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TNF is a pleiotropic cytokine required for normal development and function of the immune system; however, TNF overexpression also induces inflammation and is associated with autoimmune diseases. TNF exists as both a soluble and a transmembrane protein. Genetic studies in mice have suggested that inflammation in disease models involves soluble TNF (solTNF) and that maintenance of innate immune function involves transmembrane TNF (tmTNF). These findings imply that selective pharmacologic inhibition of solTNF may be anti-inflammatory and yet preserve innate immunity to infection. To address this hypothesis, we now describe dominant-negative inhibitors of TNF (DN-TNFs) as a new class of biologics that selectively inhibits solTNF. DN-TNFs blocked solTNF activity in human and mouse cells, a human blood cytokine release assay, and two mouse arthritis models. In contrast, DN-TNFs neither inhibited the activity of human or mouse tmTNF nor suppressed innate immunity to *Listeria* infection in mice. These results establish DN-TNFs as the first selective inhibitors of solTNF, demonstrate that inflammation in mouse arthritis models is primarily driven by solTNF, and suggest that the maintenance of tmTNF activity may improve the therapeutic index of future anti-inflammatory agents. *The Journal of Immunology,* 2007, 179: 1872–1883.

Tumor necrosis factor is a multifunctional cytokine known to regulate inflammation, autoimmunity, cancer, and infection (1–5). TNF is synthesized and presented on the cell surface as a trimeric transmembrane protein that can be cleaved by TNF-α converting enzyme (TACE)[2] (2) protease to generate soluble TNF (solTNF). Both forms of TNF are known to induce cell signaling events, with transmembrane TNF (tmTNF) acting through cell-cell contacts to promote juxtacrine signaling and solTNF acting in a paracrine fashion. Mounting evidence from clinical observations in humans and experimental studies in rodents suggests that paracrine signaling by solTNF is associated with chronic inflammation (6, 7), whereas juxtacrine signaling by tmTNF plays an essential role in resolving inflammation (8–10) and maintaining immunity to pathogens including *Listeria monocytogenes* (11, 12) and *Mycobacterium tuberculosis* (13–16). It has been suggested that different signaling cascades induced by these two forms of TNF may explain the contrasting beneficial and detrimental roles of the cytokine seen in animal models and human diseases (2, 6, 17–19).

TNF sequestration by monoclonal anti-TNF Abs (infliximab and adalimumab) and the IgG1 Fc-TNF receptor (TNFR) 2 fusion protein (etanercept) has been a successful treatment strategy for several autoimmune diseases, including rheumatoid arthritis. These two classes of biologics effectively control disease progression and in some cases can induce remission. Their use has also been linked to serious adverse events such as congestive heart failure, demyelinating disease, and lupus (20). Most notably, the use of anti-TNF biologics has been associated with an increased risk of infection (20–24). A significant common feature of these biologics is that they inhibit both paracrine (solTNF) and juxtacrine (tmTNF) cell signaling (25–28). Taken together with the mouse genetic data, these findings suggest that a solTNF-selective inhibitor that spares tmTNF activity might inhibit inflammation without suppressing the immune response to infection.

A previous report described the invention of a novel class of anti-TNF biologics (DN-TNFs) that work through a unique dominant-negative mechanism (29). These engineered variants of human TNF neither bind to nor signal through the NF receptors TNFR1 or TNFR2, but rapidly exchange subunits with native TNF homotrimers to form inactive mixed heterotrimers, effectively eliminating native TNF. DN-TNFs inhibit multiple functions of TNF, including NF-κB nuclear translocation and transcriptional activation and caspase-mediated apoptosis (29); they also reduce disease severity in rat models of arthritis and Parkinson's disease (30).

We now report that DN-TNFs are selective inhibitors of solTNF. DN-TNFs blocked solTNF paracrine signaling in numerous assays, including caspase activation in human and mouse cell lines and proinflammatory cytokine production in human whole blood. Absolute efficacy and mass potency in these assays were comparable to the Ab and decoy receptor classes of anti-TNF biologics. In contrast, DN-TNFs failed to

*Xencor, Monrovia, CA 91016; and †Institut Transgenose, Centre National de la Recherche Scientifique, Orléans, France

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[1] Address correspondence and reprint requests to Dr. David E. Szymkowski, Xencor, 111 W. Lemon Avenue, Monrovia, CA 91016. E-mail address: david.szymkowski@xencor.com

[2] Abbreviations used in this paper: TACE, TNF-α converting enzyme; CAIA, collagen-antibody induced arthritis; CIA, collagen-induced arthritis; CHO, Chinese hamster ovary; DN-TNF, dominant-negative inhibitor of TNF; PEG, polyethylene glycol; solTNF, soluble TNF; tmTNF, transmembrane TNF.

Table I. *DN-TNF biologics: sequence modifications from native human solTNF*[a]

DN-TNF	Tag[b]	Improves Expression (V1M)	Impairs Binding to TNFR1, TNFR2						Allows Site-Specific Pegylation (C69V, C101A, R31C)[c]	Improves In Vivo Half-Life (Mod C31)[d]
			L57Y	S86Q	Y87H	I97T	Y115Q	A145R		
XENP246	X					X	X			
XENP249	X			X			X			
XENP257	X		X				X			
XENP267	X				X			X		
XENP268	X					X		X		
XENP344		X			X			X	X	5-kDa mPEG[e]
XENP345		X				X		X	X	5-kDa mPEG[e]
XENP346		X				X		X	X	10-kDa mPEG[e]
XENP1595		X			X			X	X	10-kDa mPEG[e]
XENP1596		X			X			X	X	Ethyl

[a] Sequence changes are based on 157-aa native human solTNF (UniProtKB/Swiss-Prot database entry P01375).
[b] N-terminal MHHHHHH.
[c] R31C also impairs TNFR binding.
[d] Posttranslational modification (Mod) of amino acid C31.
[e] mPEG, monomethoxy-polyethylene glycol (for clarity, mPEG is not included in indicated concentrations).

inhibit juxtacrine signaling mediated by tmTNF, whereas nonselective biologics retained activity in these assays. DN-TNF biologics were as effective as a decoy receptor in reducing inflammation in two mouse models of arthritis. However, treatment with DN-TNFs did not suppress the resistance of mice to *L. monocytogenes* infection as measured by survival, bacterial load, and histopathology, whereas nonselective inhibition by decoy receptor led to marked immunosuppression. Decoy receptors also sensitized mice expressing only tmTNF (tmTNF knockins) to *Listeria* infection, whereas a DN-TNF biologic did not, providing further in vivo evidence that DN-TNFs are selective inhibitors of solTNF.

Materials and Methods

Preparation of anti-TNF biologics

DN-TNF proteins were prepared, processed, and handled as described previously (29). Multiple DN-TNF variants were engineered and assessed in in vitro and in vivo studies. These variants contained different combinations of six key mutations that disrupt TNFR binding (see Table I). Additional mutations were introduced in some compounds to facilitate purification, improve expression, or permit site-specific pegylation for in vivo use. Etanercept, infliximab, and adalimumab were obtained from a pharmacy (RxUSA).

Soluble TNF production and signaling assays

To measure solTNF signaling, increasing concentrations of TNF inhibitors were incubated with the indicated concentrations of recombinant human or mouse solTNF or mouse lymphotoxin α (R&D Systems) for 60 min at 37°C in PBS supplemented with 0.02% Igepal CA-630 (Sigma-Aldrich). This time period (60 min) was chosen to assure complete interaction with TNF for all biologics based on a previous report that DN-TNFs exchanged efficiently with native TNF within 20 min (29). These mixtures were applied to either human U937 cells or mouse WEHI-13VAR cells in the presence of 1 μg/ml actinomycin D (ICN Pharmaceuticals). After an additional 3-h incubation, a fluorescently labeled caspase-3/7 substrate (DEVD-R110; Roche) was used as a marker of TNF-induced apoptosis (31). Physiologically produced and processed solTNF was generated by treating either human THP-1 cells or murine RAW264.7 cells with 1 μg/ml LPS (Sigma-Aldrich). Conditioned medium was harvested and solTNF was measured using a commercial anti-human or anti-mouse TNF ELISA kit. Conditioned medium was supplemented with TNF inhibitors and 0.02% Igepal CA-630 and incubated for 60 min at 37°C. These mixtures were then added to cells as described above to induce caspase signaling. Levels of caspase activation induced by human and mouse solTNF were comparable in human U937 and mouse WEHI-13VAR cells. To permit a direct comparison of the efficacy and potency of DN-TNFs and control biologics against murine and human TNF irrespective of potential differences in cell assay formats, caspase activity is expressed as relative units normalized to maximum velocity (V_{max}). To facilitate comparisons of different DN-TNF molecules, the polyethylene glycol (PEG) groups on several DN-TNF biologics (see Table I) are not included in the indicated concentrations. All protein concentrations are given by mass; to convert to molar concentrations, note the following molecular masses: DN-TNF biologics, 52 kDa; etanercept ~105 kDa; adalimumab, ~148 kDa; and infliximab, ~149 kDa.

Native TNF:DN-TNF heterotrimer detection

Conditioned medium from LPS-treated RAW264.7 cells was supplemented with XENP267 and screened for native murine solTNF:DN-TNF heterotrimer formation using an indirect sandwich ELISA as previously described (29). ELISA plates were coated overnight with 2 μg/ml anti-mouse TNF Ab (BioSource International) and blocked the next day with BSA (ICN Pharmaceuticals). Conditioned medium samples were applied and heterotrimers detected with anti-penta-His Ab-biotin conjugate (Qiagen). Alkaline phosphatase-conjugated NeutrAvidin coupled with a luminescent enzyme substrate was used for detection. Under these conditions, the ELISA signal is produced exclusively from XENP267:mouse solTNF heterotrimers; control mixtures of etanercept and mouse solTNF are at background.

Transmembrane TNF production and signaling assays

Standard molecular biology techniques were used to delete amino acids 77–89 from full-length human TNF to generate Δ1–12proTNF (32). This construct was transiently transfected into Chinese hamster ovary (CHO) cells using Lipofectamine (Invitrogen Life Technologies); the next day the cells were trypsinized and transferred into 96-well plates to reduce well-to-well variation. After an overnight incubation with increasing doses of TNF inhibitors, U937 cells and 1 μg/ml actinomycin D were added; cells were cocultured for an additional 3 h to induce tmTNF-dependent caspase activity. No human solTNF bioactivity was detected under these experimental conditions. To generate endogenous mouse tmTNF, RAW264.7 cells were treated with 1 μg/ml LPS (Sigma-Aldrich) in the presence of 10 μM TACE inhibitor TAPI-2 (BIOMOL International). These cells were mixed with 1 μg/ml actinomycin D and U937 target cells to induce tmTNF-mediated caspase activity. No mouse solTNF bioactivity was detected under these experimental conditions. Caspase assay data are expressed as described above for solTNF assays; statistical analysis was by one-way ANOVA.

Microscopy for mouse tmTNF-induced caspase assay

RAW264.7 cells were plated onto glass coverslips; the next day, the medium was replaced and cells were untreated, incubated with 1 μg/ml LPS (Sigma-Aldrich), or incubated with 1 μg/ml LPS and 10 μM TAPI-2 (BIOMOL International) for 1 h at 37°C. Cells were washed in PBS, placed on ice, and mixed with 5 μg/ml anti-mouse TNF Ab (R&D Systems) for an additional 1 h. Cells were maintained on ice during this incubation to minimize internalization of the Ag complex. Cells were then washed in PBS, fixed, and processed for microscopy as described by the Ab supplier. Epifluorescent imaging was done using an Eclipse TS100 (Nikon) equipped with a charge-coupled device camera (Media Cybernetics).

Human whole blood culture

Blood samples for the TNF-induced IL-8 assays were supplied by HemaCare Blood Services following protocols approved by its institutional review board. Blood was drawn from healthy donors by venipuncture and immediately aliquoted into vials containing endotoxin-free sodium heparin (Sigma-Aldrich). Whole blood cultures were performed in flat-bottom microtiter plates; reagents were prepared in PBS containing 0.1% endotoxin-free BSA (Sigma-Aldrich). For TNF inhibition studies, biologics were combined with human solTNF and incubated for 2 h at 37°C in PBS supplemented with 0.02% Igepal CA-630. These mixtures were diluted 20-fold, added to blood specimens, and incubated an additional 6 h. Triton X-100 was then added to a final concentration of 0.5% to lyse cells and release any cell-bound cytokines. IL-8 levels were measured using a commercial ELISA (R&D Systems). Dose-ranging studies with blood from multiple donors were first done to establish the optimal solTNF concentration for antagonist studies.

Mouse collagen Ab-induced arthritis (CAIA) model

Three mouse CAIA studies were performed by MD Biosciences following protocols approved by its institutional review board. Male BALB/c mice ($n = 8$/group) were treated prophylactically with TNF inhibitors or vehicle delivered i.p., s.c., or i.p. by osmotic pump. In the first study, mice were given daily i.p. injections of vehicle or 10 mg/kg XENP345, XENP346, XENP1595, or etanercept. Injections started on day −3 and continued to day 12. Experimental arthritis was induced on day 0 by i.v. injection of four anti-collagen mAbs (MD Biosciences) at 25 mg/kg each followed by 2.5 mg/kg LPS given i.p. 72 h later. Arthritis severity was measured in a blinded fashion by measuring hind paw thickness (average of left and right) using a digital caliper. The second study was conducted similarly, except that etanercept and XENP1595 were given at a lower dose and frequency (1.0 mg/kg every 3 days) using the projected clinical formulation and s.c. route. Injections started on day −1 and continued to day 14. In the pump study, 14-day osmotic pumps (Alzet) were primed, loaded, and surgically implanted i.p. on day −3 and replaced with a fresh pump on day 11 using standard methods. Pumps delivered either XENP1595 at 3, 10, or 30 mg/kg/day, etanercept at 10 mg/kg/day, or a vehicle control (PBS (pH 8.0) and 10% glycerol). Hind paw thickness was recorded by caliper as described above. Steady-state serum levels of DN-TNFs and etanercept were determined at the end of the study by using a custom sandwich ELISA for DN-TNFs and a commercial TNFR2 ELISA for etanercept. For all CAIA studies, statistical analysis of hind paw thickness data was by one-way ANOVA with Dunnett's posttest.

Mouse collagen-induced arthritis (CIA) model

Mouse CIA studies were performed by Washington Biotechnology following protocols approved by its institutional review board. Arthritis was induced in male DBA/1J mice by two s.c. injections of an emulsion containing 5 mg/kg bovine collagen in CFA on day 0 and in IFA on day 21. Disease onset was observed by day 27 and therapeutic dosing was begun on day 33. Mice ($n = 10$/group) were treated therapeutically s.c. with vehicle, XENP1595, or etanercept. Disease symptoms were measured by summing arthritis score from four paws (0, no visible effects of arthritis; 1, edema and/or erythema of one digit; 2, edema and/or erythema of two joints; 3, edema and/or erythema of more than two joints; and 4, severe arthritis of the entire paw and digits); statistical significance was assessed using a Kruskal-Wallis nonparametric test with Dunn's posttest for multiple comparisons.

Generation of TNF knockout and tmTNF knockin mice

Knockout mice deficient in TNF ($TNF^{-/-}$) and tmTNF knockin mice were bred and housed in a pathogen-free facility at the Centre National de la Recherche Scientifique (Orléans, France). tmTNF knockin mice expressing only tmTNF were generated directly on a C57BL/6 background by replacing the endogenous TNF allele with the Δ1–9,K11E TNF allele to ensure the complete loss of TACE-mediated cleavage but normal cell surface expression and function of tmTNF (7). TNF knockout mice were backcrossed onto the C57BL/6 background as described (12).

Mouse infection model

The initial *L. monocytogenes* infection survival studies in normal $TNF^{+/+}$ mice and in tmTNF knockin mice were performed at the Centre National de la Recherche Scientifique following protocols approved by its institutional review board. In the normal mouse study, vehicle or 30 mg/kg XENP345, XENP1595, or etanercept was administered s.c. on days −1 and 2 to C57BL/6 mice ($n = 6$/group) infected on day 0 with 10^4 CFU *L. monocytogenes* i.v. as described (33). *L. monocytogenes*-infected TNF knockout and tmTNF knockin mice that were not treated with anti-TNF biologics were used as genetic controls. Survival and body weight were monitored and, in an additional four mice per satellite group, liver histology and bacterial load (CFU) were assessed 3 days after infection (33). In the tmTNF knockin study, mice ($n = 10$/group) were infected as described above and treated with vehicle or 10 mg/kg XENP1595 or etanercept s.c. on days −1, 2, 6, 9, and 13. *L. monocytogenes*-infected TNF knockout mice not treated with anti-TNF biologics served as genetic positive controls. Survival and bacterial load in the liver were monitored in an additional four mice per satellite group as described above.

Another *Listeria* survival study was performed by Washington Biotechnology following protocols approved by its institutional review board. Female BALB/c mice ($n = 8$/group) were injected i.p. daily with vehicle or 10, 30, or 50 mg/kg XENP1595 or etanercept. After three days, mice were food-deprived for 24 h and orally infected with *L. monocytogenes* at the doses indicated. Food was restored and TNF inhibitors were administered for an additional 3 days. Mice were then euthanized, and bacterial loads in spleen (total CFU) and blood (CFU/ml) were determined by standard methods. In all *Listeria* studies, survival data (Kaplan-Meier plots) were compared using a log rank test for comparison of groups, and bacterial load data were analyzed using a one-way ANOVA with Dunnett's posttest.

Results

DN-TNFs contain key mutations that prevent binding and activation of TNFR1 and TNFR2 (29, 34) yet maintain their structural integrity and ability to rapidly exchange monomer subunits with native solTNF. When mixed with solTNF, DN-TNFs sequester and effectively eliminate native cytokine by forming inactive mixed heterotrimers. Table I shows the sequences of the 10 DN-TNF biologics described in this report, including the combinations of six key mutations that impair TNFR binding. Modifications made to enhance expression, allow site-specific pegylation, and improve half-life are also shown. The 10 DN-TNF variants fall into several groups possessing the same critical mutations that impair TNFR1 and TNFR2 binding (Table I). For example, XENP267, XENP344, XENP1595, and XENP1596 all include Y87H and A145R mutations to inhibit receptor binding and differ only in the presence or absence of an N-terminal tag or PEG side chain. Multiple DN-TNF variants were included in some studies to assess whether the results were unique to individual compounds or broadly associated with this class of biologics. The variants included in each study were selected based on the nature of the assay; typically, multiple variants were included for in vitro studies on human cells, and fewer were used for in vitro and in vivo mouse studies. For example, initial human in vitro studies tested multiple DN-TNF variants containing different TNFR binding mutations; all showed similar efficacy (Fig. 1, *A* and *C*). In subsequent studies, subsets of these variants were tested (e.g., pegylated variants were used in mouse models).

DN-TNF biologics inhibit solTNF-induced caspase activation

In previous studies we showed that DN-TNFs inhibit several TNF activities, including NF-κB transcriptional activation and nuclear translocation, and caspase-mediated apoptosis (29). In this study, we compared DN-TNF inhibition of solTNF activity via subunit exchange to the TNF sequestration mechanism of other biologics by using recombinant and endogenously produced solTNF as targets. A caspase activation assay in human cells was selected because it is a well-established and sensitive method for quantifying TNF bioactivity (35, 36). In preparation for in vivo pharmacology studies and to characterize the species-specificity of DN-TNF, an analogous cell signaling assay was developed using mouse TNF and TNFRs. U937 and WEHI-13VAR cells were chosen as human and murine target cell lines, respectively, because both have been shown to express TNFRs and both are sensitive to TNF-induced caspase activation (37–40). We have also confirmed by flow cytometry that U937 cells express TNFR1 and TNFR2 (data not shown).

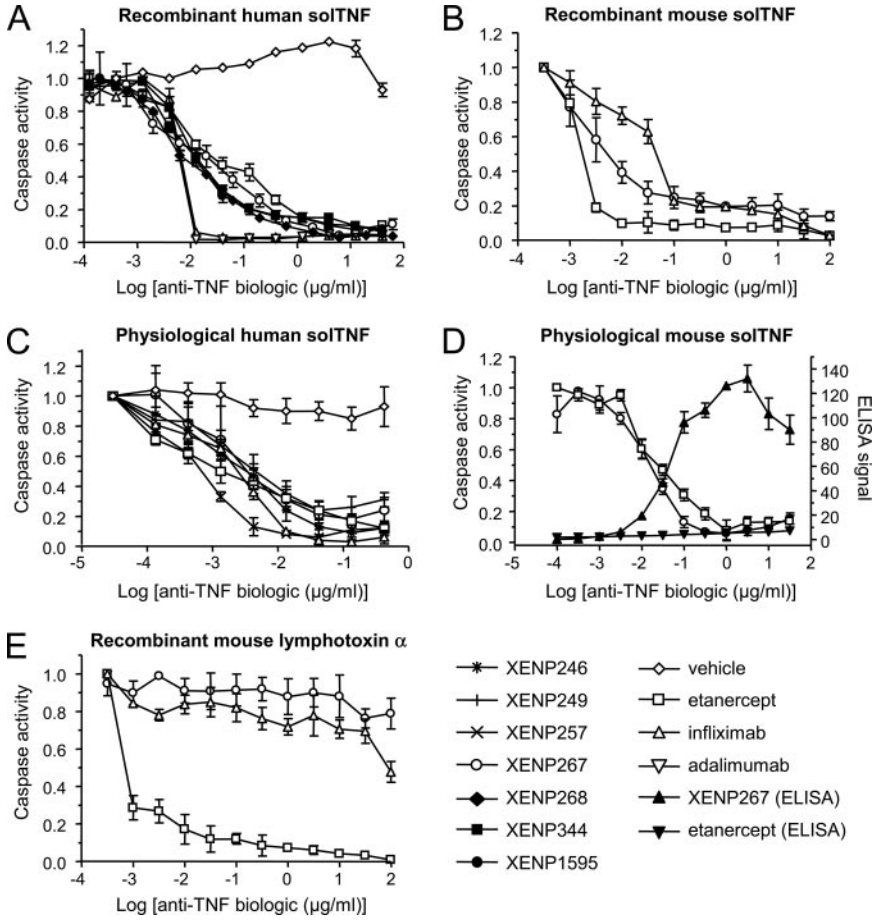

FIGURE 1. Inhibition of recombinant and physiological solTNF by DN-TNF biologics. *A*, Inhibition of recombinant human solTNF-induced cell signaling. Human solTNF (5 ng/ml) was incubated with vehicle or increasing concentrations of DN-TNFs (XENP267, XENP268, XENP344, and XENP1595), decoy receptor (etanercept), or neutralizing mAbs (infliximab and adalimumab) and then mixed with human U937 cells to induce caspase activity (mean ± SEM, n = 4). IC_{50} values: mAbs, 2 ng/ml; DN-TNFs, 11 ng/ml; etanercept, 20 ng/ml. *B*, Inhibition of recombinant mouse solTNF-induced cell signaling. Mouse solTNF (5 ng/ml) was incubated with increasing concentrations of XENP267, etanercept, or infliximab and then mixed with mouse WEHI-13VAR cells to induce caspase activity (mean ± SD, $n = 3$). IC_{50} values: infliximab, 37 ng/ml; XENP267, 5 ng/ml; etanercept, 2 ng/ml. *C*, Inhibition of LPS-induced physiological human solTNF activity. Human THP-1 cells were stimulated with LPS to generate conditioned medium containing 6 ng/ml secreted solTNF. Conditioned medium was incubated with vehicle or increasing concentrations of DN-TNFs (XENP246, XENP249, XENP257, and XENP267), etanercept, or infliximab and then processed as described in *A* (mean ± SD, $n = 3$). IC_{50} values: infliximab, 7 ng/ml; DN-TNFs, 0.5–8 ng/ml; etanercept, 2 ng/ml. *D*, Inhibition of LPS-induced physiological mouse solTNF activity. Conditioned medium from LPS-treated mouse RAW264.7 cells containing 10 ng/ml murine solTNF was incubated with increasing doses of XENP267 or etanercept. Mixtures were split and aliquots were used to stimulate caspase activation in human U937 cells, whereas the remainder was assayed for mouse TNF:DN-TNF heterotrimer formation by using a sandwich ELISA (mean ± SEM, $n = 3$). Caspase IC_{50} values: XENP267, 14 ng/ml; etanercept, 36 ng/ml. Inhibitory activity of DN-TNF correlates with subunit exchange into endogenous mouse solTNF. *E*, DN-TNF and infliximab do not inhibit lymphotoxin α (solTNFβ) activity. Mouse recombinant lymphotoxin α (5 ng/ml) was incubated with increasing concentrations of XENP267, etanercept, or infliximab and then mixed with mouse WEHI-13VAR cells to induce caspase activity (mean ± SD, $n = 3$). IC_{50} value: etanercept, <1.0 ng/ml.

In the human U937 cells, anti-TNF mAbs, decoy receptors, and multiple DN-TNF biologics inhibited recombinant human solTNF-induced caspase activation with similar maximal efficacy (Fig. 1*A*), with the two Abs being more potent by mass. In the murine cell line WEHI-13VAR, the three classes of TNF inhibitors also blocked recombinant mouse solTNF-induced caspase activation (Fig. 1*B*). Infliximab was less effective against mouse TNF relative to human TNF, probably due to the species-specificity of the Ab; this precluded its use in subsequent mouse models of arthritis and infection. The finding that a DN-TNF biologic derived from human solTNF is an effective antagonist of mouse solTNF is in agreement with published observations showing that human solTNF can exchange subunits with mouse solTNF to form heterotrimers (41).

TNF is naturally produced as a transmembrane protein that is processed by TACE to generate solTNF. Because solTNF produced in *Escherichia coli* is posttranslationally processed differently than in human or mouse cells (e.g., mouse solTNF is glycosylated), it was important to demonstrate DN-TNF inhibition against endogenously produced solTNF. LPS was therefore used to induce the production of physiological human or mouse solTNF. Briefly, human THP-1 cells or mouse RAW264.7 cells were treated with LPS, the conditioned medium was collected, and solTNF levels were determined by ELISA. The conditioned medium was then supplemented with increasing concentrations of TNF inhibitors and used to stimulate caspase activation in U937 cells. The three classes of TNF inhibitors showed similar activity against endogenously produced human solTNF (Fig. 1*C*). Similar results were obtained with mouse TNF; etanercept and DN-TNF inhibited endogenous mouse solTNF to a similar extent (Fig. 1*D*, *left axis*). In addition, a sandwich ELISA was used in this study to demonstrate that DN-TNF activity correlates with heterotrimer formation between DN-TNF and mouse solTNF (Fig. 1*D*, *right axis*).

Because many proteins in the TNF superfamily share conserved structural features, it was important to determine whether the exchange mechanism of DN-TNF is specific for TNF; therefore, a signaling assay was established to measure inhibition of lymphotoxin α (solTNFβ), the most closely related cytokine. As described above, the three classes of TNF inhibitors blocked recombinant mouse solTNF (Fig. 1*B*). However, DN-TNF and Ab failed to block recombinant mouse lymphotoxin α (each showed a >1000-fold loss of potency relative to solTNF), whereas the decoy receptor retained activity (Fig. 1*E*) in agreement with a previous report (26). Taken together, the five studies shown in Fig. 1 demonstrate that DN-TNF biologics effectively antagonize recombinant or endogenously produced solTNF with comparable overall efficacy to that of a decoy receptor and anti-TNF Abs; however, DN-TNF does not block the activity of lymphotoxin α.

DN-TNF biologics do not inhibit tmTNF-induced caspase activation

Based on the structural differences between solTNF and tmTNF, we reasoned that solTNF might dissociate more readily than the membrane-bound form and, thus, a DN-TNF biologic might be a less effective inhibitor of tmTNF. To test this hypothesis, we developed a heterologous tmTNF expression system to quantify the effects of DN-TNF biologics on tmTNF-induced juxtacrine signaling. Such heterologous cocultures are established tools for assessing tmTNF bioactivity and its inhibition by biologics (26, 42). Expression of both human and mouse tmTNF was elicited in cell lines, and signaling was again monitored by using caspase activation in human U937 target cells.

Briefly, to generate human tmTNF-expressing cells, CHO cells were transfected with a human TNF construct lacking the TACE cleavage site (Δ1–12proTNF), thus generating bioactive tmTNF that is not shed from the cell surface (26, 32). These tmTNF-expressing cells were incubated overnight with increasing concentrations of TNF inhibitors and cocultured the next day with U937 target cells. In this system, human tmTNF on CHO cells engages human TNFRs on U937 cells to stimulate caspase activity 3- to 4-fold over background. In agreement with the literature (25–28), mAbs and decoy receptors inhibited human tmTNF-induced caspase activation (IC_{50} values in this assay were ~0.12 μg/ml for the mAbs and ~1.2 μg/ml for etanercept). In contrast, DN-TNF biologics failed to inhibit tmTNF-induced activation even at >100 μg/ml (Fig. 2*A*), which is ~10,000-fold greater than the DN-TNF dose shown to effectively inhibit human solTNF (Fig. 1, *A* and *C*). Control experiments were conducted to confirm that no solTNF is generated by Δ1–12proTNF-transfected CHO cells (Fig. 2*B*) and to demonstrate that caspase activation is detectable above background only when human tmTNF-expressing CHO cells (but not mock-transfected cells) are mixed with human U937 target cells (Fig. 2*C*).

Similar results were obtained in cocultures by using cells expressing murine tmTNF. LPS-stimulated mouse RAW264.7 cells were treated with TAPI-2, a small-molecule TACE inhibitor that traps uncleaved tmTNF on the cell surface (43). These mouse tmTNF-expressing cells were incubated with TNF inhibitors and cocultured with human U937 target cells to induce caspase activation as described above. In this juxtacrine signaling assay, 10 μg/ml DN-TNF had no effect on murine tmTNF-mediated caspase activation, whereas decoy receptor and anti-mouse TNF Ab reduced caspase activation to background levels (Fig. 3*A*). Controls for this assay were also performed to confirm that no solTNF is generated in the medium (Fig. 3*B*) and that tmTNF is expressed at high levels on the RAW264.7 cell membrane (Fig. 3, *C–E*) only in the presence of LPS and TAPI-2.

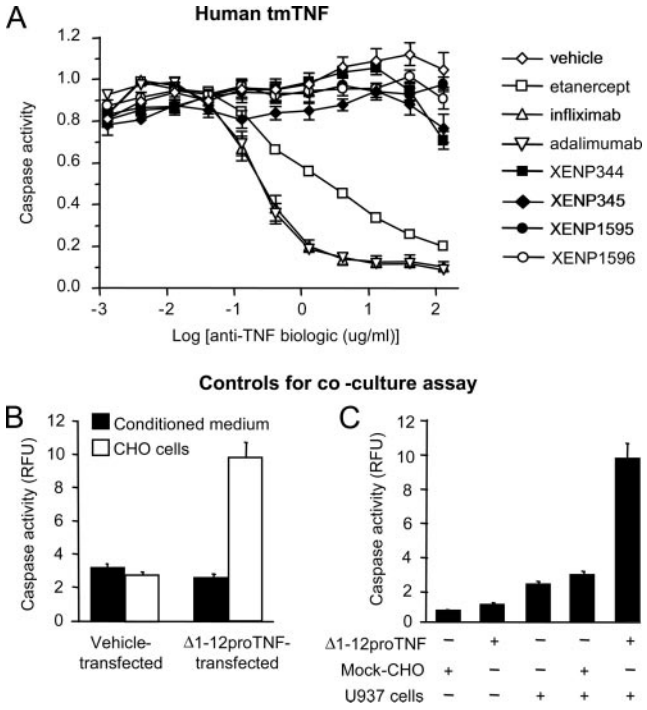

FIGURE 2. DN-TNFs do not inhibit activity of heterologously expressed human tmTNF. *A*, CHO cells expressing human tmTNF lacking a TACE cleavage site (Δ1–12proTNF) were incubated overnight with vehicle or increasing concentrations of DN-TNFs, etanercept, infliximab, or adalimumab. U937 cells were added and cocultured an additional 3 h to induce caspase activation via juxtacrine signaling. Etanercept and mAbs inhibited tmTNF-induced caspase activation, whereas four DN-TNFs did not (mean $\pm$ SEM, $n = 6$). IC_{50} values: infliximab and adalimumab, 120 ng/ml; etanercept, 1170 ng/ml. *B* and *C*, Controls for human Δ1–12proTNF coculture assays. To correctly interpret the results of the CHO and U937 cell coculture assays, it was necessary to determine: 1) whether detectable solTNF was generated by human Δ1–12proTNF-transfected CHO cells; and 2) the background contribution of each cell type to caspase activity measured during the coculture. *B*, Coculture assay in which CHO cells were either mock transfected with vehicle or transfected with the Δ1–12proTNF expression construct. U937 cells were incubated with either CHO cells or with conditioned medium obtained from the same CHO cells. Only CHO cells expressing Δ1–12proTNF protein induced caspase activity in U937 cells; no detectable TNF activity was present in conditioned medium from Δ1–12proTNF-transfected CHO cells or mock-transfected controls. *C*, Background effect of CHO and U937 cells on the tmTNF coculture assay. CHO cells alone, U937 cells alone, Δ1–12proTNF-transfected CHO cells alone, and coculture of mock-transfected CHO cells plus U937 cells established background levels of caspase activity. Coculture of Δ1–12proTNF-transfected CHO plus U937 cells stimulated a 3- to 4-fold increase in caspase activity over controls. All experimental procedures were as described in *Materials and Methods*. For *B* and *C*, values represent mean $\pm$ SD ($n = 24$).

These findings indicate that the DN-TNF class of inhibitors is highly selective for solTNF whether of human or mouse origin and whether recombinantly or physiologically produced. Unlike other classes of TNF inhibitors, DN-TNF biologics do not inhibit solTNFβ or tmTNF. Based on these observations, DN-TNF biologics are suitable tools to explore the role of solTNF in primary human cell assays as well as in mouse models of inflammation and immunity.

DN-TNF biologic inhibits solTNF-induced release of IL-8 in human blood

Translational studies were performed using a human whole blood assay to assess the anti-inflammatory effects of DN-TNF. This assay has previously been used to show that anti-TNF agents, including decoy receptor and mAbs, suppress TNF-induced IL-8

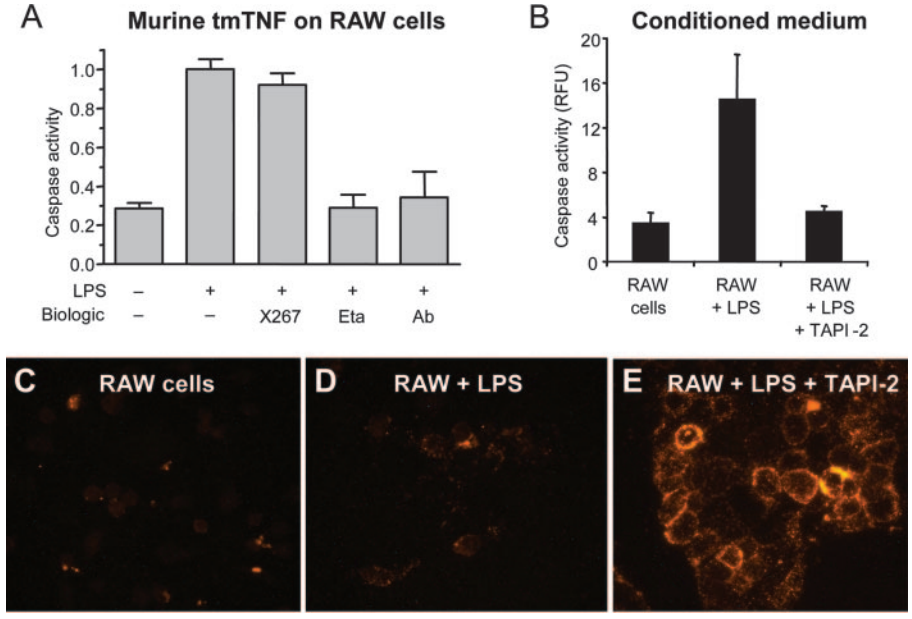

FIGURE 3. DN-TNF does not inhibit mouse tmTNF activity on human TNF receptors. *A*, Mouse RAW264.7 (RAW) cells were treated with 1 μg/ml LPS in the presence of 10 μM TACE inhibitor TAPI-2 to generate cells expressing tmTNF. Cells were incubated with 10 μg/ml XENP267 (X267), etanercept (Eta), or anti-mouse TNF neutralizing Ab (Ab) and then cocultured with human U937 cells to induce caspase activity. All columns contain RAW264.7 and U937 cells plus TAPI-2; differing LPS and biologics treatments are as shown. Etanercept and Ab decreased activity to background levels while DN-TNF had no effect (mean $\pm$ SEM, $n = 18$; $p < 0.01$ for etanercept and Ab vs vehicle). *B–E*, Controls for murine tmTNF coculture assays. *B*, U937 caspase assay using conditioned medium obtained from untreated, LPS-treated, or LPS- and TAPI-2-treated RAW264.7 cells. The addition of TAPI-2 to LPS-treated cells effectively inhibits the production of solTNF (mean $\pm$ SD, $n = 6$). *C–E*, Endogenously expressed murine TNF is trapped on the surface of LPS-stimulated RAW264.7 cells treated with TACE inhibitors. RAW264.7 cells were untreated (*C*), incubated with 1 μg/ml LPS to induce TNF (*D*), or incubated with 1 μg/ml LPS and 10 μM TACE inhibitor TAPI-2 (*E*). In the absence of LPS no TNF is produced (*C*); in the presence of LPS there is only a slight increase in cell surface TNF (*D*) because as TNF is expressed it is processed by TACE and shed into the medium as solTNF. LPS stimulation combined with TACE inhibition generates a significant amount of tmTNF trapped on the surface of these cells (*E*). All caspase procedures were as described in *Materials and Methods*.

release (44, 45). It therefore serves as an independent confirmation of the caspase assays shown in Fig. 1 and, more importantly, assesses the effects of DN-TNF on primary immune cells in the complex signaling environment of human whole blood. To determine the donor variability of solTNF-induced cytokine release, blood was collected from five human donors and treated with escalating doses of recombinant human solTNF (Fig. 4*A*). All donors responded with maximal IL-8 release ranging from 3 to 7.5 ng/ml, with EC_{50} values ranging from ~1 to 6 ng/ml TNF. Based on these results, inhibition studies were done in five additional donors using 3 and 6 ng/ml solTNF with increasing doses of DN-TNF, anti-TNF Abs, or decoy receptor. In agreement with the caspase assays described above, DN-TNF efficacy and mass potency were similar to that of other TNF inhibitors, demonstrating that in a physiologically relevant whole blood environment anti-TNF agents efficiently block IL-8 release, regardless of inhibitory mechanism (Table II and Fig. 4*B*).

DN-TNF biologics reduce inflammation in two mouse models of arthritis

To assess the in vivo anti-inflammatory activity of solTNF-selective inhibition, DN-TNFs were investigated in two established mouse models of arthritis. In the first model (collagen Ab-induced arthritis (CAIA); Fig. 5), mice were treated prophylactically with TNF inhibitors, and experimental arthritis was induced by passive immunization using a mixture of four anti-collagen mAbs on day 0 followed by LPS on day 3. The severity of arthritis was monitored throughout the study by measuring hind paw thickness. This model has the advantages of being rapid (7 days to the peak of inflammation) and robust in arthritic phenotype, with typically 100% incidence of affected animals (46, 47); furthermore, inhibition of TNF by biologics is efficacious in this model (48).

Four independent studies were performed to determine DN-TNF efficacy in the CAIA model; these studies used different DN-TNF biologics, dosing regimens, and routes of administration (i.p., s.c., and i.p. by osmotic pump). All arthritis studies included etanercept as a positive control. Results were consistent for all four studies as measured by hind paw edema and arthritis score; representative edema data from three of these studies are discussed below (arthritis score data not shown). In the first study, three DN-TNFs given daily at 10 mg/kg i.p. reduced hind paw inflammation compared with vehicle (Fig. 5*A*). Note that vehicle is an appropriate control for DN-TNF agents because these biologics are variants of human solTNF and therefore do not possess Ab Fc domains and their associated effector functions. The decoy receptor etanercept, administered using the same dosing regimen, also reduced hind paw inflammation to a similar extent as that by DN-TNF compounds.

Several DN-TNF biologics were pegylated to improve in vivo half-life (29), allowing less frequent dosing. In the second CAIA study (Fig. 5*B*), we found that a pegylated DN-TNF was efficacious when given s.c. at one-tenth the dose and one-third the frequency used in Fig. 5*A*. Histological examination of bone and cartilage at day 28 showed a trend toward reduced pathology (scores for bone and cartilage were decreased by 24–29% with both DN-TNF and etanercept), although differences among all groups were not statistically significant at this early time point (data not shown). Longer studies are necessary to determine whether inhibition of acute inflammation in the CAIA

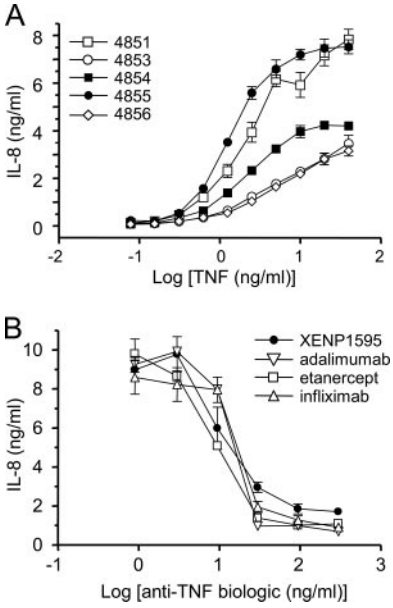

FIGURE 4. DN-TNF biologic inhibits solTNF-induced release of IL-8 in human blood. *A*, TNF dose responses for induction of IL-8 release in whole blood from multiple human donors. Fresh whole blood was obtained from five human donors; IL-8 release after solTNF treatment was then determined as described in the *Materials and Methods* (mean ± SEM, $n = 3$). The absolute level of solTNF-induced IL-8 release varied among donors; EC_{50} values ranged from 1 to 6 ng/ml TNF. *B*, Inhibition of solTNF-induced IL8 release. Recombinant human solTNF (3 ng/ml) was incubated with increasing amounts of XENP1595, etanercept, infliximab, or adalimumab and then mixed with human whole blood for 6 h at 37°C. Blood cells were lysed with Triton-X100 and IL-8 production was determined by sandwich ELISA (mean ± SEM, $n = 4$). Donor shown is number 5134 from Table II; see the table for IC_{50} values for whole blood assays of five human donors.

model by DN-TNFs will translate into reduced bone and cartilage degradation.

To address potential differences in pharmacokinetics among different classes of anti-TNF biologics, a third CAIA study was done using osmotic pumps to deliver inhibitors. Pump delivery provides a constant infusion of drug and allows better correlation of steady-state blood levels and inflammation endpoints. Three increasing doses of DN-TNF caused a progressive reduction in inflammation, with decoy receptor at 10 mg/kg showing comparable activity to the same dose of DN-TNF. Proportional increases in serum DN-TNF levels at 3, 10, and 30 mg/kg (measured at the end of the

Table II. *Inhibition of TNF-induced IL-8 release in human whole blood by anti-TNF biologics*

	IC_{50} (ng/ml)					
Donor	5129	5131	5132	5133	5134	Mean ± SD
3 ng/ml TNF						
XENP1595	15.5	6.8	17.1	11.8	11.5	12.5 ± 4.0
Etanercept	13.7	10.9	10.7	9.7	9.1	10.8 ± 1.8
Adalimumab	15.9	14.6	19.2	15.3	13.4	15.4 ± 2.2
Infliximab	19.5	16.2	16.5	16.5	19.5	17.5 ± 1.7
6 ng/ml TNF						
XENP1595	61.4	25.0	36.3	28.8	26.0	35.5 ± 15.1
Etanercept	26.9	26.2	27.1	17.9	17.2	23.1 ± 5.0
Adalimumab	47.5	34.4	89.3	46.5	41.0	51.7 ± 21.6
Infliximab	45.0	39.4	69.3	45.1	39.5	47.7 ± 12.4

Values are mean IC_{50} ($n = 4$) from a six-point dose response as shown in Fig. 4*B* for donor 5134.

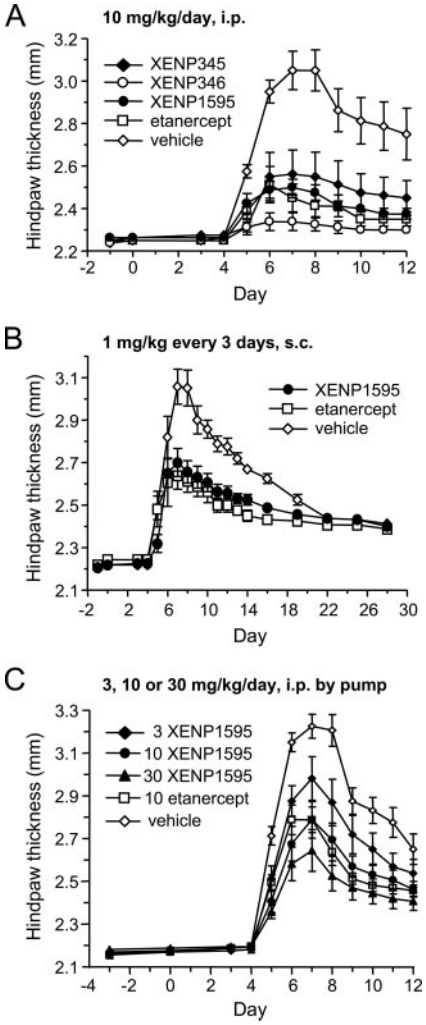

FIGURE 5. DN-TNFs and decoy receptor reduce arthritis in the mouse collagen Ab-induced arthritis model. *A*, Intraperitoneal route, prophylactic dosing. Beginning on day −3, mice ($n = 8$/group) were dosed daily i.p. with vehicle or 10 mg/kg etanercept or DN-TNF (XENP345, XENP346, or XENP1595). Joint inflammation was stimulated by i.v. injection of 25 mg/kg each of four anti-collagen Abs on day 0, followed by 2.5 mg/kg LPS i.p. on day 3. DN-TNFs and etanercept significantly reduced disease symptoms as measured by hind paw thickness (mean ± SEM): XENP345, 61.4% reduction in area under the curve; XENP346, 88.6%; XENP1595, 72.4%; and etanercept, 74.8% ($p < 0.01$ for all groups vs vehicle). At the time of sacrifice, drug levels were measured and found to be as follows: XENP345, 0.8 ± 0.4; XENP346, 4.2 ± 3.5; XENP1595, 7.0 ± 4.6; and etanercept, 35 ± 11 μg/ml. *B*, Subcutaneous route, prophylactic dosing. Beginning on day −3, mice ($n = 8$/group) were dosed s.c. every 3 days with vehicle or 1.0 mg/kg etanercept or XENP1595. Experimental arthritis was induced as in *A* and hind paw thickness (mean ± SEM) was monitored for 28 days. XENP1595 and etanercept significantly reduced hind paw thickness compared with vehicle-treated controls: XENP1595, 35.6% reduction; etanercept, 46.9% ($p < 0.01$ for both groups vs vehicle area under the curve). *C*, Dose response to DN-TNF treatment. Mice were implanted on day 3 with osmotic pumps to deliver vehicle, 10 mg/kg/day etanercept, or XENP1595 at 3, 10, or 30 mg/kg/day i.p. Experimental arthritis was induced as in *A*; disease symptoms were measured using hind paw thickness (mean ± SEM), and serum drug levels were measured at the conclusion of the study by using a sandwich ELISA. DN-TNF rescued mice from arthritic symptoms in a dose-dependent manner: XENP1595 at 3, 10, and 30 mg/kg/day = 10.9, 27.5, and 39.1% reduction; etanercept at 10 mg/kg/day = 26.8% reduction ($p < 0.05$ vs vehicle for all groups except 3 mg/kg XENP1595). Average steady-state serum levels were 7.0 ± 4.1, 24.6 ± 7.1, and 220 ± 161 μg/ml for the 3, 10, and 30 mg/kg/day dose levels of XENP1595 and 14.6 ± 13.1 μg/ml for etanercept (mean ± SD).

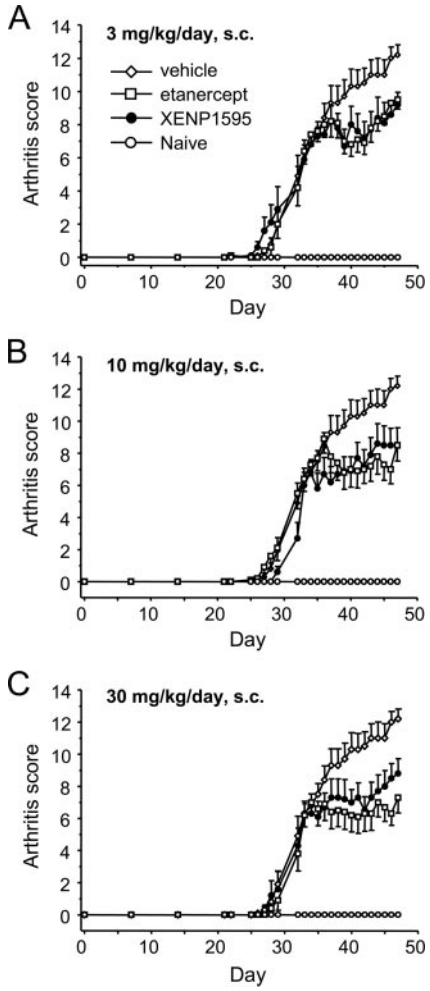

FIGURE 6. DN-TNF and decoy receptor reduce arthritis in the mouse collagen-induced arthritis model. Arthritis was induced using a 5 mg/kg s.c. administration of bovine collagen type II on days 0 and 21. Joints were inflamed by day 27 and therapeutic dosing was begun on day 33 using vehicle, XENP1595, or etanercept at 3, 10, and 30 mg/kg/day s.c. in panels *A*, *B*, and *C*, respectively. Untreated animals served as a baseline. Disease symptoms were measured using the sum of arthritis scores (0–4) from four paws (mean ± SEM, $n = 8$/group). Biologics were efficacious at doses of 3, 10, and 30 mg/kg, reducing the area under the curve (using start of dosing as baseline) relative to vehicle by 55, 67, and 68% for XENP1595 and 53, 63, and 86% for etanercept ($p < 0.05$ for 10 and 30 mg/kg XENP1595 and $p < 0.001$ for 30 mg/kg etanercept; there were no significant differences between XENP1595 and etanercept at any dose).

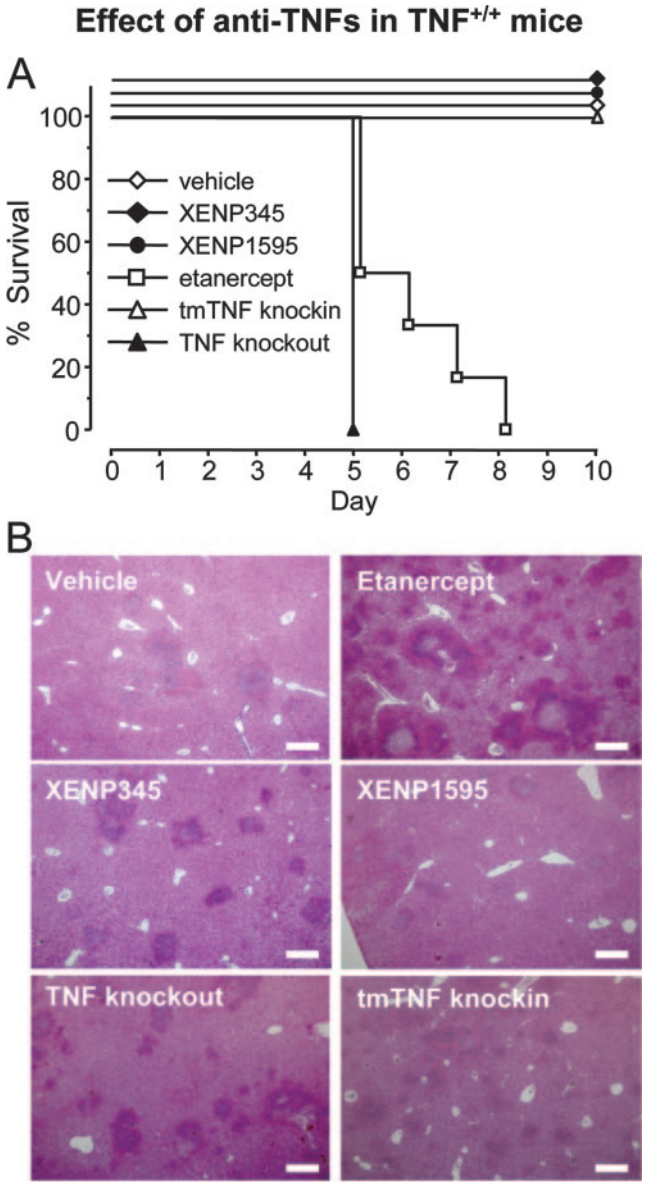

FIGURE 7. Selective inhibition of solTNF by DN-TNFs does not increase mortality of $TNF^{+/+}$ mice after *Listeria* challenge. C57BL/6 mice were given two s.c. doses (days −1 and 2) of vehicle or 30 mg/kg XENP1595, XENP345, or etanercept. *L. monocytogenes* (10^4 CFU) was administered i.v. to all mice on day 0. *Listeria*-infected TNF knockout and tmTNF knockin mice not treated with TNF inhibitors were used as genetic controls. Survival was monitored for 10 days (*A*, $n = 6$/group), and liver histology (*B*, $n = 4$ per group) was assessed 3 days after infection. In *A*, survival in etanercept and TNF knockout groups is significantly reduced relative to vehicle, tmTNF knockin, and XENP1595 groups ($p < 0.005$). In *B*, note the increased size and number of liver microabscesses (darker regions) in the etanercept-treated group. Scale bars, 1 mm.

study) correlated with efficacy (Fig. 5*C*). These results suggest that in the mouse the comparable efficacy of DN-TNF and decoy receptor can be attributed to solTNF inhibition at similar systemic drug exposures.

CIA is another established experimental arthritis model and has been used to demonstrate anti-inflammatory activity of Ab and decoy receptor anti-TNF biologics (49–51). In previous studies we showed that DN-TNFs are anti-inflammatory in the rat CIA model when dosed soon after the onset of symptoms (29). To extend these observations, DN-TNF was investigated in an advanced CIA mouse model. An aggressive dosing strategy was adopted; animals were permitted to develop advanced disease before TNF inhibitors were administered therapeutically on day 33, 5 to 7 days after a detectable increase in the arthritis score. Fig. 6 shows that DN-TNF at 3, 10, and 30 mg/kg given daily s.c. suppressed arthritis score comparably to decoy receptor, indicating that inhibition of tmTNF by a decoy receptor generates no additional anti-inflammatory activity relative to selective inhibition of solTNF. For DN-TNF, the 10 and 30 mg/kg groups showed significant reductions ($p < 0.05$); for etanercept, only the 30 mg/kg group achieved significance ($p < 0.001$). The dose response for both compounds was modest, possibly because drugs were dosed therapeutically in advanced disease rather than prophylactically. There was no significant difference in the histopathology of bone and cartilage for any treatment group vs vehicle; as with the CAIA studies above, longer studies are required to determine whether the

Table III. *Effect of treatment with anti-TNF biologics on* L. monocytogenes *load in mice 3 days postinfection*[a]

Listeria Dose (CFU, Oral)	3.9×10^9	1.0×10^{10}	3.2×10^{10}
Spleen (CFU $\times 10^4$)			
Vehicle	5 ± 1	8 ± 1	16 ± 5
XENP1595 (10 mg/kg)	5 ± 4	7 ± 1	16 ± 5
XENP1595 (30 mg/kg)	10 ± 4	6 ± 1	21 ± 9
XENP1595 (50 mg/kg)	18 ± 5	53 ± 13	8 ± 2
Etanercept (10 mg/kg)	460 ± 230*	720 ± 390*	2,700 ± 1,300***
Etanercept (30 mg/kg)	650 ± 270*	730 ± 350**	170 ± 70
Etanercept (50 mg/kg)	25,000 ± 19,700***	330 ± 110*	190 ± 60*
Blood (CFU/ml)			
Vehicle	13 ± 8	3 ± 3	5 ± 5
XENP1595 (10 mg/kg)	5 ± 5	0 ± 0	18 ± 18
XENP1595 (30 mg/kg)	13 ± 13	8 ± 8	25 ± 17
XENP1595 (50 mg/kg)	10 ± 10	0 ± 0	3 ± 3
Etanercept (10 mg/kg)	370 ± 190*	140 ± 54*	1,655 ± 410**
Etanercept (30 mg/kg)	315 ± 160	138 ± 36**	1,306 ± 300**
Etanercept (50 mg/kg)	4,900 ± 4,200***	88 ± 21*	1,903 ± 420**

[a] Values are mean ± SEM ($n = 8$); see Fig. 8 for data on individual mice.
*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

acute anti-inflammatory effects in CIA will result in reduced joint damage at later time points.

These in vivo pharmacology studies demonstrate that DN-TNF biologics are anti-inflammatory in well-characterized murine models of arthritis, showing comparable activity to that of a nonselective anti-TNF agent. The similar results seen for solTNF-selective inhibition relative to nonselective solTNF and tmTNF inhibition further suggest that solTNF is the major contributor to inflammation in these experimental models.

DN-TNF biologics maintain immunity to Listeria *infection in normal and tmTNF knockin mice*

Genetic evidence has shown that TNF knockout mice are immunosuppressed and therefore sensitized to mycobacterial and listerial infection, whereas normal mice or mice expressing tmTNF exclusively (tmTNF knockins) mount a sufficient immune response to delay or overcome infection (10–14, 16, 52). One hypothesis suggested by these genetic data is that a solTNF-selective

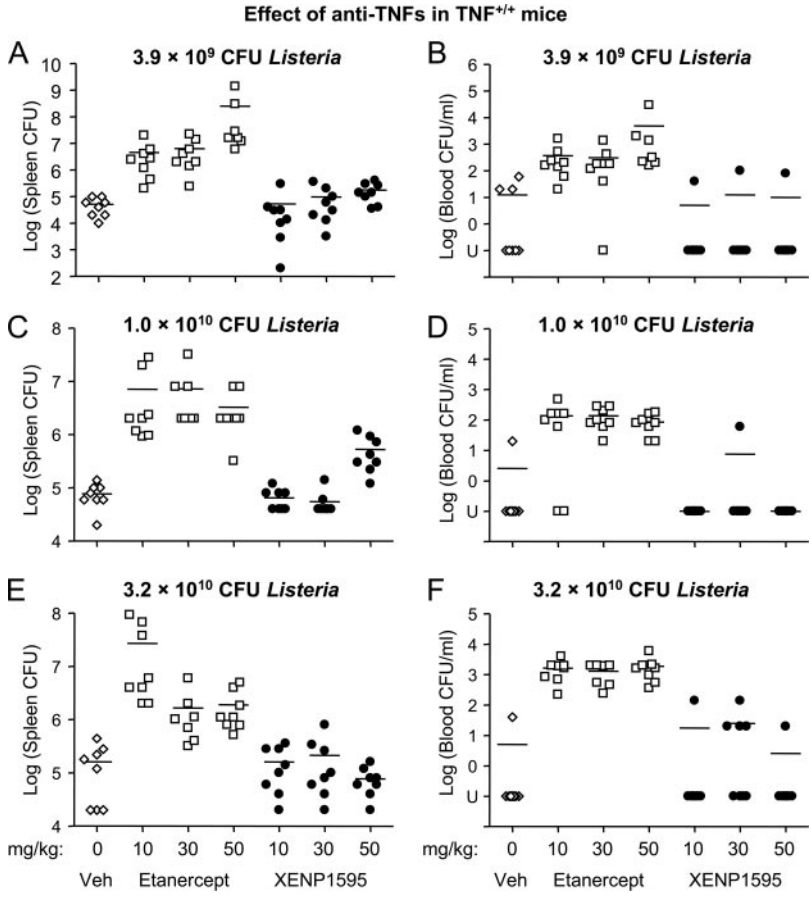

FIGURE 8. Effect of selective solTNF and nonselective TNF inhibition on innate immunity to *Listeria* challenge. Female BALB/c mice ($n = 8$/group) were dosed daily i.p. with vehicle (Veh) or 10, 30, or 50 mg/kg of either etanercept or XENP1595. On day 4, mice were orally infected with 3.9×10^9 (*A* and *B*), 1.0×10^{10} (*C* and *D*), or 3.2×10^{10} (*E* and *F*) CFU of *L. monocytogenes*. On day 7, mice were euthanized and their spleens (*A*, *C*, and *E*) and blood (*B*, *D*, and *F*) were evaluated for bacterial load (bars represent means; U, undetectable). See Table III for *p* values for each group. Regardless of bacterial inoculum or dose of biologic, etanercept increased CFUs in blood and spleen relative to vehicle and XENP1595 groups.

and tmTNF-sparing anti-inflammatory biologic would similarly preserve innate immunity to infectious challenge, whereas a nonselective TNF inhibitor would not. We tested this hypothesis in mice treated with DN-TNFs or a decoy receptor to determine whether either class of biologic compromised innate immunity to bacterial infection. *L. monocytogenes* was chosen as the model in part because it has been the organism of choice for previous TNF genetic studies. In addition, *Listeria* is an intracellular pathogen that causes granulomatous inflammation and is a risk factor associated with the clinical use of nonselective anti-TNF biologics (20, 21), yet this organism does not require the containment facilities and extended time periods necessary for *M. tuberculosis* models.

The effects on survival of two DN-TNFs and decoy receptor were first determined in normal $TNF^{+/+}$ mice. Vehicle or TNF inhibitors were administered s.c. on day −1 and day 2 to mice infected i.v. on day 0 with 10^4 CFU *L. monocytogenes*. TNF knockout and tmTNF knockin mice not treated with anti-TNF agents were used as genetic controls. Survival was monitored for 10 days and liver histology was assessed in satellite groups 3 days after infection. The two DN-TNFs did not suppress host resistance to challenge relative to the vehicle (Fig. 7*A*). In contrast, all six mice treated with decoy receptor died by day 8. As expected, all TNF knockout controls died by day 5, whereas knockin mice expressing only tmTNF were fully protected. Histological assessment of livers revealed an increase in the size and number of microabscesses in the decoy receptor-treated group (Fig. 7*B*); livers from mice treated with DN-TNFs appeared similar to those from tmTNF knockins. These results indicate that inhibition of tmTNF, whether genetically via knockout or pharmacologically by treatment with nonselective biologic, results in sensitization of mice to *Listeria* infection. In contrast, inhibition of solTNF alone, whether genetically in tmTNF knockin mice or pharmacologically by DN-TNF, does not suppress innate immunity.

To investigate whether the preservation of immunity to *Listeria* was a simple dosage effect that could be overwhelmed by increasing infectious challenge and/or TNF inhibition, we extended the study using higher doses of both *Listeria* and anti-TNF biologics. Groups were orally inoculated with three increasing doses of *Listeria*. Starting 3 days before infection, vehicle, DN-TNF, or decoy receptor was given daily i.p. at 10, 30, or 50 mg/kg. All eight mice per group were sacrificed on day 3 postinfection, and bacterial loads were determined in spleen and blood. At all *Listeria* doses the decoy receptor greatly increased the bacterial load in both the spleen (~10- to 5000-fold) and blood (~25- to 400-fold), whereas DN-TNF-treated mice were comparable to the vehicle-treated controls (Table III). There is a statistically insignificant small increase in spleen bacterial load in one XENP1595-treated group at the middle inoculum of *Listeria* (Fig. 8*C*); however, all mice in this group had undetectable levels of *Listeria* in the blood (Fig. 8*D*), and this result was not repeated in the low or high inoculum groups (Fig. 8, *A* and *E*). Two of 72 decoy receptor-treated mice died before day 3, whereas all 24 vehicle- and 72 DN-TNF-treated mice survived. Because *Listeria* is an intracellular pathogen, detectable listeremia is evidence of an uncontrolled infection. Notably, although all mice had detectable bacterial loads in the spleen (Fig. 8, *A*, *C*, and *E*), few of the DN-TNF (10 of 72) or vehicle-treated mice (5 of 24) had any detectable bacteria in the blood, whereas almost all (67 of 70) of the etanercept-treated mice did, regardless of *Listeria* inoculum or drug dose (Fig. 8, *B*, *D*, and *F*). These results indicate that DN-TNF-treated mice mounted an innate immune response indistinguishable from that of vehicle-treated control mice.

TmTNF knockin mice serve as a useful model for dissecting the functions of solTNF and tmTNF in immunity and for investigating possible differential effects of anti-TNF biologics on these two

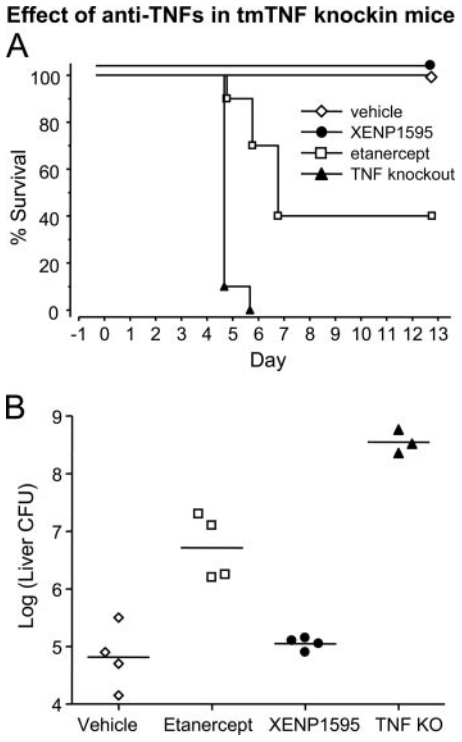

FIGURE 9. DN-TNF does not increase mortality of tmTNF knockin mice after *Listeria* challenge. The tmTNF knockin mice (n = 10/group) were treated with vehicle or 10 mg/kg XENP1595 or etanercept s.c. on days −1, 2, 6, 9, and 13 and infected with 10^4 *L. monocytogenes* i.v. on day 0. Survival (*A*) was monitored over 13 days, and bacterial load in the liver (*B*) was measured at day 4 in a satellite group of four mice. *Listeria*-infected TNF knockout mice (n = 10) were used as a genetic control for the survival study. In *A*, survival in etanercept and TNF knockout groups is significantly reduced relative to vehicle and XENP1595 groups ($p < 0.005$). In *B*, bacterial load in etanercept and TNF knockout groups is significantly higher than in vehicle and XENP1595 groups ($p < 0.01$). In addition, liver microabscess sizes were significantly increased in the TNF knockout and decoy receptor-treated satellite groups: vehicle, 3.2 ± 1.1 μm; XENP1595, 3.5 ± 0.71 μm; TNF knockout, 46.5 ± 5.7 μm; and etanercept, 38.5 ± 12.2 μm (mean ± SD; $p < 0.01$ for etanercept and TNF knockout compared with vehicle and XENP1595).

ligands. *Listeria* infection studies were therefore next performed in tmTNF knockin mice to determine whether the immunosuppression observed with decoy receptor in $TNF^{+/+}$ mice could be reproduced in the absence of solTNF. tmTNF knockin mice were treated with vehicle or 10 mg/kg DN-TNF or decoy receptor twice weekly s.c., and infected i.v. with 10^4 *Listeria* the day after the first drug dose. All DN-TNF and vehicle-treated mice survived to the end of the study (Fig. 9*A*). In contrast, six of 10 mice in the decoy receptor-treated group died by day 7. As controls, TNF knockouts were also infected and, as expected, all died by day 6. In a satellite study with four mice per group, similar results were observed for the bacterial load in the liver at day 3, with a 23-fold increase in CFU in decoy receptor-treated mice compared with the vehicle and a 13-fold increase compared with DN-TNF-treated mice (Fig. 9*B*). In addition, liver microabscess sizes were significantly increased in the TNF knockout and decoy receptor-treated groups (not shown). These results demonstrate that decoy receptor sensitized mice to *Listeria* even in the absence of solTNF and further support the hypothesis that DN-TNF has no effect on tmTNF-dependent innate immunity.

Discussion

These studies show that DN-TNF biologics selectively inhibit solTNF activity in vitro and in vivo. DN-TNFs blocked paracrine

(solTNF) signaling in human and mouse cell lines and in a human whole blood cytokine release assay and were as effective as a nonselective decoy receptor in reducing inflammation in two mouse models of arthritis. In contrast, DN-TNFs failed to block juxtacrine signaling induced by human or mouse tmTNF in the same assays. DN-TNF biologics also maintained innate resistance to *L. monocytogenes* infection in normal and tmTNF knockin mice, while nonselective TNF inhibition by decoy receptor led to a marked immunosuppression and increased mortality.

SolTNF selectivity appears to be an intrinsic property of the dominant-negative mechanism of DN-TNFs. Although the molecular basis for solTNF ligand selectivity remains to be explored, one explanation is that solTNF homotrimers are more labile than tmTNF homotrimers. Trimer dissociation is obligatory for exchange and is likely to be impeded by the large transmembrane and intracellular domains of tmTNF (32, 53) and by the membrane lipid anchor at Cys^{47} (54). The DN-TNF mechanism is not unprecedented in genetics; for example, a recent study describes a mutation in mouse TNF that suppresses TNF activity in vivo by a dominant-negative mechanism (55). However, in this system both solTNF and tmTNF are suppressed because endogenous heterozygotic expression results in the formation of heterotrimeric tmTNF. In contrast to DN-TNFs, anti-TNF Abs and decoy receptor only require TNF binding for activity, explaining the ability of these biologics to inhibit both solTNF and tmTNF.

Ligand selectivity allows DN-TNFs to be used as pharmacologic tools to explore the roles of solTNF and tmTNF in mediating the multiple functions attributed to this cytokine. For example, these are the first data exploiting selective and nonselective pharmacologic inhibitors to demonstrate the minimal contribution of tmTNF to inflammation in mouse arthritis models. Selective inhibition with DN-TNFs was similarly useful in dissecting out the roles of the two forms of TNF in maintaining immunity to infection. Pharmacologic inhibition in *Listeria* challenge models indicates that tmTNF plays a protective role in innate immunity, whereas solTNF appears less important. These results support the hypothesis that tmTNF and solTNF have nonredundant and perhaps complementary roles in inflammation and immunity (2, 7, 10, 19), which was first proposed based on evidence from mouse genetic models. These roles are possibly mediated by differential signaling through TNFR1 and TNFR2 (56–59), potentially coupled with reverse signaling events that can only be induced in cells expressing tmTNF (60). Future studies with DN-TNF biologics may help elucidate the complex signaling events initiated by solTNF and tmTNF ligands acting on TNFR1 and/or TNFR2.

Two reports using tmTNF knockin mice have suggested that tmTNF alone can drive inflammation (61, 62). However, attributing inflammation solely to tmTNF signaling in these mice is problematic, because the two studies report high levels of solTNF expression in homozygous solTNF knockouts. In addition, tmTNF expression in such knockins is abnormally high; such dysregulation of tmTNF levels may lead to nonphysiological in vivo functions. Finally, a complication of genetic models is the lack of temporal control of TNF activity; in unconditional knockouts, TNF is absent throughout development of the immune system, which may lead to compensatory physiological effects. For example, it is known that tmTNF drives normal lymphoid development (7); therefore, genetic models do not allow separation of direct effects on innate immunity of tmTNF from its indirect effects in ensuring normal development of the immune system. This developmental aspect could help explain why some reports indicate that tmTNF knockin mice possess intermediate sensitivity to acute *M. tuberculosis* infection relative to total TNF knockout and $TNF^{+/+}$ mice (13, 14), while other reports suggest that tmTNF knockin mice are as resistant to tuberculosis as $TNF^{+/+}$ mice (16, 52). Another possible explanation for differences between DN-TNF and decoy receptor biologics is the differential inhibition of lymphotoxin α activity by these agents (Fig. 1*E*). Comparative pharmacology studies of the three classes of anti-TNF biologics (decoy receptors, Abs, and dominant negatives) may help explain the signaling differences that underlie their disparate effects on inflammation and immunity, and such studies are in progress.

In conclusion, we have demonstrated that DN-TNF biologics selectively inhibit solTNF activity in vitro and in vivo. TNF ligand selectivity allows these agents to serve as discriminating tools to refine our understanding of the multiple functions of this pleiotropic cytokine and to shed light on the conflicting roles reported for TNF in the immune system and in disease. The use of DN-TNFs in mice, combined with studies in genetic models, suggests that solTNF-selective inhibitors may effectively reduce inflammation while preserving adequate host immunity to infection. Because increased risk of listeriosis and other infections is a liability of nonselective anti-TNF drugs, DN-TNF compounds may have potential for the treatment of rheumatoid arthritis and other inflammatory and autoimmune diseases.

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Disclosures

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