells interact with the recipient mouse’s B cells, which consequently produce autoantibodies that replicate the antigenic specificities characteristic of SLE (20). The advantages of the cGVH-induced lupus model are that: 1) it is inducible in the B6 background; 2) disease development is relatively quick and reproducible; and 3) onset of disease can be determined with certainty. We studied the development of autoantibodies and renal injury in Fn14-deficient mice with induced disease, as well as in Fn14 sufficient (wild-type, WT) mice with induced cGVH that were treated with neutralizing murine anti-TWEAK mAbs.

Materials and Methods

Mice

Female B6 Fn14 knockout (KO) mice (backcross generation no. 10) and WT littermates were bred at Biogen Idec and transferred to the Albert Einstein College of Medicine at 6 wk of age. For induction of cGVH-induced lupus, female bm12 mice, also at 6 wk of age, were purchased from The Jackson Laboratory. For the treatment studies with anti-TWEAK Abs, 6 wk old female B6 and bm12 mice were purchased from The Jackson Laboratory. All animal studies were conducted at, and approved by, Institute for Animal Studies at the Albert Einstein College of Medicine.

Induction of cGVH

cGVH was induced as previously described (21). In brief, disease was induced in 6-wk-old recipient mice by an intraperitoneal injection of a single cell suspension in HBSS of 10<sup>8</sup> donor spleen cells, prepared by pressing spleens through a wire mesh screen.Recipient and donor mice were sex- and age-matched within each independent experiment.

Blood samples were obtained from the experimental mice on the day before the transplant and every two weeks thereafter. Sera were stored at −80°C for later analysis. Proteinuria was measured on the day before splenocyte injection and every two weeks thereafter with reagent strips for urinalysis, where +1 is 30 mg/dl, +2 is 100 mg/dl, +3 is 300 mg/dl, and +4 is ≥2000 mg/dl (Bayer Corporation).

FIGURE 1. Prevention of TWEAK signaling does not affect serum IgG1 levels. At +11 wk after splenocyte injection, serum IgG1 and IgG2a (IgG2c in B6 mice) levels were quantified by ELISA. Left, Total IgG1 levels. Right, Total IgG2c levels. Due to the relatively lower affinity of B6 IgG2c to the anti-IgG2a capture Abs used in the assay, results for this isotype are shown in OD rather than absolute units. Shown here are the mean ± SE values. B6→B6, n = 8; B6→B6KO, n = 8; bm12→B6, n = 15; bm12→B6KO, n = 18. The data displayed in this figure are representative of two independent experiments.

ELISA for autoantibodies

ssDNA (100 μg/ml), dsDNA (100 μg/ml), histone (20 μg/ml), and chromatin (5 μg/ml) in PBS were separately coated onto Immulon II plates at 4°C overnight. Plates were blocked with 3% FCS for 1 h at 37°C, washed, and incubated with 1/250 dilutions of mouse sera for 1 h at 37°C. The plates were washed, and a 1/1000 dilution of alkaline phosphatase-linked secondary Ab (Southern Biotechnology) in PBS was added for 1 h at 37°C, followed by phosphatase substrate (Sigma-Aldrich) for 30 min at 37°C.

Quantitation ELISA for total IgG, IgG1, and IgG2c

Goat anti-mouse IgG, IgG1, or IgG2a Abs (Southern Biotechnology) at 5 μg/ml in PBS were coated onto Immulon II plates at 4°C overnight. Plates were blocked with 3% FCS for 1 h at 37°C, washed, and incubated with a serial dilution of Ab standards from 200 to 1.6 ng/ml and serial dilutions of mouse serum from 1/25000 to 1/100000 for 1 h at 37°C. The plates were washed, and a 1/1000 dilution of isotype specific alkaline phosphatase-linked secondary Abs (Southern Biotechnology) in PBS was added for 1 h at 37°C, followed by phosphatase substrate (Sigma-Aldrich) for 30 min at 37°C. Note that the anti-IgG2a Ab reagent was used to give a relative measure of IgG2c levels among the various experimental groups. The concentrations were calculated by comparing the OD level with the standard curve, as described (22).

Real-time PCR

Real-time PCR was performed as described (23). In brief, PCR primers were designed using Primer3 (www-genome.wi.mit.edu/cgi-bin/primer/primer3.cgi), and published sequence data from the Ensembl database (www.ensembl.org/Mus_musculus/). At least one intron was included to avoid genomic DNA amplification. Amplicons ranged from 80 to 120 bp. The genes that were amplified included IL-α, IFN-γ, IL-1, IL-4, IL-6, IL-10, IL-12, IL-18, CXCL11, CCL19, MCP-1, RANTES, CXCR3, CXCR4, CXCL11, and IP-10.

Total RNA was isolated from mouse kidneys using the TRIzol reagent (Invitrogen Life Technologies), reverse transcribed using oligo(dT), and real-time PCR performed in triplicate using the SYBR Green PCR Master mix 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. The depicted fold changes represent the mean of normalized expression ratios relative to GAPDH (used as a control gene), obtained in two independent experiments.

Histopathology and immunohistochemistry

Following sacrifice, one kidney was snap frozen at −80°C for later extraction of RNA; the other kidney was fixed in 10% formalin. Mouse kidneys were not perfused before harvest. For immunohistochemical staining, paraffin slides were deparaffinized in xylene and rehydrated in serial ethanol solutions. The sections were then treated by 0.02 mg/ml proteinase K in TE buffer (pH 8.0) at 37°C for 15 min in humidified chambers and cooled down to room temperature for 10 min. Sections were washed twice by PBS and blocked with 2% BSA in PBS containing avidin blocker (Vector Laboratories) for 1 h at room temperature, followed by the primary Abs anti-MCP-1, anti-RANTES, and anti-IP-10 (R&D Systems) at 10 μg/ml, anti-IL-6 (Pierce) at 20 μg/ml, and anti-mouse F4/80 (Serotec) at 1/100 dilution in PBS containing avidin blocker (Vector Laboratories) for 1 h at room temperature. The appropriate biotin-labeled secondary Abs were applied for 30 min, followed by the Vectastain ABC kit for 30 min and DAB substrate (Vector Laboratories) for 5 min. Sections were stained by hematoxylin and dehydrated in serial ethanol and xylene. One drop of Permount mounting medium (Fisher) was applied to the section and a cover glass.
mounted. IgG deposition in the kidney was similarly detected as previously described (24), and scored blindly on a scale of 0 to 4 (0, negative; 4, heavy glomerular deposition).

Protein array

Mouse sera were assayed for autoantigen binding by a multiplex proteome array system at the University of Texas/Southwestern Medical Center microarray core facility (Dallas, TX), as described in detail previously (25). The protein array chip used contains ~70 common lupus and control Ags printed in duplicate on coated glass slides. Each slide contains multiple duplicates of the same array, allowing for simultaneous testing of up to 16 samples on one slide. This multiplex autoantigen array system has been used extensively for detecting autoantibody profiles in human and mouse samples, with high specificity and sensitivity (25).

Statistical analysis

For continuous measurements (Ab titers), testing between any two experimental groups was performed by the Student t test, whereas three or more groups were tested by one-way ANOVA, which, if significant, was followed by pair wise comparisons between the groups using t tests with Bonferroni correction for multiple comparisons. When measurements were not continuous (proteinuria, staining intensity), the corresponding nonparametric tests were used to compare the experimental groups. The Mann-Whitney U test was used to compare two groups, whereas the Kruskal-Wallis test was used to compare three or more groups. Values of p < 0.05 were considered significant. The statistics program used for data analysis was Intercooled Stata version 9.2 (StataCorp). Data is shown as mean ± SE.

Results

Induction of cGVH in B6 Fn14 WT and B6 Fn14 KO mice

To determine whether TWEAK/Fn14 interactions are instrumental in the pathogenesis of LN, we compared the development of induced lupus-like disease (autoantibodies and nephritis) in WT and Fn14-deficient mice. Four groups of mice were studied: group 1, injection of B6 spleen cells to B6 mice (“B6→B6”) (negative control) (n = 8); group 2, B6 spleen cells to B6 Fn14 KO mice (“B6→B6KO”) (negative control) (n = 8); group 3, bm12 spleen cells to B6 mice (“bm12→B6”) (n = 15); and group 4, bm12 spleen cells to B6 Fn14 KO mice (“bm12→B6KO”) (n = 18). Mice were sacrificed at 11 wk following induction of cGVH. The experiments described below were repeated in an independent cohort of mice sacrificed at 13 wk, with very similar results (B6→B6: n = 4; B6→B6KO: n = 4; bm12→B6: n = 12; bm12→B6KO: n = 13).

Prevention of TWEAK signaling does not affect serum IgG levels

To evaluate whether deficiency in TWEAK signaling affects serum Ig levels or the relative distribution of the different IgG isotypes, we quantified total serum IgG1 and IgG2c Ig levels when the mice were sacrificed at 11 wk following the induction of cGVH. The bm12 recipient groups (bm12→B6 and bm12→B6KO) had significantly higher levels of total IgG1 as compared with the B6 recipient control groups (B6→B6 and B6→B6KO) (p < 0.01 and p < 0.001, respectively), but there were no significant differences in IgG1 levels between bm12 recipients dependent upon Fn14 WT and KO status (Fig. 1). Similarly, the bm12 recipient groups had significantly higher total IgG2c levels as compared with the B6 recipient control groups (p < 0.001 and p < 0.03, respectively); but whether the bm12 recipient was Fn14 WT or KO did not affect those levels (Fig. 1). When total IgG levels were measured, we similarly found that total IgG levels increased significantly (p < 0.00001) in the bm12→B6 vs the B6→B6 group, whereas a similar trend was observed in bm12→B6KO as compared with B6→B6KO (p = 0.06). However, total IgG levels were not significantly different between the two groups of bm12 injected mice (p = 0.6) (data not shown).

TWEAK deficiency has no effect on the titters of autoantibodies in cGVH-induced Lupus

Mice injected with allogeneic splenocytes in the cGVH model develop high titers of lupus associated autoantibodies including Abs to ssDNA, dsDNA, histone, and chromatin. These autoantibodies are believed to deposit in the kidney to induce the renal disease observed in this model. We compared autoantibody titers following induction of experimental lupus in the bm12→B6 group and the bm12→B6KO group in the cohort sacrificed at 11 wk post transplant. The groups receiving B6 splenocytes (B6→B6 and B6→B6KO) were used as controls, as these groups are not expected to develop autoantibodies. We found that two weeks after splenocyte injection, high titers of IgM (data not shown) and IgG anti-ssDNA, anti-dsDNA, anti-histone, and anti-chromatin Abs were induced in both bm12 recipient groups, as compared with the B6 recipient control groups (Fig. 2). Autoantibody titers to nuclear Ags in the bm12 recipient groups peaked at 2 to 8 wk following transplant, depending on the particular Ag, and declined thereafter.
However, at all time points there were no significant differences in IgM or IgG autoantibody titers against ssDNA, dsDNA, histone, and chromatin between the Fn14 KO or WT bm12 recipient groups (Fig. 2).

Fn14-deficient mice have reduced renal IgG deposition, cytokine expression, and macrophage infiltration following induction of experimental lupus.

Renal disease, including glomerular IgG deposition, is a major manifestation of cGVH-induced lupus. We determined whether Fn14 deficiency might influence renal disease by evaluating renal Ig deposition by immunohistochemistry. Although both bm12 recipient groups (bm12→B6 and bm12→B6KO) displayed glomerular IgG deposition at sacrifice (11 wk post transplant), the degree of deposition was significantly reduced in the bm12→B6KO group, in which TWEAK signaling was prevented (Fig. 3). No significant Ig deposition was seen in the control groups receiving B6 splenocytes (data not shown).

Previously, we had shown that TWEAK stimulates kidney cells to secrete cytokines and chemokines that are instrumental in the pathogenesis of LN. Accordingly, we studied cytokine and chemokine expression levels in kidneys of transplanted mice. We also analyzed cells that infiltrate the kidneys in LN (macrophages, neutrophils, T cells, and B cells), which are attracted in response to high local chemokine concentrations. By immunohistochemistry, we found that bm12→B6KO mice with induced lupus had significantly down-regulated levels of IL-6, MCP-1, RANTES, and IP-10 as compared with the bm12→B6 group (Fig. 3, A and B). Furthermore, staining for macrophages was significantly less prominent in the B6KO mice injected with bm12 splenocytes, as compared with the bm12→B6 group (Fig. 3, A and B), whereas there was no significant difference in the number of macrophages between bm12→B6KO and B6→B6KO mice. There were no differences between Fn14 WT and KO bm12 recipient groups in the degree of neutrophil or lymphocyte (B or T) infiltration (data not shown).

Besides inducing proinflammatory cytokines and chemokines, TWEAK can also affect cell proliferation and survival (13, 14, 26). As both kidney cell proliferation and apoptosis has been described in LN (27–29), we assessed renal cell proliferation and apoptosis by Ki-67 and caspase 3 staining, respectively. Renal cell proliferation (seen in both glomerular and tubular compartments) increased significantly in bm12→B6KO mice (p = 0.0001 for each comparison to the respective control group). Moreover, bm12→B6KO mice had significantly decreased renal cell proliferation as compared with bm12→B6 mice (p < 0.03) (Fig. 4). We could not detect significant renal or infiltrating cell apoptosis in any of the B6 or bm12 injected mice groups (data not shown).

TWEAK deficiency reduces proteinuria in cGVH-induced lupus.

To assess whether decreased kidney cytokine expression and macrophage infiltration results in decreased renal damage, we measured urinary protein levels in transplanted mice. As expected, proteinuria was evident in the bm12→B6 group by 2 wk after transplant, peaking at 8 wk. At 2 wk (p = 0.002), 4 wk (p = 0.007), 6 wk (p = 0.002), and 10 wk (p = 0.003) after splenocyte injection, proteinuria was significantly higher in the bm12→B6 as compared with the bm12→B6KO injected mice. Furthermore, there was no significant difference between proteinuria levels in bm12→B6KO mice and the control B6 recipient groups at weeks 2, 4, 8, and 10 (Fig. 5). No significant difference in the renal histopathology at the light microscopy level (which was not severe in either group) was found between bm12 recipient Fn14 WT and KO mice with cGVH induced renal disease (data not shown).
The effect of TWEAK deficiency on autoantibody fine specificity

Although anti-DNA and anti-chromatin Abs are not the only specificity of Abs deposited in the kidney in LN (30), these autoantibodies are believed to play a major role in pathogenesis, either directly or via cross-reactivity with renal Ags such as α-actinin (31–34). However, although we did not find significant differences in circulating autoantibodies between the bm12 injected groups (Fig. 2), bm12→B6KO mice had significantly reduced glomerular IgG deposition (Fig. 3). One explanation for this intriguing discrepancy might be that the enhanced local inflammation observed in bm12→B6 mice, and/or TWEAK itself (35, 36), may have led to increased vascular permeability, resulting in a nonspecific rise in the amount of deposited Ig. Alternatively, we questioned whether TWEAK deficiency may subtly alter Ab fine specificity such that Abs generated in an Fn14 deficient background may display differential antigenic affinity. Using the multiplex glomerular proteome we described recently (25), we compared IgG and IgM Ab titers to 70 common autoantigens between selected sera from the B6 and bm12 recipient groups. Fig. 6 shows a subset of these data for illustrative purposes. We found that the titer of IgG Abs against collagen IV, an important cross-reactive glomerular Ag in LN (37), was significantly reduced in the bm12→B6KO group when compared with the bm12→B6 group (Fig. 6). No significant differences were found in IgG titers against any other Ag on this array. Although it is important to acknowledge that due to the large number of Ags examined for each isotype a significant difference in a single Ag may very well be due to chance alone, it is interesting to note that collagen IV is one of the known specificities of autoantibodies in cGVH-induced lupus (20).

Anti-TWEAK Ab treatment of cGVH-induced lupus

To preclude the possibility that the decreased nephritis observed in Fn14 knockout mice is a result of deficiency in TWEAK signaling during development and/or potential compensatory changes, we assessed the therapeutic potential of blocking TWEAK/Fn14 interactions with Abs to confirm our results. We induced cGVH in B6 Fn14 sufficient mice by transplanting bm12 spleen cells, and evaluated the effects of treatment with a murine neutralizing anti-TWEAK mAb. Mice received intraperitoneal injections of PBS alone (n = 12), P5G9 (mouse IgG2a anti-TWEAK antibody) (13) (n = 12), and P1.17 (mouse IgG2a isotype control antibody) (n = 12) each at 200 μg twice a week after the transplant, and until the mice were sacrificed at 7 wk post transplant. A repeat experiment was also performed using separate cohorts of mice (PBS, n = 10; P1.17, n = 10; P5G9, n = 10), sacrificed at 11 wk post transplant.

Anti-TWEAK mAb therapy did not alter serum Ig levels

Total IgG1 and IgG2c levels were quantified by ELISA at 7 wk post transplantation in mice with cGVH-induced lupus treated with PBS, P5G9, and P1.17, and compared with the levels found in age and gender matched unmanipulated B6 mice (n = 6). Mice with induced GVH developed hypergammaglobulinemia, with higher levels of total IgG1 and IgG2c than normal B6 mice (Fig. 7). However, total IgG (data not shown), IgG1, and IgG2c levels (Fig. 7) in P5G9-treated mice were no different from those in mice treated with the P1.17 isotype matched control Ab. Similarly, no differences in total IgG, IgG1, and IgG2c levels between PBS, P1.17, and P5G9-treated mice were found in the second, independent cohort of mice in serum obtained at 11 wk after transplant (data not shown).

Anti-TWEAK Ab treatment does not affect autoantibody titers

We compared autoantibody titers in mice with induced lupus treated with PBS, P1.17, or P5G9 and sacrificed at 11 wk post transplant. Two weeks after transplantation, high titers of IgM anti-chromatin Abs were induced in all groups. At all time points, there were no significant differences in the titers of these Abs generated in an Fn14 deficient background compared with the B6 group (Fig. 8). Furthermore, in contrast to our results with Fn14 KO
mice, anti-TWEAK Ab-treated mice did not display a significant diminution in titers to collagen IV (data not shown).

Treatment with P5G9 reduces kidney cytokines and chemokines in mice with cGVH-induced lupus

To evaluate the effect of blocking TWEAK signaling in the cGVH-induced model of LN, we studied the gene expression of inflammatory cytokines, chemokines, and chemokine receptors in total kidney by real-time PCR at the height of disease (week 7), and when the disease was resolving spontaneously (week 11). At 7 wk after transplant, we found that IL-6, IP-10, CXCL1 (KC), and CXCR4 mRNA levels in kidneys of P5G9-treated mice were significantly reduced as compared with P1.17-treated mice. In fact, we found that P5G9 treatment decreased the levels of these proinflammatory mediators to those of age matched, normal B6 mice (Fig. 9). Similarly, when mice were sacrificed at week 11 post transplant, we found that kidney expression levels of IL-6, IP-10, and CXCR4 were still significantly reduced in P5G9 as compared with P1.17-treated mice. Furthermore, kidney expression levels of MCP-1, IL-1, IL-4, IL-12, IL-18, CXCL11, CCL19, and CXCR3 at week 11 were significantly decreased as well (data not shown).

The reduction in proinflammatory cytokines observed in mice treated with an anti-TWEAK mAb was confirmed by immunohistochemical staining of kidneys from cGVH mice at week 7 post transplant. Similar to the gene expression data, P5G9-treated mice showed significantly reduced kidney staining for IL-6 and IP-10 (Fig. 10). Significantly decreased kidney staining was observed for IL-6, IP-10, CXCR3, and CXCR4 in mice treated with P5G9 as compared with P1.17-treated mice. Furthermore, kidney expression levels of MCP-1, IL-1, IL-4, IL-12, IL-18, CXCL11, CCL19, and CXCR3 at week 11 were significantly decreased as well (data not shown).

Proteinuria was evaluated over time in the various treatment groups. In the cohort sacrificed after 11 wk, there were significantly diminished levels of proteinuria in P5G9 as compared with P1.17-treated mice at week 4 (p < 0.01), week 6 (p = 0.004), and week 8 (p = 0.05) post transplant (Fig. 11). We assessed proteinuria also in the second cohort of mice sacrificed at the height of disease (7 wk after transplantation), and found very similar results. At week 4 and week 6 following splenocyte injection, there were significantly decreased levels of proteinuria in P5G9 as compared with P1.17-treated mice (data not shown).

Discussion

Experimental models of SLE are characterized by high titers of autoantibodies against nuclear Ags, and varying degrees of autoantibody-mediated glomerulonephritis. The cGVH system is one such established animal model of lupus that has been valuable in dissecting various aspects of disease pathogenesis (21, 38–41). In this system, we found that deficiency of the TWEAK receptor Fn14 decreased renal damage as indicated by proteinuria, without down-regulating hypergammaglobulinemia or the titers of IgM and IgG autoantibodies against ssDNA, dsDNA, histone, and chromatin. Nevertheless, glomerular Ig deposition and kidney cytokine expression (including IL-6, MCP-1, and IP-10) were significantly decreased in Fn14 deficient mice as compared with WT mice. Results were highly consistent in an independent set of studies, in which mice, P5G9 treatment in Fn14 sufficient mice with induced lupus resulted in significantly reduced glomerular IgG deposition as compared with P1.17 and PBS-treated mice (Fig. 10).

**FIGURE 7.** Anti-TWEAK mAb therapy does not alter serum Ig levels. Serum IgG1 and IgG2a (IgG2c in B6 mice) levels at week +7 were quantified by ELISA (n = 12 in each of the PBS, P1.17, and P5G9 groups). Serum from age and gender matched normal B6 mice were assayed as well (n = 6). Left, Total IgG1 levels. Right, Total IgG2c levels. Due to the relatively lower affinity of B6 IgG2c to the anti-IgG2a capture Abs used in the assay, results for this isotype are shown in OD rather than absolute units. Shown here are the mean ± SE values. There are no significant differences in the IgG1 and IgG2c levels between P1.17- and P5G9-treated groups.
treatment of cGVH-induced lupus with an anti-TWEAK Ab significantly decreased renal inflammation and proteinuria but without affecting serum autoantibody levels.

Although TWEAK mediates a variety of important biological effects, including promotion of cell survival, cell death, and angiogenesis, a major pathogenic mechanism of TWEAK/Fn14 in this LN disease model appears to be through induction of cytokines and chemokines. TWEAK significantly increases PGE2, MMP-1, IL-6, IL-8, RANTES, and IP-10 in fibroblasts and synoviocytes (42), and MCP-1, IL-8, and ICAM-1 in endothelial cells (43). Similar proinflammatory effects have also been observed in bronchial epithelial cells (44), and keratinocytes (45). More recently, TWEAK has also been shown to induce chondrocyte production of MMPs (46), astrocyte production of MMP-9 (35), and in a model culture system to promote proliferation, inhibit differentiation, and induce invasion of mammary epithelial cells, via up-regulation of MMP-9 (47). Clearly, TWEAK has potent inflammatory effects, particularly via induction of chemokines and other potentially harmful mediators. These proinflammatory effects of TWEAK have recently been shown to be instrumental in the pathogenesis of disease in vivo, in collagen induced arthritis and experimental allergic encephalomyelitis (EAE). In collagen induced arthritis, anti-TWEAK blocking Abs reduce disease severity in both mouse and rat disease models without detectably affecting adaptive immune responses (46, 48, 49). In addition, transgenic mice hyperexpressing TWEAK had enhanced EAE severity (50), whereas neutralizing anti-TWEAK Abs lessened disease scores and decreased CNS leukocyte infiltration in myelin oligodendrocyte glycoprotein-induced EAE in B6 mice (11).

Little had been previously known about the role of TWEAK in the pathogenesis of SLE. In the BXSB lupus model, TWEAK mRNA is highly expressed in kidneys of male mice early in the disease process, and is down-regulated as mice age and nephritis becomes manifest (51). However, male NZB.Yaa mice have much lower levels of kidney TWEAK mRNA expression than female NZB mice despite similar degrees of nephritis, suggesting that the decrease in TWEAK expression in these models may be due to male specific genes, rather than a function of disease activity. In SLE patients, Kaplan et al. (52) showed that activated human T cells overexpress TWEAK and thereby mediate monocyte apoptosis. Kaplan et al. (52) suggested that increased monocyte killing mediated by TWEAK expressing T cells may have a double contribution to lupus pathogenesis: increasing the load of nuclear autoantigens released by apoptotic cells, while concurrently decreasing the clearance of apoptotic debris. However, it is not clear whether increased apoptosis is indeed a key feature in the pathogenesis of human SLE.

We recently reported that murine (13) and human (53) mesangial cells and podocytes express cell surface Fn14, and respond to TWEAK stimulation by producing multiple potent inflammatory
mediators via activation of the NF-κB signaling pathway. The proinflammatory effects of TWEAK in vitro were prevented by treatment with the same anti-TWEAK mAb used in the experiments described here. Importantly, in vivo overexpression of TWEAK induced a similar array of proinflammatory mediators in Fn14 WT but not Fn14 KO mice (13). As reviewed (12), several of these TWEAK-inducible chemokines in kidney cells, in particular MCP-1 and RANTES, are instrumental in the pathogenesis of LN through local recruitment of monocytes and activated T cells. For example, in the lupus-prone MRL-lpr/lpr mouse strain, overexpression of RANTES accelerates renal disease (54), whereas inhibiting MCP-1 signaling using a small molecule inhibitor (55) or by genetic approaches (56) ameliorates nephritis. Furthermore, we found high urinary TWEAK levels in SLE patients with active nephritis, which correlated with disease activity (15). Thus, our findings support that TWEAK/Fn14 interactions are likely important in the pathogenesis of lupus and lupus-like glomerulonephritis.

In our current study, we showed that prevention or blockade of TWEAK-Fn14 signaling down-regulates proinflammatory chemokines in the kidney. An important question to be considered is the identity of the Fn14+ responsive cell type(s) and the source of these cytokines. As mesangial cells, podocytes, and macrophages all display Fn14 and respond robustly to TWEAK stimulation, TWEAK inhibition may act through modulating inflammatory responses of resident kidney cells, infiltrating immune cells, or both. We observed reduced macrophage infiltration in Fn14 KO as compared with WT recipients with cGVH-induced lupus. (Lymphocytes, which also infiltrate the kidney during LN, do not display Fn14). In contrast, Fn14 sufficient mice with cGVH-induced lupus treated with anti-TWEAK Abs did not display reduced macrophage infiltration as compared with control Ig treated animals. The basis for this difference is not currently known, but we assume that the effect of anti-TWEAK treatment is simply not as complete as genetic pathway ablation. Regardless, the fact that anti-TWEAK Ab-treated mice did not display decreased macrophage infiltration yet had down-regulated cytokine expression suggests that the primary source of inflammatory mediators in cGVH-induced nephritis is resident kidney cells, rather than infiltrating cells coming from outside the kidney. Future studies, including macrophage depletion experiments and bone marrow transfer experiments into irradiated mice, will allow us to conclusively determine the relative contribution of intrinsic kidney cells vs infiltrating macrophages.

How do TWEAK-Fn14 interactions actually contribute to the renal damage in this SLE model? Differences in Ab fine specificity resulting from inhibited TWEAK signaling are not likely to have contributed to decreased glomerular Ig binding, as decreased anti-collagen IV Ab titers were found only in Fn14 KO but not anti-TWEAK Ab-treated mice. It is also theoretically possible that activation of T cells in an environment in which TWEAK signaling is prevented may result in T cells which are less nephritogenic, thus contributing to the decreased renal disease seen in these mice. However, we believe this explanation to be improbable. Preliminary assessment of TWEAK knockout and Fn14 knockout mice has revealed no consistent effect on T cell dependent immunity to date, nor has anti-TWEAK Ab treatment been shown to have an effect (L. C. Burkly, personal communication; Ref. 46, 49). Nevertheless, we plan to formally exclude this possibility in future selective cell transfer experiments alluded to above.

We observed reduced chemokine expression in the kidneys of Fn14 KO and anti-TWEAK-treated mice, as compared with their respective control groups. Local up-regulation of TWEAK expressed by infiltrating activated donor T cells, macrophages, or resident renal cells likely leads to enhanced secretion of inflammatory chemokines by intrinsic kidney cells, including mesangial cells, podocytes, tubular cells, or endothelial cells. In preliminary studies, we detected TWEAK overexpression in kidneys from mice with cGVH-induced lupus (data not shown), supporting this explanation. Interestingly, however, we also observed reduced glomerular Ig deposition in both the Fn14 KO and anti-TWEAK Ab-treated mice as compared with their control groups. TWEAK has recently been reported to increase vascular permeability in the CNS, as evidenced by increased dye extravasation (35). TWEAK appears to act in the brain by inducing astrocyte production of MMP-9, resulting in degradation of the basement membrane extracellular matrix (36). Thus, in addition to TWEAK up-regulation inducing chemokines and directing infiltration of inflammatory cells to the kidney with the ensuing production of injurious cytokines and further amplification of the inflammatory response, our data suggest that TWEAK may also contribute to kidney injury through its effects on vascular permeability. Enhanced permeability would serve to increase the extravasation of IgG and subsequent glomerular IgG deposition, thereby increasing immune complex mediated activation of effector cells. Moreover, macrophages or T cells infiltrating the kidney may themselves serve as a source of membrane bound and/or secreted TWEAK, thus creating a positive feedback loop for these pathogenic mechanisms.

In a recent paper by Maeker et al. (57), previously unrecognized roles for TWEAK in immune deviation and in down-regulation of innate inflammatory responses were reported. Maeker found that B6 TWEAK KO mice have increased numbers of NK cells due to impaired activation induced cell death, and enhanced innate inflammatory responses, as shown by increased susceptibility to endotoxin. Furthermore, NK cells and macrophages from TWEAK KO mice produced less IL-10 and more γ-IFN and IL-12 than controls. Further support for a role of TWEAK in modulating the transition from an innate to a TH1 adaptive response was found in a model of anti-tumor immunity, in which both NK cells and effector T cells are important in tumor rejection. Tumor development was significantly diminished in melanoma injected TWEAK KO mice; this was associated with an expansion in NK and CD8+ T cells in KO mice. Although these results are interesting, we (L.C. Burkly, and colleagues, unpublished observations) independently generated TWEAK KO mice that do not display the increased splenomegaly with age, or the increases in percent or absolute number of T cell subsets, NK, or NK T cells, reported by Maeker et al. (57). These significant differences in the phenotype of TWEAK KO mice are remarkable, but the reasons for them are not evident at this time. In any case, in our current study, we found no evidence of immune deviation (in the form of skewed IgG1/ IgG2a ratios) or increases in autoantibody titers in Fn14 KO or anti-TWEAK Ab-treated Fn14 WT mice, suggesting that the effects of TWEAK, at least in this model, are more specific to the
local inflammation in the kidney rather than modulation of adaptive immune responses that are activated in the cGVH model system.

The induced features of anti-nuclear autoantibody formation, glomerular Ig deposition, and proteinuria found in the bm12 to B6 cGVH model combine to make this an accepted lupus model, which has been very useful for elaborating important facets of disease pathogenesis (18–21, 39, 58–60). However, it is important to acknowledge that cGVH induced in a different strain combination (B6 × DBA/2 F2) elicits higher levels of proteinuria and more severe renal disease (61, 62). Nevertheless, in the current paper, we chose to study the bm12 to B6 model in detail rather than B6 × DBA/2 F2 mice, because the former provided the important advantage of enabling us to confirm the results obtained with anti-TWEAK Ab treatment (in Fn14 sufficient mice) also in B6 Fn14 KO mice. In any case, it will be important in future studies to substantiate the effects of TWEAK neutralization in more severe lupus disease models as well, such as cGVH in B6 × DBA/2 F2 mice and in spontaneous murine lupus.

In conclusion, inhibition of TWEAK signaling, through genetic deletion of the TWEAK receptor Fn14 or by anti-TWEAK mAb treatment, significantly improved glomerulonephritis in the cGVH model of lupus without significantly altering systemic autoantibody levels. Given the expression of Fn14 in mesangial cells and podocytes, as well as on kidney-infiltrating macrophages, our results further support a role for TWEAK as an important local mediator of kidney damage in the pathogenesis of LN. Thus, TWEAK blockade may be a novel therapeutic approach to reduce renal damage in SLE, and possibly other forms of immune nephritis as well.

Disclosures
Linda C. Burkly and Jennifer S. Michaelson are employed and hold stocks in Biogen Idec, as well as have a patent application for measurement of TWEAK Ab levels in lupus serum. Linda Burkly and Jennifer S. Michaelson are employed and hold stocks in Biogen Idec, as well as have a patent application for measurement of TWEAK Ab levels in lupus serum.

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