DMD Fast Forward. Published on September 30, 2010 as doi:10.1124/dmd.110.036012

DMD # 36012

Brain-Penetrating TNFR Decoy Receptor in the Mouse

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DMD Fast Forward. Published on September 30, 2010 as DOI: 10.1124/dmd.110.036012
This article has not been copyedited and formatted. The final version may differ from this version.

Running title: Brain Penetrating IgG-TNFR Fusion Protein

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Text pages: 18

Tables: 3

Figures: 6

References: 28

Abstract: 237 words

Introduction: 553 words

Discussion: 1176 words

Abbreviations: BBB, blood-brain barrier; TNF, tumor necrosis factor; TNFI, TNF inhibitor; TNFR, TNF receptor; ECD, extracellular domain; MTH, molecular Trojan horse; MAb, monoclonal antibody; GDNF, glial derived neurotrophic factor; AD, Alzheimer's disease; PD, Parkinson's disease; TfR, transferrin receptor; cTfRMAb, chimeric MAb against the mouse TfR; HIR, human insulin receptor; HIRMAb, engineered MAb against the HIR; ID, injected dose; HC, heavy chain; LC, light chain; VH, variable region of the HC; VL, variable region of the LC; TV, tandem vector; CHO, Chinese hamster ovary; TCA, trichloroacetic acid; RRA, radio-receptor assay; VD, volume of distribution: PK, pharmacokinetics; PS, permeability-surface area; AUC, area under the curve; AA, amino acid; IV, intravenous; IP, intra-peritoneal; TBS, Tris buffered saline; ELISA, enzyme linked immunosorbent assay

Abstract

Biologic tumor necrosis factor (TNF) inhibitors (TNFI) include TNF decoy receptors (TNFR). TNFα plays a pathologic role in both acute and chronic brain disease. However, biologic TNFIs cannot be developed as brain therapeutics because these large molecule drugs do not cross the blood-brain barrier (BBB). To enable penetration of the brain via receptor mediated transport, the human TNFR type II decoy receptor was re-engineered as an IgG fusion protein, where the IgG part is a chimeric monoclonal antibody (MAb) against the mouse transferrin receptor (TfR), and this fusion protein is designated cTfRMAb-TNFR. The cTfRMAb part of the fusion protein acts as a molecular Trojan horse to ferry the TNFR across the BBB via transport on the endogenous BBB TfR. The cTfRMAb-TNFR was expressed by stably transfected Chinese hamster ovary cells and purified by affinity chromatography to homogeneity on electrophoretic gels. The fusion protein reacted with antibodies to both mouse IgG and the human TNFR, and bound TNFα with high affinity, KD = 96 ± 34 pM. The cTfRMAb-TNFR fusion protein was rapidly transported into mouse brain in vivo following intravenous administration, and the brain uptake of the fusion protein was 2.8 ± 0.5 % of injected dose/gram brain, which is >45-fold higher than the brain uptake of an IgG that does not recognize the mouse TfR. This new IgG-TNFR fusion protein can be tested in mouse models of brain diseases in which TNFα plays a pathologic role.

Introduction

Tumor necrosis factor (TNF)-α plays a pathologic role in both acute brain disorders, including stroke (Nawashiro et al, 1997), traumatic brain injury (Knoblach et al, 1999), and spinal cord injury (Marchand et al, 2009), as well as chronic brain conditions, such as Parkinson's disease (PD) (McCoy et al, 2006) or Alzheimer's disease (AD) (McAlpine et al, 2009). TNFα action in brain can be suppressed by TNFα inhibitors (TNFI), and the most potent TNFIs are biologic drugs, including the extracellular domain (ECD) of the type II TNFα receptor (TNFR) (Tansey and Szymkowski, 2009). Etanercept is a fusion protein of the TNFR-II ECD and the Fc region of human IgG1, and is a leading biologic TNFI presently used to suppress inflammation in peripheral human diseases (Tansey and Szymkowski, 2009). Etanercept, or other biologic TNFIs, cannot be developed as a drug for disorders of the CNS, because these large molecules do not cross the blood-brain barrier (BBB) (Boado et al, 2010a).

Biologic drugs such as etanercept can be re-engineered to enable brain penetration by fusion of the biologic drug to a BBB molecular Trojan horse (MTH). The latter is a peptide or peptidomimetic monoclonal antibody (MAb) that binds an endogenous peptide receptor on the BBB. This binding triggers receptor-mediated transport of the MAb across the BBB, which enables the biologic drug that is fused to the MTH to penetrate the brain (Pardridge, 2010a). The most potent BBB MTH is a MAb against the human insulin receptor (HIR). A fusion protein of the HIRMAb and the TNFR-II ECD was engineered (Hui et al, 2009), and this HIRMAb-TNFR fusion protein was shown to rapidly penetrate the BBB in vivo following intravenous (IV) injection in

the Rhesus monkey (Boado et al, 2010a). The HIRMAb part of the fusion protein cross-reacts with the insulin receptor of Old World primates, such as the Rhesus monkey, but does not cross react with the insulin receptor of lower animals, including rodents (Pardridge et al, 1995). Therefore, the HIRMAb-TNFR fusion protein cannot be tested in rat or mouse models of brain disease. A surrogate BBB MTH has been engineered, which is a chimeric MAb against the mouse transferrin receptor (TfR), and this antibody is designated the cTfRMAb (Boado et al, 2009). Biologic drugs such as a single chain Fv antibody or a neurotrophin, such as glial derived neurotrophic factor (GDNF), have been fused to the cTfRMAb and these fusion proteins rapidly penetrate the mouse brain in vivo with a brain uptake of 3-4% of injected dose/gram brain after IV administration (Zhou et al, 2010; Boado et al, 2010b). So as to develop a brain-penetrating biologic TNFI that can be tested in mouse models of CNS disease, the present studies engineered and expressed a new IgG-TNFR fusion protein. The amino terminus of the human TNFR-II ECD was fused to the carboxyl terminus of the heavy chain of the cTfRMAb, and this new fusion protein is designated the cTfRMAb-TNFR fusion protein. The structure of the cTfRMAb-TNFR fusion protein is shown in Figure 1. The present studies describe the isolation of a stably transfected line of Chinese hamster ovary (CHO) cells expressing the cTfRMAb-TNFR fusion protein, as well as the purification and biochemical properties of the fusion protein. These studies then describe the in vivo pharmacokinetics and brain uptake of the cTfRMAb-TNFR fusion protein following IV administration in the adult mouse.

Methods

Production of CHO line. A tandem vector (TV) plasmid DNA encoding the cTfRMAb heavy chain-TNFR fusion protein, the cTfRMAb light chain (LC), and the murine dihydrofolate reductase, was engineered similar to a TV described previously (Boado et al, 2010b). The cDNA encoding the 235 amino acid (AA) human mature TNFR-II ECD was amplified by polymerase chain reaction as described previously (Hui et al, 2009), and subcloned at the 3'-end of the cTfRMAb HC to form the pcTfRMAb-TNFR tandem vector. The structure of the TV has been described previously (Boado et al, 2010b). The sequence of the TV was confirmed by bi-directional DNA sequencing performed at Eurofins MWG Operon (Huntsville, AL) using custom sequencing oligodeoxynucleotides synthesized at Midland Certified Reagent Co. (Midland, TX). The TV was linearized and DG44 CHO cells were electroporated, followed by selection in hypoxanthine-thymine deficient medium and amplification with graded increases in methotrexate up to 80 nM in serum free medium. High producing clones were identified by measurement of medium mouse IgG concentrations by enzyme-linked immunosorbent assay (ELISA). The CHO line was stable through multiple generations, and produced medium IgG levels of 5-10 mg/L in shake flasks at a cell density of 1-2 million cells/mL.

Protein purification. The CHO cells were propagated in 1 L bottles, until 2.4L of conditioned serum free medium was collected. The medium was ultra-filtered with a 0.2 um Sartopore-2 sterile-filter unit (Sartorius Stedim Biotech, Goettingen, Germany), and applied to a 25 mL column of protein G Sepharose 4 Fast Flow (GE Life Sciences, Chicago, IL) equilibrated in 25 mM Tris/25 mM NaCl/5 mM EDTA/pH=7.1. The column

was washed with 25 mM Tris/1 M NaCl/5 mM EDTA/pH=7.1, and the fusion protein was eluted with 0.1 M glycine/pH=2.8. The acid eluate was pooled and neutralized to pH=6.5 with 1M Tris base, and concentrated with an Ultra-15 microconcentrator (Millipore, Bedford, MA) and stored sterile-filtered at 4ºC or at -70ºC.

SDS-PAGE and Western blotting. The homogeneity of the cTfRMAb-TNFR fusion protein was evaluated with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing and non-reducing conditions using 12% and 7.5% Tris-HCl gels (Biorad Life Science, Hercules, CA), respectively. Western blot analysis was performed with a goat anti-mouse IgG (H+L) antibody (Bethyl Labs, Montgomery, TX) for the mouse IgG Western, and with a goat anti-human TNFR-II antibody (R&D Systems, Minneapolis, MN) for the TNFR Western. The immunoreactivity of the cTfRMAb-TNFR fusion protein was compared with the cTfRMAb described previously (Boado et al, 2009), and the 20 kDa human TNFR-II ECD (amino acids Pro-24 to Thr-206) expressed in *E.coli* (R&D Systems).

Amino terminal amino acid sequence analysis. Following SDS-PAGE under reducing conditions, the purified cTfRMAb-TNFR fusion protein was electro-blotted to a 0.45 um polyvinylidene fluoride polyvinylidene fluoride filter (Immobilon-P Transfer Membrane, Millipore, Billerica, MA), followed by staining with Coomassie Blue. Edman sequence analysis of the heavy chain was performed at the Protein/Peptide Micro Analytical Laboratory at the California Institute of Technology (Pasadena, CA) through 7 cycles. The amino acid sequence, Glu-Val-Gln-Leu-Val-Glu-Ser, was a 100% match with the amino acid sequence deduced from nucleotide sequence analysis of the heavy chain of the cTfRMAb (Boado et al, 2009).

TNFα binding ELISA. The binding of the cTfRMAb-TNFR fusion protein to human TNFα was determined by ELISA. The capture reagent was human TNFα from Peprotech (Rocky Hill, NJ). The negative control was mouse IgG1/κ from Sigma Chemical Co. (St Louis, MO). The TNFα was dissolved in 0.1 M NaHCO3/pH=9.0 and plated overnight at 4ºC in 100 uL/well (0.2 ug/well). After washing with 0.01 M Tris/0.15 M NaCl/pH=7.4 (TBS), the wells were blocked with 1% bovine serum albumin in TBS for 30 min. A volume of 100 uL/well of the cTfRMAb-TNFR fusion protein, or mouse IgG1k was plated for 60 min at room temperature. After washing with TBS plus 0.05% Tween-20, a goat anti-mouse kappa light chain antibody-alkaline phosphatase conjugate (Bethyl Labs, Montgomery, TX) was incubated (0.2 ug/well) for 60 min. Following washing with TBS plus 0.05% Tween-20, para-nitrophenylphosphate for color development and chromagen detection at 405 nm was performed with an ELISA plate reader using following termination of the reaction with 1.2 M NaOH.

TNFα radio-receptor assay. The saturable binding of human TNFα to the cTfRMAb-TNFR fusion protein was determined with a radio-receptor assay (RRA), as described previously (Hui et al, 2009). A goat anti-mouse IgG1 Fc antibody (Bethyl Labs) was plated in 96-well plates (0.2 ug/well) with an overnight incubation in 0.1 M NaHCO3/pH=8.3, followed by washing, and blocking with 1% bovine serum albumin in 0.01 M Na2HPO4/0.15 M NaCl/pH=7.4 (PBS). The cTfRMAb-TNFR fusion protein was plated (100 ng/well), followed by a 1 hour incubation at room temperature. The wells were then washed with PBS, followed by the addition of 100 uL/well of a co-mixture of [^{125}I]-human TNFα (Perkin Elmer, Boston, MA; specific activity=91 uCi/ug) at a concentration of 0.01 uCi/well (0.1 uCi/mL; 1.1 ng/mL; 60 pM) and various

concentrations of unlabeled human TNFα, followed by a 3 hour incubation at room temperature. The wells were emptied by aspiration, washed with cold PBS, and 250 uL/well of 1 N NaOH was added, followed by heating at 60C for 30 min. Radioactivity was counted in Ultima-Gold (PerkinElmer, Downers Grove, IL) in a Tricarb 2100TR liquid scintillation counter (PerkinElmer), and the fractional binding per well was computed. The half-saturation constant, KD, of TNFα binding to the cTfRMAb-TNFR fusion protein was determined by non-linear regression analysis using the BMDP2007e software (Statistical Solutions, Cork, Ireland), following fitting of binding data to the following equation: Bound=[(Bmax)(C)]/(KD+C), where Bmax is the maximal binding and C= the concentration of TNFα. The radiochemical purity of the [^{125}I]-TNFα was tested with precipitation with 10% cold trichloroacetic acid (TCA), which was >99%.

Radio-labeling of fusion protein. The cTfRMAb-TNFR fusion protein, which was injected into mice for a pharmacokinetics analysis, was radiolabeled with [^{3}H]-N-succinimidyl propionate from American Radiolabeled Chemicals (St. Louis, MO). The labeled protein was purified with a 1x28 cm Sephadex G-25 gel filtration column, with an elution buffer of 0.01 M sodium acetate/0.14 M NaCl/pH=5.5. The cTfRMAb-TNFR fusion protein was labeled to a specific activity of 0.8 uCi/ug and a TCA precipitability of 99%.

Pharmacokinetics and brain uptake in the mouse. Adult male C57BL/6J mice, weighing 25 grams, were obtained from Jackson Labs (Bar Harbor, ME). All procedures were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health. Mice in groups of 4 were anesthetized with intra-peritoneal (IP) ketamine (100

mg/kg) and xylazine (10 mg/kg), and injected either intravenously (IV) in the tail vein or IP with 0.1 mL (7 uCi) of [^{3}H]-cTfRMAb-TNFR fusion protein. The injection dose (ID) in each mouse of the cTfRMAb-TNFR fusion protein was 350 μg/kg. Since the fusion protein is 26% TNFR by amino acid content (Results), the ID of TNFR is 90 μg/kg. An aliquot (50 uL) of venous blood was collected at 0.25, 2, 5, 15, 30, and 60 min after injection by sampling the orbital vein. The blood was centrifuged for collection of plasma, which was analyzed for radioactivity. At 60 min after injection, the mice were euthanized and the cerebral hemispheres were removed and weighed for each mouse. One hemisphere was used for total radioactivity after solubilization in Soluene-350 (PerkinElmer), and one hemisphere was homogenized for capillary depletion analysis. Plasma and tissue samples were analyzed for ^{3}H radioactivity with Optifluor-O (PerkinElmer) and a Tricarb 2100TR liquid scintillation counter (PerkinElmer). Brain uptake data was expressed as a volume of distribution (VD), which is the ratio of the 60 min organ radioactivity [disintegrations per minute (DPM)/gram) divided by the 60 min plasma radioactivity (DPM/uL), or as % of injected dose (ID)/gram tissue. The plasma radioactivity that was precipitable with cold 10% TCA was determined at each time point.

The plasma radioactivity, DPM/mL, was converted to % injected dose (ID)/mL, and the %ID/mL was fit to a bi-exponential equation,

$$\%ID/mL = A1e^{-k1t} + A2e^{-k2t}$$

The intercepts (A1, A2) and the slopes (k1, k2) were used to compute the pharmacokinetics parameters, including the median residence time (MRT), the central volume of distribution (Vc), the steady state volume of distribution (Vss), the area under

the plasma concentration curve (AUC), and the systemic clearance (CL). Non-linear regression analysis used the AR subroutine of the BMDP Statistical Software. Data were weighted by $1/(\%ID/mL)^2$.

The organ clearance (μL/min/g), also called the BBB permeability-surface area (PS) product, is computed from the terminal brain uptake (%ID/g) and the 60 min plasma AUC (%IDmin/mL) as follows:

$$PS\ product = [(\%ID/g)/AUC]x1000$$

Capillary depletion method. One cerebral hemisphere from each mouse was processed for the capillary depletion method as described previously (Triguero et al, 1990), which separates the brain into 3 fractions: the total brain homogenate, the microvascular capillary pellet, and the post-vascular supernatant. The volume of distribution (VD) was determined for each of the 3 fractions from the ratio of total ^3H radioactivity in the fraction, DPM/gram brain, divided by the ^3H radioactivity in the 60 min terminal plasma, DPM/uL. A high VD in the post-vascular supernatant, as compared to the VD in the capillary pellet, is evidence for transport of the protein through the BBB into brain parenchyma.

Results

A tandem vector was engineered, which contained the expression cassettes for the heavy chain fusion gene, the light gene, and the DHFR gene on a single plasmid DNA. DNA sequence analysis showed the 3 expression cassettes spanned 9,337 nucleotides (nt). The light chain was comprised of 234 AA, which included a 20 AA signal peptide, a 108 AA variable region of the light chain (VL) of the cTfRMAb, and a 106 AA mouse kappa light chain constant region, which is 100% identical to AA 113-218 of BAA06141. The predicted molecular weight of the light chain is 23,554 Da with a predicted isoelectric point of 5.73.The fusion protein of the cTfRMAb heavy chain and the TNFR was comprised of 698 AA, which included a 19 AA signal peptide. The predicted molecular weight of the heavy chain, without glycosylation, is 74,073 Da with a predicted pI of 6.34. The domains of the fusion heavy chain include a 118 AA variable region of the heavy chain (VH) of the cTfRMAb, a 324 AA mouse IgG1 constant region, which is 100% identical to AA 140-463 of BAC44885, a 2 AA linker (Ser-Ser), and the 235 AA human TNFR-II ECD, which is 100% identical to AA 23-257 of NP_001057. The HC contains 3 N-linked glycosylation sites: 1 site in the constant region of the IgG heavy chain and 2 sites in the TNFR domain. The predicted molecular weight, without glycosylation, of the hetero-tetrameric cTfRMAb-TNFR fusion protein is 195,200 Da with an isoelectric point of 6.23.

The CHO-derived cTfRMAb-TNFR fusion protein was homogeneous on both non-reducing (Figure 2A) and reducing (Figure 2B) SDS-PAGE. The size of the HC of the cTfRMAb-TNFR fusion protein on the reducing gel, 85 kDa, was greater than the size of the HC of the cTfRMAb, 55 kDa, owing to fusion of the 30 kDa TNFR (Figure

2A). The cTfRMAb-TNFR fusion protein migrated at approximately 230 kDa on the non-reducing gel (lane 2, Figure 2B). Western blot analysis with a primary antibody against mouse IgG detected both the HC and the LC of the cTfRMAb (lane 2, Figure 3A) and the cTfRMAb-TNFR fusion protein (lane 3, Figure 3A) but not the recombinant TNFR-II ECD (lane 1, Figure 3A). Western blot analysis with a primary antibody against human TNFR-II reacted with the HC of the cTfRMAb-TNFR fusion protein (lane 3, Figure 3B), and the TNFR-II ECD (lane 1, Figure 3B), but did not react with HC of the cTfRMAb (lane 2, Figure 3B).

The design of the TNFα ELISA is shown in Figure 4A. The cTfRMAb-TNFR fusion protein bound to the plated TNFα, whereas there is no binding by the mouse IgG1 control (Figure 4B). The design of the TNFα radio-receptor assay is shown in Figure 5A. TNFα bound to the cTfRMAb-TNFR fusion protein with high affinity characterized by a KD of 96 ± 34 pM (Figure 5B).

The cTfRMAb-TNFR fusion protein was radiolabeled with the ^{3}H-N-succinimidyl propionate reagent (Methods), and injected into adult male C57Bl/6J mice via either IV or IP administration. The clearance of the [^{3}H]-cTfRMAb-TNFR fusion protein from plasma after IV administration, and the appearance of plasma radioactivity after IP administration of the fusion protein, is plotted in Figure 6. For the IV injection studies, the plasma radioactivity decay curve was fit to a bi-exponential equation to yield the pharmacokinetics (PK) parameters shown in Table 1. The [^{3}H]-cTfRMAb-TNFR fusion protein was metabolically stable after IV administration, as the plasma radioactivity that was precipitable by TCA was 99 ± 1, 96 ± 1, 92 ± 3, 88 ± 3, 86 ± 3, and 84 ± 4 %, respectively at 0.25, 2, 5, 15, 30, and 60 min after IV injection. Following IP injection,

the concentration of the cTfRMAb-TNFR fusion protein in blood increases with time (Figure 6). Based on the trapezoid rule, the plasma AUC of the cTfRMAb-TNFR fusion protein at 60 min after IP injection is 158 %ID•min/mL, which is 20% of the plasma AUC following IV injection at the 60 min time point (Table 1). The plasma AUC after IP injection may approach the AUC after IV injection at later time points.

The capillary depletion analysis showed the VD of the cTfRMAb-TNFR fusion protein in the brain homogenate was high, 431 ± 39 uL/gram (Table 2). The VD of the fusion protein in the post-vascular supernatant, 326 ± 19 uL/gram (Table 2), is 76% of the homogenate VD, indicating the majority of the cTfRMAb-TNFR fusion protein had completed transcytosis through the BBB by 60 min after IV injection.

The uptake of the [^{3}H]-cTfRMAb-TNFR fusion protein by brain was determined at 60 min after IV injection, and was 2.8 ± 0.5 %ID/gram brain (Table 3). The cTfRMAb-TNFR fusion protein was also taken up by spinal cord, and the uptake was 2.6 ± 0.3, 3.1 ± 0.4, and 3.6 ± 0.3 %ID/gram in cervical, thoracic, and lumbar spinal cord, respectively. The BBB permeability-surface area (PS) product of the cTfRMAb-TNFR fusion protein in brain, which is equal to the ratio of the 60 min %ID/g (Table 3) and the 60 min plasma AUC (Table 1), is 3.6 ± 0.6 uL/min/g. The concentration of the cTfRMAb-TNFR fusion protein in brain at 60 min after IV injection is 250 ± 45 ng/gram (Table 3). Since the TNFR content of the fusion protein is 26% by amino acid content, the brain concentration of TNFR at 60 min after IV injection of the fusion protein is 65 ± 12 ng/gram (Table 3).

Discussion

Biologic TNFIs include TNFR decoy receptors and anti-TNFα monoclonal antibodies, and these biologic drugs are potent anti-inflammatory agents for the treatment of peripheral tissues (Tansey and Szymkowski, 2009). Biologic TNFIs could also prove to be important new treatments for brain disorders (Pardridge, 2010b), as TNFα plays a pro-inflammatory role in both acute disorders of the brain such as traumatic brain injury, spinal cord injury, or stroke (Nawashiro et al, 1997; Knoblach et al, 1999); Marchand et al, 2009), as well as chronic brain diseases such as Parkinson's disease or Alzheimer's disease (McCoy et al, 2006; McAlpine et al, 2009). However, the biologic TNFIs cannot be developed for the brain, because these large molecule drugs do not cross the BBB, as recently demonstrated for the TNFR decoy receptor in the primate (Boado et al, 2010a).

The CNS drug development of the biologic TNFIs requires in vivo validation in animal models. However, in vivo animal studies cannot go forward until the biologic TNFI is re-engineered to cross the BBB. The purpose of the present study was to re-engineer the TNFR decoy receptor as the first biologic TNFI that is brain-penetrating in the mouse. The TNFR was re-engineered as a fusion protein with the cTfRMAb, which acts as a molecular Trojan horse to ferry the TNFR decoy receptor across the BBB. This re-engineering of the TNFR decoy receptor was accomplished by fusion of the amino terminus of the human TNFR-II ECD to the carboxyl terminus of the heavy chain of the cTfRMAb, as illustrated in Figure 1. The human TNFR decoy receptor was fused to the cTfRMAb rather than the mouse TNFR, because the human TNFR is the receptor that will ultimately be used for human therapeutics, and because the human TNFR

binds human and murine TNFα with the same high affinity (Scallon et al, 2002). The binding of murine TNFα by the human TNFR is compatible with the high, 78%, amino acid identity between the monomeric human TNFα (NP_000585) and the monomeric mouse TNFα (NP038721). The fusion of the TNFR decoy receptor to the carboxyl terminus of the IgG heavy chain is a departure from the genetic engineering of all other decoy receptors. The engineering of all prior decoy receptor:Fc fusion proteins involves the fusion of the carboxyl terminus of the decoy receptor to the amino terminus of the human IgG Fc fragment (Peppel et al, 1991; Scallon et al, 1995). The structure shown in Figure 1 was chosen because the amino terminus of the TNFR receptor is not involved in ligand binding (Banner et al, 1993), and this design leaves free the amino terminus of the receptor-specific MAb. The variable regions of the antibody that bind to the BBB receptor are located in the amino terminal parts of the IgG chains (Boado et al, 2009), and fusion of proteins to the antibody amino terminus impairs binding of the antibody to the target BBB receptor (Boado and Pardridge, 2010).

The structure of the cTfRMAb-TNFR fusion protein shown in Figure 1 was validated by DNA sequencing and the deduced amino acid sequence (Results). The SDS-PAGE (Figure 2) and the mouse IgG and human TNFR Western blots (Figure 3) also verify the structure of the fusion protein. The cTfRMAb-TNFR fusion protein is a bi-functional molecule that binds both TNFα and the murine TfR with high affinity characterized by low nM binding constants. The cTfRMAb-TNFR fusion protein binds human TNFα in the ELISA (Figure 4) and in the radio-receptor assay (Figure 5). The KD of fusion protein binding to human TNFα is low, 96 ± 34 pM (Figure 5B), and this high binding will enable sequestration of TNFα in brain at pM concentrations.

The cTfRMAb binds the mouse TfR with a KD of 2.6 ± 0.3 nM (Boado et al, 2009), and fusion of therapeutics to the cTfRMAb does not impair fusion protein binding to the murine TfR (Zhou et al, 2010; Boado et al, 2010b). Evidence for the high affinity binding of the cTfRMAb-TNFR fusion protein to the TfR on the mouse BBB is the high brain uptake of the cTfRMAb-TNFR fusion protein, 2.8 ± 0.5 % ID/gram brain (Table 3). In contrast, the brain uptake of a MAb that does not recognize the mouse TfR is 47-fold lower, 0.06 ± 0.01 % ID/gram brain (Lee et al, 2000). The binding of the cTfRMAb-TNFR fusion protein to the BBB TfR triggers transcytosis through the BBB and penetration of the brain parenchyma. This is demonstrated with the capillary depletion method (Table 2). The VD of the fusion protein in the post-vascular supernatant, 326 ± 19 uL/gram, is many-fold above the brain blood volume in the mouse, 10 ± 1 uL/gram (Lee et al, 2000), and is 76% of the fusion protein VD in the brain homogenate (Table 2). Measurement of the specific activity of capillary specific enzymes such as γ-glutamyl transpeptidase and alkaline phosphatase, shows the microvasculature of brain is 95% removed from the post-vascular supernatant with the capillary depletion method (Triguero et al, 1990). Therefore, the high VD in the post-vascular supernatant represents fusion protein penetration into the brain parenchyma. The findings with the capillary depletion method have been verified with emulsion autoradiography of brain, which show rapid penetration of brain parenchyma following fusion protein targeting of the BBB receptor (Boado and Pardridge, 2009). The neuroprotection in experimental PD observed following IV administration of the cTfRMAb-GDNF fusion protein is pharmacologic evidence for fusion protein penetration of brain parenchyma from blood (Fu et al, 2010).

The concentration of the TNFR decoy receptor in mouse brain following the IV injection of 350 ug/kg is 65 ± 12 ng/gram (Table 3), which is equivalent to a brain TNFR concentration of 3.7 nM, which is high compared to the concentration of TNFα in brain. Under normal conditions, the brain TNFα concentration is 20-100 pg/gram, or 1-5 pM (Zhao et al, 2007; Liu et al, 2009). In experimental stroke, the brain TNFα concentration increases to about 0.5 nM (Shohami et al, 1994). Therefore, a relatively low dose of fusion protein, 350 ug/kg, is sufficient to sequester even the very high concentrations of TNFα that occur immediately after stroke. This dose of IgG-TNFR fusion protein is small compared to the dose of TNFR decoy receptor that is required to induce immune suppression in peripheral tissues (Boado et al, 2010a). Therefore, chronic conditions such as AD or PD could be treated with brain-penetrating biologic TNFIs such as IgG-TNFR fusion proteins. Several lines of investigation confirm the primary role played by TNFα in the pathogenesis of both PD (Ferger et al, 2004; McCoy et al, 2006) and AD (Janelsius et al, 2008; McAlpine et al, 2009).

In summary, the present studies describe the genetic engineering, CHO cell expression, purification, and biochemical analysis of a new IgG-TNFR fusion protein that is specific for the mouse. The cTfRMAb-TNFR fusion protein is the first biologic TNFI that is capable of penetration of the BBB following peripheral administration in the mouse. Future investigations of murine models of acute and chronic brain disease may evaluate the efficacy of brain-penetrating biologic TNFIs such as the cTfRMAb-TNFR fusion protein.

Acknowledgements

Winnie Tai and Phuong Tram provided technical assistance.

Authorship Contribution

Participated in research design: Boado, Pardridge

Conducted experiments: Zhou, Boado, Hui, Lu

Contributed new reagents or analytic tools: Boado

Performed data analysis: Zhou, Boado, Hui, Lu, Pardridge

Wrote or contributed to writing of the manuscript: Zhou, Boado, Hui, Lu, Pardridge

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Footnotes

This work was supported by the National Institutes of Health [R01-AG032244].

Legends to Figures

Figure 1. The cTfRMAb-TNFR fusion protein is comprised of 2 heavy chains and 2 light chains. The heavy chain is formed by fusion of the variable region of the heavy chain (VH) of the rat 8D3 MAb against the mouse transferrin receptor (mTfR) to the amino terminus of mouse IgG1 constant (C)-region, and fusion of human TNFR-II ECD to the carboxyl terminus of the heavy chain C-region. The light chain is formed by fusion of the variable region of the light chain (VL) of the rat 8D3 MAb to the mouse kappa light chain C-region (CL). The heavy chain C-region is comprised of 4 domains: CH1, hinge, CH2, and CH3.

Figure 2. Reducing (A) and non-reducing (B) SDS-PAGE of the cTfRMAb (lane 1) and the cTfRMAb-TNFR fusion protein (lane 2).

Figure 3. Western blotting with a primary antibody against mouse IgG (A) or against human TNFR-II (B). The anti-mouse antibody reacts with the HC and LC of both the cTfRMAb (lane 2, panel A) and the cTfRMAb-TNFR fusion protein (lane 3, panel A), but not with the TNFR-II ECD (lane 1, panel A). The anti-TNFR antibody reacts only with the HC of the cTfRMAb-TNFR fusion protein (lane 3, panel B), and the TNFR-II ECD (lane 1, panel B), but does not react with cTfRMAb (lane 2, panel B).

Figure 4. (A) Format of ELISA used to measure binding of the cTfRMAb-TNFR fusion protein to human TNFα. Human TNFα is the capture agent and a conjugate of a goat anti-mouse (GAM) IgG and alkaline phosphatase (AP) is the detector reagent. (B) The

cTfRMAb-TNFR fusion protein binds to TNFα, whereas there is no binding to TNFα by mouse IgG1 (mIgG1).

Figure 5. (A) Outline of radio-receptor assay binding of TNFα to the cTfRMAb-TNFR fusion protein. A goat anti-mouse (GAM) IgG1 Fc was plated, which bound the Fc region of the cTfRMAb-TNFR fusion protein. The TNFR extracellular domain (ECD) region of the fusion protein then bound the [^{125}I]-TNFα, which was displaced by the addition of unlabeled TNFα. (B) The saturable binding was analyzed by a non-linear regression analysis to yield the concentration, K_D, that gave 50% inhibition of TNFα binding to the cTfRMAb-TNFR fusion protein

Figure 6. Plasma concentration, expressed as % of injected dose (I.D.)/mL, of the [^{3}H]-cTfRMAb-TNFR fusion protein after intravenous or intraperitoneal injection in the mouse. Data are mean ± S.E. (n=4 mice/point).

Table 1. Pharmacokinetic parameters of cTfRMAb-TNFR in the mouse

Parameter	Units	cTfRMAb-TNFR
A1	%ID/mL	14.8 ± 1.3
A2	%ID/mL	16.1 ± 0.7
k1	min^{-1}	0.38 ± 0.06
k2	min^{-1}	0.0094 ± 0.0011
MRT	min	105 ± 12
Vc	mL/kg	130 ± 5
Vss	mL/kg	238 ± 10
AUC(60 min)	%ID·min/mL	778 ± 13
AUCss	%ID·min/mL	1761 ± 144
Cl	mL/min/kg	2.3 ± 0.2

MRT=mean residence time; Vc=plasma volume; Vss=steady state volume of distribution; AUC(60 min)=area under the curve first 60 min; AUCss=steady state AUC; Cl=clearance from plasma. The injection dose was 350 μg/kg, and the body weight of the mice was 0.058 kg. The parameters were determined from the plasma profile shown in Figure 6 for IV administration.

DMD Fast Forward. Published on September 30, 2010 as DOI: 10.1124/dmd.110.036012
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Table 2. Capillary depletion analysis for brain uptake of cTfRMAb-TNFR fusion protein

Fraction	**VD (μL/g)**
Brain homogenate	431 ± 39
Post-vascular supernatant	326 ± 19
Vascular pellet	155 ± 18

Mean ± S.E. (n=4 mice). The fusion protein was administered by IV injection, and brain measurements made 60 min following injection.

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Table 3. cTfRMAb-TNFR brain uptake parameters

Parameter	Value	Units
Brain uptake	2.8 ± 0.5	%ID/gram
BBB PS Product	3.6 ± 0.6	μL/min/gram
Injection dose	350 ± 5	μg/kg
cTfRMAb-TNFR brain concentration	250 ± 45	ng/gram
TNFR brain concentration	65 ± 12	ng/gram

Mean ± S.E. (n=4 mice). The fusion protein was administered by IV injection, and brain measurements made 60 min following injection.

Figure 1

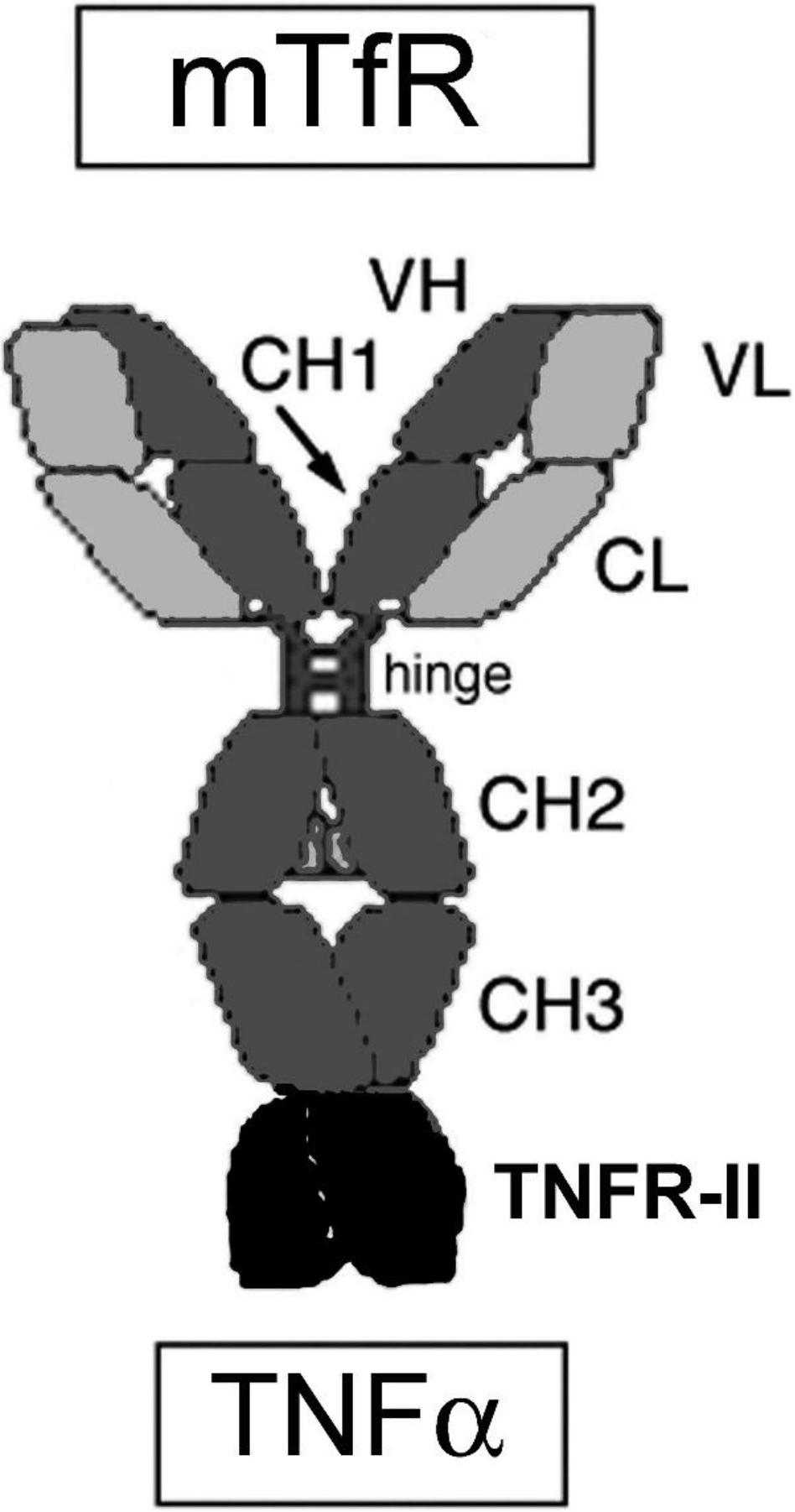
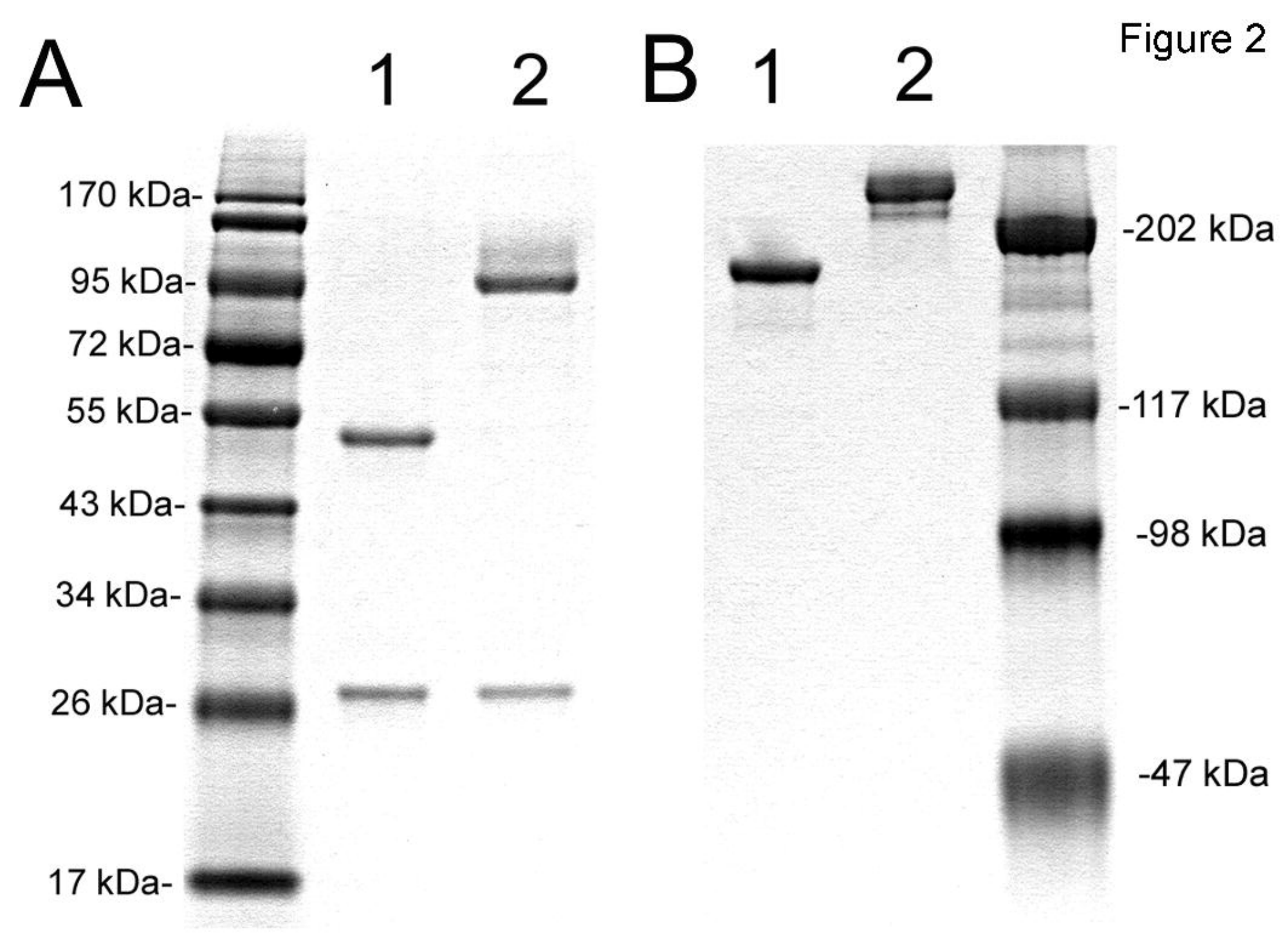


Figure 2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95 kDa-
72 kDa-
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B
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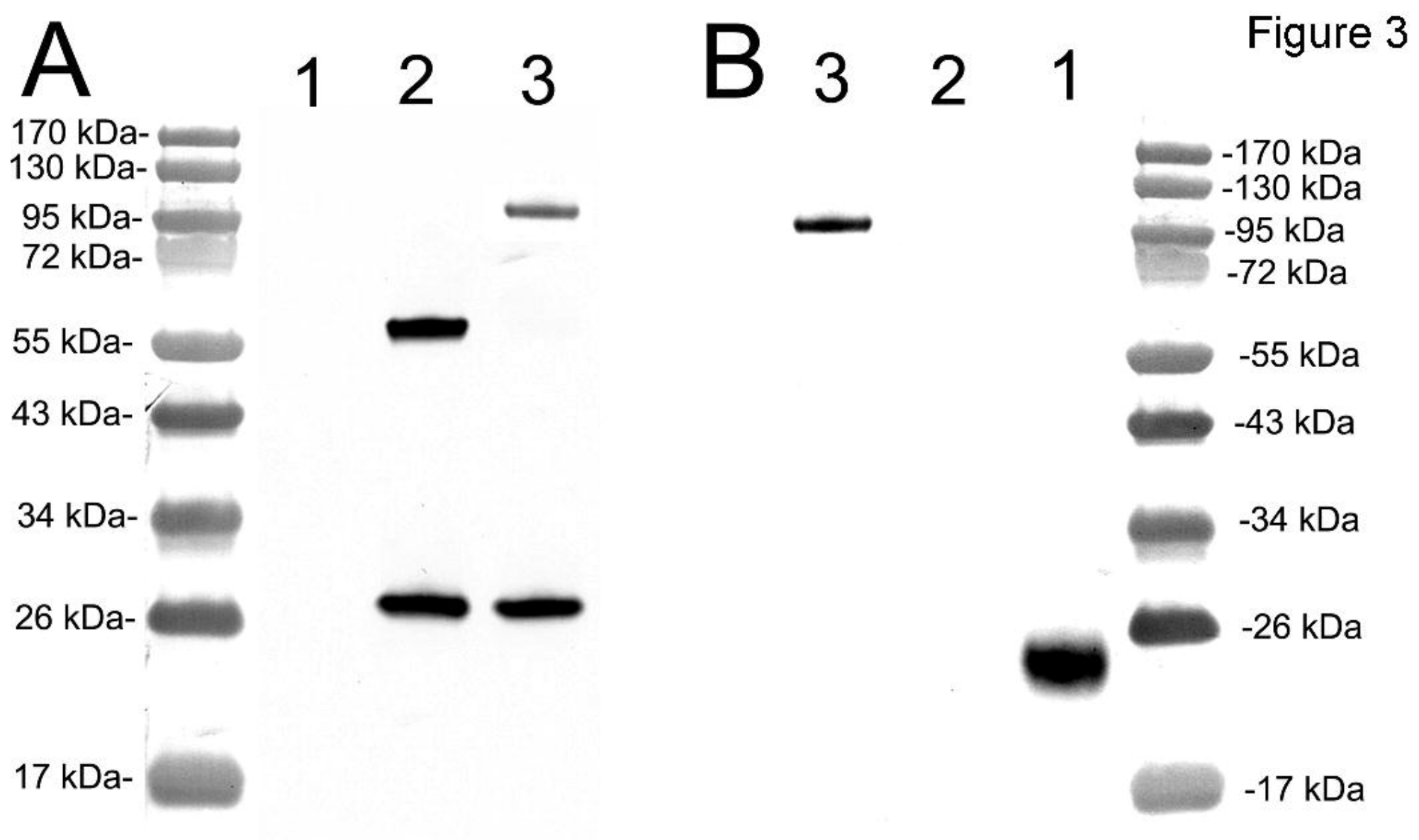
2
-202 kDa
-117 kDa
-98 kDa
-47 kDa

Figure 3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2
3
170 kDa-
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17 kDa-
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2
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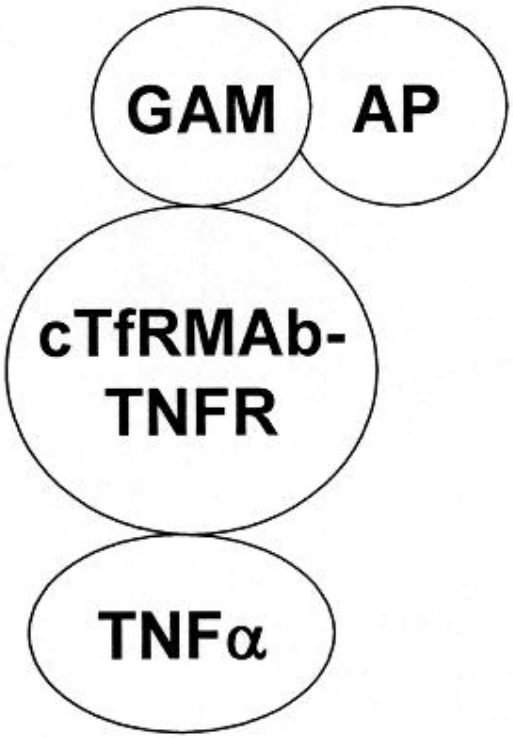
-95 kDa
-72 kDa
-55 kDa
-43 kDa
-34 kDa
-26 kDa
-17 kDa

Figure 4

A


GAM
AP
cTfRMAb-
TNFR
TNFα

B

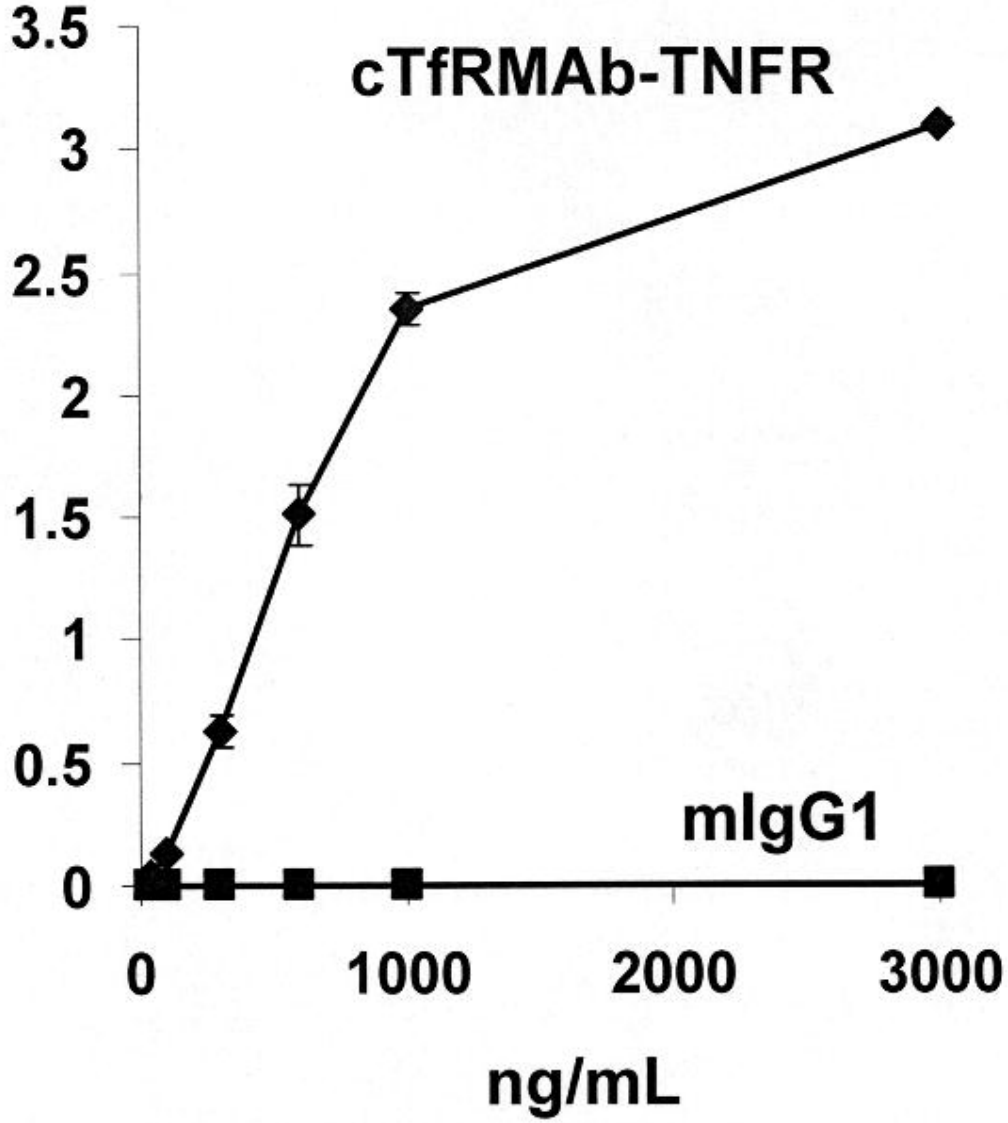

3.5
3
2.5
2
1.5
1
0.5
0
cTfRMAb-TNFR
mIgG1
0
1000
2000
3000
ng/mL

Figure 5

A


GAM-Fc
cTfR MAb
TNFR ECD
TNFα
-[125I]

B

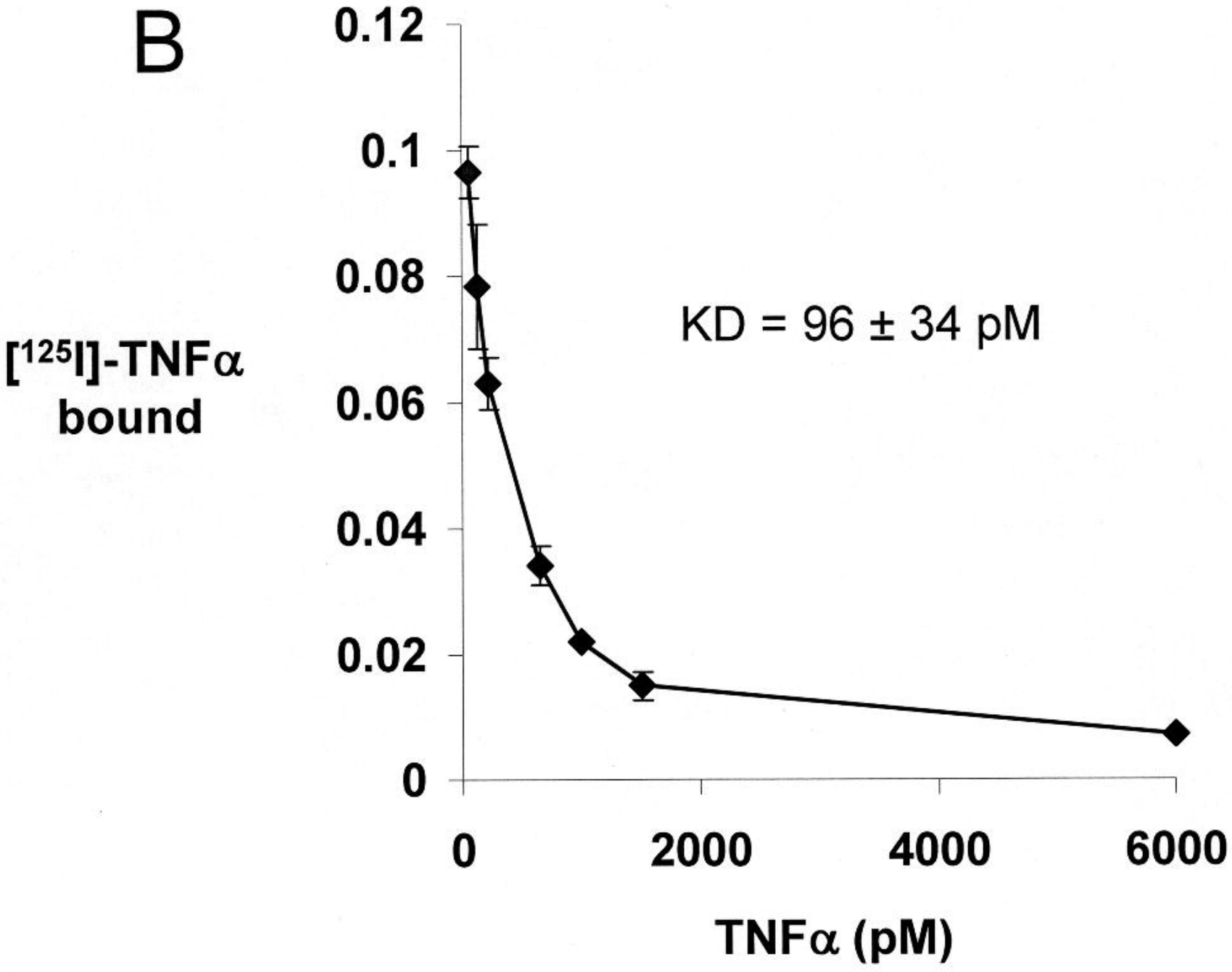
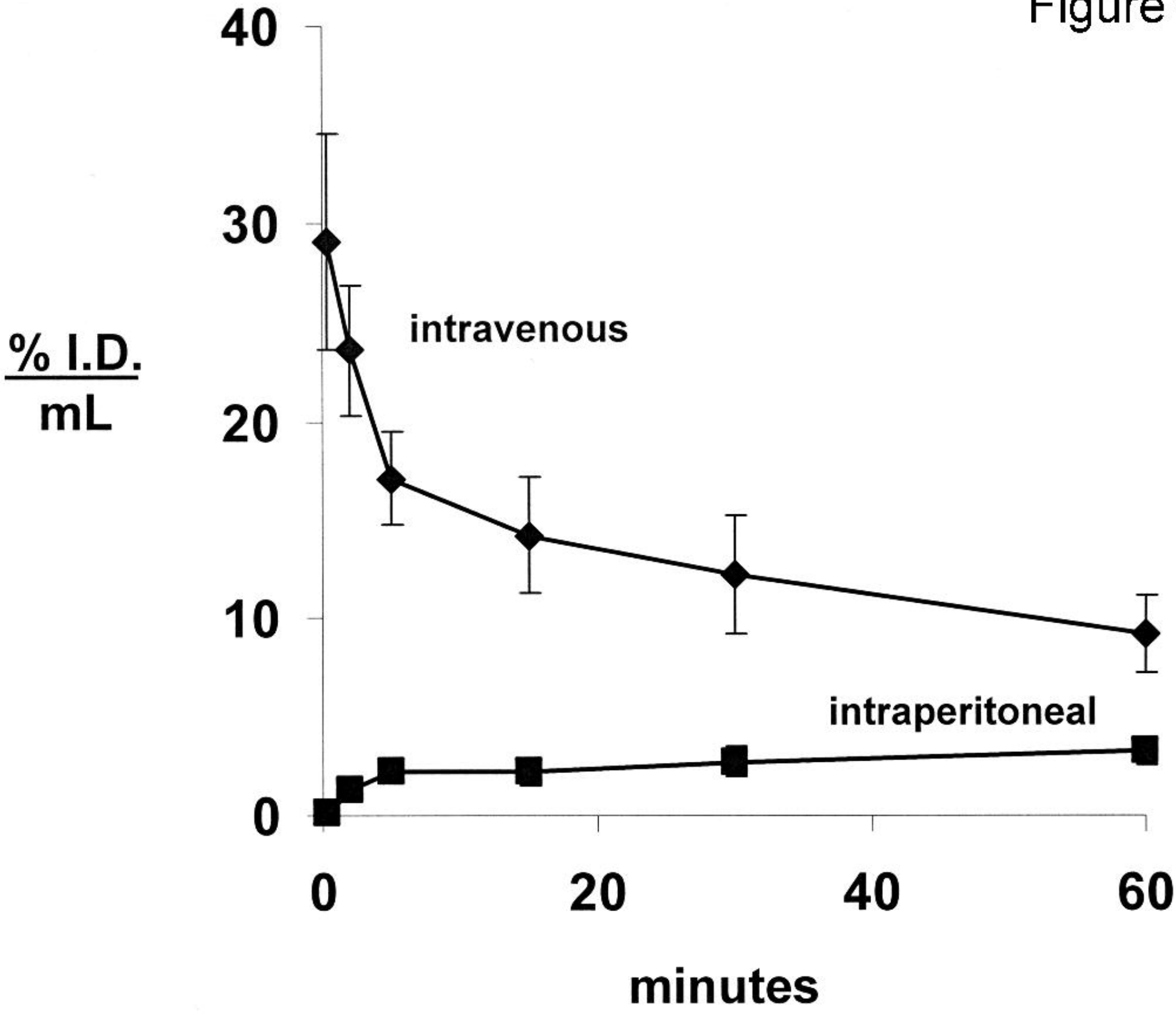

0.12
0.1
0.08
0.06
0.04
0.02
0
[125I]-TNFα bound
KD = 96 ± 34 pM
0
2000
4000
6000
TNFα (pM)

Figure 6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10
0
% I.D.
mL
intravenous
intraperitoneal
0
20
40
60
minutes