Immunopathology and Infectious Diseases

Fn14-TRAIL, a Chimeric Intercellular Signal Exchanger, Attenuates Experimental Autoimmune Encephalomyelitis

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Hallmarks of the pathogenesis of autoimmune encephalomyelitis include perivascular infiltration of inflammatory cells into the central nervous system, multifocal demyelination in the brain and spinal cord, and focal neuronal degeneration. Optimal treatment of this complex disease will ultimately call for agents that target the spectrum of underlying pathogenic processes. In the present study, Fn14-TRAIL is introduced as a unique immunotherapeutic fusion protein that is designed to exchange and redirect intercellular signals within inflammatory cell networks, and, in so doing, to impact multiple pathogenic events and yield a net anti-inflammatory effect. In this soluble protein product, a Fn14 receptor component (capable of blocking the pro-inflammatory TWEAK ligand) is fused to a TRAIL ligand (capable of inhibiting activated, pathogenic T cells). Sustained Fn14-TRAIL expression was obtained in vivo using a transposon-based eukaryotic expression vector. Fn14-TRAIL expression effectively prevented chronic, nonremitting, paralytic disease in myelin oligodendrocyte glycoprotein-challenged C57BL/6 mice. Disease suppression in this model was reflected by decreases in the clinical score, disease incidence, nervous tissue inflammation, and Th1, Th2, and Th17 cytokine responses. Significantly, the therapeutic efficacy of Fn14-TRAIL could not be recapitulated simply by administering its component parts (Fn14 and TRAIL) as soluble agents, either alone or in combination. Its functional pleiotropism was manifest in its additional ability to attenuate the enhanced permeability of the blood-brain barrier that typically accompanies autoimmune encephalomyelitis. (Am J Pathol 2009, 174:460–474; DOI: 10.2353/ajpath.2009.080462)

Despite a steadily expanding set of treatment options for multiple sclerosis (MS), there remains a pressing need for more effective therapeutic agents to address this debilitating autoimmune disorder of the central nervous system (CNS). Although the precise etiology of MS is unknown, key features of its pathogenesis and clinical evolution are emerging.1–3 Among various immune cellular effectors that have been implicated, pathogenic T cells loom large as pivotal drivers of the disease. As a consequence, various therapeutic paths are converging on T effectors as targets, with complementary goals of blocking their activation and re-activation, eliminating them from the larger T-cell reservoir, and interfering with their transit to sites of pathogenesis within the CNS. A complex interplay of positive and negative intercellular signals regulates activation and maintenance of T-cell effector functions. Proteins of the tumor necrosis factor (TNF) superfamily figure prominently in this matrix of signals, bridging various cells of the immune system, as well as to cells of other organ systems. In so doing, TNF superfamily members contribute to both tissue homeostasis and pathogenesis via effects on cell survival and death, cellular differentiation, and inflammation.4,5 From the standpoint of autoimmune pathogenesis, two especially interesting members of the TNF superfamily are the cell surface ligands TWEAK (TNF-related weak inducer of apoptosis)6,7 and TRAIL (TNF-related apoptosis-inducing ligand).8–11 TWEAK, a TNF superfamily ligand, and its counter-receptor Fn14 (fibroblast growth factor-inducible 14-kDa protein) are expressed in a range of immune and nonimmune cell types. TWEAK, which is expressed on cells such as macrophages, dendritic cells, NK cells, endothelial cells, microglial cells, and astrocytes,6,12,13 stimulates proliferation of astrocytes.

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A patent is being filed around Fn14-TRAIL and its uses, and there are active efforts ongoing to license this intellectual property.

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and endothelial cells, as well as production of various inflammatory cytokines, chemokines, and adhesion molecules. Moreover, the TWEAK:Fn14 signaling axis has pro-inflammatory effects that go beyond promoting cell proliferation and cytokine production, some of which tie into autoimmune pathogenesis. TWEAK, whose endogenous expression is elevated in the CNS during experimental autoimmune encephalitis (EAE), a murine model for MS, increases the permeability of the neurovascular unit, contributing in this way to perivascular inflammatory cell infiltration. Moreover, TWEAK has pro-angiogenic activity, which is of interest given the association between angiogenesis and autoimmune pathogenesis. TWEAK increases EAE severity and associated neurodegeneration, and circulating TWEAK levels are significantly increased in patients with MS and other chronic inflammatory diseases. The induction of inhibitory anti-TWEAK or Fn14 antibody (Ab) in vivo, via vaccination with the extracellular domains of either TWEAK or Fn14, ameliorates EAE manifestations in rat and mouse models.

TRAIL, a TNF superfamily ligand, binds to several different cognate TNF receptor superfamily receptors, some activating and others decoy. The activating receptors in humans are TRAIL-R1 (DR4), TRAIL-R2 (DR5), and osteoprotegrin, whereas in mice, the sole activating receptor is DR5. Virtually all cells of the immune system (including T cells, B cells, natural killer cells, dendritic cells, monocyes, and granulocytes) up-regulate surface TRAIL ligand and/or release soluble TRAIL in response to interferon and other activation signals. TRAIL receptors are primarily expressed on immune cells, such as activated T cells. Native TRAIL expression attenuates EAE, as is evident from experiments involving genetic deletion of TRAIL (in TRAIL-/- mouse) or administration of TRAIL blocking agents (soluble TRAIL receptor (sDR5) or neutralizing anti-TRAIL mAb). Moreover, embryonic stem cell-derived dendritic cells with enforced co-expression of TRAIL and pathogenic myelin oligodendrocyte glycoprotein (MOG) protein suppress EAE induction. Interestingly, soluble TRAIL has emerged as a response marker in MS patients undergoing interferon (IFN)-β therapy, with those most likely to respond to treatment showing early and sustained soluble TRAIL induction after therapeutic cytokine administration.

Based on this intriguing constellation of activities associated with the TWEAK:Fn14 and TRAIL:TRAIL-R signaling axes, which impact immunological responses and inflammatory processes, we have now designed an Fn14-TRAIL fusion protein that bridges them. The Fn14 component of this fusion protein has the capacity to bind and block endogenous TWEAK, whereas the TRAIL ligand component, once anchored to TWEAK-bearing cells via the Fn14 bridge, can direct intercellular inhibitory signals to its cognate receptors on TRAIL receptor-bearing cells, such as activated T cells.

Fn14-TRAIL is in essence exchanging TWEAK pro-inflammatory signals into immunoinhibitory TRAIL-driven ones. However, in contrast to another fusion protein that alters intercellular signals, the trans signal converter protein CTLA-4-Fasl, Fn14-TRAIL is designed to redirect an exchanged negative signal to third-party (TRAIL receptor-bearing) cells. Furthermore, by virtue of the highly pleiotropic functionality of the TRAIL:TRAIL-R and TWEAK:Fn14 signaling axes, Fn14-TRAIL has inherently greater potential for higher order functionality with a net anti-inflammatory output. The present study begins to explore this fusion protein’s functional repertoire by demonstrating Fn14-TRAIL’s capacity to ameliorate MOG-induced EAE.

Materials and Methods

Mice

Four- to six-week-old C57BL/6 female mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and were maintained under pathogen-free conditions at the University of Pennsylvania Animal Facility. All animal experiments were approved by the University of Pennsylvania Animal Care and Use Committee.

Reagents

Plasmids pT2/BH and pNEB193 UbC-SB11 were provided by Dr. Perry Hacket (University of Minnesota, Minneapolis, MN), and murine TRAIL and TWEAK cDNAs were obtained from Dr. Hideo Yagita (Juntendo University School of Medicine, Tokyo, Japan). The plasmid pmFneo was obtained from Dr. Herman Waldmann (University of Oxford, Oxford, UK). Pertussis toxin was purchased from EMD Biosciences (San Diego, CA). Mouse MOG38–50 peptide (GWYRSPFSRWVHL) was synthesized using F-moc solid phase methods and purified by high performance liquid chromatography at Invitrogen Life Technologies (Carlsbad, CA). Pertussis toxin was purchased from EMD Biosciences (San Diego, CA). The following reagents were purchased from BD Pharmingen (San Diego, CA): enzyme-linked immunosorbent assay (ELISA) Ab pairs for mouse interleukin (IL)-2, IL-4, IL-6, IFN-γ, and recombinant mouse IL-2, IL-4, IL-6, IFN-γ. An IL-17 ELISA Ab pair was obtained from Southern Biotechnology (Birmingham, AL), and recombinant mouse IL-17 was purchased from Biosource (Camarillo, CA). PE-anti-mouse TRAIL and PE-anti-mouse TWEAK were purchased from eBioscience (San Diego, CA). Recombinant TRAIL (Super Killer TRAIL) was purchased from Axxora Platform (San Diego, CA).

Plasmid Construction

Chimeric Fn14-TRAIL and Fn14-IgG1(mut) coding cassettes were constructed by polymerase chain reaction (PCR), using partially overlapping synthetic oligonucleotides. cDNA encoding amino acids 1 to 79 of murine Fn14 (Swiss-prot accession number Q9CR75) was joined to cDNA encoding either amino acids 118 to 291 of murine TRAIL (Swiss-prot accession number P50592) or a mutated human IgG1 Fc (Fcγ1) segment, respectively. For the latter, a cDNA encoding human Fcγ1 was modified by PCR-based site-directed mutagenesis, using oligonucleotides configured to mutate C220→S, C226→S, C229→S, N297→A, E233→P, L234→V, and L235→A.
To express soluble TRAIL, cDNA encoding amino acids 118 to 291 of murine TRAIL was used. All of these cDNA segments were subcloned into a pMFneo eukaryotic expression vector downstream of an EF1-α promoter region. Coding sequence for luciferase was mobilized with HindIII and BamH1 from pTAL-Luc (BD Biosciences, San Jose, CA), and subcloned into the respective sites of pMFneo. cDNA encoding full-length murine TWEAK was generated by PCR and subcloned into the pcDNA3 eukaryotic expression vector (Invitrogen Life Technologies).

To generate a derivative Sleeping Beauty expression vector that incorporates within the same plasmid both transposon and transposase cassettes, a transposase coding sequence flanked upstream by a ubiquitin C promoter was generated by PCR from pNEB193 UbC-SB11 (base pairs 432 to 2958) and then ligated between the Apal and Xhol sites of pt2/BH vector, which contains a transposon cassette. This new expression vector, incorporating both transposase and transposon expression cassette, was designated pSBC21. Next, cDNAs corresponding to Fn14-TRAIL, soluble Fn14, Fn14-IgG1(mut), soluble TRAIL, or luciferase, each linked to the EF1-α promoter, were subcloned from their respective pMFneo expression constructs into the transposon cassette of pSBC21, downstream of the transposase expression module. All subcloned cDNAs were oriented in the same direction as the transposase.

Cell Culture and Transfection

Human 293 kidney cells and CHO cells were cultured in Dulbecco’s modified Eagle’s medium and Ham’s F-12, respectively, supplemented with 100 μg/ml of penicillin, 100 U/ml of streptomycin, and 2% and 10% heat-inactivated fetal bovine serum. 293 cells were transiently transfected with the Fn14-TRAIL, soluble Fn14, Fn14-IgG1(mut), and soluble TRAIL pMFneo expression plasmids, using LipofectAMINE reagent (Invitrogen Life Technologies). Proteins in conditioned media were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and detected by Western blot analysis. Anti-mouse Ab used for detecting Fn14 and TRAIL were purchased from eBioscience and R&D Systems (Minneapolis, MN), respectively. CHO cells were transiently transfected with a pcDNA3-based murine TWEAK expression construct. TWEAK expression on transfectants was verified by immunofluorescence and flow cytometry.

Induction and Disease Evaluation of EAE

EAE was induced according to a standard induction protocol. Briefly, female C57BL/6 mice were challenged with a total of 300 μg of MOG38-50 peptide (divided into two subcutaneous injections, one on each dorsal flank) in 0.1 ml of phosphate-buffered saline (PBS), emulsified in an equal volume of Complete Freund’s Adjuvant (CFA) containing 4 mg/ml of Mycobacterium tuberculosis H37RA (Difco, Detroit, MI). These mice were simultaneously injected intravenously with 100 ng of pertussis toxin in 0.2 ml of PBS. A second intravenous injection of pertussis toxin (100 ng/mouse) was given 48 hours later. Mice were examined daily for signs of EAE and scored as follows: 0, no disease; 1, tail paralysis; 2, hind limb weakness; 3, hind limb paralysis; 4, hind limb plus forelimb paralysis; 5, moribund or dead.

Cytokine and Proliferation Assays

For cytokine assays, splenocytes were cultured at 1.5 × 10⁶ cells per well in 0.2 ml of Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum, in the presence or absence of different concentrations of MOG38-50 peptide, or 1 μg/ml of Con A (Sigma-Aldrich, St. Louis, MO). Conditioned media were collected 40 hours later, and cytokine concentrations were determined by quantitative ELISA using paired mAb specific for the corresponding cytokines per the manufacturer’s recommendations (BD Pharmingen). Proliferation assays were performed using 0.5 × 10⁶ cells per well in 96-well plates. [³H] thymidine was added to the cultures at 48 hours, and cells were harvested 16 hours later. Radioactivity was determined using a flatbed β-counter (Wallac-Perkin Elmer, Waltham, MA).

Hydrodynamic Injection

Mice were injected with pSBC21 vector alone or pSBC21-based expression constructs incorporating Fn14-TRAIL, soluble Fn14, Fn14-IgG1(mut), soluble TRAIL, or luciferase coding sequences. Expression plasmids were dissolved in saline in a volume (in ml) equivalent to 10% of body weight (in g). The entire volume for each animal was injected within 5 seconds via tail veins, according to a published protocol. Retro-orbital blood samples were collected using heparinized glass capillaries. After centrifugation, plasma was recovered and kept at −20°C until ELISA assays were performed. For gene transfer experiments, plasmid concentrations were measured spectrophotometrically, and plasmid copy numbers were calculated by using the equation: pmol DNA = (μg DNA) × 10⁶/333g/mol × (no. bp × 2).

Measurement of Recombinant Proteins in Serum

ELISA assays were performed in 96-well microtitration plates. For Fn14-TRAIL, soluble Fn14, and Fn14-IgG1(mut), purified anti-human/mouse Fn14/TWEAK receptor Ab from eBioscience was used as capture Ab; for soluble TRAIL, anti-mouse TRAIL Ab from R&D Systems was used as capture Ab. Detecting Abs were: biotin-anti-mouse TWEAK receptor Ab from eBioscience for Fn14; biotin-anti-mouse TRAIL Ab from eBioscience for Fn14-TRAIL and soluble TRAIL; anti-human IgG, Fcγ fragment-specific Ab from Jackson ImmunoResearch Laboratories (West Grove, PA) for Fn14-IgG1(mut).

Capture Ab diluted in coating buffer (0.1 mol/L carbonate, pH 8.2) was distributed in microtitration plates and incubated at 4°C overnight. After washing twice with 0.05% Tween-20 in PBS, wells were incubated for an
additional 2 hours at room temperature with PBS-3% albumin to block nonspecific binding sites. After washing twice again, 100 μl of serum samples were added and incubated at 4°C overnight. After incubation, wells were rinsed four times and incubated for 1 hour with biotinylated detection Ab. For the enzymatic reaction, avidin peroxidase and TMB Microwell peroxidase substrate (KPL, Gaithersburg, MD) were applied sequentially.

In Vivo Bioluminescence Imaging
All of the imaging work was performed at the Small Animal Imaging Facility in the Department of Radiology at the University of Pennsylvania. Images were acquired at 5 hours, 24 hours, 5 days, 34 days, 51 days, and 1 year after injection of the luciferase expression plasmid.40 Briefly, mice were sacrificed and brains were collected. For quantitative measurements, the chimeric Fn14-TRAIL coding sequence was produced using a pMFneo eukaryotic expression system. The chimeric Fn14-TRAIL coding sequence contained the full extracellular domains of the Fn14 type I and TRAIL type II fusion protein. To generate a hybrid soluble type I · type II fusion protein. To generate

Molecular Modeling of the Chimeric Fn14-TRAIL Protein
A three-dimensional model of the Fn14-TRAIL protein was generated using the crystal structure of TRAIL (pdb code: 1D0G)41 and a modeled Fn14 molecule. A threedimensional model of the ligand binding domain of Fn14 was generated using MODELLER.42 Briefly, the starting model of the ligand binding domain of Fn14 was obtained based on the template structure of human TACI (1XU1) and BCMA (1XU2).43 The extended region of Fn14 was treated as a linker between Fn14 and TRAIL. To obtain a stereochemically and energetically favored model, the linker conformation was optimized by short molecular simulation studies using InsightII (Accelrys, Inc., San Diego, CA) as described before.44

Flow Cytometry and MTT Assays
Immunostaining was performed at 4°C with specified Ab suspension in PBS containing 0.5% bovine serum albumin and 0.05% sodium azide (NaNO3). All flow cytometric analyses were performed on a FACS Calibur apparatus with Cell Quest software and dual laser (488 and 633 nm) excitation (BD Biosciences). The MTT assay was performed according to the manufacturer’s protocol (American Type Culture Collection, Manassas, VA).

Statistical Analysis
The Student’s t-test or Mann-Whitney U-test was used to determine the statistical significance of differences. A P value of <0.05 was considered to be statistically significant.

Results
Production of Functional Fn14-TRAIL Protein
Recombinant Fn14-TRAIL, along with related control proteins [soluble Fn14, Fn14-IgG1(mut), soluble TRAIL], were produced using a pMFeuo eukaryotic expression system. The chimeric Fn14-TRAIL coding sequence linked the full extracellular domains of the Fn14 type I and TRAIL type II membrane proteins, thereby creating a hybrid soluble type I · type II fusion protein. To generate
The functionality of the TRAIL component of Fn14-TRAIL was determined by evaluating its capacity to induce apoptosis in L929 cells, using an MTT assay. As shown in Figure 1C, Fn14-TRAIL induces apoptosis of L929 cells in a dose-dependent manner in the presence of actinomycin D. Recombinant TRAIL (Super Killer TRAIL) was used as a positive control in this experiment. Of note, no TWEAK was detected by immunofluorescence and flow cytometry on these L929 cells (not shown), arguing against the possibility that the Fn14 component of Fn14-TRAIL drives apoptosis through some kind of back-signaling through surface TWEAK.

Development of a Transposon-Based Expression System for Sustained in Vivo Expression of Fn14-TRAIL

To enable sustained in vivo expression of Fn14-TRAIL (and control proteins), we invoked the transposon-based Sleeping Beauty (SB) expression system. This system combines the advantages of plasmid-mediated gene delivery together with an ability to integrate into the chromosome and provide for sustained transgene expression. To optimize the efficiency of this expression system, we generated a derivative expression vector, designated pSBC21, that combines within a single plasmid both transposon (accommodating the transgene of interest) and transposase expression cassettes (Figure 2A). Because the relative expression level from the two cassettes is important, we screened a number of promoter combinations, and determined that a combination of UBC promoter (driving the transposase) and EF1-α promoter (driving the transposase cassette), arrayed in tandem, affords strong transgene expression (not shown).

The functionality of this unique dual-cassette transposon/transposase vector derivative (with a UBC/EF1-α promoter combination) was validated using a luciferase reporter. A pLuciferase·SBC21 plasmid, at varying concentrations, was administered by hydrodynamic injection to C57BL/6 mice. Hydrodynamic injection of transposon-based expression constructs provides for sustained gene expression in mouse hepatocytes in vivo.38 Bioluminescent images acquired after administration of luciferase’s substrate, d-Luciferin, revealed luciferase expression at 22 days (Figure 2B), with significant levels still detected after 6 months (not shown).

Having documented the functionality of our derivative expression vector using a luciferase reporter, we next invoked this vector for expressing Fn14-TRAIL, specifically asking whether levels of Fn14-TRAIL in serum correlate with the dose of injected pFn14-TRAIL·SBC21 plasmid. C57BL/6 mice (in experimental groups of four) were challenged with MOG38-50 peptide in CFA supplemented with M. tuberculosis, and on day 2 were each treated with a single hydrodynamic injection of empty pSBC21 vector or pFn14-TRAIL·SBC21 plasmid, in escalating doses (10, 20, 50, or 100 μg of plasmid). Serum levels of Fn14-TRAIL were measured by ELISA 20 days

The various pM-Fneo-based expression constructs were transiently transfected into 293 cells, and expression and secretion of the respective proteins were demonstrated by Western blot analysis of conditioned media (Figure 1A).

To validate the identity of expressed Fn14-TRAIL, we next documented its ability to bind to Fn14’s ligand, TWEAK. To this end, we transiently transfected CHO cells with a murine TWEAK cDNA expression construct (in the pcDNA3 vector), and after 48 hours, transfectants were incubated at 4°C with purified Fn14-TRAIL or soluble TRAIL. Immunofluorescence and flow cytometric analysis of these cells, using anti-mouse TWEAK and anti-mouse TRAIL as detecting Ab, showed significant binding of Fn14-TRAIL, but not soluble TRAIL, to cell surface TWEAK on transfectants (Figure 1B).

The fn14-IgG1(mut) coding sequence, several amino acids within the human IgG1 component were mutated (see Material and Methods) to block FcγR binding (and consequent nonspecific depletion of lymphocytes) and to interfere with N-glycosylation (which is important for in vivo effector function of human IgG1).45 The various pM-Fneo-based expression constructs were transiently transfected into 293 cells, and expression and secretion of the respective proteins were demonstrated by Western blot analysis of conditioned media (Figure 1A).

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after plasmid administration, and a dose-dependent increase in serum Fn14-TRAIL levels was observed, starting with the 10-μg plasmid dose (Figure 2C). Of note, Western blot analysis 20 days after plasmid administration verified that the fusion protein in the serum of Fn14-TRAIL-treated mice was intact (not shown).

**Fn14-TRAIL Suppresses MOG-Induced Autoimmune Encephalomyelitis**

We next investigated the therapeutic potential of Fn14-TRAIL in a murine EAE disease model. To this end, a single encephalitogenic dose of MOG 38-50 peptide was administered to C57BL/6 mice. Two days after peptide injection, we administered by hydrodynamic injection a single dose of pFn14-TRAIL·SBC21 plasmid (50 μg/mouse), or one of four control plasmids [pFn14·SBC21, pFn14-IgG1(mut)·SBC21, pTRAIL·SBC21, and pSBC21]. Of note, because the expression cassettes represent relatively small components of these various plasmids, plasmid copy numbers for them (within the 50-μg aliquots) fell within a narrow range [Fn14·pSBC21 = 11.89 pmol, Fn14-TRAIL·pSBC21 = 11.0 pmol, TRAIL·pSBC21 = 11.28 pmol, Fn14-IgG1(mut)·pSBC21 = 10.71 pmol]. By ELISA, we detected comparable serum levels of expressed proteins in animals hydrodynamically injected with each of the respective plasmids (Figure 3A).

Disease progression in the treated mice was monitored by both physical examination (Figure 3 and Table 1) and histological analysis of recovered spinal cords. Fn14-TRAIL expression significantly attenuated EAE manifestations, with decreases in both mean clinical scores (calculated throughout a 43-day period after MOG administration; Figure 3B, top) and cumulative clinical score (calculated throughout a 40-day period after MOG administration; Table 1). Maximum disease score was...
also significantly lower in the Fn14-TRAIL-treated group, as compared with the various controls (Table 1). Similarly, the disease score at day 35 was also significantly lower for the Fn14-TRAIL group (Table 1).

The therapeutic benefit of Fn14-TRAIL was also evident from an analysis of day of disease onset and disease incidence. Fn14-TRAIL extended the mean day of disease onset (14.8 ± 4.7 days), compared with control mice treated with empty vector (12.8 ± 4 days), and typically the latter mice developed EAE starting at ~10 days after MOG peptide administration (Table 1). The mean day of disease onset for mice receiving pFn14·SBC21, pFn14-IgG1(mut)·SBC21, pTRAIL·pSBC21 plasmids were 13.63 ± 2.72 days, 11.66 ± 1.2 days, and 13.42 ± 2.2 days, respectively. Although all expressed proteins reduced disease incidence to some extent, remarkably only 50% of Fn14-TRAIL-treated animals showed signs of disease during the course of this experiment (Figure 3B, bottom; Table 1). Of note, because there were no animal deaths in the experiment of Figure 3B (Table 1), the changes in disease incidence that were observed are linked to late onset of disease in some animals and recovery of others.

Fn14-TRAIL Is More Effective than Its Component Parts, in Combination

Having shown that neither of the components of Fn14-TRAIL, when administered as soluble agents one at a time, are as effective as Fn14-TRAIL in suppressing EAE, we next asked whether the Fn14-TRAIL fusion protein’s therapeutic efficacy can be recapitulated by administering soluble Fn14 and TRAIL proteins simultaneously. This was evaluated in the same EAE model. Two days after administering a single encephalitogenic challenge of MOG38-50 peptide to C57BL/6 mice, single doses of either pFn14·SBC21 plasmid (25 μg/mouse) or a mixture of pFn14·SBC21 and pTRAIL·SBC21 plasmids (25 μg each/mouse) were hydrodynamically injected into the animals. Although Fn14-TRAIL significantly suppressed EAE as before, with decreases in both mean clinical scores (Figure 4, A and B) and cumulative mean clinical score (Figure 4C), the combination of soluble Fn14 and soluble TRAIL showed no significant therapeutic effect. Serum levels of these various proteins, expressed by hydrodynamic injection of the respective transposon-based expression plasmids, were comparable, as measured by ELISA (Figure 4D). Taken together, these data establish that Fn14-TRAIL has substantial therapeutic benefit in preventing EAE induction, and this effect cannot be replicated by simply administering this fusion protein’s two component elements as soluble agents in combination.

Fn14-TRAIL Blocks Proliferation and Differentiation of Autoreactive T Cells

We next assessed the effect of Fn14-TRAIL on the proliferation and differentiation of myelin-specific T cells recovered from treated animals. To this end, splenocytes were recovered 43 days after MOG challenge from both Fn14-TRAIL-treated and control mice receiving vector only. These splenocytes were evaluated in vitro for their proliferation and cytokine production in response to MOG38-50 peptide. Splenocytes from control animals proliferated vigorously in response to MOG peptide (Figure 4E) and produced significant amounts of Th1 (IL-2 and IFN-γ), Th2 (IL-10, IL-4, and IL-6), and Th17 (IL-17) cytokines (Figure 5). In contrast, splenocytes from Fn14-TRAIL-treated animals proliferated to a lesser extent in response to MOG stimulation (Figure 4E) and produced significantly lower levels of these various cytokines (Figure 5). Taken together, these results suggest that both T-cell proliferation and the expression of an array of T-cell cytokines are attenuated by in vivo treatment with Fn14-TRAIL.

Fn14-TRAIL Reduces Inflammation in the CNS

A key pathological feature of EAE is the infiltration of inflammatory cells into the CNS. We next determined Fn14-TRAIL’s effect on this inflammatory process. To this end, we recovered inflammatory cells from CNS tissues, and used immunofluorescence and flow cytometry to characterize these cells in terms of their expression of the early activation marker CD69, the inflammatory cytokine IL-17, and the lymphoid subset markers CD4 and CD8.

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>Day of onset mean ± SD*</th>
<th>Day of onset Incidence†</th>
<th>Maximum clinical score mean ± SEM</th>
<th>Maximum clinical score median (range)</th>
<th>Score (d35) mean ± SEM</th>
<th>Score (d35) median (range)</th>
<th>Cumulate score mean ± SEM§</th>
<th>Cumulate score median (range)</th>
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<tr>
<td>Vector (n = 8)</td>
<td>12.80 ± 4.18</td>
<td>8/8</td>
<td>2.62 ± 0.42</td>
<td>3 (1–4)</td>
<td>1.93 ± 0.29</td>
<td>2 (1–3.5)</td>
<td>48.00 ± 9.78</td>
<td>47.75 (12–89)</td>
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<tr>
<td>Fn14 (n = 8)</td>
<td>13.62 ± 2.72</td>
<td>6/9</td>
<td>2.44 ± 0.44</td>
<td>2.5 (1–4)</td>
<td>1.750 ± 0.154</td>
<td>1 (1–3.5)</td>
<td>44.00 ± 12.52</td>
<td>35.5 (2.5–96.5)</td>
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<td>Fn14 TRAIL (n = 8)</td>
<td>14.80 ± 4.7</td>
<td>4/9</td>
<td>1.22 ± 0.465</td>
<td>0.5 (0-3)</td>
<td>0.611 ± 0.261</td>
<td>0.75 (0–2.5)</td>
<td>18.60 ± 7.45</td>
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<td>Fn14-IgG (mut) (n = 9)</td>
<td>11.66 ± 1.2</td>
<td>8/9</td>
<td>3.1 ± 0.351</td>
<td>5.5 (1–4)</td>
<td>1.944 ± 0.306</td>
<td>2 (1–3.5)</td>
<td>60.05 ± 7.44</td>
<td>66 (10.5–88)</td>
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<tr>
<td>TRAIL (n = 8)</td>
<td>13.42 ± 2.2</td>
<td>6/8</td>
<td>2.0 ± 0.535</td>
<td>2 (0–4)</td>
<td>1.438 ± 0.448</td>
<td>1 (1–3.5)</td>
<td>42.06 ± 11.08</td>
<td>4 (0–82)</td>
</tr>
</tbody>
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*Day of onset is the first day mice showed signs of EAE (after immunization).
†Incidence is calculated as number of sick mice/number of mice immunized for each group at day 40.
§Cumulative score is the sum of each mouse’s clinical score.

In the calculations of parameters within this table, the scores of all mice are included except for day of onset in which only mice that exhibited signs of EAE were included.
In the case of infiltrating CD8+ T cells, MOG challenge increased the percentage of these inflammatory cells in spinal cord/cerebellum/brain stem from 0.3% (in naïve mice, with no MOG challenge) to 1.3%, and Fn14-TRAIL reduced this to 0.8% (Figure 6B). In contrast, for brain, MOG challenge did not lead to an increase in CD8+ T cells. This lymphoid subset analysis was extended to encompass a broader range of cytokines, encompassing both Th1 and Th2 types. Because the effect of Fn14-TRAIL treatment is more pronounced in spinal cord (as opposed to brain), we focused on inflammatory cells derived from this site. In this experiment, the absolute number of inflammatory cells was measured, along with the percentage of early-activated CD4+ T cells and of IFN-γ, IL-17-, and IL-10-expressing cells, in the spinal cords of Fn14-TRAIL- versus vector-treated EAE mice on days 7 and 14 after MOG challenge. On day 7, all of these cell categories were markedly reduced in the Fn14-TRAIL-treated group (Figure 7, A–D). At day 14 (peak of the disease), the Fn14-TRAIL-associated reduction in absolute numbers of cytokine-expressing cells was still manifest (Figure 7, E and F). These findings with
TWEAK is known to increase the permeability of the neurovascular basement membrane by inducing MMP-9 (matrix metalloproteinase-9) expression. We posited that the decreased infiltration of inflammatory cells into the CNS seen in Fn14-TRAIL-treated mice could be a consequence, at least in part, of a reduction in TWEAK-dependent enhancement of blood brain barrier (BBB) permeability. BBB integrity was evaluated by a conventional dye enhancement of blood brain barrier (BBB) permeability assay, as described in the Material and Methods. Live lymphocytes were gated based on forward and side scatter. CD8 and CD69 markers were measured on gated lymphocytes, and analysis of cells producing each of the cytokines shown was for gated CD4+ lymphocytes. Absolute cell numbers were calculated by multiplying the total number of live mononuclear cells times the percentage of each indicated cell type (determined by flow cytometry). Data are representative of three independent experiments with similar results.

Figure 6. Fn14-TRAIL/pSBC21 treatment reduces early activated CD4+ IL-17-producing cells and CD8+ cells in the CNS of MOG-challenged mice. MOG-challenged mice, treated with either pSBC21 vector only or Fn14-TRAIL/pSBC21 (three mice per group), or naïve mice (no MOG challenge), were sacrificed on day 7 after challenge, and flow cytometry was performed on mononuclear cells isolated from their brains or pooled spinal cords/cerebellums/brain stems. Live lymphocytes were gated based on forward and side scatter. A: Left: Early activated (CD69+) IL-17-producing cells, gated on CD4+ lymphocytes. B: Left: CD8+ cells, gating on live lymphocytes, right: depiction of percentage of CD8+ lymphocytes. The data shown are representative of three independent experiments.

Figure 7. Fn14-TRAIL/pSBC21 treatment reduces the number of activated and cytokine-producing cells in spinal cords of MOG-challenged mice on days 7 and 14 of disease. Mice were sacrificed on days 7 or 14 of disease, and flow cytometry was performed on the mononuclear cells isolated from spinal cords of pSBC21 vector-only or Fn14-TRAIL/pSBC21-treated mice (three mice per group), as described in the Material and Methods. Live lymphocytes were gated based on forward and side scatter. CD8 and CD69 markers were measured on gated lymphocytes, and analysis of cells producing each of the cytokines shown was for gated CD4+ lymphocytes. Absolute cell numbers were calculated by multiplying the total number of live mononuclear cells times the percentage of each indicated cell type (determined by flow cytometry). Data are representative of three independent experiments with similar results.

Fn14-TRAIL Attenuates BBB Permeability

We next correlated the concentration of dye penetrated into the spinal cords with the respective EAE mean clinical scores on the day of dye application. Significantly, even for mice with the same EAE mean clinical scores (0 or 1) on day 13, control vector-only-treated mice showed higher concentrations of dye in their spinal cords compared with Fn14-TRAIL-treated mice (Figure 9C). This finding, coupled with the findings of reduced inflammatory cell infiltration in Fn14-TRAIL-treated spinal cords recovered from spinal cords were consistent with histopathological examination of spinal cord tissues recovered 43 days after MOG challenge. Whereas control vector-only-treated animals uniformly displayed multiple inflammatory foci within their spinal cords, Fn14-TRAIL-treated mice exhibited a dramatic reduction of inflammatory cell infiltration and demyelination in their spinal cords (Figure 8, A–C).
cords, supports the notion that Fn14-TRAIL attenuates infiltration of inflammatory cells across the endothelial BBB by suppressing the progressive increase in BBB permeability that accompanies encephalomyelitis.

Molecular Model of the Chimeric Fn14-TRAIL Protein

We next asked whether Fn14-TRAIL’s significant therapeutic efficacy might stem, at least in part, from the way in which it engages and bridges TWEAK ligand and TRAIL receptor (DR5) molecules. To this end, we modeled the Fn14-TRAIL protein, both as a monomer (Figure 10A) and as a trimer (Figure 10B and C). Moreover, we visualized the putative interaction of trimeric Fn14-TRAIL at its opposite ends with TWEAK and DR5, respectively (Figure 10D). Significantly, the modeled TWEAK:Fn14-TRAIL:DR5 complex showed that: i) the TWEAK-binding domains of Fn14, when forced into an artificial trimeric configuration by the chimerized, naturally trimeric TRAIL component, are favorably positioned to interact with their cognate partners within trimeric TWEAK; ii) the DR5-binding domains of trimeric TRAIL are favorably positioned to interact with their cognate partners within trimeric DR5; and iii) the carboxy-terminus of the Fn14 component within the fusion protein serves as a surrogate linker that is sufficiently rigid to keep the Fn14 and TRAIL domains apart, with no propensity for collapsing. Interestingly, the ligand binding domains of Fn14 and TRAIL are separated by ~60 Å, raising the possibility that this fusion protein could act like a spacer between the interacting cells and limit local cell-to-cell contact. Taken...
together, the modeling analysis verified that FN14-TRAIL can indeed simultaneously engage both TWEAK and DR5 on opposing cells. Moreover, the enforced FN14 neo-trimer assumes a configuration that allows for binding to the natural TWEAK trimer, perhaps creating a particularly stable higher order structure.

**Discussion**

Many immunotherapeutic proteins feature a capacity to bind and block molecules essential for immunological responses, for example, co-stimulators, pro-inflammatory cytokines, and adhesins. However, their efficacy is limited by the inherently transient nature of passive blockade, as well as by functional redundancies that often permit bypass of individual blocked signals. This limitation beckons the development of new classes of immunotherapeutic proteins with higher order functionality. In this vein, we previously introduced trans signal converting proteins (TSCPs) as a new class of immunomodulatory fusion proteins that combine passive blockade with active inhibition. Such proteins alter the cross talk between two interacting cells, for example, converting an activating APC-to-T cell signal into an inhibitory one. The paradigmatic TSCP for us was CTLA-4-FasL and its efficacy, along with the overall robustness of the intercellular signal conversion concept, have been documented in the contexts of both alloimmunity and autoimmunity.

In the present study, we describe yet another type of fusion protein that can impact intercellular signaling. This one exchanges intercellular signals, and furthermore, redirects converted neo-signals to other cell types for therapeutic benefit. Hence, such proteins might be designated trans signal redirecting proteins (TSRPs). The TSRP described here, FN14-TRAIL, bridges two prominent intercellular signaling axes: TWEAK-to-Fn14 and TRAIL-to-TRAIL receptor (TRAIL-R; in mice, DR5). Pro-inflammatory TWEAK signals, emanating from a range of TWEAK-bearing immune and nonimmune cell types, are converted by FN14-TRAIL into inhibitory TRAIL ones. Significantly, the opposing (anti-inflammatory TRAIL) neo-signals are by definition turning the TWEAK-bearing cell’s attention, and in effect redirecting signaling, from FN14 “TRAIL-R” to FN14 “TRAIL-R” activated T cells driving autoimmune pathogenesis. The functional pleiotropism of the TWEAK:FN14 and TRAIL:TRAIL-R signaling axes, especially the former, spanning an array of immune and nonimmune cell types, in particular inFN14-TRAIL extensive functional possibilities for impacting and expanding cellular networking.

Key findings of the present study include: i) both building blocks of FN14-TRAIL retain their functionality after chimerization, as documented in vitro by the fusion protein’s ability to bind to enforced TWEAK on transfected CHO cells (through its FN14 end) and induce apoptosis in actinomycin D-treated, TRAIL-R activated T cells. ii) FN14-TRAIL bridges two prominent intercellular signaling axes: TWEAK-to-Fn14 and TRAIL-to-TRAIL receptor (TRAIL-R; in mice, DR5). Pro-inflammatory TWEAK signals, emanating from a range of TWEAK-bearing immune and nonimmune cell types, are converted by FN14-TRAIL into inhibitory TRAIL ones. Significantly, the opposing (anti-inflammatory TRAIL) neo-signals are by definition turning the TWEAK-bearing cell’s attention, and in effect redirecting signaling, from FN14 “TRAIL-R” to FN14 “TRAIL-R” activated T cells driving autoimmune pathogenesis. The functional pleiotropism of the TWEAK:FN14 and TRAIL:TRAIL-R signaling axes, especially the former, spanning an array of immune and nonimmune cell types, in particular inFN14-TRAIL extensive functional possibilities for impacting and expanding cellular networking.

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inflammatory cells and/or activation of resident innate immune cell types. Similarly, Fn14 is induced dramatically in injured and diseased tissues, such as in spinal cord after induction of autoimmune encephalomyelitis. Moreover, endothelial cells secrete CCL2, MCP-1, and IL-8, as well as up-regulate ICAM-1 and E-selectin, in response to TWEAK treatment in vitro. Interestingly, the CCL2 chemokine and its receptor CCR2 have been shown to play crucial nonredundant roles in the pathogenesis of EAE. CCL2 expression in general correlates with the severity of inflammation and has been shown to increase during the course of chronic relapsing EAE. Hence, Fn14-TRAIL, by blocking TWEAK, could potentially impact more than one step involved in inflammatory cell transit into the CNS.

A key observation was a reduction in the absolute number of inflammatory cells, including early activated T cells, in the spinal cords of Fn14-TRAIL-treated animals on day 7 after immunization. Moreover, later on at the peak of disease, the absolute number of inflammatory cytokine-expressing cells remained lower for animals treated with the fusion protein. CNS inflammation correlated with BBB permeability in an intriguing way. Not only did spinal cords, brain stems, and cerebellums of Fn14-TRAIL-treated mice show attenuated BBB permeability, but these mice exhibited less BBB permeability even when compared with vector-only-treated mice with matched disease scores. This dovetails nicely with our observation that Fn14-TRAIL-treated mice, even when compared with vector-only-treated mice with comparable disease scores at the onset of disease, stay healthier in the later stages of disease and exhibit lower cumulative disease scores. Hence, although this EAE model does not permit one to tease apart the contributions of lymphoid cell suppression from reduced lymphoid cell transit into the CNS, the data certainly point to some contribution of the latter to suppression of inflammation that is likely attributable to Fn14-TRAIL antagonism of TWEAK.

A pivotal finding in the present study is that the fusion protein proved to be far more therapeutically active in vivo than its component protein parts (Fn14 and TRAIL), when the latter are administered as soluble entities either alone or in combination. The limited nature of the literature bearing on these particular components when used as soluble agents makes it difficult to compare our findings. Although one group has reported that Ab blockade of TWEAK improves the clinical course of EAE and decreases CNS-specific inflammation, the only study invoking Fn14 per se as a blocking agent involved a full-length Fn14, apparently encompassing the transmembrane and cytoplasmic regions of a rat Fn14 protein. In the case of TRAIL, there is again only a single report in the literature dealing with human TRAIL and its use as a soluble agent (in this case, produced in bacteria) in an EAE therapeutic context. As alternatives, however, surrogates for soluble TRAIL have been invoked, namely, agonistic anti-DR5 Ab and dendritic cells with enforced TRAIL expression. In each of these latter cases, the TRAIL receptor trigger may be achieving a higher effective valency. This, along with dosing factors, could explain why soluble TRAIL in our hands, expressed in vivo, in an AJP February 2009, Vol. 174, No. 2 context. Chimerizing the Fn14 and TRAIL components within a single fusion protein may elicit special and sometimes complementary functional properties that could augment the efficacy of the two components, mask negative effects of one or the other, which might otherwise limit their therapeutic utility, and confer particular advantages in the EAE context. These include the following:

1) By linking Fn14, a protein that is naturally monomeric, to TRAIL (naturally a trimer), one is in effect cre-
ating a trimeric variant of Fn14. As it turns out, predictive structural modeling suggests that this Fn14 neo-trimer could neatly dock with Fn14’s naturally-trimeric TWEAK counterreceptor (Figure 10). Moreover, at the other end of the Fn14-TRAIL fusion protein, the trimeric TRAIL is docking with the trimeric DR5 receptor for TRAIL. Hence, these structural complementarities, generated de novo by chimerizing monomeric Fn14 to trimeric TRAIL-DR5 molecular bridge allowing for greater potency in blocking TWEAK and driving DR5 on cells facing each other.

2) TRAIL ligand is in effect being anchored to the cell surface via an Fn14 bridge, which serves to augment its effective valency. In a sense, this is replicating the situation with dendritic cell neo-expressing surface TRAIL, which have been shown to be effective in suppressing EAE.30

3) There is evidence that TRAIL-mediated inhibition may function primarily at the EAE priming phase (during T-cell triggering and expansion),29 whereas TWEAK-blockade’s benefit may be more focused on downstream pro-inflammatory events (including preventing breakdown of the BBB).59 Thus, by combining the two, one has a single agent that could in principle impact both priming and later phases of the disease.

4) One group has reported that TRAIL may contribute to death of neurons after the priming phase,70,73 in keeping with previous studies showing that both primary human neurons74 and oligodendrocytes75 are susceptible to TRAIL-induced cell death. By coupling Fn14 to TRAIL, one is providing an agent that sustains the integrity of the BBB (via TWEAK blockade) and thereby mitigates TRAIL’s access to the CNS. Moreover, because TWEAK has been reported to trigger neuronal cell death,24 Fn14-TRAIL would be expected to protect neurons by blocking this TWEAK activity as well.

5) During EAE, the number of vessels correlates with both disease scores and pathological measures for inflammation, leukocyte infiltration, and demyelination.22 Because TWEAK promotes angiogenesis21 and TRAIL inhibits it,76 there is the intriguing possibility that Fn14-TRAIL may inhibit angiogenesis at inflammatory sites by a dual mechanism, that is, by antagonizing TWEAK’s proangiogenic activity and synergistically reinforcing this effect through TRAIL’s anti-angiogenic activity.

6) Yet another synergy could stem from modulation of endothelial cell function and chemokine expression. As stated above, blockade of TWEAK may interfere with chemokine expression by, and leukocyte adhesion to, endothelial cells. TRAIL is also linked to these processes. TRAIL counteracts TNF-a-induced leukocyte adhesion to endothelial cells by down-modulating CCL8 and CXCL10 chemokine expression and release.77 Thus, Fn14-TRAIL could impact leukocyte attraction and adhesion through both its Fn14 and TRAIL ends.

Although the present study centers around the autoimmune encephalomyelitis model, Fn14-TRAIL’s therapeutic benefit may well extend to other autoimmune conditions. TWEAK has emerged as a potent arthritogenic ligand, with anti-TWEAK blocking Ab reducing disease severity in the collagen-induced arthritis model.78,79 TWEAK has also been linked to systemic lupus erythematosus, with TRAIL blocking Ab again showing a beneficial effect with respect to renal damage in mice with induced lupus.80 Adding TRAIL-mediated immunoinhibitory capacity to a TWEAK-blocking agent would be expected to augment therapeutic efficacy in these other autoimmune settings.

Fn14-TRAIL may ultimately prove to have functions that extend beyond those described so far. The observation that Fn14 can stimulate neurite outgrowth in peripheral neurons independent of TWEAK81 suggests the likelihood that there are additional ligands for the Fn14 receptor. If so, Fn14-TRAIL’s effects could go well beyond functions associated with the TWEAK ligand per se, some of which could be serving to reinforce the autoimmune benefit, as well as point to clinical applications beyond autoimmune therapies. Also, because there is now some evidence that TWEAK may bind to at least one additional receptor other than Fn14, namely CD163,82 Fn14-TRAIL would interfere with biological effects stemming from TWEAK interacting with such a non-Fn14 receptor. Furthermore, therapeutic fusion proteins can be functionally more than the mere sum of their parts. This has proved to be the case for the TSCP CTLA-4-Fasl, which was shown to be able to uniquely block the up-regulation of the anti-apoptotic c-FLIP protein after T-cell activation.83 Dissecting out just how a multifunctional therapeutic TSRP such as Fn14-TRAIL perturbs immune cellular networking from multiple angles and through this achieves a net anti-inflammatory therapeutic effect should be informative down the road. What may emerge is a sense of how fusion proteins with multifunctional potential can be best deployed to tackle diseases with multidimensional pathogenesis.

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References

12. Wiley SR, Winkles JA: TWEAK, a member of the TNF superfamily, is a multifunctional cytokine that binds the TWEAKR/Fn14 receptor. Cyto- kine Growth Factor Rev 2003, 14:241–249