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# Tumor Necrosis Factor Receptor-IgG Fusion Protein for Targeted Drug Delivery across the Human Blood−Brain Barrier

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**Abstract:** The tumor necrosis factor-alpha receptor (TNFR) extracellular domain (ECD) is a decoy receptor that could be developed as a neurotherapeutic for stroke, brain injury, or chronic neurodegeneration. However, the TNFR ECD is a large molecule therapeutic that does not cross the blood−brain barrier (BBB). Human TNFR ECD was re-engineered by fusion of the receptor protein to the carboxyl terminus of the chimeric monoclonal antibody (mAb) to the human insulin receptor (HIR). The HIRMAb-TNFR fusion protein is bifunctional, and binds both the HIR, to trigger receptor-mediated transport across the BBB, and TNFα, to sequester this cytotoxic cytokine. COS cells were dual transfected with the heavy chain (HC) and light chain fusion protein expression plasmids, and the HC of the fusion protein was immunoreactive with antibodies to both human IgG and TNFR. The HIRMAb-TNFR fusion protein bound to the extracellular domain of the HIR with an affinity comparable to the HIRMAb, and bound TNFα with a $K_D$ of $0.34 \pm 0.17$ nM. Both the TNFR:Fc fusion protein and the HIRMAb-TNFR fusion protein blocked the cytotoxic actions of TNFα on human cells in a bioassay. In conclusion, these studies describe the re-engineering of the TNFR ECD to make this decoy receptor transportable across the human BBB.

**Keywords:** Blood−brain barrier; drug targeting; monoclonal antibody; stroke; decoy receptor

## Introduction

Tumor necrosis factor (TNF)-α is a proinflammatory cytokine that plays a pathogenetic role in acute and chronic disorders of the brain. Both TNFα and the TNFα receptor (TNFR) are upregulated in brain ischemia.[1]The transcranial administration of the TNFR extracellular domain (ECD) reduces the size of the infarct in a middle cerebral artery occlusion (MCAO) model.[2] The TNFR ECD must be injected directly into brain, because the soluble decoy receptor is a large molecule that does not cross the blood−brain barrier (BBB). In spinal cord injury (SCI), the intrathecal administration of a fusion protein of human IgG1 Fc fragment and the ECD of the human TNFR type II reduces the neuropathic pain associated with the SCI.[3] Similarly, the transcranial administration of the TNFR-II: Fc fusion protein (etanercept) in a traumatic brain injury (TBI) model is therapeutic.[4] However, the intravenous administration of the TNFR:Fc fusion protein in TBI is not therapeutic,[4] because the molecule does not cross the BBB. In addition to acute brain disorders, such as ischemia or brain

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or spinal cord injury, the use of TNFR:Fc fusion proteins may also be therapeutic in chronic neurodegeneration.[5]

The drug development of soluble decoy receptors, such as the TNFR-II ECD, for brain diseases is limited by the BBB. A new approach to BBB drug delivery of large molecules is the re-engineering of the pharmaceutical as a fusion protein with a BBB molecular Trojan horse.[6] The latter is a peptidomimetic monoclonal antibody (mAb) to an endogenous BBB peptide receptor transport system, such as the insulin receptor. The most potent BBB molecular Trojan horse is a mAb against the BBB human insulin receptor (HIR). A humanized HIRMAb has been genetically engineered for brain drug delivery in humans.[7] Moreover, the HIRMAb retains high affinity for the BBB HIR following fusion of proteins to the carboxyl terminus of the constant region of the heavy chain of the HIRMAb, including neurotrophins,[8,9] enzymes,[10,11] or single chain Fv antibodies.[12]

The fusion of the TNFR-II ECD to the carboxyl terminus of the HIRMAb heavy chain is depicted in Figure 1. The structure in Figure 1 represents a departure from prior Fc: decoy receptor fusion proteins. In previous work, the TNFR ECD was fused to the amino terminus of the IgG Fc fragment.[13] Similarly, other decoy receptor ECDs have been fused to the amino terminus of the IgG Fc fragment, including the vascular endothelia growth factor (VEGF) receptor,[14] the TNF-like weak inducer of apoptosis (TWEAK) receptor, Fn14,[15] or the lymphotoxin $\beta$ receptor.[16] However, in the present study the TNFR decoy receptor ECD is fused to the carboxyl terminus of the IgG heavy chain Fc region,

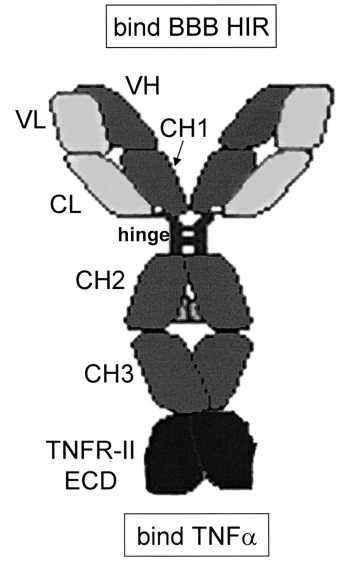


***Figure 1.*** The HIRMAb-TNFR fusion protein is formed by fusion of the amino terminus of the TNFR ECD to the carboxyl terminus of the CH3 region of the heavy chain of the chimeric HIRMAb. The fusion protein is a bifunctional molecule: the fusion protein binds the HIR, at the BBB, to mediate transport into the brain, and binds TNF$\alpha$, to suppress the inflammatory properties of this cytokine.

as shown in Figure 1. If the TNFR was fused to the amino terminus of the variable region of the IgG chain, there could be a significant loss of affinity for the HIR, as the binding site is near the amino terminus of the variable region of the HIRMAb heavy and light chains. The problem of loss of activity of the HIRMAb part of the fusion protein is eliminated by fusion of the TNFR ECD to the carboxyl terminus of the heavy chain. Moreover, this design places the TNFR ECD in a dimeric configuration, which promotes

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***Table 1.*** Oligodeoxynucleotide (ODN) Primers Used in the RT-PCR Cloning of Human TNFR-II and in the Engineering of the HIRMAb-TNFR Expression Vector

| ODN name | DNA sequence |
|---|---|
| human TNFR FWD | phosphate-CCTTGCCCGCCCAGGTGG |
| human TNFR REV | phosphate-TCAGTCGCCAGTGCTCCCTTC |

high affinity binding to the cognate ligand, TNFα.[17] When the TNFR is fused to the carboxyl terminus of the heavy chain, the HIRMAb part of the fusion protein folds in the host cell first, followed by folding of the TNFR within the carboxyl terminal portion of the fusion protein. Therefore, it is not clear if the TNFα binding properties of the TNFR would be retained in the fusion protein configuration shown in Figure 1. The purpose of the present study was to genetically engineer the HIRMAb-TNFR fusion protein shown in Figure 1, and to express this protein in mammalian host cells. The affinity of the HIRMAb-TNFR fusion protein for both the HIR and TNFα was then examined.

## Materials and Methods

**Cloning of TNFR cDNA.** The human TNFR-II extracellular domain (ECD) corresponds to amino acids 23−257 of NP_001057, and was cloned by the polymerase chain reaction (PCR) using the oligodeoxynucleotides (ODNs) described in Table 1 and cDNA derived from reverse transcription of polyA+RNA isolated from human U87 glial cells. The TNFR cDNA was cloned by PCR using 25 ng polyA+RNA-derived cDNA, 0.2 $\mu$M forward and reverse ODN primers (Table 1), 0.2 mM deoxynucleosidetriphosphates, and 2.5 U PfuUltra DNA polymerase (Stratagene, San Diego, CA) in a 50 $\mu$L Pfu buffer (Stratagene). The amplification was performed in a Mastercycler temperature cycler (Eppendorf, Hamburg, Germany) with an initial denaturing step of 95 °C for 2 min followed by 30 cycles of denaturing at 95 °C for 30 s, annealing at 55 °C for 30 s and amplification at 72 °C for 1 min; followed by a final incubation at 72 °C for 10 min. PCR products were resolved in 0.8% agarose gel electrophoresis, and the expected major single band of ~0.6 kb corresponding to the human TNFR cDNA was produced (Figure 2A). The amino acid sequence of the TNFR ECD was deduced from the nucleotide sequence of the cloned TFR ECD cDNA, and encompassed $Leu^{23}$-$Asp^{257}$ (NP_001057).

**Engineering of HIRMAb-TNFR Expression Vector.** For the engineering of the pHIRMAb-TNFR heavy chain (HC) expression plasmid, the mature human TNFR cDNA corresponding to amino acids $Leu^{23}$-$Asp^{257}$ of the human TNFR-II ECD (NP_001057) was inserted at the *Hpa*I site of the pHIRMAb-HC expression plasmid (Figure 2B) to produce a new plasmid designated pHIRMAb-TNFR (Figure 2B). The pHIRMAb-HC plasmid encodes the HC of the chimeric HIRMAb, and dual transfection of COS cells with this plasmid and a light chain (LC) expression plasmid, pHIRMAb-LC, allows for transient expression of either the chimeric HIRMAb, or a fusion protein. The TNFR forward (FWD) PCR primer (Table 1) introduces "CA" nucleotides to maintain the open reading frame and to introduce a Ser-Ser

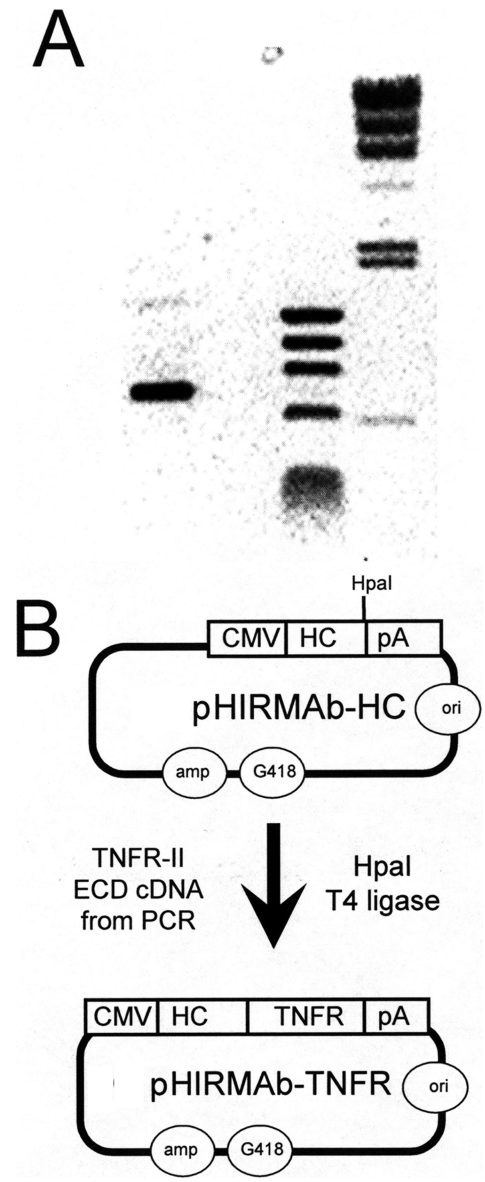


***Figure 2.*** (A) Ethidium bromide stain of agarose gel of human TNFR ECD cDNA (lane 1), which was produced by PCR from cDNA produced by reverse transcription of RNA from human U87 glial cells, and TNFR-specific ODN primers (Table 1). Lanes 2 and 3: DNA sizing standards. (B) Genetic engineering of pHIRMAb-TNFR, the eukaryotic expression plasmid encoding the fusion protein of TNFR ECD and the heavy chain (HC) of the chimeric HIRMAb. The fusion gene is 5′-flanked by the cytomegalovirus (CMV) promoter and 3′-flanked by the bovine growth hormone polyA (pA) sequence.

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linker between the carboxyl terminus of the CH3 region of the HIRMAb HC and the amino terminus of the TNFR ECD minus its signal peptide. The fusion of the TNFR monomer to the carboxyl terminus of each HC is depicted in Figure 1. This design sterically restricts the TNFR to a dimeric configuration, which is a preferred conformation of the TNFR ECD, which crystallizes as receptor dimer.[18] The TNFR reverse (REV) PCR primer (Table 1) introduces a stop codon, "TGA," immediately after the terminal aspartic acid of the TNFR ECD protein. The engineered pHIRMAb-TNFR expression vector was validated by DNA sequencing.

The HIRMAb HC and LC cDNA expression cassettes are driven by the cytomegalovirus (CMV) promoter and contain the bovine growth hormone (BGH) polyadenylation (pA) sequence (Figure 2B). The engineering of the universal pHIRMAb-HC vector was performed by insertion of a single *Hpa*I site at the end of the HIRMAb HC CH3 open reading frame (orf) by site directed mutagenesis (SDM), as described previously.[8]

**Transient Expression of HIRMAb-TNFR Fusion Protein in COS Cells.** COS cells were dual transfected with pHIRMAb-LC and pHIRMAb-TNFR using Lipofectamine 2000, with a ratio of 1:2.5, $\mu$g of DNA:$\mu$L of Lipofectamine. Following transfection, the cells were cultured in serum free VP-SFM (Invitrogen, Carlsbad, CA). COS cells were initially plated in 6-well cluster dishes for screening for expression with a human IgG specific ELISA. Subsequently, the transfection was scaled up for plating of transfected COS cells in 10xT500 flasks. The conditioned serum free medium was collected at 3 and 7 days. The fusion protein was purified by protein A affinity chromatography.

**Human IgG ELISA.** Human IgG ELISA was performed in Immulon 2 high binding plates (Dynex Tech., Chantilly, VA) with COS cell conditioned medium. A goat anti-human IgG primary antibody (Zymed-Invitrogen, Carlsbad, CA) was plated in 0.1 M $NaHCO_3$ (100 $\mu$L, 2 $\mu$g/mL) and incubated overnight at 4 °C. Plates were washed with 0.01 M $Na_2HPO_4$/0.15 M NaCl/pH = 7.4/0.05% Tween-20 (PBST), and blocked with 1% gelatin in PBST for 30 min at 22 °C. Plates were incubated with 100 $\mu$L/well of either human IgG1 standard or the fusion protein for 60 min at room temperature (RT). After washing with PBST, a goat anti-human kappa LC antibody conjugated to alkaline phosphatase (Sigma Chemical Co., St. Louis, MO) was plated for 60 min at 37 °C. Color development was performed with *p*-nitrophenyl phosphate (Sigma) at pH = 10.4 in the dark. The reaction was stopped with NaOH, and absorbance at 405 nm was measured in a BioRad ELISA plate reader.

**SDS−PAGE and Western Blotting.** The homogeneity of protein A purified fusion protein produced by COS cells was evaluated with a reducing 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), followed by Coomassie Blue staining. For Western blotting, human IgG immunoreactivity was tested with a primary goat antibody to human IgG (H+L) (Vector Laboratories, Burlingame, CA), and human TNFR immunoreactivity was evaluated with a mouse monoclonal antibody to the human TNFR-II ECD (Santa Cruz Biotechnology, Santa Cruz, CA).

**HIR Receptor Assay.** The affinity of the fusion protein for the HIR extracellular domain (ECD) was determined with an ELISA using the lectin affinity purified HIR ECD. CHO cells permanently transfected with the HIR ECD were grown in serum free media (SFM), and the HIR ECD was purified with a wheat germ agglutinin affinity column, as previously described.[19] The HIR ECD (0.2 $\mu$g/well) was plated on Immulon 2 high binding 96-well plates, and the binding of the chimeric HIRMAb, the HIRMAb-TNFR fusion protein, or human IgG1 to the HIR ECD was detected with a biotinylated goat anti-human IgG (H+L) antibody (0.3 $\mu$g/well), and the ABC Elite detection system (Vector Laboratories). The concentration that caused 50% binding to the HIR ECD, the $ED_{50}$, was determined by nonlinear regression analysis using the BMDP2007e software (Statistical Solutions, Cork, Ireland).

**TNFα Binding ELISA.** Binding of the HIRMAb-TNFR fusion protein to TNFα was determined with an ELISA. The capture reagent was human TNFα from Peprotech (Rocky Hill, NJ). The positive control in the assay was recombinant human TNFR-II:human IgG1 Fc fusion protein, designated TNFR:Fc (R&D Systems, Minneapolis, MN, catalogue no. 726-R2), and the negative control was human IgG1/$\kappa$ from Sigma Chemical Co. (St Louis, MO). The TNFα was dissolved in 0.1 M $NaHCO_3$/pH = 9.0 and plated overnight at 4 °C in 100 $\mu$L/well (0.2 $\mu$g/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 (BSA) in TBS for 30 min. A volume of 100 $\mu$L/well of HIRMAb-TNFR, TNFR:Fc, or human IgG1k was plated for 60 min at room temperature. After washing with TBS plus 0.05% Tween-20 (TBST), a goat anti-human IgG−alkaline phosphatase conjugate (Bethyl Laboratories, Montgomery, TX) was incubated (0.2 $\mu$g/well) for 60 min. Following washing with TBST, color detection at 405 nm was performed with an ELISA plate reader after color development with *p*-nitrophenylphosphate and termination of the reaction with 1.2 M NaOH. The TNFR:Fc, or the HIRMAb-TNFR, bound to the plated TNFα in a linear relationship that did not saturate within the tested concentration range of 0−600 ng/mL. Therefore, the data were fit to a linear regression analysis to compute the slope of the binding curve; the inverse of the slope is equal to the $K_D/A_{max}$ ratio, where $K_D$ is the binding constant of TNFR binding to the plated TNFα, and $A_{max}$ is the maximum absorbance. In this analysis, the slope of the binding curve is proportional to the affinity of the TNFR fusion protein for the TNFα.

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**TNFα Radio-Receptor Assay.** The saturable binding of human TNFα to the HIRMAb-TNFR fusion protein was determined with a radio-receptor assay (RRA). A mouse anti-human IgG1 Fc antibody (Invitrogen/Zymed) was plated in 96-well plates (0.4 μg/well) with an overnight incubation in 0.1 M $NaHCO_3$/pH = 8.3, followed by washing, and blocking with 1% bovine serum albumin (BSA) in 0.01 M $Na_2HPO_4$/0.15 M NaCl/pH = 7.4 (PBS). Then, one of the following solutions was plated at 100 μL/well: (a) 1% BSA in PBS, (b) 100 ng/well of human IgG1/kappa, or (c) 100 ng/well of the HIRMAb-TNFR fusion protein, followed by a 1 h incubation at room temperature. The wells were then washed with PBS, followed by the addition of 200 μL/well of a comixture of [$^{125}$I]-TNFα (Perkin-Elmer, Boston, MA) at a concentration of 0.01 μCi/well (0.2 ng/well) and various concentrations of unlabeled human TNFα, followed by a 3 h incubation at room temperature. The wells were emptied by aspiration and washed with cold PBS, and 250 μL/well of 1 N NaOH was added, followed by heating at 60 °C for 30 min. Radioactivity was counted in Ultima-gold (Perkin-Elmer) in a Perkin-Elmer liquid scintillation counter, and the fractional binding per well was computed. The half-saturation constant, $K_D$, of TNF binding to the HIRMAb-TNFR fusion protein was determined by nonlinear regression analysis using the BMDP2007e software.

**TNFα Bioassay.** Human WEHI-13 VAR cells (CRL-2148) were obtained from the American Type Culture Collection (Manassas, VA) and used as a bioassay of TNFα cytotoxicity.[20] The cells were plated in 24-well cluster dishes at 300,000 cells/well in RPMI-1640 medium with 10% fetal bovine serum (FBS). Following growth overnight, half of the medium was removed by aspiration, and was replaced by 200 μL of fresh RPMI-1640 medium, 50 μL/well of 10 μg/mL of actinomycin D (final concentration = 1.0 μg/mL), and final concentrations of human recombinant TNFα ranging from 1 to 100 pg/mL. In some wells, the TNFα was complexed to recombinant TNFR:Fc, or the HIRMAb-TNFR fusion protein, for 30 min prior to addition to the wells. The final concentration of the TNFR:Fc or the HIRMAb-TNFR fusion protein was 1.4 nM. After overnight incubation (20 h) at 37 °C in a humidified incubator, the medium was supplemented with thiazolyl blue tetrazolium bromide (MTT, Sigma) to a final concentration of 0.5 mg/mL. After a 3 h incubation at 37 °C, the reaction was terminated by the addition of solubilizing solution (48% isopropanol, 52% 1 N HCl). The absorbance at 570 and 650 nm and the $A_{570} - A_{650}$ difference were computed. MTT is oxidized by mitochondria in healthy cells to formazan crystals, and this reaction is inversely related to cell viability.

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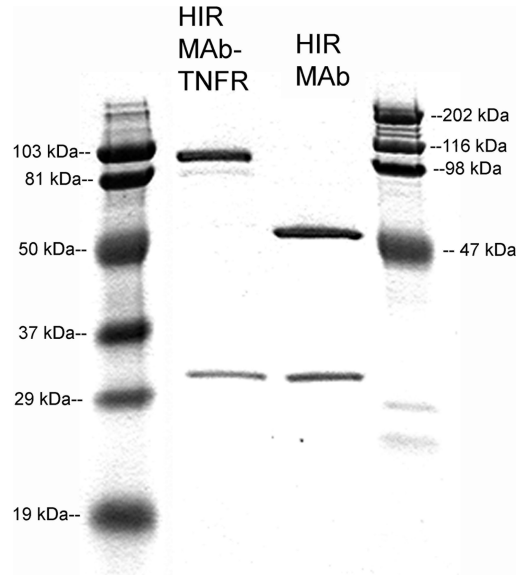


***Figure 3.*** Reducing SDS−PAGE and Coomassie blue staining of protein A affinity purified chimeric HIRMAb and the HIRMAb-TNFR fusion protein. Both are purified to homogeneity and are comprised of a heavy chain and a light chain.

## Results

The cDNA corresponding to the 235 amino acid TNFR-II ECD was amplified by PCR (Figure 2A), and this cDNA was subcloned into the *Hpa*I site of the pHIRMAb-HC plasmid, as outlined in Figure 2B. DNA sequencing of the expression cassette of the pHIRMAb-TNFR plasmid encompassed 3,193 nucleotides (nt), including a 714 nt CMV promoter, a 9 nt full Kozak site (GCCGCCACC), a 2,100 nt HIRMAb HC-TNFR fusion protein open reading frame, and a 370 nt BGH sequence. The plasmid encoded for a 699 amino acid protein, composed of a 19 amino acid IgG signal peptide, the 443 amino acid HIRMAb HC, a 2 amino acid linker (Ser-Ser), and the 235 amino acid human TNFR-II ECD minus its signal peptide. The predicted molecular weight of the heavy chain fusion protein, minus glycosylation, is 73,900 Da, with a predicted isoelectric point (p*I*) of 8.45. The deduced amino acid sequence of the TNFR ECD portion of the fusion protein included the presence of 22 cysteine residues and 2 N-linked consensus glycosylation sites within the TNFR ECD.

Dual transfection of COS cells with the pHIRMAb-TNFR and the pHIRMAb-LC resulted in medium human IgG levels of about 100 ng/mL, as determined with a human Fc specific ELISA. The HIRMAb-TNFR fusion protein was purified by protein A affinity chromatography. Following SDS−PAGE and Coomassie blue staining, the size of the light chain (LC) is the same for both the HIRMAb and the HIRMAb-TNFR fusion protein (Figure 3). The size of the heavy chain (HC) of the fusion protein is about 30 kDa larger than the HC of the HIRMAb (Figure 3). On Western blotting, the LC of either the HIRMAb or the HIRMAb-TNFR fusion protein reacts

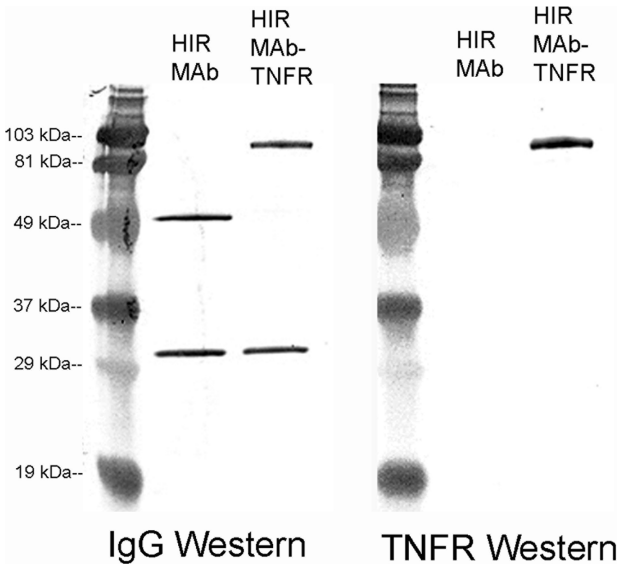


***Figure 4.*** Western blot with either anti-human (h) IgG primary antibody (left panel) or an anti-human TNFR-II primary antiserum (right panel). The immunoreactivity of the HIRMAb-TNFR fusion protein is compared to the chimeric HIRMAb. Both the HIRMAb-TNFR fusion protein and the HIRMAb have identical light chains on the anti-hIgG Western. The HIRMAb-TNFR fusion heavy chain reacts with both the anti-hIgG and the anti-human TNFR antibody, whereas the HIRMAb heavy chain only reacts with the anti-hIgG antibody. The size of the HIRMAb-TNFR fusion heavy chain is about 30 kDa larger than the size of the heavy chain of the HIRMAb, owing to the fusion of the 30 kDa TNFR ECD to the 55 kDa HIRMAb heavy chain.

equally on the Western blot with a primary antibody directed against the human IgG (H+L), as shown in Figure 4 (left panel). The size of the HC of the fusion protein is about 30 kDa larger than the size of the HC of the HIRMAb on both Western blots using either the anti-human IgG primary antibody (Figure 4, left panel) or the anti-human TNFR primary antibody (Figure 4, right panel).

The affinity of the fusion protein for the HIR extracellular domain (ECD) was determined with a ligand binding assay using lectin affinity purified HIR ECD (Materials and Methods). There is comparable binding of the chimeric HIRMAb and the HIRMAb-TNFR fusion protein for the HIR ECD with $ED_{50}$ of 0.44 ± 0.19 nM and 0.62 ± 0.06 nM, respectively (Figure 5).

The affinity of either recombinant TNFR:Fc or the HIRMAb-TNFR fusion protein for human TNFα was measured with an ELISA. The TNFR:Fc bound to the TNFα with a $K_D/A_{max}$ ratio 0.88 ± 0.02 nM, whereas human IgG1-kappa (hIgG1$\kappa$) does not bind to TNFα (Figure 6A). The HIRMAb-TNFR fusion protein bound to the TNFα with a comparable $K_D/A_{max}$ ratio of 1.40 ± 0.08 nM (Figure 6B). Saturable binding of TNFα to the HIRMAb-TNFR fusion protein was detectable with the radio-receptor assay, which is outlined in Figure 7A. Nonlinear regression analysis of

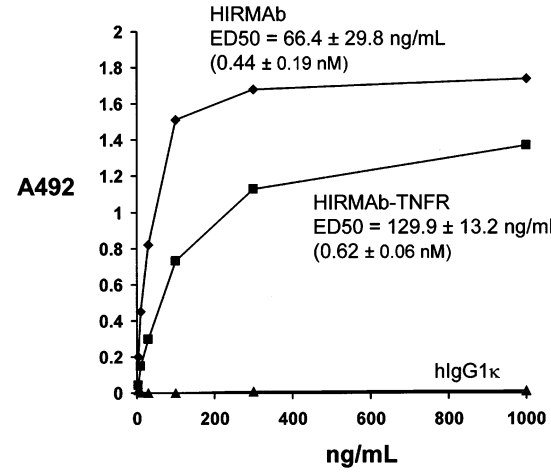


***Figure 5.*** Binding of either the chimeric HIRMAb or the HIRMAb-TNFR fusion protein to the HIR extracellular domain (ECD) is saturable. The $ED_{50}$ of HIRMAb-TNFR binding to the HIR ECD is comparable to the $ED_{50}$ of the binding of the chimeric HIRMAb.

the binding data (Materials and Methods) indicated the $K_D$ of binding was 0.34 ± 0.17 nM (Figure 7B).

The inhibition of TNFα biological activity by either the TNFR:Fc fusion protein or the HIRMAb-TNFR fusion protein was evaluated in a bioassay using human WEHI-13 VAR cells treated with 1 $\mu$g/mL of actinomycin D (Materials and Methods). In the absence of actinomycin D, the TNFα, at a concentration of 1−100 pg/mL, was not toxic to the cells. However, in the presence of actinomycin D, these concentrations of TNFα produced a dose-dependent cytotoxicity with an $ED_{50}$ of 5−10 pg/mL human TNFα (Figure 8). However, the cytotoxic effect of the TNFα was blocked by the coincubation of the cytokine with 1.4 nM concentrations of either the TNFR:Fc fusion protein or the HIRMAb-TNFR fusion protein (Figure 8).

## Discussion

The results of this study are consistent with the following conclusions. First, a bifunctional IgG-TNFR fusion protein has been genetically engineered, wherein mature human TNFR-II ECD is fused to the carboxyl terminus of the heavy chain (HC) of a chimeric HIRMAb (Figure 1), and expressed and secreted in COS cells (Figure 3 and 4). Second, the HIRMAb-TNFR fusion protein is bifunctional and binds the HIR (Figure 5) and human TNFα (Figures 6 and 7) with high affinity. Third, the HIRMAb-TNFR fusion protein has activity in a bioassay of human cells comparable to recombinant TNFR:Fc (Figure 8).

The present study describes a novel formulation of a soluble decoy receptor:IgG fusion protein, wherein the amino terminus of the decoy receptor, minus the receptor signal peptide, is fused to the carboxyl terminus of the IgG Fc region, as depicted in Figure 1. All prior decoy receptor:Fc fusion proteins fuse the carboxyl terminus of the decoy

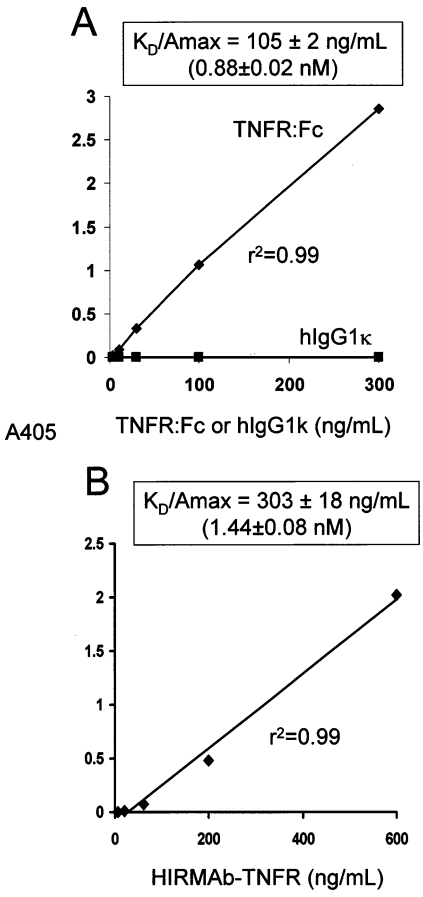


***Figure 6.*** Binding of either the TNFR:Fc fusion protein (A) or the HIRMAb-TNFR fusion protein (B) to the TNFα is linear. There is no binding of human IgG1 to the TNFα, as shown in panel A. The slope of the linear regression analysis yields the $K_D/A_{max}$ ratio, where $K_D$ is the binding constant for TNFα and $A_{max}$ is the maximal absorbance, and is a relative index of the $K_D$ of binding for TNFα. Both the TNFR:Fc fusion protein and the HIRMAb-TNFR fusion protein bind with comparable affinity to TNFα.

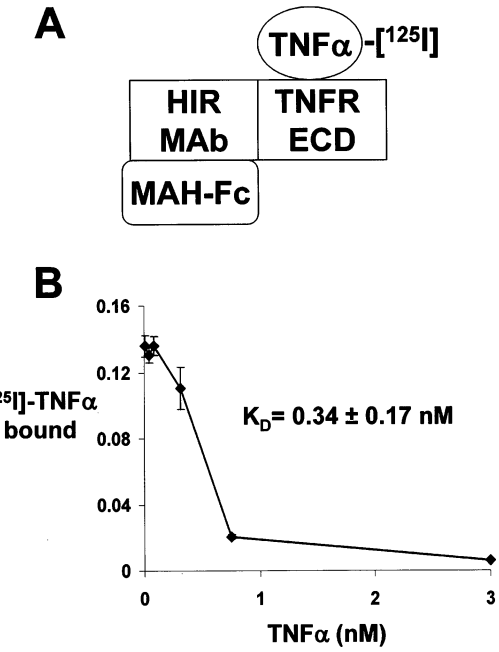


***Figure 7.*** (A) Outline of radio-receptor assay binding of TNFα to the HIRMAb-TNFR fusion protein. A mouse anti-human (MAH) IgG1 Fc was plated, which bound the Fc region of the HIRMAb-TNFR fusion protein. The TNFR extracellular domain (ECD) region of the fusion protein binds to the [$^{125}$I]-TNFα, which is displaced by the addition of unlabeled TNFα. (B) The saturable binding was analyzed by a nonlinear regression analysis to yield the concentration, $K_D$, which produced 50% inhibition of TNFα binding to the HIRMAb-TNFR fusion protein.

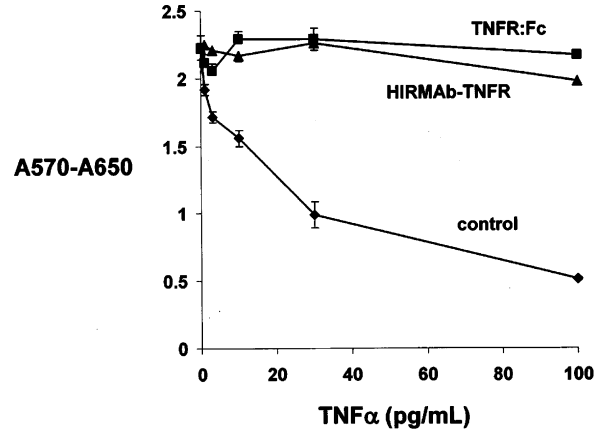


***Figure 8.*** TNFα causes cytotoxicity in actinomycin D-treated human WEHI-13 VAR cells with an $ED_{50}$ of about 10 pg/mL. However, in the presence of either 1.4 nM TNFR:Fc or 1.4 nM HIRMAb-TNFR, there is no cytotoxicity caused by the high concentrations of TNFα.

receptor to the amino terminus of the IgG Fc fragment.[13−17,21] However, fusion of proteins to the amino terminus of the heavy chain of the HIRMAb could result in an unacceptable loss of affinity of the HIRMAb for the HIR. The portion of the HIRMAb that binds the HIR is located within the near amino terminus of the heavy and light chains of the HIRMAb. Therefore, fusion of the carboxyl terminus of the decoy receptor to the amino terminus of the HIRMAb chains is not the preferred orientation. Fusion of the amino terminus of the decoy receptor to the carboxyl terminus of the IgG chain had not been done previously. However, the crystal structure of the TNFR:TNFa complex shows the amino terminus of the TNFR ECD does not bind to the ligand.[22] This suggests it may be possible to fuse the amino terminus of the TNFR ECD to the carboxyl terminus of the IgG chain, and still retain high affinity binding for TNFα. High affinity for TNFα binding is retained following fusion of the TNFR ECD to the carboxyl terminus of the HIRMAb heavy chain

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(Figure 6−8). Fusion of the TNFR ECD to the carboxyl terminus of the HIRMAb heavy chain has minimal effect on the affinity of the HIRMAb part of the fusion protein for the HIR (Figure 5).

The HIRMAb-TNFR fusion protein described in this study is a first in class molecule, as prior fusion proteins involved the fusion of either a receptor ligand, e.g., a neurotrophin,[8,9] an enzyme,[10,11] or a single chain Fv antibody.[12] The present work demonstrates it is also possible to construct biologically active bifunctional IgG fusion proteins with the soluble decoy receptor ECD (Figure 1). The HIRMAb-mediated delivery into brain of the TNFR could have beneficial effects in multiple brain disorders, including stroke,[2] traumatic brain injury,[4] spinal cord injury,[3] or neurodegeneration.[5] The HIRMAb-mediated delivery across the BBB of other soluble decoy receptors could also be therapeutic in brain disease. The decoy VEGF receptor could be used for angiogenesis blockade in brain tumors.[14] The decoy TWEAK receptor, Fn14, could reduce stroke volume in brain ischemia.[15] The lymphotoxin $\beta$ receptor (Lt$\beta$R) could be therapeutic in multiple sclerosis.[16]

In conclusion, these studies describe the genetic engineering, expression, and validation of an IgG decoy receptor fusion protein, wherein the decoy receptor is fused to the carboxyl terminus of the IgG heavy chain (Figure 1). The IgG used in the construction of this fusion protein undergoes receptor-mediated transport across the BBB via transport on the endogenous BBB insulin receptor.[6] The HIRMAb-TNFR fusion protein represents a re-engineering of the TNFR decoy receptor to enable transport of this biotherapeutic across the human BBB in vivo. The HIRMAb decoy receptor fusion protein can be administered by systemic injection to humans for treatment of multiple neurologic disorders, including stroke, acute brain or spinal cord injury, chronic neural repair, or multiple sclerosis.

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