Identification of potent human anti-IL-1R\textsubscript{I} antagonist antibodies

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Interleukin-1 (IL-1) blockade by IL-1 receptor antagonist benefits some arthritis patients by reducing joint damage. This fact inspired us to develop antagonist human therapeutic antibodies against IL-1R\textsubscript{I} using phage libraries that display single-chain variable fragment (scFv) antibody fragments. Panning libraries against human IL-1R\textsubscript{I} generated 39 unique scFv-phage whose binding to IL-1R\textsubscript{I} was competed by IL-1 ligands. Fifteen of these scFv-phage, identified using IL-1R\textsubscript{I}-binding assays and dissociation rate ranking, were reformatted as scFv-Fc and IgG\textsubscript{4} molecules. The ease of producing antibodies in the scFv-Fc format permitted rapid identification of four lead clones (C10, C13, C14, C15) that inhibit NF-\kappa B nuclear translocation induced by IL-1. Reformatting these clones as IgG\textsubscript{4} molecules increased their inhibition potency by \textgammasy 24-fold. C10 IgG\textsubscript{4} is the most potent antagonist of IL-1\alpha (26 nM IC\textsubscript{50}) and IL-1\beta (18 nM IC\textsubscript{50}) in the NF-\kappa B bioassay, although less potent than IL-1ra (~0.4 nM IC\textsubscript{50}). C10 is the highest affinity clone for human IL-1R\textsubscript{I} (K\textsubscript{D} ~60 nM). Flow cytometry indicates that several lead clones bind cell-surface cynomolgus or murine IL-1R\textsubscript{I}, characteristics advantageous for preclinical toxicology and efficacy studies. This study demonstrates the utility of scFv-Fc fusion proteins for rapid screening of clones derived from phage libraries to identify antibody leads with therapeutic potential.

Keywords: antibody/IL-1/interleukin-1 receptor/phage display/rheumatoid arthritis/scFv

Introduction

The cytokines interleukin-1\alpha (IL-1\alpha) and IL-1\beta are naturally occurring agonists of the type I IL-1 receptor (IL-1R\textsubscript{I}). Binding of these agonist molecules to IL-1R\textsubscript{I} activates the receptor and triggers recruitment of a second receptor component known as the IL-1R\textsubscript{I} accessory protein (AcP). The resultant ternary complex (IL-1/IL-1R\textsubscript{I}/AcP) initiates a complex intracellular signaling cascade that includes activation and subsequent nuclear translocation of the transcription factor, NF-\kappa B. Activation of NF-\kappa B transcription results in the expression of many cytokine and other genes involved in inflammation and immune responses (Barnes, 1997).

Over-expression of the proinflammatory cytokines IL-1 and tumor necrosis factor \alpha (TNF-\alpha) has been shown to play a major role in the pathogenesis of rheumatoid arthritis (RA), a common chronic autoimmune disorder characterized by inflammation of synovial tissues, joint swelling, stiffness and pain that may progress to joint destruction (Bingham, 2002). Although the etiology of RA is unknown, a plethora of conventional disease modifying antirheumatic, corticosteroid and non-steroidal anti-inflammatory drugs exist to treat those suffering from RA. Recently, several protein pharmaceuticals that specifically target TNF have been approved for the treatment of RA including the TNF receptor immunoadhesin, etanercept (Enbrel\textregistered), and the anti-TNF-\alpha antibodies infliximab (Remicade\textregistered) and adalimumab (Humira\textsuperscript{TM}). These biologics act by specifically blocking cytokine-receptor binding and have been shown to significantly improve the clinical signs and symptoms of RA and reduce the rate of disease progression (Arend, 2002; Weinblatt \textit{et al}, 2003). Despite these recent advances some RA patients continue to suffer from this disease and there is still a significant unmet need for new therapies (Hallegra and Weisman, 2002). These observations have inspired the exploration of IL-1 antagonists to block the pathological effects of IL-1 ligands.

There are two known natural inhibitors of IL-1, namely, the type II IL-1R (IL-1R\textsubscript{II}) and the IL-1 receptor antagonist (IL-1ra). IL-1R\textsubscript{II} is a so-called ‘decoy receptor’ that has a short cytoplasmic tail and exists in both membrane-associated and secreted forms (Colotta \textit{et al}, 1993). Both IL-1\alpha and IL-1\beta can bind IL-1R\textsubscript{II}; however, these agonists are not able to initiate intracellular signaling (McMahan \textit{et al}, 1991). Thus, this receptor can sequester IL-1\alpha and IL-1\beta and block their biologic effects. The other naturally occurring antagonist, IL-1ra, binds to IL-1R\textsubscript{I} with high affinity but is unable to activate the receptor and trigger a biological response. IL-1ra is a competitive antagonist of IL-1R\textsubscript{I} that directly blocks IL-1\alpha and IL-1\beta binding to IL-1R\textsubscript{I} thereby inhibiting the pro-inflammatory effects of these cytokines. Clinical demonstration of the therapeutic benefit of antagonizing IL-1\alpha and IL-1\beta using IL-1ra is provided by anakinra (Kinere\textsuperscript{TM}). Anakinra is a recombinant, non-glycosylated form of human IL-1ra that can be used to treat moderate to severe cases of RA. Anakinra treatment of RA patients has led to a significant reduction in the frequency and severity of joint damage (Bresnihan, 2002; St. Clair, 2002).

An anti-IL-1R\textsubscript{I} antagonist antibody offers potential advantages over anakinra for IL-1 antagonism. Therapeutic antibodies can have terminal half-lives in patients of up to several weeks, allowing daily or less frequent dosing (Presta, 2002). In contrast, anakinra has a terminal half-life of 4–6 h in patients necessitating daily dosing (Campion \textit{et al}, 1996). It seems likely that the terminal half-life of an anti-IL-1R\textsubscript{I} antibody will be substantially greater than that of anakinra and may obviate the need for daily dosing. Furthermore, the expected elevation in trough levels of circulating antagonist due to increased half-life of the antibody also has the potential to increase efficacy. Support for the notion of using an antibody
to antagonize IL-1 is provided by M147, a hamster anti-mouse IL-1R\(\alpha\) antibody (Rogers et al., 1992). Administration of this anti-IL-1R\(\alpha\) monoclonal Ab (mAb) has proved highly efficacious in prophylaxis and therapy models of collagen-induced arthritis (Barone et al., 2002). Unfortunately, M147 does not cross-react with human IL-1R\(\alpha\) and thus does not provide a tractable starting point for drug development.

Here we describe the use of phage display (Smothers et al., 2002) and single-chain variable fragment (scFv) phage libraries to identify potential antagonist antibodies against human IL-1R\(\alpha\) that block in vitro binding of IL-1\(\alpha\) and IL-1\(\beta\). Although antibody fragment phage libraries are valuable tools for isolating antibodies with the desired binding properties (Quan and Carter, 2002), antibody phage are not considered as potential therapeutic agents. Isolated scFv-phage clones that met specific biochemical criteria were therefore converted to a more clinically relevant format, namely IgG\(_4\). The process of generating recombinant IgG\(_4\) starting from scFv clones is somewhat slow and arduous, as it requires separate sub-cloning of variable heavy and light chains (\(V_H\) and \(V_L\)) into heavy and light chain expression vectors, respectively. Moreover, four individual primers are required for each scFv—two each for \(V_H\) and \(V_L\). An alternative antibody format, scFv-Fc, was explored to potentially increase the throughput of screening putative IL-1R\(\alpha\) antagonist antibody candidates and mimic the bivalent properties of IgG molecules. An scFv-Fc is an engineered immunoglobulin-like molecule formed by dimerization of an scFv molecule linked to a human \(\gamma 1\) Fc molecule (Shu et al., 1993; Hayden et al., 1994). Dimerization of scFv-Fc molecules yields two identical antigen-binding sites per molecule. Conversion of scFv fragments to scFv-Fc molecules can be accomplished using a universal set of PCR primers and a single judiciously designed expression vector as demonstrated here. scFv-Fc and IgG\(_4\) proteins were compared in their potency of IL-1\(\alpha\) and IL-1\(\beta\) antagonism in biochemical and cellular assays, as well as in their binding affinity for IL-1R\(\alpha\). This study permitted us to identify potent IL-1R\(\alpha\) antagonist antibodies and investigate the utility of scFv-Fc molecules as a screening tool.

### Materials and methods

**Materials**

All proteins used in these studies are human unless otherwise noted. Recombinant proteins including IL-1\(\alpha\), IL-1\(\beta\), IL-1ra, IL-1R\(\alpha\) ectodomain (residues 1–331) and murine IL-1R\(\alpha\) ectodomain (residues 1–339) were generated at Immunex, now part of Amgen. Receptor ectodomains were also genetically fused to human IgG\(_4\), Fc to facilitate their expression and purification. Cynomolgus IL-1R\(\alpha\) ectodomain was cloned from an activated T-cell cDNA library from cynomolgus monkeys (B.Renshaw, GenBank accession No. AY497008) and then expressed both as an Fc fusion protein and as a monovalent receptor containing C terminal FLAG epitope and His\(\sigma\)-tag (RSGSSDYKDDEDDKGSSHHHHHHH). K299 (KARPAS-299) cells, used in FACS analysis studies, are a human non-Hodgkin’s T-cell lymphoma cell line (DSMZ, Braunschweig, Germany) that constitutively expresses IL-1R\(\alpha\). Stable CHO cells expressing murine IL-1R\(\alpha\) were generated by M.Kubin (Amgen) and used in NF-kB assays. Neutralizing and weakly neutralizing mouse and rat anti-human IL-1R\(\alpha\) mAb, M1 and M8, respectively, have previously been described (McMahan et al., 1991).

Biotinylated human IL-1R\(\alpha\) was generated using vendor protocols (Pierce Biotechnology, Rockford, IL) with some modifications. Briefly, biotinylation was performed at 25°C for 30 min followed by 15 min on ice using a 1:2 molar ratio of receptor to biotinylation reagent (EZ-Link Sulfo-NHS-SS-Biotin; Pierce Biotechnology). Receptors were biotinylated to a stoichiometry approaching 2 mol biotin/mol receptor, as estimated by HABA titration (Bayer and Wilchek, 1990). Biotinylation of the receptor was not found to be detrimental to the IL-1 binding site as surface plasmon resonance (SPR) studies determined IL-1\(\alpha\) to have the same binding affinity to biotinylated receptor (1 nM \(K_D\), data not shown) as unmodified receptor.

Europium (Eu) labeling of lysine residues on anti-M13 bacteriophage mAb (Amersham Biosciences, Piscataway, NJ), IL-1\(\alpha\) and IL-1\(\beta\) was performed by PerkinElmer Life Sciences (Akeron, OH) using Eu-N1-ITC. Anti-M13 mAb and receptor ligands were labeled to a stoichiometry of approximately 6.0 and 2.3 Eu residues/molecule, respectively.

**Affinity selection of IL-1R\(\alpha\)-binding scFv clones**

A panel of human IL-1R\(\alpha\)-specific scFv clones was isolated by panning three different human scFv antibody libraries against biotinylated soluble human IL-1R\(\alpha\) ectodomain. These scFv libraries (Cambridge Antibody Technology, Cambridge, UK) were constructed from the V-gene segments of non-immunized human donors with a total diversity of \(\sim 1 \times 10^8\) scFv fragments (Vaughan et al., 1996). Prior to panning, scFv-phage particles and streptavidin M-280 magnetic beads (Dynal Biotech, Oslo, Norway), preblocked in buffer A [PBS containing 0.1% (v/v) Tween-20 and 3% (w/v) dry non-fat milk], were incubated together to deplete streptavidin-binding phage from each library. ‘Precleared’ libraries were then mixed with 100 nM biotinylated IL-1R\(\alpha\) and allowed to bind for 1 h. The first round of selection was performed under low stringency conditions (100 nM IL-1R\(\alpha\)) to recover as many different IL-1R\(\alpha\) binding candidates as possible. The phage library-antigen complex mixture was added to streptavidin beads and gently mixed for 15 min. Complex-bound beads were collected and washed several times with buffer B [PBS containing 0.1% (v/v) Tween-20]. IL-1R\(\alpha\) specific-scFv-phage molecules were eluted from the beads using 50 mM dithiothreitol. *Escherichia coli* TG1 cells (Carter et al., 1985) were infected with the eluted phage (Harrison et al., 1996), plated onto 2YTG/Carb [2YT broth containing 4% (w/v) glucose and 100 µg/ml carbencillin] and incubated at 30°C overnight. Selected scFv phage clones were amplified by superinfecting TG1 cells with M13 K07 helper phage and collected and concentrated by PEG precipitation (Harrison et al., 1996). In an effort to enrich for higher affinity clones, this selection process was repeated for rounds 2 and 3 with increased selection stringency (5 and 0.1 nM receptor, respectively) using phage isolated from the previous round. After three rounds of panning, individual clones were randomly selected from each library for further analysis.

**Phage binding and inhibition assays**

scFv-displaying phage binding specifically to IL-1R\(\alpha\) were identified by plate-binding assays. Briefly, 1 pmol of biotinylated IL-1R\(\alpha\) was immobilized to pre-blocked streptavidin-coated 96 well plates. Plates containing streptavidin alone were used as controls to allow identification and subsequent elimination of phage that bound non-specifically to streptavidin or plastic. To prepare phage supernatants (Harrison et al.,

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*Z.L.Fredericks et al.*
1996), TG1 cells in 2YTG/Carb were inoculated with individual phage clones, grown at 37°C to an OD_{600} of ~0.6, then superinfected with helper phage at a multiplicity of infection of 10. Cells were pelleted by centrifugation (1900 g, 10 min at 4°C), resuspended in 2YT media containing 50 µg/ml kanamycin and grown at 30°C for >5 h. The cells were then pelleted again by centrifugation and phage-containing supernatants were diluted 2- to 4-fold into buffer A and incubated with immobilized receptor for 1 h at 25°C. Unbound phage and ligand were removed by washing with buffer B. Eu-labeled anti-M13 mAb was then added and allowed to bind the remaining phage for 1 h. Plates were washed again and incubated with DELFIA enhancement solution for 10 min. The fluorescence signal was read at 615 nm using a VICTOR3 plate reader (PerkinElmer Life Sciences).

Phage clones were also examined at 25°C for inhibition of binding to receptor in the presence of IL-1α or IL-1β prebound to plate-immobilized receptor. In this assay, ~1 pmol of biotinylated IL-1R1 was immobilized on a streptavidin-coated plate and a 30-fold excess of IL-1α or IL-1β ligand was added and allowed to bind the receptor for 30 min. Phage supernatants were freshly prepared and diluted 2-fold into buffer A containing a 30-fold excess of ligand. This phage/ligand mixture was added to ligand-bound IL-1R1 and allowed to bind for 1 h. Unbound phage were washed away with buffer B and phage bound to IL-1R1 were detected by time-resolved fluorescence using Eu-labeled anti-M13 mAb as described above. Phage clones were considered to be receptor-specific if they yielded signals of ≥20-fold above the streptavidin controls. Phage were defined as potential ligand blockers if the specific signal was reduced by ≥25% in the presence of excess IL-1α or IL-1β.

**DNA sequencing of clones**

scFv-phage that bound to IL-1R1 but were blocked from binding receptor in the presence of IL-1α and IL-1β were PCR amplified and sequenced across the scFv-encoding region (VH, linker and VL) using fdtetseq and PUC19 reverse primers (Vaughan et al., 1996). Sequences were aligned using an in-house MiniPileup program to display the clonal diversity. Each V_H and V_L gene was assigned to a germline V gene segment using VBASE (www.mrc-cpe.cam.ac.uk/vbase-ok.php?menu=901).

**Dissociation rate ranking and affinity measurements**

SPR analysis was used to rank scFv clones based on their dissociation rates (k_{off} values) and to determine equilibrium binding constants (K_D values) of purified scFv-Fc and IgG proteins. All SPR experiments were performed at 25°C in binding buffer [10 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.005% (v/v) Nonidet P-20, pH 7.4] using a Biacore 3000 instrument (Biacore, Uppsala, Sweden). Data were analyzed using BiaEvaluation software version 3.02 (Biacore).

To rank the 39 unique scFv phage clones based on dissociation rates, k_{off} values were determined using ~250 resonance units (RU) of biotinylated human IL-1R1 captured on one flow cell of a streptavidin-coated chip. The signal from a reference flow cell (lacking IL-1R1) was subtracted from the IL-1R1 flow cell signal. scFv-phage supernatants were prepared by filtering and then diluted 2-fold into binding buffer. Phage supernatants (100 µl at 1.9x10^{12}-1.8x10^{14} c.f.u/ml) were coinjected as analytes at 50 µl/min followed immediately by 250 µl of binding buffer containing 4 µM unlabeled IL-1R1. Excess receptor was added as a competitor to minimize reassociation of phage once dissociated from the chip. Data from the first 100 s of the dissociation phase were fit using a 1:1 Langmuir binding model. The chip surface was regenerated between cycles with a 30 s injection of 10 mM glycine, pH 2.5.

Kinetic experiments were performed to determine the binding affinity (K_D) of human IL-1R1 (monomeric) for immobilized scFv-Fc or IgG4 molecules. These experiments were performed in this way to estimate monomeric binding affinities. For most scFv-Fc molecules, ~6000 RU of protein A (Amersham Biosciences) were coupled to two flow cells on a CM5 chip by amine coupling according to the vendor (Biacore). scFv-Fc 17, 18 and 26 were alternatively captured using ~6000 RU of goat anti-human Fc (Jackson ImmunoResearch, West Grove, PA) because these scFv-Fc molecules contain VH regions that can also bind to protein A.

The first flow cell contained a captured scFv-Fc that did not recognize IL-1R1 and was used as a reference surface for scFv-Fc captured on other flow cells. For each scFv-Fc a mean of 60 RU was captured on the flow cell surface. At least six different concentrations of monomeric IL-1R1 ectodomain (analyte) were then flowed over the chip surface at 50 µl/min using binding buffer containing 100 µg/ml BSA. Chips were regenerated between cycles with 10 mM glycine, pH 1.5. Data were fit using a Langmuir single-site model with double referencing. Analysis of IgG4 was performed in a similar manner except a goat anti-human Fc was immobilized on the flow cells and used to capture a mean of 100 RU of IgG4. The control flow cell used was coated with capture antibody only.

**Production of scFv-Fc and IgG4 proteins**

scFv-Fc expression constructs (H.Zhou and Z.-H.Hu, unpublished data) were made by ligating Ncol/NotI restriction fragments of each scFv clone into a similarly digested modified form of the mammalian expression vector pDC409 (Giri et al., 1994) called pDC409a-huG1Fc (ImmuneX/Amgen). This modified vector also contains cloned VH5 leader and IgG4 Fc regions. For IgG expression constructs, DNA encoding VH and VL domains of individual scFv fragments were generated by PCR amplification of the scFv-Fc pDC409a-huG1Fc plasmids using appropriate primers listed below in a 5’ to 3’ direction. Heavy chain forward (VHF) and reverse (VHR) direction PCR primers were customized for specific clones: VHF1, CTAGCTAGCAGTGCACTGCTG for C7, C11 and C13; VHF2, CTAGCTAGCAAGTGCACTGCTG for C6, C9, C10, C14 and C16; VH3, CTAGCTAGCCAGTGCACTGCTG for C15, C23, C26, C27; VH5, CTAGCTAGCCAGTGCACTGCTG for C4, VH1, CTAGCTAGCAGTGCACTGCTG for C18; VH2, CTAGCTAGCAGTGCACTGCTG for C19; VH3, CTAGCTAGCAGTGCACTGCTG for C16; and VH4, CTAGCTAGCAGTGCACTGCTG for all other clones that were reformatted. V_H PCR fragments were restriction digested with NheI and cloned into similarly cleaved mammalian expression vector, pDC414N-NC (ImmuneX/Amgen), a modified form of the pDC409 vector containing a minimal Epstein–Barr replication origin (Shirakata and Hirai, 1998), human IgG4 constant domains (Ellison and Hood, 1982; Brusco et al., 1998) and a modified VH5a leader sequence.

Light chain forward (VLF) and reverse (VLR) direction PCR primers were also customized for specific clones: VLF1, CTAGCTAGCAGAAATTTGTGTGTTG for C27; VLF2, CTAGCTAGCAGAAATTTGTGTGTTG for C6 and C14; VLF3, CTAGCTAGCAGAAATTTGTGTGTTG for C13 and C23; VLF4,
CTAGCTAGCCGTCTGCGCTT for C9, C10 and C11; VLF5, CTAGCTAGCCGTCTGCGCTT for C4 and C15; VLF6, CTAGCTAGCCGTCTGCGCTT for C7, C17, C18 and C26; VLF7, CTAGCTAGCCGTCTGCGCTT for C6; VLR2, CTAGCTAGCCGTCTGCGCTT for C3; VLR4, CTAGCTAGCCGTCTGCGCTT for C4, C7, C9, C10, C13–15 and C26; and VLR5, CTAGCTAGCCGTCTGCGCTT for C11 and C16–18.

Vi, PCR fragments were restriction digested with 5 ′ NheI and 3 ′ BsaI and cloned into similarly cleaved mammalian expression vector, pDC414N-LCKL (Immunex/Amgen), a modified version of the pDC409 vector containing the human κ light chain constant region and a modified A27 leader sequence.

scFv-Fc and IgG4 proteins were expressed in COS-1 or PKB E5 cells and purified using protein A affinity chromatography. Briefly, antibodies were passed over a POROS20 A column (PerSeptive Biosystems, Foster City, CA) in PBS buffer, pH 7.4, bound antibodies were eluted as 1 ml fractions using 0.1 M glycine, pH 2.7 containing 0.3 M NaCl and immediately neutralized using 1.0 M Tris, pH 8.0. Peak fractions were pooled and dialyzed into PBS, pH 7.4. Purified scFv-Fc and IgG4 proteins were flash frozen and stored long term at ~80 °C and short-term at 4 °C. Repeated freeze–thaw cycles were avoided.

**Ligand competition assay**

Competitive-binding assays were used to rank putative IL-1Rα antagonist candidates in scFv-Fc and IgG4 formats based on their relative biochemical potency as IL-1 blockers. Assays were performed at 25 °C in streptavidin-coated 96 well microtiter plates (Greiner Bio-One Inc, Longwood, FL) blocked with buffer B containing 3% (w/v) BSA. To begin, 5 microtiter plates (Greiner Bio-One Inc, Longwood, FL) containing 2 pmol of competitor (scFv-Fc or IgG4 in buffer A) containing 2 pmol of Eu-labeled IL-1α or IL-1β were added to wells and allowed to bind for 1 h. The amount of Eu IL-1 used was ~80% of the maximal IL-1β binding signal when bound to immobilized IL-1Rα. Unbound material was removed by washing. Enhancement solution was added to each well and fluorescence signals were read at 615 nm, as previously described. Data were processed using GraphPad Prism version 3.03 (GraphPad Software, San Diego, CA) and were fit by non-linear regression to a one-site competition binding equation. Plotted data points are the mean of quadruplicate measurements.

**Flow cytometry**

Fluorescence-activated cell sorting (FACS) was used to assess the ability of scFv-Fc and IgG4 proteins to recognize IL-1Rα on the cell surface. K299 cells were used to assess binding to human IL-1Rα. A stable CHO cell line expressing murine IL-1Rα (produced at Immunex/Amgen) was used to assess binding to murine IL-1Rα. Both CV-1 and HEK293 cells transiently expressing cynomolgus IL-1Rα were used to assess binding to cynomolgus IL-1Rα. Cells were incubated with 10 μg/ml scFv-Fc or IgG4 for 1 h at 4 °C. Bound scFv-Fc or IgG4 was detected using phycoerythrin-conjugated goat anti-human IgG F(ab′)2 (Jackson ImmunoResearch). Cells were analyzed using a FACScalibur flow cytometer (Becton Dickinson, Mountain View, CA). Those scFv-Fc and IgG proteins that caused a significant shift in cell fluorescence were considered receptor binders.

**NF-κB nuclear translocation assay**

When IL-1 binds to the IL-1Rα receptor complex a signaling cascade is initiated that involves the activation of cytoplasmic NF-κB and translocation to the nucleus. To access biologic activity of our putative antagonist clones, NF-κB nuclear translocation assays (Ding et al., 1998) were performed in 384 well microtiter plates using HeLa cells endogenously expressing human IL-1Rα and a NF-κB Activation HitKit (Cellomics Inc., Pittsburgh, PA). Cells were seeded at a density of 2000 cells/well and incubated overnight at 37 °C. The cells were then stimulated for 40 min at 37 °C with 12 pM human IL-1α or IL-1β in the presence of various concentrations of scFv-Fc or IgG4 clones. Ligand concentrations were experimentally determined based on dose titration experiments using HeLa cells. Concentrations selected were ~75% of maximal IL-1α or IL-1β stimulation. After ligand and clone exposure, cells were immediately washed and fixed with 3.5% (v/v) formaldehyde in PBS for 10 min at 20 °C followed by permeabilization. NF-κB and the cell nuclei were then stained according to the Cellomics protocol. Finally, cells were imaged and fluorescence measured with the ArrayScan II cytometer (Cellomics) optimizing the vendor protocol for the cells used. After subtraction of the mean cytoplasmic from mean nuclear fluorescence, data were fit using a non-linear regression variable slope dose–response using GraphPad software. Data points are the mean of quadruplicate measurements.

**Results**

**Isolation of IL-1Rα antagonists from scFv libraries**

After three rounds of affinity selection using human IL-1Rα ectodomain, individual scFv-phage candidates were randomly chosen for screening from rounds 2 and 3 of selection. Approximately 33% of the 1152 scFv-phage screened bound specifically to IL-1Rα as judged by a time-resolved fluorescence receptor-binding assay. IL-1Rα-binding candidates were next screened for their ability to bind to the same receptor-binding sites as IL-1 using phage inhibition assays. These assays were used to identify phage candidates as potential antagonists worthy of further analysis rather than to rank them by their potency as receptor antagonists (see Discussion). Initially, all candidates were tested for the ability to bind IL-1Rα prebound with IL-1α. Phage candidates that were significantly inhibited (>25% inhibition) from binding to receptor in the presence of IL-1α were then tested for inhibition of receptor binding in the presence of IL-1β. Phage candidates that were inhibited from binding in the presence of both IL-1α and IL-1β were selected for further analysis. Complete inhibition of both IL-1α and IL-1β binding is desired because only very few IL-1 molecules are required to induce a strong IL-1Rα response (Arend, 2002). Eighty-one (21%) of the 382 IL-1Rα-binding phage were signifi-
cantly and reproducibly inhibited from binding in the presence of both IL-1α and IL-1β. Diversity analysis of these 81 putative antagonist clones by nucleotide sequencing revealed 39 unique sequences (data not shown).

We hypothesize that high affinity receptor binding (low $K_d$) is a desirable property of ligand-blocking clones that will likely
contribute to their potency as antagonists. Purified phage representing each of the 39 putative antagonist clones were analyzed by SPR using a Biacore 3000. Estimation of dissociation rate constants was used as a surrogate for \( K_D \) (\( k_{off}/k_{on} \)) for the following reasons. First, \( k_{off} \) values for antibody–antigen interactions typically vary over a much wider range than do association rate constants (\( k_{on} \) values) (Lowman and Wells, 1993; Yang et al., 1995); consequently a low \( k_{off} \) value is a common hallmark of high affinity clones (low \( K_D \) values). Secondly, \( k_{off} \) measurements are concentration independent and can be measured readily with scFv-phage preparations. Thirdly, \( k_{on} \) determinations require estimates of functional scFv concentration that cannot be readily determined in phage format. Of the 39 putative antagonist clones analyzed by Biacore, 30 gave a robust signal (\( \Delta R_U \geq 60–1300 \)) upon binding to immobilized human IL-1RI ectodomain. These clones dissociate with rate constants (\( k_{off} \)) of \( 1.2 \times 10^{-3} \) to \( 3.6 \times 10^{-2} \) s\(^{-1}\). The 15 clones with the slowest dissociation rates (lowest \( k_{off} \) values) that were also inhibited from binding IL-1RI by \( \geq 50\% \) in the presence of IL-1 were selected for further analysis (Table I).

Table I lists the related V domain families and gene segments and corresponding sequences of the 15 selected clones. The heavy chains are highly diverse (Figure 1B) with eight different VH genes represented (Table I) out of a possible 54 (Tomlinson et al., 1995). The VH3 family predominates (13/15) amongst these VH sequences. Strikingly, the light chains are virtually all of the \( \kappa \) (14/15) rather than \( \lambda \) (1/15) isotype. Sequence analysis of 100 individual clones from each unselected library revealed a significant bias in favor of \( \kappa \) over \( \lambda \) in the starting libraries, which may account for the preponderance of \( \kappa \) clones in our selected clones. Comparison of the sequences of the VH and VL regions of these clones revealed that many of the clones are closely related (Figure 1).

### Table I. Properties of the lead anti-IL-1R\(_I\) scFv-phage clones

<table>
<thead>
<tr>
<th>Clone</th>
<th>Library/round(^d)</th>
<th>V domain family/V gene segment(^b)</th>
<th>( k_{off} ) (( 10^{-3} ) s(^{-1}))</th>
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<td>C23</td>
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<td>S2</td>
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</tr>
<tr>
<td>C18</td>
<td>B2</td>
<td>VH3, DP46</td>
<td>9.3</td>
</tr>
<tr>
<td>C26</td>
<td>D2</td>
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<td>9.7</td>
</tr>
<tr>
<td>C9</td>
<td>S2</td>
<td>VH3, DP38</td>
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</tr>
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<td>C17</td>
<td>B2</td>
<td>VH3, DP46</td>
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</tr>
<tr>
<td>C15</td>
<td>D3</td>
<td>VH3, DP47</td>
<td>11.0</td>
</tr>
<tr>
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<td>B2</td>
<td>VH3, DP31</td>
<td>12.0</td>
</tr>
<tr>
<td>C14</td>
<td>S2</td>
<td>VH3, DP54</td>
<td>13.0</td>
</tr>
</tbody>
</table>

The 15 scFv-phage clones with the slowest dissociation rates (lowest \( k_{off} \) values) that were also inhibited from binding IL-1RI by \( \geq 50\% \) in the presence of IL-1 are listed.

\(^a\)scFv-displaying phage libraries are denoted by S, D or B for spleen, DP47 or bone marrow libraries, respectively, whereas the numbers 2 or 3 indicate that clones were found in rounds 2 or 3 of panning, respectively.

\(^b\)V domain family and V gene segment assignments from VBASE (www.mrc-cpe.cam.ac.uk/vbase-ok.php?menu=901).

\(^c\)Off-rate ranking of purified scFv-phage clones was performed using Biacore. Clones are listed in order of increasing \( k_{off} \) values.

**Fig. 1.** Amino acid sequence comparison of 15 anti-IL-1R\(_I\) scFv-phage clones. Comparisons were made between the (A) 15 unique scFv sequences (putative IL-1R\(_I\) antagonists) with the slowest \( k_{off} \) rates and their component (B) VH and (C) VL domains. Amino acid sequence comparisons are represented as phylogram trees produced using an in-house MiniPileup program. Clone numbers are listed along the right side of the plots.
sequences (C9 and C10) differ from each other by at least 13 amino acid replacements.

**Conversion of scFv-phage clones to scFv-Fc and IgG4 formats**

More detailed characterization of the 15 selected scFv clones necessitated the expression and purification of antibody protein. We elected to reclone the 15 scFv clones listed in Table I into both scFv-Fc and IgG4 formats, each being dimeric and bivalent antibody structures. Recoveries of IgG4 proteins ranged from 0.4 to 7.0 mg/l as estimated by the absorbance at 280 nm and theoretical extinction coefficients calculated from the amino acid sequence of individual clones (data not shown).

Recovery of those same clones in scFv-Fc format was 2–14-fold greater, ranging from 0.8 to 32.6 mg/l (data not shown).

Analysis of purified proteins on reduced and non-reduced PAGE revealed that scFv-Fc and IgG4 molecules were ≥90% pure (Figure 2A and B). Under reducing and non-reducing conditions, with the exception of C14, scFv-Fc molecules gave rise to a single major band of apparent molecular weight of 55 and 120 kDa, respectively (Figure 2A), as anticipated for these reducible disulfide-linked dimers. In contrast, IgG4 preparations, with the exception of C14, migrated with two major bands of apparent molecular weight ~150 and ~75 kDa under non-reducing conditions (Figure 2B). This finding is consistent with the observation that intra-heavy chain disulfide bond

![Fig. 2. SDS-PAGE analysis of purified maxibodies and IgG proteins. Reduced (+DTT) and non-reduced (-DTT) acrylamide gels were used to resolve the purity of the most potent IL-1Rα antagonist antibodies C10, C13, C14 and C15. Both (A) scFv-Fc and (B) IgG formats are shown for each of these clones. Molecular weight markers are indicated along the left side of the gels. The C14 glycosylation site mutant, C14 N25S, was only produced as an IgG4. (C) The scFv sequence of clone C14 is shown with heavy chains (red), light chains (blue), CDRs (underlined), the linker region (bold) and a potential N-linked glycosylation site (boxed).]
formation competes with inter-heavy chain disulfide bond formation for IgG₄ molecules. Consequently, IgG₄ preparations typically contain a variable mixture of covalent and non-covalent tetramers (Angal et al., 1993; Bloom et al., 1997; Schuurman et al., 2001). For clone C10, size-exclusion chromatography was used to verify that the preparation behaved as a ~150 kDa molecule in solution, consistent with the presence of covalent and non-covalent tetramers. Under reducing conditions, IgG₄ molecules give rise to bands of ~50 kDa ~28 kDa apparent molecular weight, as expected for light and heavy chains (Figure 2B).

C14 IgG₄ is notable in that two light chain bands were observed following SDS–PAGE under reducing conditions, as judged by N-terminal sequence analysis (Figure 2B). The sequence of clone C14 is unusual in that it contains a potential N-linked glycosylation site (N25K26S27) beginning at position 25 of V₅. Within the first complementarity determining region (CDR) (Figure 2C). To investigate whether this site is glycosylated in the C14 IgG₄ molecule, an N25S variant of clone C14 (C14 N25S) was constructed in which the putative glycosylation site was removed. Under reducing conditions, C14 N25S gives rise to a single light chain band (Figure 2B). These data from C14 and C14 N25S in toto support the notion that ~50% of C14 light chains are glycosylated at the NKS site within the V₅ domain.

**Functional characterization of scFv-Fc and IgG₄ proteins**

The 15 scFv-clones converted to scFv-Fc and IgG₄ formats were assayed to identify the most potent IL-1R₁ antagonists and to assess the degree of functional correspondence between these antibody formats. First, these clones were assessed for binding to cell-surface receptors by flow cytometry using K299 cells expressing human IL-1R₁. In the scFv-Fc format, all clones except for C4, C9, C11, C16, C17 and C26 displayed significant binding to cell-surface human IL-1R₁. Likewise, in the IgG₄ format, the same clones bound to cell-surface IL-1R₁. Based on these flow cytometry data, six clones (C4, C9, C11, C16, C17 and C26) were eliminated from further analysis.

The remaining nine scFv-Fc and IgG₄ molecules (C6, C7, C10, C13, C14, C15, C18, C23 and C27) were next compared in competition-binding assays for their ability to inhibit Eu-labeled IL-1α and IL-1β binding to IL-1R₁ ectodomain. Eu-labeling of IL-1β slightly impairs its binding to human IL-1R₁ as judged by a 3-fold increase in Kᵦ to 3 nM estimated by Biacore (data not shown). With this in mind, the IC₅₀ values reported here are interpreted as relative rather than absolute estimates of binding inhibition. In the scFv-Fc format, clones C10, C13, C14, C15, C18 and C27 were the most potent inhibitors of IL-1α and IL-1β binding. Similar results were seen with these six clones in IgG₄ format. These six clones represent the lead clones and their relative IC₅₀ values are listed in Table II. Corresponding IC₅₀ values for clones as scFv-Fc and IgG₄ were within 3-fold of each other. Moreover, the most potent clones in the scFv-Fc format were also the most potent in IgG₄ format and yielded IC₅₀ values similar to those obtained using IL-1ra as a competitor (Table II). The remaining three clones (C6, C7 and C23) showed significantly lower or no inhibition in this assay. Figure 3 shows binding curves from two of the most potent clones (C10 and C14) in both scFv-Fc and IgG₄ formats alongside one of the less potent clones (C6) and the weakly neutralizing M8 mAb.

**Table II. Potency of lead anti-IL-1R₁ antibodies**

<table>
<thead>
<tr>
<th>Format/clone</th>
<th>Protein yielda (mg/l)</th>
<th>Species cross-reactivityb</th>
<th>Ligand competitionc,d</th>
<th>NF-κB nuclear translocatione,f</th>
<th>Binding affinityg</th>
<th>Potency (Kᵦ) (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>scFv-Fc</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C10</td>
<td>10.2</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>1.6</td>
<td>380</td>
</tr>
<tr>
<td>C13</td>
<td>7.8</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>1.4</td>
<td>600</td>
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<tr>
<td>C14</td>
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<tr>
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</tr>
<tr>
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<td>+</td>
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</tr>
<tr>
<td>IgG₄</td>
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<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
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<td>–</td>
<td>–</td>
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<tr>
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<td>+</td>
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<td>–</td>
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<td>+</td>
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</tr>
<tr>
<td>C27</td>
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<td>+</td>
<td>–</td>
<td>+</td>
<td>14.0</td>
<td>113</td>
</tr>
<tr>
<td>IL-1ra</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>3.1</td>
<td>0.4</td>
</tr>
</tbody>
</table>

aProtein yields estimated from A₂₈₀ and corresponding theoretical extinction coefficient estimated using the predicted amino acid sequence of clones. n.d., not determined.
bStrong (+), weak or insignificant (±) binding of antibodies to IL-1R₁ from different species was observed by flow cytometry using K299 cells (human IL-1R₁), transfected CHO (murine IL-1R₁) or transfected COS-1 (cynomolgus IL-1R₁) cells.
cRelative IC₅₀ values estimated from a non-linear least squares four-parameter fit.
dExperiments were performed using biotinylated human IL-1R₁ ectodomain immobilized on streptavidin-coated microtiter plates.
eExperiments were undertaken using human ligands and HeLa cells expressing human IL-1R₁, NI, not inhibited.
fKᵦ and IC₅₀ as determined by SPR. Experiments were conducted with scFv-Fc or IgG₄ captured on a goat anti-human Fc antibody-coupled CM5 chip to measure monovalent interactions between the IL-1 receptor (analyte) and each antagonist candidate (ligand).
gDoes not fit to a 1:1 ligand binding model.
hBinding affinity of soluble IL-1ra to receptor was determined by SPR using IL-1R₁-Fc captured on a goat anti-human Fc antibody-coupled CM5 chip (D.Friend).
The biologic potency of the six lead clones as receptor antagonists was assessed by measuring their impact on IL-1α and IL-1β induced NF-κB nuclear translocation within HeLa cells. In both scFv-Fc and IgG4 formats, four clones (C10, C13, C14, C15) were identified that significantly inhibited both IL-α and IL-β induction of NF-κB translocation. In the scFv-Fc format these clones yielded IC₅₀ values from 310 to 900 nM (Table II). These same clones in the IgG4 format were 3- to 24-fold more potent (reduction in IC₅₀ values) than scFv-Fc molecules at blocking ligand-dependent signaling (Table II).

Fig. 3. Inhibition of IL-1 binding to IL-1R₁ by anti-IL-1R₁ antibodies. Representative plots of competition-binding assays using Eu-labeled IL-1α and various competing molecules. All lead scFv clones were examined in this assay for the ability to compete with IL-1α and IL-1β for binding to immobilized IL-1R₁. IL-1α competition curves for the anti-IL-1R₁ antibody clones C6 (squares), C10 (upright triangles) and C14 (diamonds) and M8 mAb (inverted triangles) and IL-1ra (circles) competitors are shown in both the (A) scFv-Fc and (B) IgG₄ formats. Relative IC₅₀ values for each of the lead clones were calculated from these curves.
There is good agreement between IL-1α and IL-1β IC₅₀ values and none of the clones examined inhibited binding of one ligand but not the other (Table II). In this bioassay, C10 IgG4 is the most potent antagonist of IL-1α (26 nM IC₅₀) and IL-1β (18 nM IC₅₀; Table II). Figure 4 shows the binding curves for two of the lead clones that inhibit NF-κB translocation compared with the M1 neutralizing Ab and IL-1ra. Although four of the lead clones are potent antagonists of IL-1R activity in this assay, IL-1ra is significantly more potent (Figure 4 and Table II).

To determine whether glycosylation of the light chain of C14 impacts its ability to function as an antagonist, C14 IgG4 and its N25S variant were directly compared in both the ligand competition and NF-κB nuclear translocation assays. Results of the ligand competition assay indicate that C14 and C14 N25S behave similarly when competing for IL-1R₁ binding with IL-1α and IL-1β. In fact, C14 and C14 N25S yielded nearly identical IC₅₀ values in this assay (Table II). In the NF-κB assay, C14 and C14 N25S were equipotent at inhibiting IL-1 induced translocation of NF-κB (Table II). These findings indicate that glycosylation of the light chain does not impact the function of C14 IgG₄.

SPR experiments were performed on the lead antagonistic clones to determine their binding affinity for soluble IL-1R₁ and to examine whether the potency of the IgG₄ molecules in the NF-κB assay relates to binding affinity. Examination of the results of these affinity measurements revealed broadly similar K_D values for these clones in both IgG₄ and scFv-Fc formats (Table II). C10 was found to be the highest affinity clone in both formats having a K_D of ~60 nM in each case. All other clones showed significantly lower binding affinities to human IL-1R₁ compared with C10 (Table II).

These data appear consistent with the NF-κB assay in which C10 was also the most potent clone. Based on the results of these measurements, binding affinity does not offer a plausible explanation for the superior potency of IgG₄ molecules over scFv-Fc for these clones in the NF-κB assay (see Discussion; Table II).

Binding to cell-surface IL-1R₁ from rodents and primates are highly desirable properties of IL-1R₁ antagonist antibodies intended for preclinical development. These properties may allow efficacy and/or toxicology studies to be potentially undertaken with the same antibody clone selected for clinical development. For this reason, we used FACS analysis to screen our six lead clones for cross-reactivity with IL-1R₁ from cynomolgus monkey and mouse. As IgG molecules, four of six lead clones (C13, C15, C18 and C27) bound significantly to cynomolgus receptor as similarly for the lead clones in both antibody platforms with the exceptions of C10 and C27. C10 bound to receptor as an scFv-Fc, but did not significantly bind cynomolgus IL-1R₁ in IgG format (Table II). Likewise, C27 bound to cynomolgus receptor as an IgG, but not as an scFv-Fc. This high frequency of antibodies that cross-react with human and cynomolgus IL-1R₁ likely reflects the close identity between these receptor ectodomains (~94% sequence identity). In addition to the four clones that were observed to bind cynomolgus IL-1R₁ in IgG format (C14 and C18) bound murine IL-1R₁ expressed on the surface of CHO cells (Table II). This lower frequency of antibody cross-reactivity between human and murine IL-1R₁ ectodomains appears consistent with these receptor sequences being more distantly related (~64% identity). Interestingly,
C14 bound to cell surface expressed murine and human, but not cynomolgus IL-1Rα.

Discussion

Phage display technology enables the rapid identification of antibodies that bind antigens with high affinity and specificity. This technology has emerged as an important method for deriving or enhancing antibodies for treating human diseases. Evidence is provided by the recent FDA approval of the first fully human therapeutic antibody, an anti-TNF-α antibody called adalimumab (Rau, 2002; Weinblatt et al., 2003), with at least six other human antibodies derived from phage display in clinical trials (www.cambridgeantibody.com). Using phage display we generated a set of anti-IL-1Rα antibody candidates and used biochemical plate assays, cell-based assays and affinity measurements to select the most potent IL-1Rα antagonist antibodies. Our study demonstrates the utility of using phage display to generate potent antibodies in vitro with the desired biologic properties and significant therapeutic potential.

Initial identification of 39 unique scFv-phage clones whose binding to IL-1Rα was impaired by prebound IL-1 was accomplished using a modified binding inhibition assay format. This assay approach was used in place of a traditional binding competition assay because scFv concentrations on phage molecules are too low and variable to compete effectively for IL-1 binding to receptor. A priori, scFv-phage clones that are weakly inhibited from binding receptor in the presence of ligand might include highly potent clones that compete effectively with ligand as well as clones whose receptor binding is minimally impaired by ligand. Two steps were taken to reduce the risk of discarding the most potent clones in this initial screening assay. First, plates were preblocked with ligand to reduce the risk of scFv-phage out-competing ligand. Secondly, we adopted a low threshold of inhibition (>25% inhibition) to consider clones for further analysis.

In addition to selecting lead IL-1Rα antagonist antibodies, one of the objectives of our study was to determine if scFv-Fc molecules could be predictive of IgG properties. Each of the 15 scFv-phage clones listed in Table I was converted to scFv-Fc and IgG formats to allow direct comparison of these two formats. scFv-Fc molecules shown to be equipotent to their corresponding IgG molecules could be used as a rapid screening tool to eliminate low potent clones from further characterization, thus reducing the number of putative antagonist clones needed to be converted to IgG format. scFv-Fc molecules are well suited as initial screening tools for two important reasons. First, scFv-Fc molecules require much less time to produce than IgG molecules for reasons already mentioned. Secondly, an scFv-Fc gives rise to higher protein yields than its corresponding IgG (up to 14-fold greater; Table II). Furthermore, current methods for high-throughput IgG production do not consistently yield sufficient protein for functional characterization studies.

Direct comparison of the 15 scFv clones expressed as both scFv-Fc and IgG molecules revealed striking similarities between these two antibody formats. First, flow cytometry studies identified the same nine clones in both formats as binders to cell-surface human IL-1Rα (data not shown). Secondly, ligand competition-binding assays performed on these nine clones yielded similar IC50 values for both formats with C10, C13, C14, C15, C18 and C27 being most potent (Table II). Thirdly, the same clones (C10, C13, C14 and C15) in both scFv-Fc and IgG4 formats were the most potent inhibitors of NF-κB nuclear translocation (Table II). Moreover, our most potent clone, C10, demonstrated the greatest relative inhibition in both formats (Table II). Fourthly, all of our most potent antibody clones have similar monovalent binding affinities in both formats (Table II). Finally, four of the six lead clones bound to either murine or cynomolgus IL-1Rα in FACS studies both as an scFv-Fc and an IgG4 (Table II).

The most striking difference between the two antibody platforms is apparent upon quantitative examination of the NF-κB translocation assay results. Although C10, C13, C14 and C15 were observed to inhibit NF-κB translocation in both formats, these clones are significantly more potent inhibitors in IgG format (Table II). This finding is unexpected considering that these same clones have similar binding affinities (to monomeric receptor) in both scFv-Fc and IgG formats (Table II). One possible explanation for this potency discrepancy is that the geometry of an scFv-Fc may not allow efficient bivalent binding to cell-surface IL-1Rα. As an scFv-Fc, the two antigen-binding sites are closer together in three-dimensional space compared with clones in the IgG format. Therefore, in the cell environment, an scFv-Fc may only be able to form monovalent interactions, whereas the corresponding IgG readily forms bivalent interactions. This hypothesis, however, seems likely to vary with antigen and epitope rather than be a characteristic property of the scFv-Fc platform. If the geometry of an scFv-Fc impacts how it can interact with an antigen, then this hypothesis may explain why clones in the IgG format are more potent inhibitors of NF-κB translocation. Another possible explanation for this potency discrepancy may be that clones are more stable in the IgG than the scFv-Fc format. It is possible, however unlikely, that scFv-Fc molecules are more susceptible to proteolysis than IgG molecules, thus effectively decreasing the functional population of scFv-Fc molecules able to inhibit downstream NF-κB translocation. It is also possible that the differences in observed potency are a result of a larger percentage of misfolded and non-functional scFv-Fc molecules compared with the IgG populations. Despite differences in functional potency in the cell-based assay, the correspondence between anti-IL-1Rα scFv-Fc and IgG properties is sufficiently robust to warrant further exploration of the scFv-Fc format as a screening tool given the greater ease with which scFv-Fc molecules can be produced. Further engineering of the scFv-Fc format may further improve the similarities between scFv-Fc and IgG properties. For example, addition of linker regions or additional domains between the Fc and scFv portions of an scFv-Fc that more closely match the conformation of an IgG may permit bivalent interactions to IL-1Rα and increase antagonist potency.

Despite the advantages of using the scFv-Fc format to improve protein production yield and efficiency, the IgG format has a major advantage for drug development in that it is a well established therapeutic platform. Also, structural and stability differences between scFv-Fc and IgGs are unknown and require further investigation. In this study, the IgG4 isotype was chosen over other isotypes because IgG4 are typically deficient or at least inefficient in their ability to support antibody-dependent cellular cytotoxicity or complement-dependent cytotoxicity activities (Presta, 2002). By using the IgG4 isotype we avoided such secondary immune effects to
reduce the risk of killing target cells, which may potentially give rise to adverse events in patients.

Clones C10, C13, C14 and C15 in the IgG₄ format are the most potent antagonist antibody candidates with C10 exhibiting the highest binding affinity and antagonist potency. These four lead clones demonstrated IL-1-blocking properties equivalent to IL-1ra when examined in the competition assay (Table II). However, IL-1ra was significantly more potent at blocking IL-1-dependent NF-kB translocation than all four of these clones examined in the NF-kB assay (Table II). Figures 3 and 4 show examples of lead clone and IL-1ra binding curves derived from competition and NF-kB assays, respectively. Avidity effects in the ligand competition assay may explain the discrepancy between these two assays. The high density of receptors bound to microtiter plates may limit the ability to significantly distinguish between the most potent clones and IL-1ra. For this reason, the IC₅₀ values obtained from our competition assay allowed us to rank the clones relative to each other for the purpose of eliminating the least effective IL-1 blockers. The NF-kB bioassay allowed the most potent IL-1 blockers to then be further ranked as potent receptor antagonists and C10 to be identified as the most potent anti-IL-1R₁ antagonist antibody.

During this study we have focused on the in vitro identification of anti-IL-1R₁ antibodies with potent antagonistic activities, strong binding affinities and species cross-reactivity as these characteristics are likely to be of paramount importance in developing a therapeutic antibody. Results from the NF-kB assay identified IgG₄ C10 as the most potent IL-1R₁ antagonist despite relatively modest monovalent receptor binding affinity (Kᵦ ~60 nM) compared with IL-1ra (Kᵦ ~0.04 nM). Comparison of IL-1ra and IgG₄ C10 receptor affinity and antagonist potency suggest that relatively high affinity is not an absolute prerequisite for the functional potency of these clones (Table II). It is important to consider that the optimal affinity for a therapeutic antibody is not known and significantly high affinity binders are not always needed to achieve the best antibody potency (Adams et al., 2001). Additionally, the superior in vitro potency of IL-1ra in our assays does not indicate that IL-1ra will outperform a high affinity antagonist IL-1R₁ antibody in vivo. As discussed earlier, the greatest potential advantage of using an anti-IL-1R₁ antibody over IL-1ra (anakinra) as a therapeutic is the substantial increase in terminal half-life. Moreover, it is desirable and common practice to increase the binding affinity of therapeutic antibody candidates prior to clinical development using affinity maturation. This process involves introducing mutations into antibody complementarity determining regions and screening resultant antibodies for improved binding affinity and/or biological potency. Affinity maturation has been successfully used to significantly improve the binding affinity of numerous antibodies (Yang et al., 1995; Schier et al., 1996; Pini et al., 1998). Affinity maturation of our top antagonistic clones seems to be an obvious next step to possibly identify anti-IL-1R₁ antibodies with significantly improved binding affinities and antagonist potency.

Identification of lead antibody candidates that cross-react with primate and rodent systems may permit in vivo efficacy experiments and/or toxicological studies to be performed with the same antibody intended for clinical development. Use of cross-reacting antibodies would thereby obviate the need for surrogate antibodies and be a more accurate indication of the safety and effectiveness of a lead therapeutic antibody. The strong cross-reactivity observed between IgG molecules C13, C15, C18, C27 and cynomolgus IL-1R₁ and C14, C18 and murine IL-1R₁ (Table II) indicates that phase display techniques can be used to generate cross-species reactive antibodies potentially suitable for preclinical animal model studies.

Phage display techniques and rapid screening methods allowed us to quickly select for specific and potent antagonist antibodies to human IL-1R₁. While use of off-rate ranking of scFv-phage requires further investigation to validate its usefulness in choosing high affinity scFv antibodies, our most potent antibody candidate, C10, supports the use of this rapid screening method. C10 displayed the highest receptor affinity in both IgG and scFv-Fc formats and yielded one of the slowest determined kₑff rates (Table I) as an scFv-phage molecule. Screening of numerous scFv clones for receptor antagonism was accomplished by rapidly converting clones to the scFv-Fc format. Although we found that the scFv-Fc format consistently mimicked the properties of the corresponding IgG molecules, caution must be taken when using this alternative antibody platform as the success of this screening method may be epitope and antigen dependent (J.Smathers, unpublished results). Further refinement of this alternative format is under way. Nonetheless, until it becomes routine practice to produce sufficient quantities of purified IgG molecules in a high-throughput fashion, it will be necessary to utilize rapid screening methods such as those presented here.

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