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RESEARCH PAPER

Protein engineering and preclinical development of a GM-CSF receptor antibody for the treatment of rheumatoid arthritis

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BACKGROUND AND PURPOSE

For antibody therapies against receptor targets, *in vivo* outcomes can be difficult to predict because of target-mediated clearance or antigen 'sink' effects. The purpose of this work was to engineer an antibody to the GM-CSF receptor α (GM-CSFRα) with pharmacological properties optimized for chronic, s.c. treatment of rheumatoid arthritis (RA) patients.

EXPERIMENTAL APPROACH

We used an *in silico* model of receptor occupancy to guide the target affinity and a combinatorial phage display approach for affinity maturation. Mechanism of action and internalization assays were performed on the optimized antibody *in vitro* before refining the modelling predictions of the eventual dosing in man. Finally, *in vivo* pharmacology studies in cynomolgus monkeys were carried out to inform the predictions and support future clinical development.

KEY RESULTS

Antibody potency was improved 8600-fold, and the target affinity was reached. The refined model predicted pharmacodynamic effects at doses as low as 1 mg kg^{-1} and a study in cynomolgus monkeys confirmed *in vivo* efficacy at 1 mg kg^{-1} dosing.

CONCLUSIONS AND IMPLICATIONS

This rational approach to antibody drug discovery enabled the isolation of a potent molecule compatible with chronic, s.c. self-administration by RA patients. We believe this general approach enables the development of optimal biopharmaceuticals.

Abbreviations

CDR, complementarity determining region; ECD, extracellular domain; IC_{50}, half maximal inhibitory concentration; K_d, equilibrium dissociation constant; K_t, internalization rate constant; PD, pharmacodynamic; PK, pharmacokinetic; RA, rheumatoid arthritis; scFv, single chain variable fragment; $t^1/_2$, half-life; $V_{H,}$ variable heavy; $V_{L,}$ variable light

Introduction

Rheumatoid arthritis (RA), which affects 0.5–1% of the population in the industrialized world (Symmons, 2002), is a systemic autoimmune disease characterized by chronic and progressive inflammation in multiple joints, leading to irreversible tissue damage and deformity that culminates in disability, a lower quality of life and in many cases premature

death (Mikuls *et al.*, 2011). It is initially characterized by inflammatory synovitis involving a broad range of cells such as macrophages, neutrophils, B cells, T cells and synovial fibroblasts. Pioneering work by Feldman and Maini (Feldmann *et al.*, 1996) highlighted the central role of TNF-α in this disease, ultimately leading to the approval of four anti-TNF-α therapies (Taylor and Feldmann, 2009) for the successful treatment of RA.

Although targeting TNF-α in RA has provided significant clinical benefits approximately 30–40% of severe RA patients remain refractory to treatment (Singh *et al.*, 2009) and therefore new therapies that can address this area of unmet medical need are still required. Recently, a number of groups have focused their efforts on the GM-CSF pathway. GM-CSF has been shown to be elevated in synovial fluid (Bell *et al.*, 1995; Ottonello *et al.*, 2002) and membrane biopsies (Farahat *et al.*, 1993) from patients with RA. Furthermore, treatment of patients with recombinant GM-CSF for chemotherapy-induced neutropenia or Felty's syndrome was shown to result in a flare in their arthritis, suggesting that GM-CSF may modulate the immune response towards disease. These observations are supported by mechanistic studies in *in vivo* models of arthritis (Cook *et al.*, 2001; Plater-Zyberk *et al.*, 2007).

Although the immunological rationale for targeting either GM-CSF or its receptor for the treatment of rheumatoid arthritis is compelling, recent changes in treatment paradigms in this disease mean that successful new therapies need not only be efficacious with a good or improved safety profile but must also be formulated in such a way as to allow self administration by patients avoiding the need for inconvenient and costly i.v. infusions. Moreover, new therapies also have to be dosed infrequently (e.g. monthly for patient convenience). Current autoinjector devices for patient self-administration have an upper volume limit of around 1 mL, which, combined with the challenge of formulating any protein at very high concentrations, emphasizes the importance of engineering high potency in proteins used therapeutically.

A key requirement during antibody development is therefore to engineer a molecule with sufficiently high affinity to guarantee effective target occupancy over prolonged periods. Antibodies against cell membrane-associated antigens (receptors) are usually subject to target mediated clearance, or the antigen-sink effect (Roskos *et al.*, 2004). There are two major implications of the potential antigen-sink liability for a receptor antigen. First, the simple Langmuir equation (Langmuir, 1916) can no longer be used to estimate target antigen occupancy (neutralization) *in vivo*. Second, the expression and turnover rate of the target receptor may have a profound impact on the pharmacokinetics (PK) of antibodies against the target. As a result, the assessment of affinity goals for antibodies against membrane receptors is often challenging.

Here, we describe an integrated approach to engineer an optimal antibody against the GM-CSF receptor α (GM-CSFRα) suitable to meet the needs of the RA market at launch. Using mathematical modelling of receptor occupancy, we define a theoretical affinity target that would achieve high levels of GM-CSFR occupancy over prolonged periods of time. In order to meet this affinity goal, we employ a highly efficient approach of randomly recombining pools of affinity matured variable heavy (V_H) and variable light (V_L) domains to identify optimal V_H/V_L domain pairings that work in synergy to achieve large gains in affinity and potency. Using a range of *in vitro* biological assays, the antibody was then characterized, and the data were used to refine the model. Finally, the antibody was evaluated *in vivo* in cynomolgus monkeys to determine its PK and pharmacodynamic (PD) profile, both reinforcing our approach and demonstrating the suitability of the molecule for clinical evaluation.

Methods

In silico *translational simulations*

An *in silico* mechanistic biomathematical model was constructed to describe the PK of a typical human IgG, binding of the antibody to GM-CSFRα and the internalization of GM-CSFRα and the antibody–receptor complex. The model assumed 50% absolute s.c. bioavailability, 2.5 mL kg^{-1} day^{-1} IgG clearance by the reticuloendothelial system, a distribution volume of 64 mL kg^{-1}, and 20 pM GM-CSFRα with a 1 h internalization half-life for the receptor and antibody–receptor complex (Roskos *et al.*, 2004). The following differential equations describe the PK of the antibody 574D04, its binding to GM-CSFR and the internalization of the complex following s.c. administration (CL_{RES}: intrinsic clearance of endogenous IgG by reticuloendothelial system; S_0: synthesis/recycling rate of target receptor, GM-CSFRα; k_E: receptor internalization rate; k_{on}, k_{off}: association and dissociation rate constants). To avoid model overparameterization, it is assumed that the internalization rates of unoccupied receptor and 574D04-bound receptor are the same. Although no data have been reported to directly support this assumption for GM-CSF receptors, published data for EGF receptors are consistent with this assumption (Wiley *et al.*, 1991).

$$\frac{dAb_{SC}}{dt} = -(k_a \cdot Ab_{SC})$$

$$\frac{dAb}{dt} = (k_a \cdot Ab_{SC}) - \left(\frac{CL_{RES}}{V} \cdot Ab\right) - (k_{on} \cdot Ab \cdot R) + (k_{off} \cdot AbR)$$

$$\frac{dR}{dt} = S_0 - (k_E \cdot R) - (k_{on} \cdot Ab \cdot R) + (k_{off} \cdot AbR)$$

$$\frac{dAbR}{dt} = -(k_E \cdot AbR) + (k_{on} \cdot Ab \cdot R) - (k_{off} \cdot AbR)$$

In the equations, Ab_{SC} represents 574D04 at the s.c. dosing site. Bolus 574D04 is administered to this compartment with an amount of F·dose, where F is the absolute s.c. bioavailability. Ab represents 574D04 in the serum compartment. R is the target receptor, GM-CSFRα, and AbR is the antibody_receptor complex. Following antibody optimization, the model parameters were adjusted to reflect the *in vitro* binding affinity of 574D04 and the internalization half-life of 574D04/GM-CSFRα complex. Simulations were performed to predict GM-CSFRα blockade following single 0.01–10 mg kg^{-1} i.v. or s.c. administration of 574D04 in humans. The differential equations describing the disposition of 574D04 and interac-

tion with GM-CSFR following i.v. administration are similar to those shown above, except that the dose is directly given to the Ab compartment.

Expression of recombinant GM-CSFRα and phage display antibody isolation

The sequence encoding the human GM-CSFRα extracellular domain with a murine IL-3 signal sequence and an N-terminal FLAG tag was cloned into the mammalian expression plasmid pEF-BOS (Mizushima and Nagata, 1990). Following transient transfection of the plasmid into CHO cells using standard procedures, the cells were cultured and the encoded protein was expressed. The soluble extracellular domain (ECD) of GM-CSFRα was then purified from the CHO culture supernatants on an M2 affinity chromatography column and eluted with free FLAG peptide. Phage display selections were performed essentially as described previously (Vaughan *et al.*, 1996). In brief, a large single chain variable fragment (scFv) human antibody library was panned in a series of selection cycles on the purified GM-CSFRα ECD in order to enrich for scFv antibodies able to bind to the antigen. Following selections, individual scFv were confirmed as binding to GM-CSFRα by phage ELISA (Vaughan *et al.*, 1996) and then tested for their ability to inhibit the binding of recombinant GM-CSF to purified GM-CSFRα ECD in a biochemical receptor : ligand binding assay.

Construction of targeted and recombination libraries

Two V_HCDR3 and two V_LCDR3 libraries, comprising, respectively, randomization of V_H95–100, V_H100–102, and V_L89–94 and V_L94–97 (numbered according to Kabat, 1991), were prepared by oligonucleotide-directed mutagenesis, as described (Baker *et al.*, 2003) in the pCantab6 phage display vector (McCafferty *et al.*, 1994). For recombination, the V_H library variants from round 2 of selection on recombinant GM-CSFRα ECD were PCR-amplified, digested with *Sfi*I and *Xho*I restriction enzymes and cloned into plasmids harbouring the V_L variant population, also from round 2 of selection, using previously described methods (Vaughan *et al.*, 1996). Phage display selections for improved affinity were performed in principle according to the published method (Hawkins *et al.*, 1992). Briefly, rounds of phage display selection were performed on purified, biotinylated GM-CSFRα, using decreasing concentrations of antigen over successive rounds in order to stringently select for antibodies of improved K_d.

Expression of scFv and IgG4 molecules

For preparation of scFvs, plasmids containing scFv genes were cultured in *Escherichia coli*, expression induced by isopropyl-D-thiogalactoside and scFv proteins were isolated from the periplasm by osmotic shock followed by capture of the C-terminal His-tag by Ni2-nitrilotriacetic acid chromatography (Vaughan *et al.*, 1996). For IgG expression, the V_H and V_L chains of selected scFvs were cloned into human IgG expression vectors, expressed and purified as described (Persic *et al.*, 1997).

In vitro *functional assays for GMCSFR antagonism*

The TF-1 cell proliferation, granulocyte shape change, granulocyte survival and monocyte TNF-α release assays are all described in the Appendix S1.

Schild analysis

The change in forward scatter of human granulocytes was induced by increasing concentrations of GM-CSF using the described method for neutrophil shape change. This dose–response was carried out in the presence of increasing concentrations of 574D04 to produce a rightward shift of the GM-CSF dose–response curve. EC_{50} values for GM-CSF in the absence and presence of 574D04 were calculated using GraphPad PRISM software (La Jolla, CA, USA), and the dose ratio (DR) was calculated. Linear regression analysis was performed on log [574D04] M (*x*-axis) versus log [DR-1] (*y*-axis), and the pA_2 value was determined by extrapolating the line on this Schild plot to zero on the *y*-axis. If the slope of this line is unity, then the pA_2 equals the pK_b, an estimate of the drugs affinity for the target receptor. A full account of pA_2 analysis has been described previously by Kenakin (1982).

BIAcore measurements

The methods for performing BIAcore analysis are described in the Appendix S1.

Internalization measurements

Detailed methods for antibody internalization measurements are provided in the Appendix S1. Briefly, a GM-CSF-dependent mouse fibroblast cell line expressing human GM-CSF receptors (FD-hGM-CSFR), was labelled with carboxyfluorescein succinimidyl ester and stained with Alexa647-574D04, which bound to cell surface GM-CSFR. The cells were transferred to 37°C to start the reaction. The images of cells were captured over time using an OPERA LX high content confocal imaging system (Perkin Elmer, Waltham, MA, USA) and analysed using Acapella software (Perkin Elmer) and a proprietary algorithm specifically designed to quantify intracellular and membrane fluorescence. Rate of fluorescence accumulated inside the cells was calculated by model fitting of the data using SAAMII software (The Epsilon Group, Charlotteville, VA, USA).

Cynomolgus PK and PD assay

All animal care and experimental studies were approved by the SNBL Institutional Animal care and Use Committees (IACUC) and complied with the principles of the the 3Rs (replace, reduce and refine). All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). The *in vivo* studies were conducted at SNBL USA LTD. All test substances were well tolerated and the animals were returned to the colony upon study completion. Two male and two female adult cynomolgus monkeys (*Macaca fascicularis*) were dosed i.v. with a single 10 mg kg^{-1} infusion of 574D04. Samples for PK analysis were taken before dosing and then at 1 h and on days 1, 2, 4, 8, 14, 21, 28 and 35 after dosing. 574D04 levels in serum samples

were measured by antigen capture ELISA. Samples for PD analysis were collected before dosing, 1 h and days 2, 8, 14, 21 and 35 after dosing. Shape change response of eosinophils and neutrophils was assessed using an assay based on that described by Sabroe *et al*. (1999).

In vivo *blockade of GM-CSFR with 574D04*

Four treatment groups of five male cynomolgus monkeys received PBS or 574D04 (1, 10 or 30 mg kg^{-1}) as a 30 min i.v. infusion 48 h and 1 h before GM-CSF administration. The first dose of GM-CSF was given 30 min following the end of antibody dosing and animals were dosed s.c. twice daily (approximately 8 h apart) for three consecutive days with 5 μg kg^{-1} recombinant human GM-CSF. Blood for haematology (complete blood count with differential counts) and serum for determination of antibody concentration was collected prior to each antibody infusion, at 30 min and 4 h after GM-CSF dosing on day 1, 4 h after GM-CSF dosing on days 2 and 3, and on days 4, 6 and 8. Statistical analyses were performed with GraphPad Prism 5 software, using a two-way ANOVA with repeated measures. Bonferroni's post tests were performed to compare treatments with the control PBS group.

Results

Theoretical prediction of affinity goal for anti-GM-CSFRα candidate drug

To guide us in determining the affinity required for an effective anti-GM-CSFRα antibody, we developed a mathematical model to predict the theoretical affinity required to occupy greater than 99% (>99%) of the GM-CSF receptors and therefore block the activity of GM-CSF. By testing different theoretical affinities in the model, a prediction was made that an antibody affinity of 100 pM would be sufficient to achieve and maintain >99% occupancy for 28 days at a s.c. dose of 1 mg of drug per kg of patient body weight (mg kg^{-1}) (Figure 1).

Isolation of anti-GM-CSFRα scFv and affinity maturation strategy

Having identified a target affinity of 100 pM for an anti-GM-CSFRα antibody, the recombinant human extracellular domain of GM-CSFRα was presented to three different human naïve phage display antibody libraries to isolate a scFv that was specific for GM-CSFRα, i.e., that did not bind to either IL-3Rα, IL-5Rα or the common β chain (Figure S1A). This scFv could dose-dependently inhibit GM-CSF binding to GM-CSFRα (Figure S1B) and could inhibit GM-CSF induced proliferation of TF-1 erythroleukaemic cells (Figure S2). However, the potency of this scFv was low (IC_{50} > 300 nM) and even upon conversion to a human IgG molecule only achieved a mean IC_{50} of 43 nM (n = 5) in the TF-1 cell proliferation assay, 237 nM (n = 3) in a granulocyte shape change assay and was unable to inhibit GM-CSF-driven granulocyte survival at concentrations up to 3300 nM. To increase the affinity and potency of this scFv, phage display libraries were first generated to randomise amino acids in the V_H and V_L CDR3 loops of the scFv. Following affinity selection by phage display, pools of V_H and V_L genes containing mutations in the CDR3 regions were recombined into a large, combinatorial

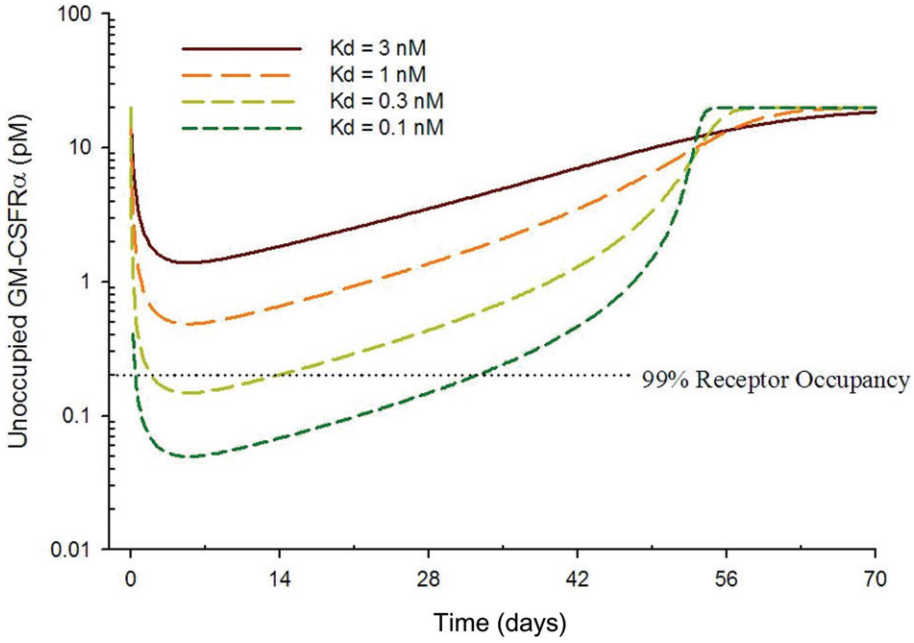

Figure 1

Antibody affinity goal predictions using *in silico* translational simulations. The biomathematical model assumed 50% s.c. absolute bioavailability, 2.5 mL kg^{-1} day^{-1} IgG clearance by the reticuloendothelial system, a distribution volume of 64 mL kg^{-1} and 20 pM GM-CSFRα with a 1 h internalization rate for the receptor and antibody–receptor complex. Simulations were performed to predict the unoccupied receptor level in humans following a single 1 mg kg^{-1} s.c. dose.

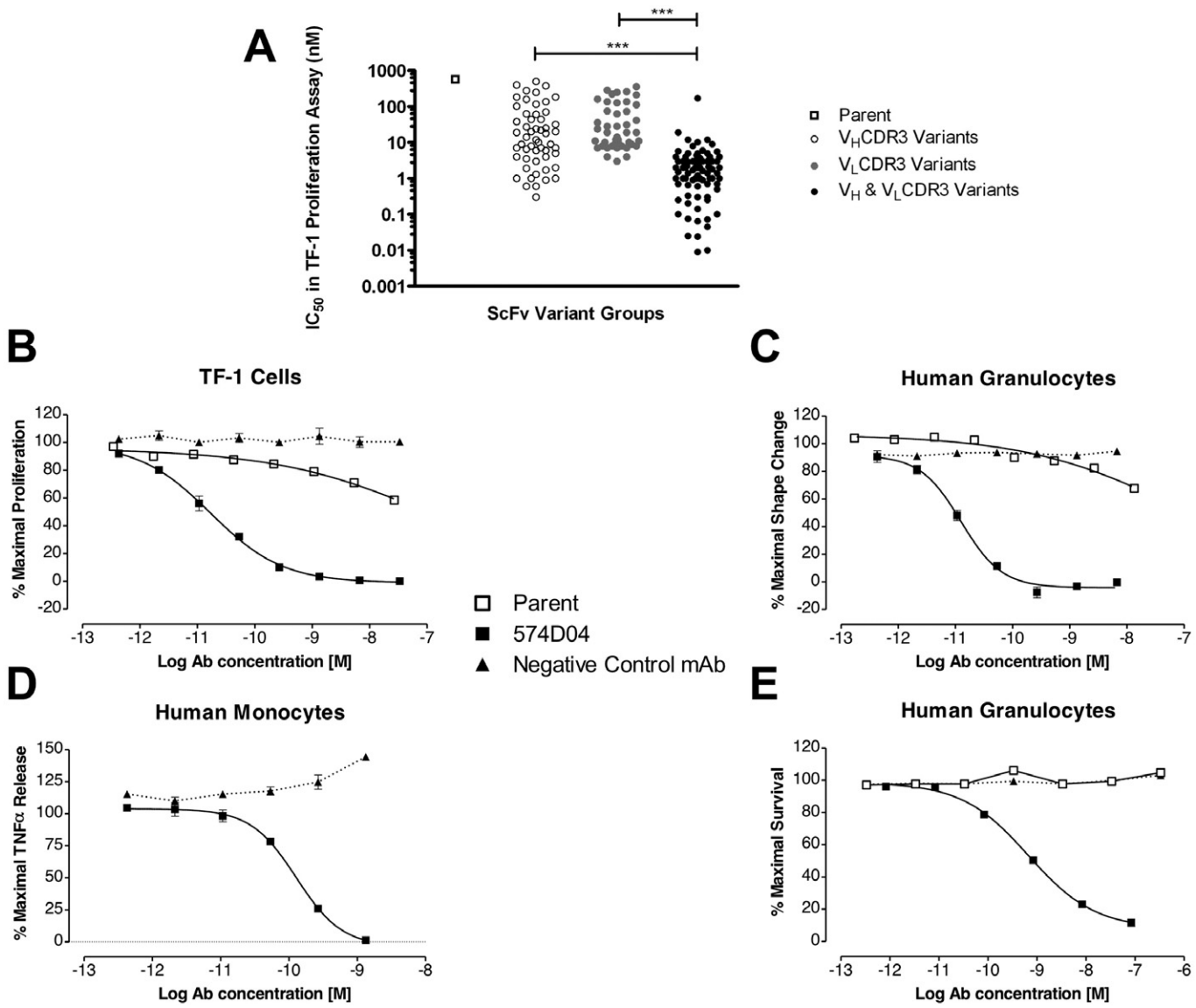

Figure 2

Potency improvements from the combinatorial affinity maturation strategy. (A) IC_{50} values of scFv antibodies in the TF-1 cell proliferation assay are plotted, showing the improvements in potency of variants compared with the parent antibody. The range of potencies observed following mutation of V_HCDR3 alone or V_LCDR3 alone are compared with the potencies observed when V_HCDR3 and V_LCDR3 diversity are combined. (B–E) Comparison of affinity matured 574D04 IgG and parent antibody 28G5 IgG for the neutralization of GM-CSF-induced TF-1 cell proliferation, IC_{50} = 15 ± 8pM (B); granulocyte shape change, IC_{50} = 41 ± 30pM (C); monocyte TNF-α release, IC_{50} = 99 ± 33pM (D); granulocyte survival, IC_{50} = 911 ± 467pM (E). (B) TF-1 cell proliferation was induced with human recombinant GM-CSF corresponding to EC_{80} concentration. Cells were incubated with ligand and a dilution series of antibody for 16 h, after which time cell proliferation was quantified by culture for 4–5 h with tritiated thymidine. (C) Human granulocytes were stimulated with human recombinant GM-CSF corresponding to EC_{80} concentration. Cells were incubated with ligand and a dilution series of antibody for 4 h, after which time cell activation was quantified by an increase in forward scatter (FSC) by flow cytometry. (D) Human monocytes were stimulated with 1 nM human recombinant GM-CSF for 18 h and TNF-α released into the supernatant was quantified by ELISA. (E) Human granulocytes were stimulated with 7 pM human recombinant GM-CSF for 68 h, and cell viability was assessed using Alamar Blue. Results are representative of at least three separate experiments. IC_{50} values were determined using Graphpad PRISM.

library that we hypothesized would contain a greater proportion of high-affinity scFv. Pre-recombination variants, with mutations in either V_H CDR3 or V_L CDR3, were compared with post-recombination variants, with mutations in both V_H CDR3 and V_L CDR3, in the TF-1 proliferation assay to determine IC_{50} values for each scFv molecule. ScFv isolated from the post-recombination library were orders of magnitude more potent than scFv from pre-recombination libraries underlining the power of this combinatorial approach (Figure 2A).

Table 1

Binding kinetics and TF-1 proliferation IC_{50} values for wild-type anti-GMCSFR antibody 28G5 and affinity matured antibody variant 574D04

Assay parameter	Wild type	574D04
k_{on}, M^{-1} s^{-1}	2.45×10^5	1.21×10^6
k_{off}, s^{-1}	1.75×10^{-2}	1.70×10^{-4}
K_d, nM	71.4	0.14
K_d wild-type/K_d variant	–	514
TF-1 IC_{50}, nM	43	0.005
TF-1 IC_{50} wild-type/TF-1 IC_{50} variant	–	8600
pH 7.4 K_d, nM	–	0.145
pH 5.7 K_d, nM	–	13.4
pH 5.7 K_d/pH 7.4 K_d	–	92

k_{on} = association rate constant; k_{off} = dissociation rate constant; K_d = equilibrium dissociation constant; IC_{50} = half maximal inhibitory concentration.

Characterization of affinity matured leads as IgG

Lead scFvs were converted to human IgG molecules and re-tested in a panel of *in vitro* assays that reflected the proposed mechanism of action. We confirmed that these antibodies were significantly improved over the parent antibody (Figure 2). In TF-1 proliferation, granulocyte shape change and monocyte TNF-α release assays, the lead antibody 574D04 had a sub-100 pM potency (15 ± 8, 41 ± 30 and 99 ± 33 pM respectively). In addition, 574D04 was able to inhibit granulocyte survival with sub-nanomolar (911 ± 467 pM) potency, whereas the parent antibody was unable to affect this response at concentrations of up to 3300 nM. The CDR3 recombination approach was able to increase the TF-1 cell proliferation potency of the IgG molecules by 8600-fold (Table 1). Although we obtained significant gains in potency, the 574D04 monovalent K_d value of 139 pM, as determined by surface plasmon resonance, was not within the requirements defined by the initial modelling. Given that measurements of monovalent antibody affinity for recombinant protein may not fully reflect the physiological antibody interaction with cell surface presented GM-CSFR, Schild analysis (Arunlakshana and Schild, 1959) was therefore used to quantify the affinity based on the functional activity of 574D04 (Figure 3A). The Schild analysis of 574D04, using GM-CSF-induced human granulocyte shape change as a suitable assay system, was calculated to be 10.6 (slope = 0.96), equating to an apparent affinity of 27 pM (Figure 3B).

Antibody internalization

As 574D04 binds to a cell surface receptor we also investigated the rate of the antibody internalization following binding. Using fluorescently labelled 574D04, we demonstrated specific binding of the antibody to the surface of FD-hGM-CSFR cells, a GM-CSF-dependent mouse fibroblast cell line that expresses human GM-CSFRα (Figure S3). Following addition to FD-hGM-CSFR, labelled 574D04 accumulated at the cell surface and was internalized within the cell in a time-dependent manner. Internalized antibody was characterized by a punctate staining of vesicles within cells (Figure 3C). The images of cells were captured and analysed using an algorithm designed to quantify intracellular and membrane fluorescence. The rate of fluorescence accumulated inside the cells was calculated to determine the half-life ($t½$) of 35 ± 6.5 min ($n = 7$) for internalization, four- to fivefold slower than that described for GM-CSF-mediated receptor internalization with a $K_e = 0.12$ min^{-1}, $t½$ of approximately 8 min (Metcalf *et al.*, 1999) or t½ of approximately 7 min (Walker and Burgess, 1987). This slower internalization rate coupled with the observation that direct binding of 574D04 to cells did not evoke any downstream cytokine release (Figure S4A,B) suggested that the antibody-evoked internalization was due to receptor turnover rather than ligand dependent internalization. In addition, we investigated the potential effects of pH changes in the endosomal compartment by measuring the pH dependence of 574D04 binding to GM-CSFRα. As the pH was lowered from pH 7.4 to pH 5.7, we saw a 92-fold reduction in the affinity of the antibody to the receptor (Figure S4C, Table 1), suggesting that antibody dissociation would be strongly favoured in endosomal vesicles. However, further work is required to fully understand the eventual fate of the antibody and receptor.

Theoretical prediction of 574D04 receptor occupancy in humans

The mathematical model was updated to incorporate the new information about affinity and internalization rate of the optimized human antibody 574D04. Simulations using the refined model predicted that a single s.c. dose of 1 mg kg^{-1} 574D04 should maintain 99% GM-CSFRα occupancy for up to 5 weeks (Figure 4).

In vivo *activity of 574D04*

In order to confirm the *in vivo* activity of 574D04, species cross-reactivity was investigated and 574D04 shown to antagonize cynomolgus monkey, but not rodent, GM-CSFR. Schild analysis of 574D04 was performed in a cynomolgus granulocyte shape change assay and was found to be 10.8 (slope = 1.1), equating to an apparent affinity of 16 pM (Figure S5), in line with the affinity against human GM-CSFRα of 27 pM. Thus, cynomolgus monkey was determined as the most pharmacologically relevant species in which to assess *in vivo* efficacy of 574D04.

PK of 574D04 in cynomolgus monkey was assessed following a single 10 mg kg^{-1} i.v. infusion (Figure 5A). The terminal half-life of 574D04 was estimated as approximately 5.6 days, within the typical range for a human monoclonal antibody in this species. Samples were also taken during the PK study to investigate the PD of 574D04 . Using a whole blood assay, a significant shape change (67%) in response to GM-CSF stimulation *ex vivo* was observed in the eosinophil population before treatment (Figure 5A). Following i.v. infusion of 574D04, responsiveness to GM-CSF was lost and this lack of response to GM-CSF was maintained until day 21. The functional blockade correlated with serum concentrations of 574D04 and suggested that a loss of effective blockade occurred between serum concentrations of 12–71 μg mL^{-1}.

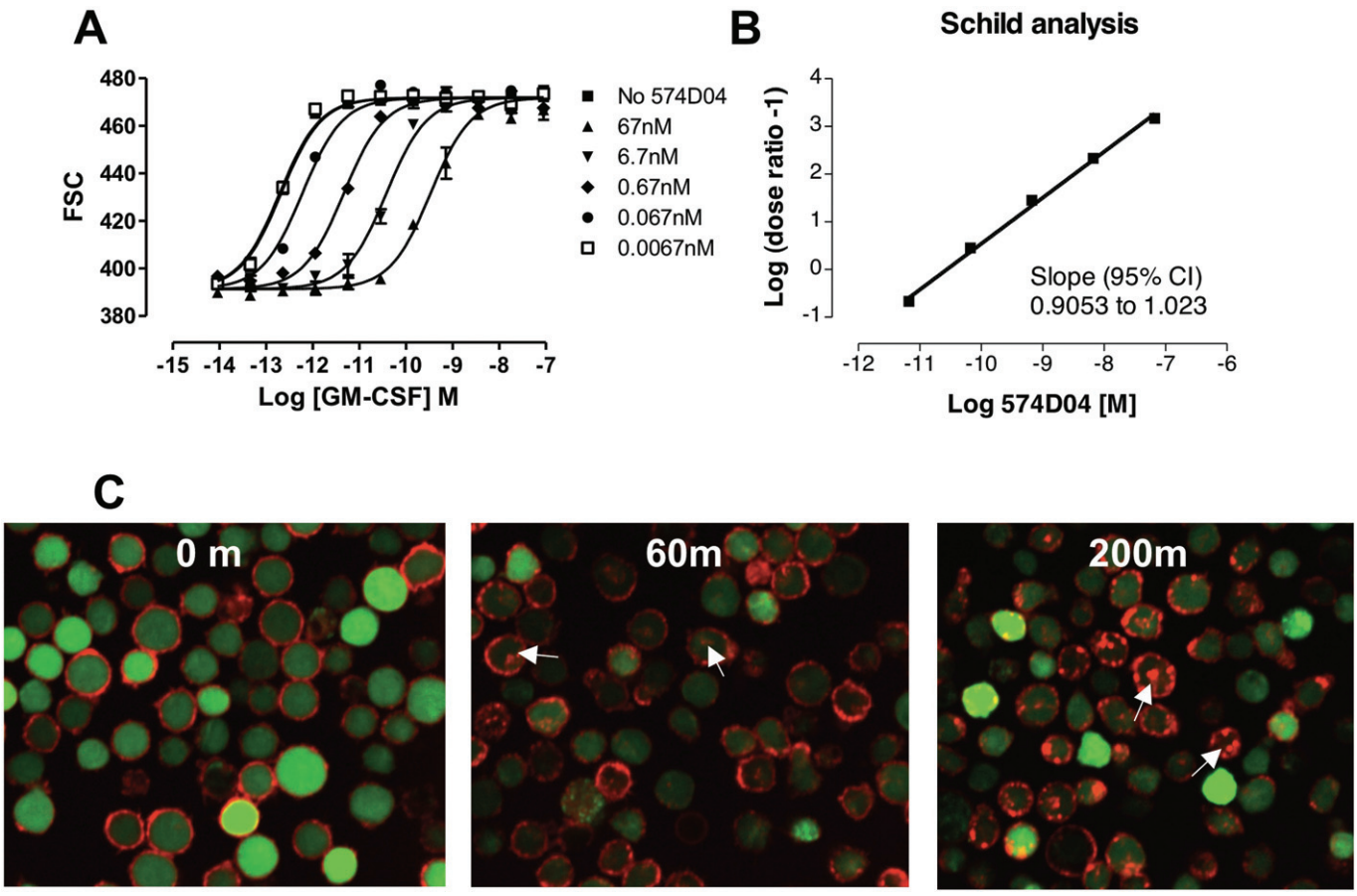

Figure 3

Characterization of 574D04 binding and internalization kinetics. (A) Schild analysis of 574D04 in an assay measuring GM-CSF-induced shape change of human granulocytes. Human peripheral blood granulocytes were stimulated with increasing concentrations of recombinant human GM-CSF (R&D Systems) for 4 h in the presence or absence of a single concentration of 574D04 (0.001, 0.01, 0.1, 1, 10 μg mL^{-1}) to produce a rightward shift in the dose–response curve. Cells were then analysed using forward scatter on a flow cytometer to monitor the change in shape. (B) Schild analysis to determine the pA2 value and thus functional affinity (K_D) of 574D04 for the receptor. (C) 574D04 internalization. 574D04 and an isotype control IgG antibody were directly conjugated with Alexa Fluor 647. Internalization studies were performed with AF647-labelled antibodies with mouse FDCP-1 cells overexpressing human GM-CSFR (FD-hGMR). Internalization over time was monitored by translocation of fluorescence from cell membrane to cytosolic compartment, visible as punctate staining of vesicles within cells. Intracellular versus membrane fluorescence was quantified using an OPERA imaging system.

Following this initial demonstration of PK/PD activity, the ability of pre-treatment with 574D04 to suppress an *in vivo* stimulation with GM-CSF was assessed. Based on the PK/PD analysis, 10 mg kg^{-1} was selected as an efficacious dose and 1 mg kg^{-1} as a predicted minimally effective dose. Recombinant human GM-CSF (5μg kg^{-1}) was administered twice daily for three consecutive days to cynomolgus monkeys that had previously received PBS, 1 or 10 mg kg^{-1} 574D04 (Figure 5B). The first dose of GM-CSF stimulated a rapid and transient drop (margination) in circulating leukocytes (69% decrease from baseline, $P < 0.001$) in the control PBS group (Figure 5C). This margination response was significantly inhibited in the presence of 574D04 after dosing at 1 and 10 mg kg^{-1} ($P < 0.001$). A similar trend was noted for neutrophils, basophils, monocytes and eosinophils (84%, 62%, 78% and 98% reductions from baseline, respectively, that were reversed by 574D04) (Figure S6). Continued administration of GM-CSF over 3 days stimulated a leukocytosis (Figure 5C), with significant increases in white blood cells at 52 h ($P < 0.01$) and 76 h ($P < 0.01$). This response was inhibited at both 10 mg kg^{-1} ($P < 0.001$) and 1 mg kg^{-1} ($P < 0.01$ at 76 h) of 574D04.

Discussion and conclusions

Over the last decade, the use of antibodies as a treatment option for chronic diseases has been widely accepted as a viable therapeutic approach by patients, clinicians, regulatory agencies and the pharmaceutical industry. However, due to the high cost of generating these molecules, increased competition and greater expectations of physicians and payers, more consideration has to be given to the initial engineering, design and pharmacology of the antibody to meet the needs of the patient and physicians at launch.

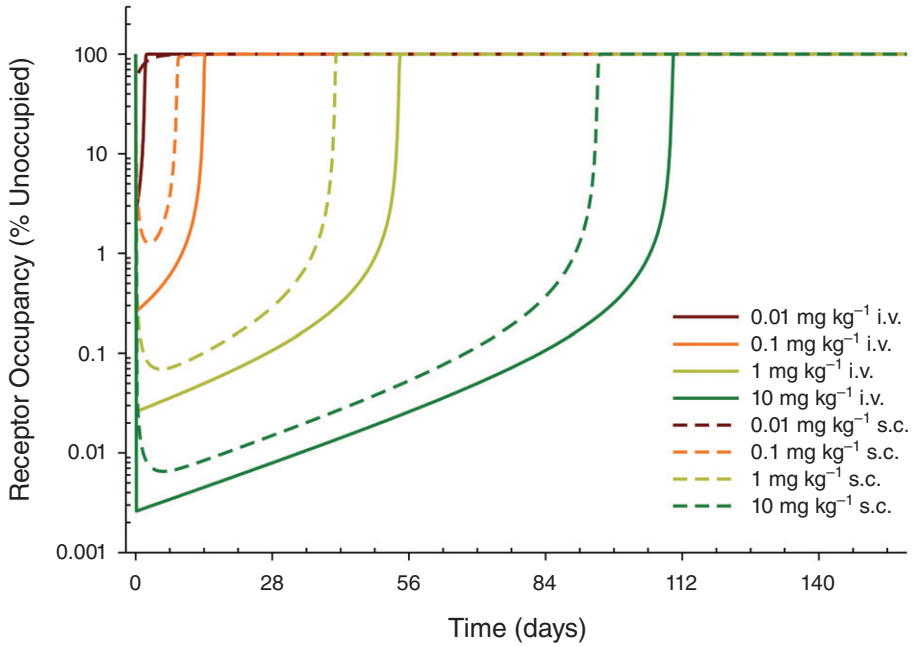

Figure 4

Predicted GM-CSFRα occupancy profiles for 574D04 first-time-in-human dose selections using the revised biomathematical model. Model parameters were adjusted to reflect the *in vitro* binding affinity of 574D04 and the internalization rate of 574D04/GM-CSFRα complex. Simulations were performed to predict GM-CSFRα blockade following single 0.01–10 mg kg^{-1} i.v. or s.c. administration of 574D04 in humans.

This work describes an integrated approach for engineering a specific, neutralising antibody compatible with s.c. delivery and infrequent dosing intervals. Self-administration by s.c. delivery using spring-driven syringes or autoinjectors is increasingly becoming a key feature for biopharmaceutical drug candidates in RA. Autoinjectors typically have a maximal holding volume of 1 mL and as monoclonal antibodies similar to other proteins tend to reach a solubility limit at around 100 mg mL^{-1} dosing at approximately 1 mg kg^{-1} is crucial to ensure device compatibility.

GM-CSF and its receptor are emerging targets of interest for the treatment of RA. Published reports suggest that only 10% of available GM-CSF receptors need to be bound by GM-CSF to elicit a maximal response (Nicola *et al.*, 1988; Hercus *et al.*, 1994). Therefore, this parameter was used in the first model to guide the target antibody affinity required to prevent GM-CSF signalling *in vivo*. To ensure complete block of GM-CSF function, we chose to aim for >99% occupancy of receptors, at which level the model predicted a target affinity of at least 100 pM for the antibody therapeutic candidate. Given the low starting affinity of our lead antibody (71 nM), which had been isolated by phage display from a naïve antibody library, and the challenging affinity requirements to achieve high receptor occupancy, we took a novel approach to improving affinity.

Previously, saturation mutagenesis has been performed on just one CDR loop (Thompson *et al.*, 1996; Baker *et al.*, 2003; Thom *et al.*, 2006) or sequentially on different loops, using the 'CDR-walking' approach (Barbas *et al.*, 1994; Yang *et al.*, 1995; Schier *et al.*, 1996). Alternatively, different CDR loops or regions of a CDR have been optimized in parallel and a small number of optimized loop region sequences recombined to achieve additive gains in potency (Yang *et al.*, 1995; Schier *et al.*, 1996). The novelty of the approach used here was to acknowledge the unpredictable nature of CDR loop synergy and include as many functional V_HCDR3 and V_LCDR3 loop sequences as possible in a large (>10^9) combinatorial library. In so doing, we allowed for the emergence of rare, synergistic combinations of CDR loop sequences that could not have been predicted and would not have been isolated by the previous approaches. The overall optimization strategy was highly successful in improving the potency of the final IgG version of the antibody by a factor of 8600-fold. The affinity of the final lead (574D04) for human GM-CSFRα was calculated using Schild analysis (Arunlakshana and Schild, 1959) to be 27 pM, meeting the previously defined affinity requirements of 100 pM.

Having identified an antibody of potentially suitable characteristics for human dosing, it was important to confirm that no further optimization of the molecule was required prior to expensive further investment in human clinical trials. To increase the accuracy of predictions of the dose to man, the PK/PD model required refinement to reflect the complexity of targeting a cell surface receptor. Internalization of receptor-bound antibody is likely to dominate the drug clearance at lower concentration levels when the target receptor is not fully saturated and could constitute a significant sink (Roskos *et al.*, 2004). Indeed, receptor binding and internalization of 574D04 was demonstrated and a quantitative imaging method used to determine the internalization half-life. Although these data were collected using a mouse fibroblast cell line overexpressing human GM-CSFR, a comparable

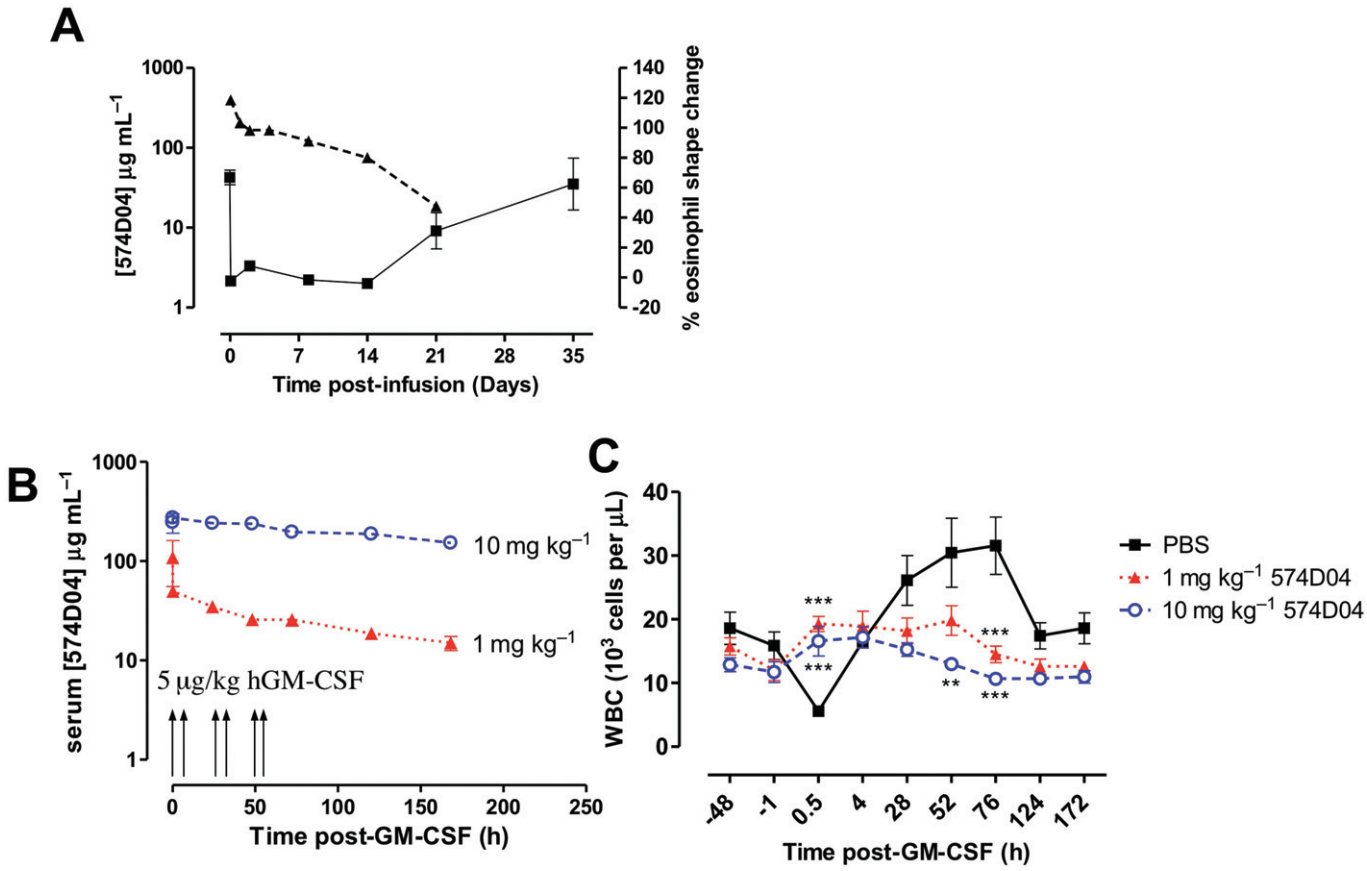

Figure 5

(A) Relationship between levels of free 574D04 and functional blockade of GM-CSFR in cynomolgus monkeys. Four adult cynomolgus monkeys were dosed i.v. with 10 mg kg^{-1} 574D04. 574D04 levels in serum samples were measured by antigen capture ELISA. 574D04 receptor blockade on eosinophils was determined by changes in forward scatter by flow cytometry (shape change) after 1 h *ex vivo* GM-CSF stimulation of erythrocyte-depleted blood samples. (B,C) *In vivo* blockade of GM-CSF-induced responses. Recombinant human GM-CSF (5 µg kg^{-1}) was administered twice daily for three consecutive days to cynomolgus monkeys that had previously received PBS, 1 mg kg^{-1} or 10 mg kg^{-1} of 574D04. (B) 574D04 levels in serum samples were measured by antigen capture ELISA. (C) Complete blood count with differential was monitored at regular intervals. Statistical analyses were performed using a two-way ANOVA with repeated measures and Bonferroni post tests. $^{**}P < 0.01$, $^{***}P < 0.001$, significantly different from control group).

internalization rate has been measured in the human TF-1 cell line, which is not transfected and therefore expresses GM-CSFR at endogenous levels. The method of using confocal microscopy to calculate antibody-receptor-mediated internalization has been previously described for an anti-EGF receptor antibody, Sym004 (Pedersen *et al.*, 2010). Like the antibody 574D04, Sym004 did not induce signalling or phosphorylation but did induce internalization. As the rate of internalization with 574D04 was four- to fivefold slower than ligand-mediated internalization (Walker and Burgess, 1987; Metcalf *et al.*, 1999), we hypothesized that the antibody-bound internalization rate was determined by receptor turnover. This was not formally characterized in this study; however, it has been shown that unoccupied GM-CSFR on mouse peritoneal macrophages internalises with a rate constant K_t of 0.021 min^{-1} and on BALB/c bone marrow cells with a K_t of 0.009 min^{-1} equivalent of a $t½$ of 47 and 111 min, respectively (Nicola *et al.*, 1988), broadly similar to the internalization of the human receptor plus the antibody 574D04. To gain an even greater understanding of this mechanism, it would be of interest to compare these data with a radioimmunoassay that would allow quantification of internalization with and without ligand. In addition, it would also be of interest to determine the fate of the antibody receptor complex once internalized. However, for the purposes of the current study, we have made the assumption that all internalized antibody was degraded.

The revised model, based on the internalization data and the other characteristics of the optimized antibody 574D04, was able to deliver predictions about the proposed clinical dosing. For s.c. dosing, which is strongly preferred as the eventual route for patient self-administration, the model suggests that receptor occupancies >99% could potentially be achieved for the potency-optimized 574D04 given at a dose of 1 mg kg^{-1} every 5 weeks.

We demonstrated equivalent *in vitro* activity against cynomolgus monkey GM-CSFR granulocytes confirming the suitability of this species to demonstrate *in vivo* activity of

574D04. Rodent studies could not be undertaken due to 574D04's lack of cross-reactivity with rodent GM-CSFRα. Therefore, we evaluated 574D04 in cynomolgus monkeys to determine the PK/PD relationship. Although it would have been informative to support our original affinity prediction by conducting a head-to-head comparison of low- and high-affinity GM-CSFR antibodies in cynomolgus monkeys, ethical considerations precluded us from doing so. However, in a single dose PK study, we confirmed that a 10 mg kg^{-1} dose of the affinity-matured antibody promoted long-term suppression (3–4 half-lives) of GM-CSF activity, and that the PD activity of 574D04 was lost when serum antibody levels dropped below 12–71 μg mL^{-1}. More critically, in a second study, where supra-physiological levels of recombinant GM-CSF were used, we fully inhibited leukocytosis at doses of 574D04 as low as 1 mg kg^{-1}. The increased levels of GM-CSF were above those observed in RA patients. These data therefore demonstrate the potential of 574D04 to fully suppress GM-CSF activity in patients with RA at doses compatible with s.c. delivery. In addition, these findings confirm the theoretical model that we built and the resulting design criteria that we used in the engineering of 574D04.

Recently, the PK and PD results profile of mavrilimumab, the germ-lined derivative of 574D04, from a single ascending dose phase I study in subjects with RA was reported (Burmester *et al.*, 2011). In this human study, it was demonstrated that mavrilimumab at 1 mg kg^{-1} i.v. significantly inhibited the *ex vivo* induction of suppressor of cytokine signalling 3 (SOCS3) mRNA by GM-CSF in peripheral blood for up to 2 weeks, suggesting maximal receptor occupancy in man and thus supporting the theoretical model.

In conclusion, we have demonstrated that close interaction between theoretical PK/PD modelling and antibody potency engineering, early in the drug dvelpopment process, resulted in the successful generation of a high-affinity candidate drug, mavrilimumab, suitable for clinical development. The validity of this approach has been borne out in a subsequent clinical study in humans that demonstrated significant inhibition of GM-CSF-mediated effects for up to 2 weeks upon dosing with 1 mg kg^{-1} mavrilimumab, a result which is well suited to the ultimate goal of patient self-administration. Whilst acknowledging that in this case the theoretical model did not alter the course of action and that further work can be done to find out how broadly this general strategy can be applied, we believe that this integrated approach will be critical for the discovery and development of new biological therapeutic agents.

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Conflict of interest

None.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 (A) Phage ELISA performed with phage-displayed parental scFv antibody 28G5, demonstrating specific binding to GM-CSFRα. 28G5 phage were tested for binding to recombinant extracellular domain of GM-CSFRα, IL-3Rα, IL-5Rα chain, the common β chain or no antigen. A separate control which omitted 28G5 phage was included for comparison. (B) Biochemical receptor : ligand inhibition assay demonstrating antibody inhibition of the recombinant GM-CSFRα interaction with recombinant GM-CSF. The inhibitory profiles of the anti-GMCSFRα antibodies 28G5 (IC_{50} = 98 pM) and the commercial mAb (IC_{50} = 59 pM) are compared with an isotype control antibody.

Figure S2 Parent scFv (28G5) IC_{50} curve. TF-1 cell proliferation was induced with human recombinant GM-CSF corresponding to EC_{80} concentration. Cells were incubated with ligand and a dilution series of antibody for 16 h, after which time cell proliferation was quantified by culture for 4–5 h with tritiated thymidine. The IC_{50} value for the anti-GMCSFRα scFv 28G5 was >300 nM.

Figure S3 Flow cytometry measurement of binding of Alexa647-labelled 574D04 to FD cells transfected with human GM-CSFRα. Flow cytometry traces and geometric mean calculations are shown for Alexa647-labelled 574D04, an Alexa647-labelled lgG4 isotype control and unstained cells. Alexa647-labelled 574D04 shows fluorescence shift as compared with Alexa647-labelled isotype and unstained cells.

Figure S4 Cytokine production by human PBMCs (mean ± SEM of four donors) in response to 574D04, 10 ng mL^{-1} human GM-CSF or 5 μg mL^{-1} PHA. (A) IL-6 production. (B) TNF-α production. (C) The pH dependence of 574D04 binding to GMCSFRα. Binding kinetics was measured by surface plasmon resonance at pH 7.4 and pH 5.7, and the k_d was recorded as 0.15 and 13.4 nM, respectively, indicating a

92-fold change in binding affinity over this pH range. Note that the higher binding at pH 5.7 reflects the higher amount of 574D04 IgG captured by the protein-G surface at pH 5.7. Additional controls are 0 nM GMCSFRα at pH 7.4 and 0 nM GMCSFRα at pH 5.7.

Figure S5 Characterization of 574D04 for cross-reactivity with cynomolgus monkey GM-CSFR. (A) Schild analysis of 574D04 in an assay measuring GM-CSF induced shape change of cynomolgus monkey granulocytes. Cynomolgus peripheral blood granulocytes were stimulated with increasing concentrations of recombinant human GM-CSF (R&D Systems) for 4 h in the presence or absence of a single concentration of 574D04 (0.00025, 0.0025, 0.025, 0.25, 2.5 μg mL^{-1}) to produce a rightward shift in the dose–response curve. Cells were then analysed using forward scatter on a flow cytometer to monitor the change in shape. (B) Schild analysis to determine the pA2 value and thus functional affinity (K_D) of 574D04 for the receptor.

Figure S6 *In vivo* blockade of GM-CSF-induced responses. Recombinant human GM-CSF (5 μg kg^{-1}) was administered twice daily for three consecutive days to cynomolgus monkeys that had previously received PBS, 1 mg kg^{-1} or 10 mg kg^{-1} of 574D04. Complete blood count with differential was monitored at regular intervals. $^{**}P < 0.01$, $^{***}P < 0.001$ versus control group.

Appendix S1 Supporting methods.