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ORIGINAL RESEARCH ARTICLE

Demonstration of Functional Similarity of Proposed Biosimilar ABP 501 to Adalimumab

Jyoti Velayudhan[1] · Yuh-feng Chen[1] · Amanda Rohrbach[1] · Christina Pastula[1] · Gwen Maher[1] · Heather Thomas[1] · Ryan Brown[1] · Teresa L. Born[1]

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Abstract

Background Due to the complex molecular structure and proprietary manufacturing processes of monoclonal antibodies (mAbs), differences in structure and function may be expected during development of biosimilar mAbs. Important regulatory requirements for approval of biosimilar products involve comprehensive assessments of any potential differences between proposed biosimilars and reference mAbs, including differences in all known mechanisms of action, using sensitive and relevant methods. Any identified structural differences should not result in differences in biofunctional or clinical activity.

Objective A comprehensive assessment comparing the Amgen biosimilar candidate ABP 501 with FDA-licensed adalimumab (adalimumab [US]) and EU-authorized adalimumab (adalimumab [EU]) was conducted to demonstrate similarity in biofunctional activity.

Methods The functional similarity assessment included testing of binding kinetics to soluble tumor necrosis factor α (TNFα) and relative binding to transmembrane TNFα. The neutralization of TNFα-induced caspase activation, TNFα- and lymphotoxin-α (LTα)-induced chemokine production, and cytotoxicity was also tested. Binding to Fc-gamma receptors FcγRIa, FcγRIIa (131H), FcγRIIIa (158V and 158F), and neonatal Fc receptor (FcRn) was compared with the reference mAbs, as was antibody-dependent cell-mediated cytotoxicity and complement-dependent cytotoxicity.

Results The data demonstrate that ABP 501 is similar to both adalimumab (US) and adalimumab (EU) with respect to evaluated biofunctional activities.

Conclusion Similarity in biofunctional activity is a critical component of the totality of evidence required for demonstration of biosimilarity. The functional similarity demonstrated for ABP 501 comprehensively assesses the known mechanisms of action of adalimumab, supporting the conclusion that ABP 501, adalimumab (US), and adalimumab (EU) are likely to be clinically similar.

Key Points

ABP 501, a biosimilar candidate to adalimumab, binds to and neutralizes tumor necrosis factor α (TNFα), a pleiotropic proinflammatory cytokine that can induce a variety of cellular effects contributing to autoimmune disease.

A comprehensive similarity assessment comparing ABP 501, adalimumab (US), and adalimumab (EU) demonstrated similarity with respect to a variety of biological properties, including binding to soluble and transmembrane TNFα; neutralization of TNFα-induced caspase activation, TNFα- and lymphotoxin-α (LTα)-induced chemokine production, and cytotoxicity; Fc receptor binding; and effector function activation.

Similarity in preclinical biological activity contributes to the foundation of the stepwise approach used to demonstrate biosimilarity, which subsequently includes human pharmacokinetic studies and clinical efficacy and safety studies.

✉ Teresa L. Born
bornt@amgen.com

[1] Amgen, Inc., One Amgen Center Drive, Thousand Oaks, CA 91320, USA

1 Introduction

Biosimilars, biologic products similar in terms of quality, efficacy, and safety to licensed biologic reference products, are being developed to provide less expensive therapeutic alternatives in an effort to reduce healthcare expenditures [1]. The high complexity in molecular structure and unique/proprietary biomanufacturing processes of biologics, however, can result in structural and functional differences, making it impossible to produce biosimilar molecules that are identical to the innovator biologics [2]. It is therefore important to demonstrate that structural and functional differences between biosimilars and reference biologics do not result in clinically meaningful differences in safety or efficacy. The US Food and Drug Administration (FDA) and European Medicines Agency (EMA) have developed guidelines for the development of biosimilars that recommend a stepwise approach emphasizing the totality of evidence for demonstration of biosimilarity, encompassing similarity in analytical studies (physiochemical product quality attributes and biological activity); relevant animal studies (preclinical pharmacokinetics, pharmacodynamics, and toxicity); and finally, clinical studies (pharmacokinetics and pharmacodynamics, immunogenicity, safety, and efficacy) (Fig. 1) [3–5]. Sensitive analytical methods capable of detecting potential differences are the foundation of the stepwise biosimilar evaluation process, and identified differences are further evaluated to confirm they do not impact clinical efficacy and safety.

ABP 501 is being developed as a biosimilar to adalimumab, a human immunoglobulin G1 (IgG1) anti-tumor necrosis factor α (anti-TNFα) monoclonal antibody (mAb) that prevents interaction of TNFα with its receptors, thereby interfering with the inflammatory signaling central

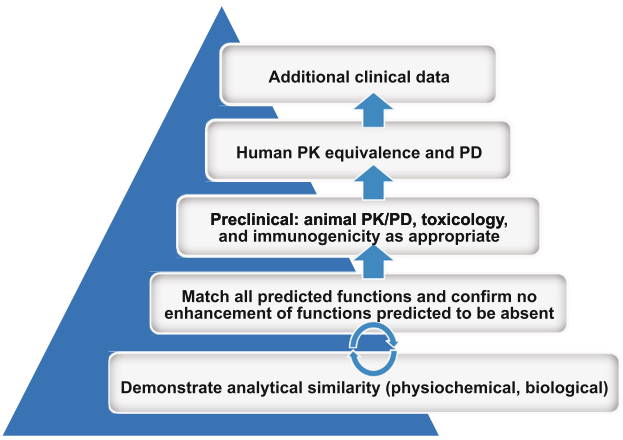

Fig. 1 Stepwise, totality-of-evidence approach to demonstrating biosimilarity [3, 4]. *PD* pharmacodynamics, *PK* pharmacokinetics

to chronic autoimmune diseases such as rheumatoid arthritis, psoriasis, ulcerative colitis, and Crohn's disease. Adalimumab is highly specific for TNFα and does not bind murine TNFα or the closely related human cytokine lymphotoxin-α (LTα) [6, 7]. Adalimumab is known to bind soluble TNFα (sTNFα) with high affinity and inhibit its bioactivity [7]. The primary bioactivity of TNFα is elicited when the soluble version of the cytokine engages TNF receptor 1 (p55TNFR). More specifically, sTNFα can ultimately induce either nuclear factor kappa B (NFκB)-dependent gene expression or (in the absence of NFκB activity) cell death, depending upon the molecular context of the responding cell [8]. The NFκB-dependent induction of gene expression, in particular, is central to the propagation of autoimmune disease pathology.

Adalimumab also binds to uncleaved transmembrane TNFα (mbTNFα) and, since it is an IgG1 capable of binding to Fc gamma receptors (FcγR) [7], mediates induction of antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) in vitro [9]. The relevance of ADCC- or CDC-mediated cytotoxicity of mbTNFα-expressing cells in relation to clinical efficacy is not well established, but may be important, particularly in inflammatory bowel diseases [7, 10]. It is well known that differences in the glycosylation pattern within the Fc region of an IgG monoclonal antibody can have significant effects on the effector functions of the molecule [11]. Since analytical differences, especially in the glycan profiles, may be expected between a biosimilar mAb, other biosimilars, and the reference mAb product, it is important to fully characterize effector functions of the candidate biosimilar mAb, even if the precise contribution to clinical efficacy is unclear.

In addition to mediating effector functions via FcγR binding, adalimumab is capable of binding to the neonatal Fc receptor (FcRn), which can influence the plasma half-life of the antibody. The amino acid sequences of the Fc region of IgG1 mAbs are identical; however, there is some evidence that some post-translational modifications, or overall mAb conformation, may confer differences in FcRn binding affinities and therefore plasma half-lives [7]. Therefore, similarity in FcRn binding is critical for providing confidence that the clinical pharmacokinetic profile will be similar between the two products.

Although ABP 501 and adalimumab share the same amino acid sequence [12], differences could be expected in product quality attributes due to inherent differences in expression systems, bioprocess, and purification [13]. Demonstrating equivalence of all functional properties is of foundational importance during the stepwise development of a biosimilar (Fig. 1). The analytical and functional equivalence will ultimately support abbreviated clinical studies and contribute to the scientific justification for

extrapolation to all approved indications [13–15]. Results of the analytical similarity assessment comparing ABP 501 to FDA-licensed adalimumab (adalimumab [US]), and EU-authorized adalimumab (adalimumab [EU]) have been previously reported (Liu et al. [16]). The objectives of these studies are to provide a comprehensive assessment of functional similarity, to address the pleiotropic effects induced by TNFα as well as the multitude of interactions mediated by the Fc region of an IgG1 mAb, and to assess both binding activities and functional outcomes.

2 Methods

2.1 Materials

The ABP 501 drug product was manufactured by Amgen Inc. (Thousand Oaks, CA, USA). Multiple lots of adalimumab (US) (Humira®, AbbVie, North Chicago, IL, USA) and adalimumab (EU) were procured and stored according to the manufacturer's instructions. For ABP 501, drug product was used for all analyses. An ABP 501 reference standard lot was included in most analyses. Recombinant sTNFα was purchased from PeproTech (Rocky Hill, NJ, USA) or R&D Systems (Minneapolis, MN, USA). Chinese hamster ovary cells expressing non-cleavable mbTNFα have been previously described, and are referred to as MT-3 [17].

2.2 Tumor Necrosis Factor α (TNFα) Binding Assays

Relative binding to sTNFα was determined by solid phase enzyme-linked immunosorbent assay (ELISA). Recombinant sTNFα was coated onto the wells of a microtiter ELISA plate and the plate was blocked using a gelatin buffer. Dilutions of reference standard and test samples were added to the appropriate wells and incubated for 90 min at ambient temperature. Bound mAb was detected with goat anti-human IgG (Fc fragment) conjugated to horseradish peroxidase (HRP). Relative binding activities were calculated based on the ratio of half-maximal effective concentration (EC_{50}; concentration at which 50 % inhibition is observed) values of the reference standard curve relative to the test sample. A total of ten lots of each test mAb were assessed.

The kinetics of binding to recombinant sTNFα were determined by surface plasmon resonance (SPR) using a Biacore™ T200 (GE Healthcare Bio-Sciences, Piscataway, NJ, USA) with single-cycle kinetics methodology. Goat anti-human IgG Fc-specific capture antibody (Jackson ImmunoResearch, West Grove, PA, USA) was immobilized to the sensor chip, allowing for capture of ABP 501, adalimumab (US), and adalimumab (EU). Soluble TNFα was tested at escalating concentrations (0.18–60 nM). The double-referenced data from the single-cycle kinetic run was fitted locally to a 1:1 binding model with Biacore kinetics software. The association rate constant (k_a), the dissociation rate constant (k_d), and the dissociation equilibrium binding constant (K_d) were fitted globally. The relative binding affinity of each of the samples was calculated based on the K_d value as compared with the ABP 501 reference standard. A total of three lots of each test mAb were assessed.

Binding to mbTNFα was assessed in a competitive cell-based binding assay using MT-3 cells and Alexa Fluor® 488 (Thermo Fisher Scientific, Waltham, MA, USA) dye (Alexa-488)-labeled ABP 501. A dose titration of reference standard, ABP 501, adalimumab (US), or adalimumab (EU) test samples and a fixed concentration of the labeled mAb were incubated with cells for 4–6 h at room temperature. Binding was assessed by measuring cell-bound fluorescence on an Acumen® eX3 imaging cytometer (TTP Labtech, Hertfordshire, UK), wherein binding of the test mAbs to mbTNFα was reflected in the decreased binding of the labeled mAb. After assessing parallelism of the dose–response curves, the test-sample binding relative to the reference standard was determined using a 4-parameter logistic model fit (SoftMax® Pro Software, Molecular Devices, Sunnyvale, CA, USA). A total of three lots each of ABP 501, adalimumab (US), and adalimumab (EU) were tested, with three independent determinations for each lot.

2.3 Neutralization of TNFα Bioactivity

Monocytic (U937) cells were stimulated with 3 ng/mL TNFα in the presence of a dose titration of reference standard, ABP 501, adalimumab (US), or adalimumab (EU) for approximately 2 h. Caspase-Glo 3/7® reagent (Promega, Madison, WI, USA) was added to the samples, with an additional 30- to 60-min incubation. Luminescence, measured in relative luminescence units (RLUs), was determined as a measure of the degree of caspase activation. After assessing parallelism of the dose–response curves, the test-sample activity relative to the reference standard was determined using a 4-parameter logistic model fit (SoftMax Pro). Multiple lots (≥10) of ABP 501, adalimumab (US), and adalimumab (EU) were tested, with three independent determinations each.

Human umbilical vein endothelial cells (HUVEC) were cultured in a 96-well culture plate prior to stimulation with 3 ng/mL TNFα or 10 ng/mL LTα for 4 h in the presence of a dose titration of ABP 501, adalimumab (US), or adalimumab (EU). Supernatants were collected from each well, and the concentration of interleukin-8 (IL-8) was quantified

using a single-spot immunoassay (Meso Scale Diagnostics, Rockville, MD, USA). Percent of control (POC) was calculated based on IL-8 produced in unstimulated cells and in cells that were stimulated in the absence of added test antibody. The EC_{50} was calculated for each sample using Prism® sigmoidal dose–response-curve-fitting software (GraphPad Software, La Jolla, CA, USA). A total of three lots of each test mAb were compared.

Fibrosarcoma (L929) cells were cultured in 96-well culture plates and sensitized with actinomycin D (2 μg/mL) for 2 h, then stimulated with TNFα (0.75 ng/mL) in the presence of a dose titration of ABP 501, adalimumab (US), or adalimumab (EU). Following overnight incubation, cytotoxicity was measured using the viability indicator dye alamarBlue® (Invitrogen/Thermo Fisher Scientific, Carlsbad, CA, USA), which was added to the cells during the final 4 h of stimulation. Plates were analyzed for fluorescence on an EnVision® 2101 Multilabel Reader (PerkinElmer, Waltham, MA, USA). Percent viability was calculated based on viability of unstimulated cells and of cells stimulated in the absence of added test antibody. The EC_{50} was calculated for each sample using sigmoidal dose–response-curve-fitting software (GraphPad Prism). A total of three lots of each test mAb were compared.

2.4 Fc Receptor Binding

Relative binding to human FcγRIa, FcγRIIa (131H), and FcγRIIIa (158V and 158F) was determined by AlphaLISA® (reagents from PerkinElmer, Waltham, MA, USA). FcγRIa-HIS-GST protein (1 nM final), FcγRIIa (131H)-GST-H6 protein (2 nM final), FcγRIIIa (158V)-GST-His6 protein (1 nM final), or FcγRIIIa (158F)-GST-His6 protein (4 nM final) were pre-incubated with Glutathione AlphaLISA Acceptor beads biotinylated IgG1 mAb competitor (1 nM final for FcγRIa, 0.4 nM final for FcγRIIa, and 2 nM final for FcγRIIIa), and a dose titration of reference standard, ABP 501, adalimumab (US), and adalimumab (EU). In one experiment, recombinant sTNFα was included at an equimolar concentration. Samples were incubated at ambient temperature for approximately 22 h. Streptavidin-coated donor beads were added to each well and incubated for approximately 22 h. Samples were read for luminescence (in RLUs) with an EnVision plate reader, using an AlphaScreen® protocol (PerkinElmer, Waltham, MA, USA). After assessing parallelism of dose–response curves, the test-sample binding relative to the reference standard was determined using a 4-parameter logistic model fit (SoftMax Pro). Multiple lots (≥10) of ABP 501, adalimumab (US), and adalimumab (EU) were tested, with three independent determinations each. A single lot of each test mAb was assessed for FcγRIIIa (158V) binding in the presence of sTNFα.

Relative binding to FcRn was determined using a competitive image cytometry-based assay using engineered 293T cells (293T-7A1) overexpressing human FcRn. Dose titrations of reference standard, ABP 501, adalimumab (US), and adalimumab (EU) were incubated with 293T-7A1 cells and a fixed concentration of recombinant IgG1 Fc labeled with Alexa-488 (0.1 μg/mL) for approximately 4 h at ambient temperature at pH 6. After incubation, the cell-bound fluorescence was read on an Acumen eX3 imaging cytometer wherein binding of the test mAbs to FcRn was reflected in the decreased binding of the labeled mAb. After assessing parallelism of dose–response curves, the test-sample binding relative to the reference standard was determined using a 4-parameter logistic model fit. Multiple lots (≥10) of ABP 501, adalimumab (US), and adalimumab (EU) were tested, with three independent determinations each.

2.5 Induction of Effector Function

To determine ADCC activity, MT-3 target cells were labeled with calcein-AM dye (Sigma-Aldrich, St. Louis, MO, USA) prior to incubation with a dose titration of reference standard, ABP 501, adalimumab (US), or adalimumab (EU). Effector cells (NK-92M1 cells stably transfected with human FcγRIIIa [158V] licensed from Conkwest [Cardiff-by-the-Sea, CA, USA], now NantKwest) were then added to the opsonized target cells at an effector-to-target ratio of 25:1 and incubated for approximately 1 h. Calcein released from lysed target cells was determined by measuring the fluorescence of the supernatant. After assessing parallelism of dose–response curves, the percent cytotoxicity of ABP 501, adalimumab (US), or adalimumab (EU) test samples relative to the reference standard was determined using a 4-parameter logistic model fit (SoftMax Pro). Multiple lots (≥10) of ABP 501, adalimumab (US), and adalimumab (EU) were tested, with three independent determinations for each lot.

CDC activity was measured using MT-3 cells. Target cells were labeled with calcein-AM prior to incubation with a dose titration of ABP 501, adalimumab (US), or adalimumab (EU). Baby rabbit complement (Cedarlane, Burlington, ON, Canada) was added to opsonized target cells for approximately 1 h to allow complement-mediated lysis. Calcein released from lysed target cells was determined by measuring the fluorescence of the supernatant. After assessing parallelism of dose–response curves, the percent cytotoxicity of ABP 501, adalimumab (US), or adalimumab (EU) test samples relative to the reference standard was determined using a 4-parameter logistic model fit (SoftMax Pro). Multiple lots (≥10) of ABP 501, adalimumab (US), and adalimumab (EU) were tested, with three independent determinations for each lot.

2.6 Statistical Analysis

For the TNFα-binding (ELISA) assay and potency (U937 apoptosis inhibition) assay, similarity was assessed using statistical equivalence. Under this approach, similarity was achieved when the confidence interval (CI) for the difference in means between the products was contained within an equivalence acceptance criterion of ±1.5 times the standard deviation of the adalimumab lots tested. For secondary mechanisms of action, including ADCC, CDC, FcγRIIIa (158V) binding, and FcRn binding, results were considered statistically similar when 90 % of the ABP 501 lots fell within a pre-defined quality range established based on the adalimumab lots tested; the quality range was defined as the mean of the adalimumab lots tested ±3 standard deviations. For characterization assays, three lots each of ABP 501 and adalimumab were tested, and with this number of tested lots, statistical assessment of similarity was not performed, and similarity was determined by a qualitative comparison of the results.

3 Results

3.1 Binding to Soluble and Transmembrane TNFα is Similar Between ABP 501 and Adalimumab

An ELISA assay was performed to compare the binding of ABP 501, adalimumab (US), and adalimumab (EU) to (immobilized) recombinant sTNFα. Relative binding to TNFα was similar between the tested mAbs (Fig. 2) and the mean relative binding by ABP 501 was statistically similar to the binding observed for adalimumab, based on equivalence acceptance criteria. Specifically, the mean ABP 501 relative binding was 108.10 % and mean adalimumab (US) relative binding was 111.83 %, with the difference between means of −3.74 % (90 % CI −11.03 to 3.55). The equivalence acceptance criterion (EAC) for the difference was ±15.02 % for adalimumab (US) and thus the products are considered statistically equivalent. The mean relative binding for adalimumab (EU) was 111.33 %, with a difference from ABP 501 of −3.23 % (90 % CI −9.39 to 2.93). The EAC for adalimumab (EU) similarity was a ±15.58 % difference, and thus ABP 501 is also statistically equivalent to adalimumab (EU).

To further characterize the binding characteristics, biacore SPR was used to provide a comparison of the binding kinetics of ABP 501, adalimumab (US), and adalimumab (EU) to sTNFα. The on rates, off rates and K_d for soluble TNFα binding to three different lots each of ABP 501, adalimumab (US), and adalimumab (EU) were similar (Table 1). The equilibrium binding affinity of ABP 501 to sTNFα was 52, 48, and 51 pM for the three lots tested. The equilibrium binding affinity was 53, 48, and 53 pM for the tested lots of adalimumab (US), and was 54, 46, and 51 pM for the tested lots of adalimumab (EU) (Table 1).

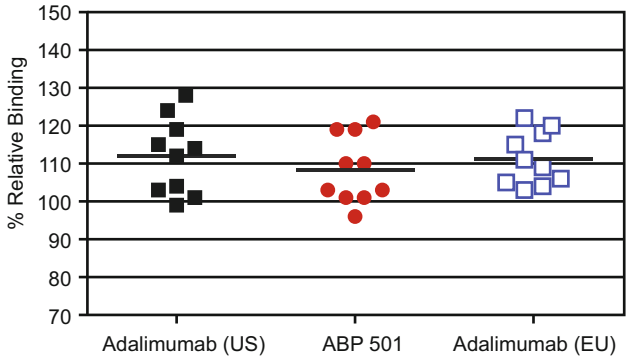

Fig. 2 Similarity of ABP 501, adalimumab (US), and adalimumab (EU) with respect to binding to sTNFα. The relative binding values for ABP 501 (*red*), adalimumab (US) (*black*), and adalimumab (EU) (*blue*) to immobilized recombinant TNFα in an ELISA format are shown. Each point represents results from testing a unique lot. The lines represent the mean relative binding for that sample. *Adalimumab (EU)* EU-authorized adalimumab, *adalimumab (US)* FDA-licensed adalimumab, *ELISA* enzyme-linked immunosorbent assay, *sTNFα* soluble tumor necrosis factor α

Table 1 Binding kinetics of ABP 501, adalimumab (US), and adalimumab (EU) to sTNFα. Kinetics of binding were determined by surface plasmon resonance, testing three unique lots of each test mAb

Sample	On rate k_a (1/ms)	Off rate k_d (1/s)	K_d (pM)
ABP 501	7.62 E+5	3.94 E−5	52
ABP 501	7.69 E+5	3.73 E−5	48
ABP 501	8.35 E+5	4.28 E−5	51
Adalimumab (US)	7.45 E+5	3.94 E−5	53
Adalimumab (US)	8.34 E+5	3.98 E−5	48
Adalimumab (US)	8.12 E+5	4.27 E−5	53
Adalimumab (EU)	8.08 E+5	4.38 E−5	54
Adalimumab (EU)	8.58 E+5	3.90 E−5	46
Adalimumab (EU)	8.65 E+5	4.44 E−5	51

Adalimumab (EU) EU-authorized adalimumab, *Adalimumab (US)* FDA-licensed adalimumab, k_a association rate constant, k_d dissociation rate constant, K_d dissociation equilibrium binding constant, *M* molar, *mAb* monoclonal antibody, *ms* milliseconds, *s* seconds, *sTNFα* soluble tumor necrosis factor α

Adalimumab can bind mbTNFα as well as sTNFα, ultimately blocking signaling induced by mbTNFα or potentially mediating cellular effects directly by engaging mbTNFα. Modulation of cellular activities subsequent to binding mbTNFα have been proposed to be relevant to efficacy in inflammatory bowel disease [18]. In order to further characterize the binding characteristics, similarity in binding to mbTNFα was determined in a competitive cell-based assay using MT-3 cells [17]. The mean (from three independent experiments) percent relative binding to

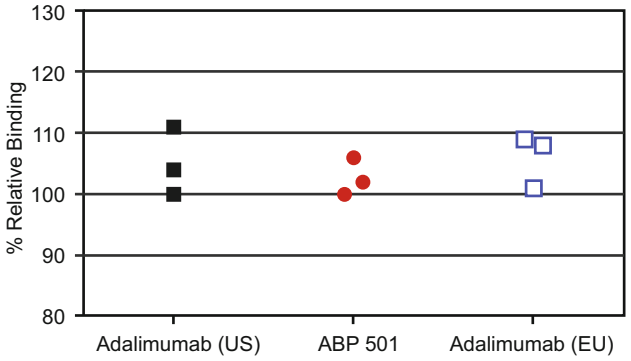

Fig. 3 Similarity of ABP 501, adalimumab (US), and adalimumab (EU) with respect to binding to mbTNFα. The relative binding values for ABP 501 (*red*), adalimumab (US) (*black*), and adalimumab (EU) (*blue*) in a competitive binding assay to mbTNFα using MT-3 cells are shown. Each point represents results from testing a unique lot. *Adalimumab (EU)* EU-authorized adalimumab, *adalimumab (US)* FDA-licensed adalimumab, *mbTNFα* transmembrane tumor necrosis factor α, *MT-3* Chinese hamster ovary cells expressing non-cleavable mbTNFα

mbTNFα for three tested lots of ABP 501 was 100–106 %, for three lots of adalimumab (US) was 100–111 %, and for three lots of adalimumab (EU) was 101–109 % (Fig. 3). With the observed overlap in the results when comparing ABP 501 with adalimumab (US) and adalimumab (EU), the binding to mbTNFα was concluded to be similar.

3.2 Neutralization of TNFα Bioactivity Is Similar Between ABP 501 and Adalimumab

The primary mechanism of action of adalimumab is the inhibition of proinflammatory signaling induced by sTNFα. TNFα has been shown to induce apoptosis in cells, especially under conditions in which NFκB activity is reduced [8]. As a well established measure of TNFα activity in vitro, the potency of ABP 501 was compared with the potency of adalimumab (US) and adalimumab (EU) by testing the inhibition of TNFα-induced apoptosis in U937 cells. Apoptosis was assessed as caspase 3/7 activation. As shown in Fig. 4a, the potency of ABP 501 is similar to that of adalimumab (US) and adalimumab (EU). The mean ABP 501 relative potency was 103.77 % and mean adalimumab (US) relative potency was 105.50 %, with the difference between means of −1.73 % (90 % CI −5.17 to 1.72), which is within the EAC of ±8.64 % for the difference from adalimumab (US). The mean relative potency for adalimumab (EU) was 102.83 %, with a difference from ABP 501 of 0.94 % (90 % CI −4.42 to 6.29), which is within the ±14.04 % EAC for the difference from adalimumab (EU). Therefore, APB 501 is statistically equivalent to adalimumab (US) and to adalimumab (EU).

TNFα is known to induce a proinflammatory cascade of cytokine and chemokine induction, which largely explains the effectiveness of TNFα inhibition in treating autoimmune disease. To further characterize the similarity in neutralization of TNFα, the ability of ABP 501, adalimumab (US), and adalimumab (EU) to inhibit TNFα-induced IL-8 secretion in HUVEC was assessed, testing three lots of each test mAb. ABP 501 inhibited TNFα-induced IL-8 secretion from HUVEC with EC_{50} values ranging from 192 to 294 pM, which were of a similar range to that observed for adalimumab (US) (131–253 pM) and adalimumab (EU) (168–225 pM). Dose response results from a representative assay are shown in Fig. 4b. In order to confirm the specificity of ABP 501, adalimumab (US), and adalimumab (EU), the mAb samples were shown to be unable to inhibit LTα-induced IL-8 production (Fig. 4b). The inhibition of chemokine induction, including specificity against LTα, is similar between ABP 501, adalimumab (US), and adalimumab (EU).

In addition to inducing proinflammatory cytokine/chemokine production and apoptosis, TNFα can induce non-apoptotic cell death. As further characterization, the ability of ABP 501, adalimumab (US), and adalimumab (EU) to inhibit TNFα-induced cell death in L929 cells was tested. Three lots of each mAb were compared. ABP 501 inhibited recombinant human TNFα-induced cell death with EC_{50} values ranging from 240 to 511 pM for the three lots, compared with 284–544 pM for the lots of adalimumab (US) and 294–407 pM for the lots of adalimumab (EU). A representative dose–response curve from one lot of each of the test mAbs for the inhibition of TNFα-induced cytotoxicity is presented in Fig. 4c. The ability of ABP 501, adalimumab (US), and adalimumab (EU) to inhibit TNFα-induced cell death is similar.

3.3 Fc Receptor Binding is Similar Between ABP 501 and Adalimumab

FcγRs play a critical role in regulating immune responses. Signaling through this receptor family can result in cytokine release, modulation of cell activation, apoptosis, phagocytosis, and ADCC. FcγRIa is the only high-affinity FcγR able to bind monomeric IgG [19]. Crosslinking of FcγRIa results in classical spleen tyrosine kinase (Syk)-mediated downstream signaling [20], but the ultimate functional consequence of FcγRIa signaling is not well understood, so the implications of this binding activity for clinical mechanism of action are not established. The similarity in binding to FcγRIa was compared between ABP 501, adalimumab (US), and adalimumab (EU) in a competitive AlphaLISA binding assay. Mean (from three independent experiments) percent relative binding values for the FcγRIa AlphaLISA binding assay ranged from 96 to 99 % for the ABP 501 lots, 92–96 % for the adalimumab (US) lots, and 92–94 % for the adalimumab (EU) lots.

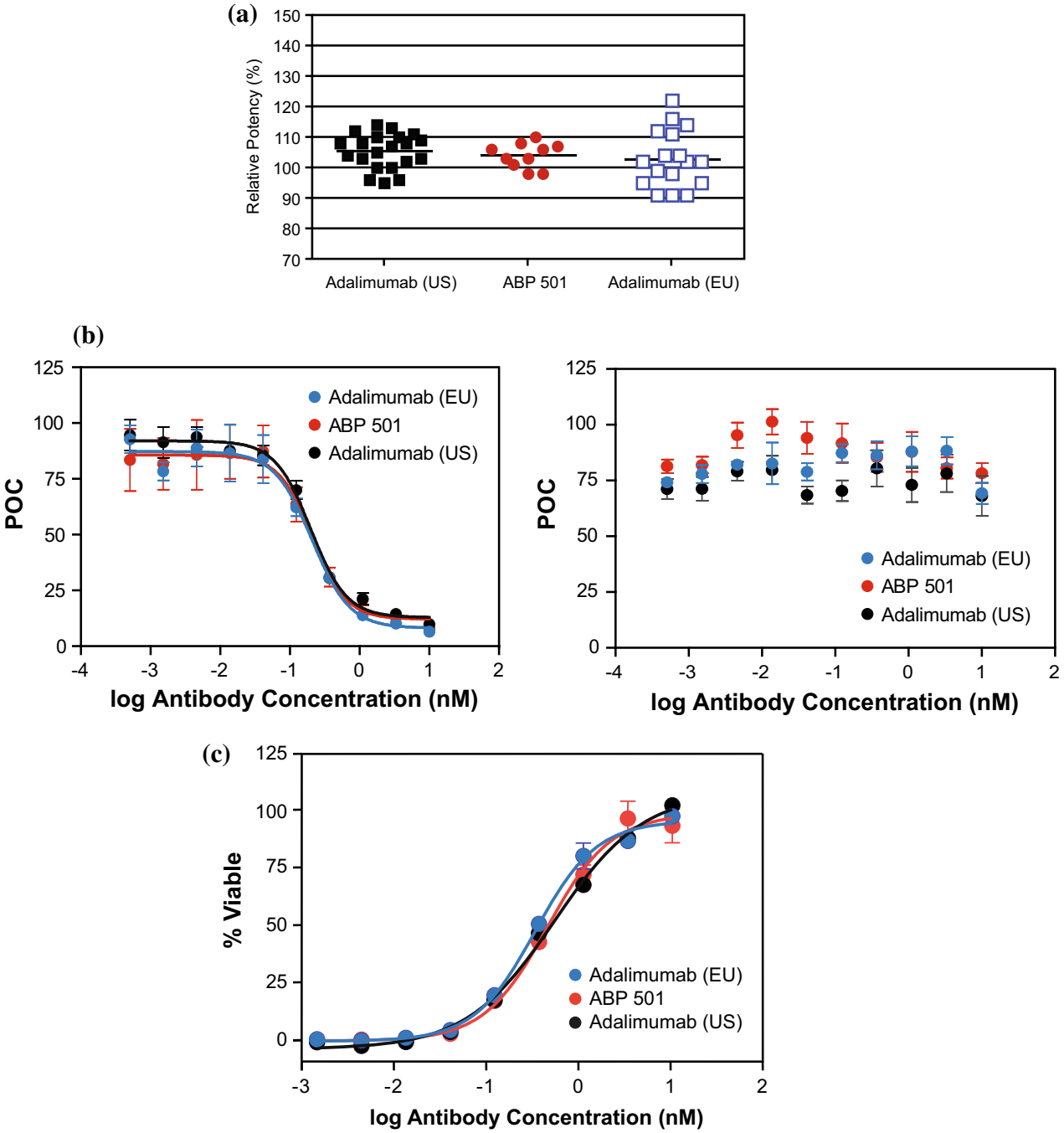

Fig. 4 Similarity of ABP 501, adalimumab (US), and adalimumab (EU) in TNFα-induced functional assays. **a** Inhibition of TNFα-induced caspase activation in U937 by ABP 501 (*red*), adalimumab (US) (*black*), and adalimumab (EU) (*blue*). Each point represents results from testing a unique lot. The lines represent the mean relative potency for that sample. **b** Inhibition of human TNFα-induced IL-8 secretion in HUVEC. Titration of ABP 501 (*red*), adalimumab (US) (*black*), and adalimumab (EU) (*blue*) against 3 ng/mL TNFα-stimulated IL-8 production in HUVEC (left) or 10 ng/mL LTα-stimulated IL-8 production in HUVEC (right). Results depicted are from a representative assay. Each point represents the mean POC of triplicates ± SEM. **c** Inhibition of human TNFα-induced cytotoxicity in L929 cells. Dose-responsive inhibition of 0.75 ng/mL TNFα-stimulated cytotoxicity in L929 cells, showing a titration of ABP 501 (*red*), adalimumab (US) (*black*), and adalimumab (EU) (*blue*). Representative dose–response curves are shown with each point representing the mean percent viable cells for triplicates ± SEM. *Adalimumab (EU)* EU-authorized adalimumab, *adalimumab (US)* FDA-licensed adalimumab, *HUVEC* human umbilical vein endothelial cells, *IL-8* interleukin-8, *LTα* lymphotoxin-α, *POC* percent of control, *SEM* standard error of the mean, *TNFα* tumor necrosis factor α

Representative (from one of three experiments) dose response overlay curves are shown in Fig. 5a. The results demonstrate that the relative FcγRIa binding activities of ABP 501, adalimumab (US), and adalimumab (EU) are similar.

FcγRIIa is a low-affinity FcγR expressed on myeloid and some lymphoid cells, including mast cells, macrophages, monocytes, dendritic cells, neutrophils, and platelets. There are two allelic variants of FcγRIIa, expressing either arginine (high responder, low affinity) or histidine (low responder, high affinity) at position 131 [19]. Since FcγRIIa can mediate phagocytosis and platelet activation, and the affinity of FcγRIIa binding can be impacted by post-translational modifications and aggregate levels, an FcγRIIa (131H) competitive AlphaLISA binding assay was used to compare ABP 501, adalimumab (US), and adalimumab (EU). The mean (from three independent experiments) percent relative binding values for the FcγRIIa AlphaLISA binding assay ranged from 95 to 107 % for the ABP 501 lots, 101–105 % for the adalimumab (US) lots, and 96–100 % for the adalimumab (EU) lots. Representative (one of three experiments) dose–response curves from the FcγRIIa binding assay are shown in Fig. 5b. The results demonstrate that the relative FcγRIIa binding activities of ABP 501, adalimumab (US), and adalimumab (EU) are similar.

FcγRIIIa is a pro-inflammatory receptor expressed on human natural killer cells, and is involved in the induction of ADCC. A genetic polymorphism in FcγRIIIa results in expression of valine (V, high affinity) or phenylalanine (F, low affinity) at amino acid 158. Binding of ABP 501, adalimumab (US), and adalimumab (EU) to both variants was tested. The binding of ABP 501, adalimumab (US), and adalimumab (EU) to FcγRIIIa (158V), the high-affinity allotype, was determined for $\geq$10 lots of each test mAb. Similar activity was observed as illustrated in the representative dose response curve in Fig. 5c. The mean (from three independent experiments) percent relative binding values for the FcγRIIIa (158V) AlphaLISA binding assay ranged from 67 to 113 % for the ABP 501 lots, 76–114 % for the adalimumab (US) lots, and 86–104 % for the adalimumab (EU) lots. Furthermore, ABP 501 was statistically similar in that the relative binding values of the ABP 501 lots fell within the quality range established based on the adalimumab lots tested.

Human monoclonal antibodies to TNFα, including adalimumab, have been shown to form higher-order complexes with TNFα [21, 22]. These complexes can form because bivalent antibodies can bind two different TNFα molecules and trimeric TNFα molecules can bind multiple antibody molecules. The higher-order complexes that form can increase the apparent affinity to Fc receptors involved in mediating effector functions due to the increase in avidity. In order to assess the impact of antibody/TNFα complex formation on the Fc effector function potential of ABP 501, adalimumab (US), and adalimumab (EU), the FcγRIIIa AlphaLISA binding assay was performed in the presence of an equimolar concentration of recombinant sTNFα, testing a single lot of each mAb. In the presence of TNFα, the mean (three independent experiments) percent relative binding to FcγRIIIa (158V) was 108 % for ABP 501, 101 % for adalimumab (US), and 113 % for adalimumab (EU). Representative dose–response curves from the binding assays with and without TNFα addition are shown in Fig. 5c. As expected, an affinity shift is observed in the presence of TNFα, but the binding of ABP 501, adalimumab (US), and adalimumab (EU) are similar to each other in each experiment.

Relative binding to the FcγRIIIa (158F) allotype was also determined. Mean percent relative binding in the FcγRIIIa (158F) AlphaLISA assay (in the absence of TNFα) ranged from 73 to 93 % for the three ABP 501 lots; 83–95 % for the three adalimumab (US) lots; and 88–98 % for the three adalimumab (EU) lots. Representative (one experiment of the three performed) dose–response curves from the binding assay are shown in Fig. 5d. The results demonstrate that the relative binding of ABP 501, adalimumab (US), and adalimumab (EU) to FcγRIIIa (158F) is similar.

The FcRn binds IgG heavy chains in the Fc region under mildly acidic conditions (pH 6) and releases IgG at neutral pH (7.4). It is through this highly pH-dependent interaction that FcRn mediates IgG homeostasis in human adults through recycling of IgG back into the serum. A cell-based FcRn binding assay using a variant of the human embryonic kidney cell line overexpressing human FcRn (293T-7A1) was used to test the binding of the Fc moiety of ABP 501, adalimumab (US), and adalimumab (EU) to FcRn. Mean (from three independent experiments) percent relative binding of ABP 501, adalimumab (US), and adalimumab (EU) to FcRn was similar, with relative binding values ranging from 86 to 101 % for the ABP 501 lots, 91–114 % for the adalimumab (US) lots, and 81–116 % for the adalimumab (EU) lots. Representative (one of three experiments performed) dose–response overlay results are shown in Fig. 5e. Statistical evaluation of the results demonstrate that the relative binding to FcRn is similar between ABP 501, adalimumab (US), and adalimumab (EU), since relative binding of the ABP 501 lots fell within the quality range established based on the adalimumab lots tested.

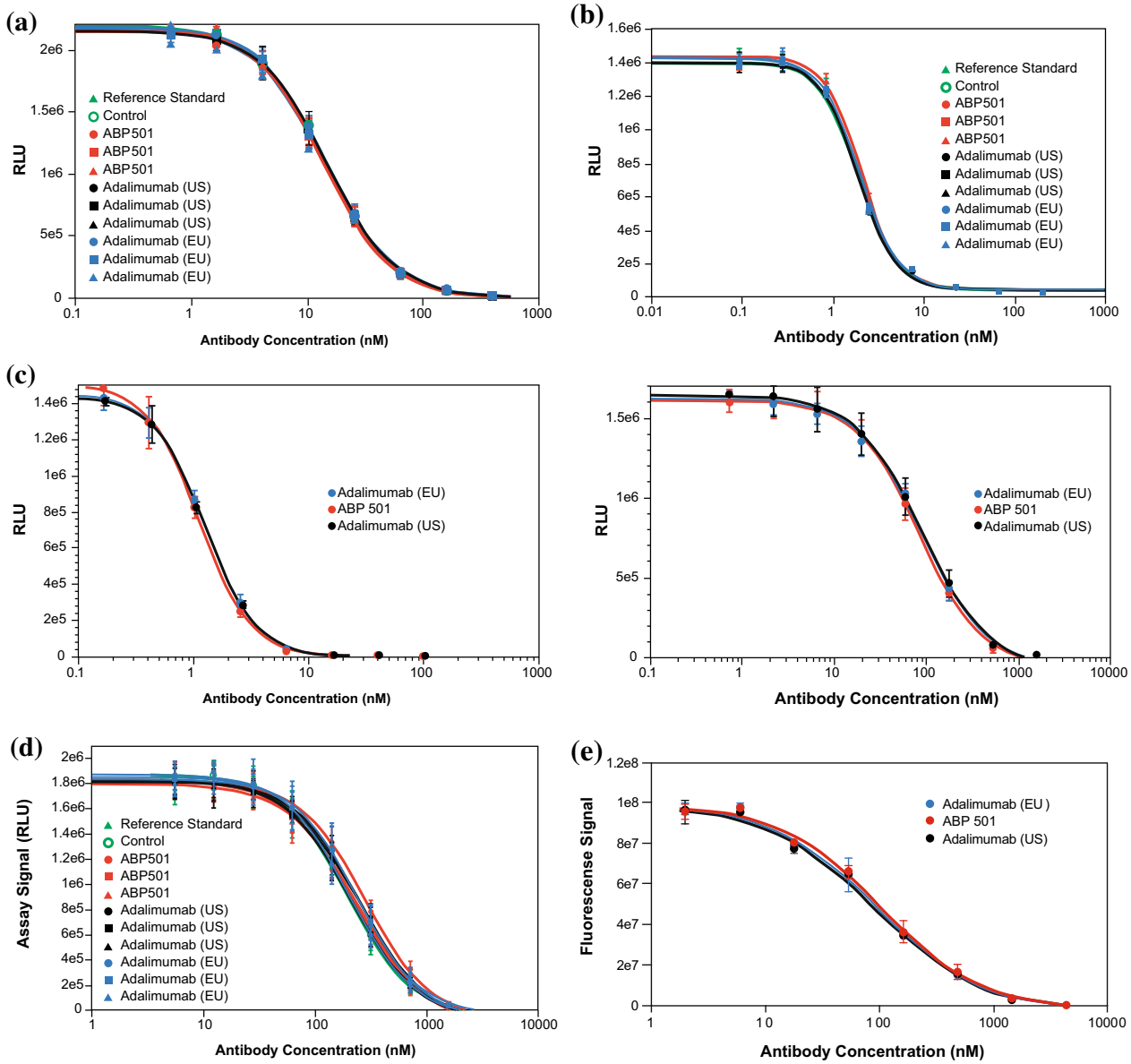

Fig. 5 Similarity of ABP 501, adalimumab (US) and adalimumab (EU) in Fc-gamma receptor (FcγR) binding. **a** Representative non-constrained dose–response curves of reference standard and control (*green*), ABP 501 (*red*), adalimumab (US) (*black*), and adalimumab (EU) (*blue*) showing binding to FcγRIa. Three different lots of each mAb were tested and each dose point represents the mean of three intra-assay replicates ± standard deviation. A total of three independent assays were conducted and the mean percent relative activity is reported (see text). A dose response from a single assay of the three is presented here as representative data. **b** Representative nonconstrained dose–response curves of reference standard and control (green), ABP 501 (*red*), adalimumab (US) (*black*), and adalimumab (EU) (*blue*) showing binding to FcγRIIa. Three different lots of each mAb were tested and each dose point represents the mean of three intra-assay replicates ± standard deviation. A total of three independent assays were conducted, and the mean percent relative activity is reported (see text). A dose response from a single assay of the three is presented here as representative data. **c** Representative nonconstrained dose–response curves of ABP 501 (*red*), adalimumab (US) (*black*), and adalimumab (EU) (*blue*) showing FcγRIIIa (158V) binding with (left) and without (right) TNFα. Each dose point represents the mean of three intra-assay replicates ± standard deviation. **d** Representative nonconstrained dose–response curves of reference standard and control (*green*), ABP 501 (*red*), adalimumab (US) (*black*), and adalimumab (EU) (*blue*) showing binding to FcγRIIIa (158F). Three different lots of each mAb were tested and each dose point represents the mean of three intra-assay replicates ± standard deviation. A total of three independent assays were conducted, and the mean percent relative activity is reported (see text). A dose response from a single assay of the three is presented here as representative data. **e** Representative nonconstrained dose–response curves of ABP 501 (*red*), adalimumab (US) (*black*), and adalimumab (EU) (*blue*) showing binding to FcRn. Each point is a mean of three replicates ± standard deviation. *Adalimumab (EU)* EU-authorized adalimumab, *adalimumab (US)* FDA-licensed adalimumab, *FcRn* Fc neonatal receptor, *mAb* monoclonal antibody, *RLU* relative luminescence units, *TNFα* tumor necrosis factor α

3.4 Induction of Effector Function is Similar Between ABP 501 and Adalimumab

Adalimumab is able to mediate ADCC in vitro [9, 17, 23]. Although the contribution of ADCC activity to clinical efficacy is unclear, it is important to characterize all activities of the candidate mAb, especially those that can be affected by differences in post-translational modifications, such as glycosylation. The ability of ABP 501 to induce ADCC was assessed using MT-3 cells as target cells, and NK-92M1 cells stably transfected with human FcγRIIIa (158V) as effector cells. Mean (three independent experiments) percent relative ADCC activities were determined for ABP 501, adalimumab (US), and adalimumab (EU). As shown in Fig. 6a with the quality range depicted by dotted lines, statistical similarity was demonstrated since all of the ABP 501 lots fell within the quality range established based on the adalimumab (US) lots tested.

Another mechanism for inducing cell death is the induction of CDC in cells expressing mbTNFα. A comparison of the CDC activity of ABP 501 to that of adalimumab (US) and adalimumab (EU) using MT-3 cells as target cells was conducted. Mean (three independent experiments) percent relative CDC activities were similar (Fig. 6b) and since the ABP 501 relative activity results were within the quality range established by the adalimumab (US) lots, the activity is considered statistically similar.

4 Discussion

TNFα is a pleiotropic cytokine that is able to mediate diverse cellular functions in order to finely control the immune response in vivo. Among its cellular functions, TNFα is able to induce cytokines, chemokines, proliferation, and also cell death. The induction of pro-inflammatory versus death signals depends upon the molecular context of the responding cell, and specifically whether NFκB is involved [8]. Adding to the complexity of signaling, it is also reported that TNFα exists in both soluble and transmembrane forms, with differing reported activities in each case. Not surprisingly, given the multifaceted nature of TNFα, many diverse mechanisms of action have been reported for adalimumab and other approved anti-TNF agents [7, 10]. Therefore, a comprehensive assessment of the bioactivity of adalimumab should include assessment of multiple in vitro endpoints (NFκB-dependent and NFκB-independent) and should include binding to both soluble and transmembrane TNFα. ABP 501 has been shown to be similar to adalimumab in its ability to neutralize TNFα-induced caspase activation, chemokine production, and cytotoxicity, functions inclusive of both NFκB-dependent and NFκB-independent pathways. Additionally, similarity has been demonstrated in binding to soluble and transmembrane TNFα, including an assessment of binding kinetics by SPR. Demonstrating no gain of function is also an important aspect in biosimilar

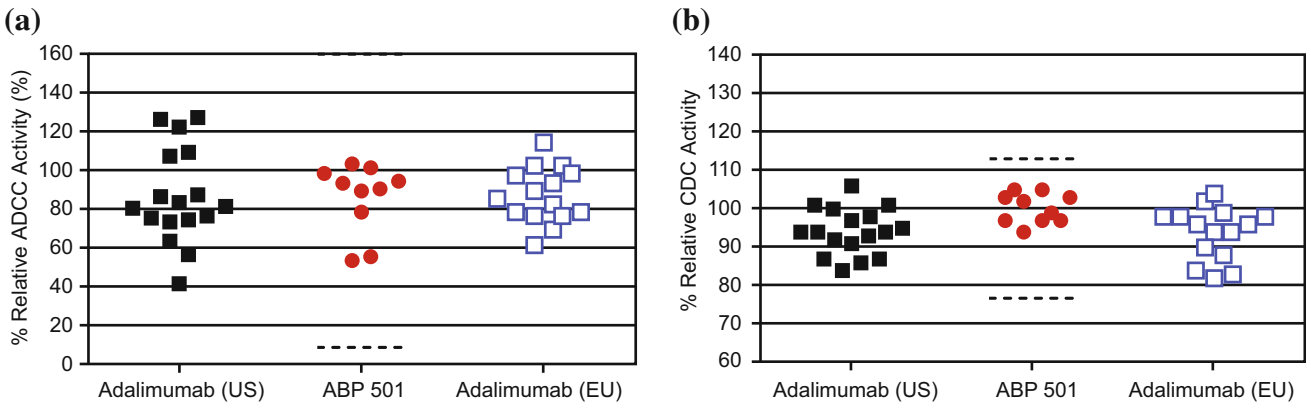

Fig. 6 Similarity of ABP 501, adalimumab (US), and adalimumab (EU) in effector function activity. **a** Induction of ADCC by ABP 501 (*red*), adalimumab (US) (*black*), and adalimumab (EU) (*blue*). Each point represents results from testing a unique lot. The dotted lines represent the quality range established based on the adalimumab (US) lots tested (mean ± 3 standard deviations). *Adalimumab (EU)* EU-authorized adalimumab, *adalimumab (US)* FDA-licensed adalimumab, *ADCC* antibody-dependent cell-mediated cytotoxicity. **b** Induction of CDC by ABP 501 (*red*), adalimumab (US) (*black*), and adalimumab (EU) (*blue*). Each point represents results from testing a unique lot. The dotted lines represent the quality range established based on the adalimumab (US) lots tested (mean ± 3 standard deviations). *Adalimumab (EU)* EU-authorized adalimumab, *adalimumab (US)* FDA-licensed adalimumab, *CDC* complement-dependent cytotoxicity

development and the results presented here show that both ABP 501 and adalimumab are unable to neutralize the bioactivity of LTα, the most closely related cytokine to TNFα. The methods used to demonstrate functional similarity in the fragment antigen-binding (Fab) region of the mAb were shown to be able to discriminate a thermally degraded sample (data not shown), demonstrating that the utilized assays are sensitive to detect differences in activity, if they did exist.

It is well established that IgG1 mAbs are efficient mediators of effector function, and are able to bind to many of the known FcγRs. A sensitive comparison of these Fc-dependent activities is important in biosimilar development, since glycosylation and other product quality attribute differences are to be expected between biosimilars and reference products due to inherent cell line differences (even in the same cell expression system) and the proprietary nature of manufacturing. ABP 501 has been shown to be similar to adalimumab with respect to binding to a panel of Fc receptors, including FcγRIa, FcγRIIa, FcγRIIIa (158V) (with and without TNFα), and FcγRIIIa (158F). Importantly, effector function activation (ADCC and CDC) was also demonstrated to be similar between ABP 501 and adalimumab using highly sensitive methods. The ADCC and CDC methods have been demonstrated to be sensitive to detect differences in the glycan profile of the mAbs (data not shown). Although effector function is not known to be directly associated with the clinical efficacy of adalimumab, it cannot be ruled out conclusively, especially in inflammatory bowel disease. The efficacy of the TNFα-neutralizing Fab certolizumab is reduced in Crohn's disease relative to the efficacy observed with adalimumab [24, 25], which suggests that Fc-mediated effector functions may be important. Binding of mAbs to FcRn affects clearance, so a similarity assessment of biosimilars should also include sensitive methods to assess binding to FcRn. ABP 501 was shown to have similar binding to FcRn as compared with adalimumab.

Given the similarity in analytical characteristics as reported separately (Liu et al. [16]) and biofunctional activity demonstrated here, ABP 501 is expected to be clinically similar to the adalimumab reference product without any clinically meaningful differences. Clinical data will help to confirm the safety and efficacy profile of ABP 501. In a phase I human pharmacokinetic study, ABP 501 has been shown to be similar to adalimumab (US) and adalimumab (EU) [26, 27]. Clinical studies designed to assess the similarity of ABP 501 relative to the adalimumab reference product for the treatment of moderate to severe plaque psoriasis [28, 29] and moderate to severe rheumatoid arthritis [30, 31] have been completed.

5 Conclusion

During the stepwise development of a biosimilar mAb, it is crucial that analytical and biofunctional similarity to the reference product be demonstrated with sensitive, state-of-the-art methods that exhaustively examine all potential regions of the mAb and mechanisms of action. This is often an iterative process in which analytical similarity is tested and then further explored in preclinical biofunctional tests to determine whether minor differences, should they exist, result in differences in the biological activity of the molecule (Fig. 1). Confidence in the similarity of the molecule at this foundational step allows for a reduction in residual uncertainty as the molecule progresses into clinical testing for equivalence [2, 13, 15, 32]. The current data, as presented, demonstrate that ABP 501 is similar to adalimumab (US) and adalimumab (EU) with respect to multiple analytical (Liu et al. [16], includes additional data on potency as measured in multiple lots) and biofunctional parameters. The biofunctional parameters tested include an assessment of both binding and function within both the Fab and Fc portions of the mAb.

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Compliance with Ethical Standards

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Conflict of interest Jyoti Velayudhan, Amanda Rohrbach, Christina Pastula, Heather Thomas, and Ryan Brown are former employees and stockholders of Amgen, Inc. Yuh-feng Chen, Gwen Maher, and Teresa L. Born are employees and stockholders of Amgen, Inc.

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