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Differences in binding and effector functions between classes of TNF antagonists

Taruna Arora [a,*], Rupa Padaki [a], Ling Liu [a], Agnes E. Hamburger [a], Aaron R. Ellison [a], Seth R. Stevens [a], James S. Louie [b], Tadahiko Kohno [a]

[a] Amgen Inc., One Amgen Center Drive, Thousand Oaks, CA 91320-1799, USA
[b] UCLA Rheumatology, 1000 Veteran Ave., Rm 32-59, Los Angeles, CA 90095-1670, USA

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ABSTRACT

There are currently two Food and Drug Administration-approved classes of biologic agents that target tumor necrosis factor-α (TNF-α): anti-TNF monoclonal antibodies (mAbs) (adalimumab and infliximab), and soluble TNF receptors (etanercept). This study examined the ability of the TNF antagonists to: (1) bind various polymorphic variants of cell surface-expressed Fc receptors (FcγRs) and the complement component C1q, and (2) mediate Ab-dependent cellular cytotoxicity (ADCC) and complement-mediated cytotoxicity (CDC) killing of cells expressing membrane-bound TNF (mTNF) *in vitro*. Both mAbs and the soluble TNF receptor demonstrated low-level binding to the activating receptors FcγRI, FcγRIIa, and FcγRIIIa, and the inhibitory receptor FcγRIIb, in the absence of exogenous TNF. However, upon addition of TNF, the mAbs, but not etanercept, showed significantly increased binding, in particular to the FcγRII and FcγRIII receptors. Infliximab and adalimumab induced ADCC much more potently than etanercept. In the presence of TNF, both mAbs bound C1q in *in vitro* assays, but etanercept did not bind C1q under any conditions. Infliximab and adalimumab also induced CDC in cells expressing mTNF more potently than etanercept. Differences in the ability to bind ligand and mediate cell death may account for the differences in efficacy and safety of TNF antagonists.

1. Introduction

There are currently two Food and Drug Administration (FDA)-approved classes of biologic therapeutic agents that target tumor necrosis factor (TNF) bioavailability: soluble TNF receptors (etanercept) and anti-TNF monoclonal antibodies (mAbs) (adalimumab and infliximab). Although all three agents bind to TNF, they differ in clinical efficacy and safety [1,2]. Etanercept is FDA-approved for the treatment of patients with moderate to severe active rheumatoid arthritis (RA), polyarticular juvenile RA, psoriatic arthritis, ankylosing spondylitis, and chronic moderate to severe plaque psoriasis [3]. Adalimumab is indicated for the treatment of moderate to severe RA, polyarticular juvenile RA, psoriatic arthritis, ankylosing spondylitis, psoriasis, and moderate to severe Crohn's disease [4]. Infliximab is approved for treatment of moderate to severe RA, psoriatic arthritis, ankylosing spondylitis, moderate to severe ulcerative colitis, and moderate to severe Crohn's disease [5].

All three currently approved agents bear the Fc portion of complement-activating human immunoglobulin G1 (IgG1). The Fc region is a native component of the mAbs, whereas, to construct etanercept, the Fc region is genetically fused to a soluble portion of the TNF receptor p75.

Fc regions bind to Fc receptors (FcγRs), a family of Ig-binding molecules with different patterns of expression on immune cells, including monocytes, macrophages, granulocytes, natural killer (NK) cells, B cells, and platelets [6]. There are several different classes of FcγR in humans: FcγRI (CD64), FcγRIIa (CD32), FcγRIIb (an inhibitory receptor) and FcγRIII (CD16) [7]. Types of FcγRIII include high affinity (V/V) and low affinity (F/F) polymorphic variants, associated with certain autoimmune disorders [8,9]. FcγRI receptors have high affinity for IgG1 and can bind monomeric IgG1. The other classes have lower affinity and generally bind aggregated antibody–antigen complexes [10], resulting in trapping and clearance of immune complexes. In general, triggering of activating FcγRs leads to effector functions: opsonization, phagocytosis, and downstream cytokine release and immunomodulation. Activation through FcγRIII (CD16) on NK cells can result in triggering of lytic mechanisms through Ab-dependent cellular cytotoxicity (ADCC), in which cells coated with antigen:antibody complexes are targeted for killing by NK cells via lytic and apoptotic cascades [6]. Signal triggering through the inhibitory FcγRIIb receptor results in inhibition of antibody secretion and down-modulation of B cell response and antibody production [7].

The cytoplasmic FcR common gamma chain protein associates with the transmembrane and intracellular regions of FcγRI and

* Corresponding author. Fax: +1 805 499 7464.
E-mail addresses: tarora@amgen.com (T. Arora), jlouie@mednet.ucla.edu (J.S. Louie).

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FcγRIII [7], and possesses tyrosine-based activation motifs (ITAM motifs) for signaling through the FcγR complex. It also induces optimal cell surface expression of these FcR complexes [7,11]. FcγRII receptors induce signaling events through ITAM motifs (on activating FcγRIIa) or ITIM motifs (on inhibitory FcγRIIb) within their own gamma-like cytoplasmic domains.

IgG1 Fc also binds to the classical complement component C1q, and can trigger the lytic complement cascade. In addition, C1q has also been shown to increase FcR-based immune complex uptake and phagocytosis [12].

There are many clinically relevant outcomes to individual or combination Fc-mediated events. A balance between stimulation by activating and inhibitory Fc receptors may determine outcome of immune response, based on affinity for receptors, and levels of receptor expression [7,11]. The role of the TNF antagonist Fc regions in mediating FcγR-related events has not been thoroughly investigated. In previous *in vitro* studies [13], we have shown that the mAbs, but not the p75 TNF soluble receptor etanercept, form large protein complexes. The close proximity of many Fc regions may enable mAbs to cross-link and activate low affinity FcγRII and FcγRIII. In this report, we investigate the ability of the TNF antagonists to bind to membrane-bound TNF (mTNF), FcγR and C1q, and to induce cell death via ADCC and complement-mediated cytotoxicity (CDC)-mediated pathways.

2. Materials and methods

2.1. TNF antagonists

Etanercept was supplied in a buffer containing 25 mM sodium phosphate, 25 mM L-arginine, 98 mM sodium chloride, and 1% sucrose. Adalimumab was supplied in 105 mM sodium chloride, 5.5 mM monobasic sodium phosphate dihydrate, 8.6 mM dibasic sodium phosphate dihydrate, 1 mM sodium citrate, 6.2 mM citric acid monohydrate, 66 mM mannitol, 0.1% polysorbate 80, pH 5.2. Infliximab was supplied in 1.6 mM monobasic sodium phosphate monohydrate, 3.4 mM dibasic sodium phosphate dehydrate, 5% sucrose, 0.005% polysorbate 80, pH 7.2.

2.2. Construction of a stable mTNF-expressing CHO cell line

A membrane-bound mutant TNF-α, with amino acids 77 through 88 removed, was constructed from wild-type TNF DNA sequence (Ultimate ORF clone IOH21536; Invitrogen Corp., Carlsbad, CA), based on previous studies [14]. Site-directed mutagenesis was performed using a QuikChange kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. The primers used were: 5′-CTA ATC AGC CCT CTG GCC CAA GCA GTA GCC CAT GTT GTA GCA AAC CCT C- 3′ (forward) and 5′-TGC TTG GGC CAG AGG GCT GAT TAG- 3′ (reverse). The mutated TNF DNA sequence was cloned into a lentiviral expression vector using Gateway cloning methods and reagents (Invitrogen Corp.). The lentiviral vector contains the CMV promoter driving expression of the TNF gene and an internal ribosomal entry site-neomycin resistance marker for stable selection in mammalian cells. Lentiviruses were generated by transient co-transfection into HEK293T cells (transformed human embryonic kidney cell cultures, American Type Culture Collection (ATCC), Manassas, VA), and were concentrated and purified by ultracentrifugation. Chinese hamster ovary (CHO) cells (ATCC) were transduced with the lentiviruses containing the mutated TNF sequence at a multiplicity of infection of 20. The stable cell line named MT-3 was obtained by selection with 500 μg/ml G418, and single cell clones were obtained by cell sorting (FACSAria, BD Biosciences).

2.3. Binding of TNF antagonists to membrane TNF (mTNF)

FITC-conjugated agents (0.3 μg/ml) were incubated with mTNF-expressing MT-3 cells alone or in the presence of 100 nM soluble TNF for 1 h at 4 °C. The cells were washed with flow buffer (PBS with 0.2% BSA) to remove unbound agents. Binding of TNF antagonists to MT-3 cells was analyzed by flow cytometry (FACSCalibur, BD Biosciences, San Jose, CA).

2.4. FcR ^{125}I-binding assays

THP-1 cells (ATCC) a human monocytic cell line that expresses the Fc receptors FcγRI (CD64) and FcγRII (CD32), was used to evaluate the FcγR-binding characteristics of the TNF antagonists [15]. THP-1 cells were plated in binding medium (1% FBS/0.1% sodium azide in RPMI1640 medium) at 2.4×10^5 cells/well in a 96-well plate (Falcon U-bottom plates). TNF antagonists were radiolabeled with ^{125}I using Iodo-gen (Pierce Biotechnology Inc., Rockford, IL) according to the manufacturer's recommendations. The specific activities of radiolabeled etanercept, adalimumab, and infliximab were 22 μCi/μg, 24 μCi/μg, and 21 μCi/μg, respectively. Radiolabeled agents (ranging from 49 pM to 25 nM) were incubated with THP-1 cells alone, in the presence of 0.8-fold molar excess human TNF (Amgen Inc.), 200-fold molar excess human Fc (CHO cell-derived human IgG1; Amgen Inc.), or 200-fold molar excess cold agent. After incubating for 15 h at room temperature, the cells were washed with binding medium, harvested in 100 ml of 2N sodium hydroxide (Sigma Chemical Co.), and the amount of cell-bound iodinated agent was measured using a gamma counter (COBRA II, PerkinElmer, Waltham, MA). Rates of dissociation for each agent were determined by the quantification of cell-bound radiolabeled agent following the addition of 200-fold molar excess human Fc over time.

2.5. Generation of FcR-expressing HEK293T cells

cDNAs for human FcγRIa, FcγRIIa, FcγRIIIa 158V (higher affinity) and 158F (lower affinity), and FcR gamma chain, including translation termination codons, were obtained from DNA 2.0 (Menlo Park, CA) or Blue Heron Biotechnology (Bothell, WA). FcγR cDNAs were subcloned into pUB6/V5-H6-A vector (Invitrogen) and FcR gamma chain cDNA was cloned into pEF4/V5-H6-A vector (Invitrogen). HEK293T cells (ATCC) that stably expressed cell surface FcγR chains were created by co-transfection of full-length human FcγRs and FcR gamma chains at a 1:1 ratio, using Lipofectamine 2000 (Invitrogen), according to manufacturer's instructions, followed by antibiotic selection. Approximately 2 weeks after transfection, antibiotic-resistant cell clones were stained with murine fluorescein isothiocyanate (FITC)-conjugated anti-human CD64, CD32 or CD16 (BD Biosciences, San Jose, CA), and individual clones were sorted for FcγR expression by flow cytometry, using a FACSAria cell sorter (BD Biosciences).

2.6. FITC conjugation of TNF antagonists

Etanercept, adalimumab, and infliximab were prepared at 2 mg/ml in 0.1 M sodium carbonate. 10 μl FITC in DMSO was added per ml of TNF antagonist, and the reaction was incubated in the dark for 8 h. After quenching with 50 mM ammonium chloride and a further 2 h incubation FITC-conjugated proteins were purified by gel filtration (NAP-5, GE Healthcare, Chicago, IL).

2.7. FcR fluorescence binding assays

Stably transfected HEK293T cells were plated in PBS/2% FBS at 1×10^6 cells/well in a 96-well plate. To assess binding to FcγRIIb,