Target-independent variable region mediated effects on antibody clearance can be FcRn independent

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ABSTRACT

The importance of the neonatal Fc receptor (FcRn) in extending the serum half-life of monoclonal antibodies (mAbs) is well demonstrated, and has led to the development of multiple engineering approaches designed to alter Fc interactions with FcRn. Recent reports have additionally highlighted the effect of nonspecific interactions on antibody pharmacokinetics (PK), suggesting an FcRn-independent mechanism for mAb clearance. In this report we examine a case study of 2 anti-interleukin-12/23 antibodies, ustekinumab and briakinumab, which share the same target and Fc, but differ in variable region sequences. Ustekinumab displayed near baseline signal in a wide range of early stage developability assays for undesirable protein/protein interactions, while briakinumab showed significant propensity for self- and cross-interactions. This phenotypic difference correlates with faster clearance rates for briakinumab in both human FcRn transgenic and FcRn knockout mice. These findings support a dominant contribution for FcRn-independent clearance for antibodies with high nonspecificity, and highlight a key role for early stage developability screening to eliminate clones with such high nonspecific disposition PK.

Introduction

Improvements in antibody selection technologies have led to robust development of high affinity antibodies against arbitrary targets; however, the process of advancing a lead candidate toward the clinic entails risks beyond bioactivity. An extensive but largely unwritten history of promising drugs failing due to poor developability supports a considerable need to develop monoclonal antibodies (mAbs) that display not only desirable binding characteristics, but also favorable biophysical properties. One key consideration is antibody pharmacokinetics (PK), which correlates to therapeutic efficacy.1,2 Antibody PK can be largely influenced by dose-dependent target mediated drug disposition (TMDD), as well as dose-independent recycling as a result of interaction with FcRn.3,4 FcRn mediates the recycling of IgG by binding tighter to the Fc portion in the endosome at pH (6-6.5) and then releasing IgG quickly into plasma at neutral pH (7-7.5).5 The residues of the IgG1 Fc portion that interact with FcRn are well established.6-8 and as such many efforts have focused on improving FcRn affinity to improve PK.9-14 In addition to Fc engineering efforts, multiple studies have reported disparate PK parameters in antibodies with identical Fc regions but different variable regions.15-20 In particular, clearance rates are increased in antibodies with polyreactive21-23 or cross-reactive24,25 profiles.

Studies analyzing how the variable region may influence FcRn affinity with PK consequences have generally concluded that a slower dissociation at neutral pH (7.3) is predictive of shorter in vivo half-life.17,19,20 Affinity measurements in many of these experiments use surface plasmon resonance (SPR) or biolayer interferometry (BLI), conjugating FcRn to the sensor surface. As has been noted in previous studies, measurements for equivalent antibodies can vary significantly if great care is not taken.14,26 and additionally the presence of a very small amount of aggregate can have a noticeable effect on apparent affinity measurement.27

In this study, we aimed to establish whether Fv region nonspecificity can accelerate systemic clearance in vivo by entirely FcRn-independent mechanisms. We focused on 2 mAbs, briakinumab and ustekinumab, which share the same target, the p40 subunit of human interleukin (IL)-12 and IL-23, and here are expressed as an IgG1 isotype using the same heavy chain constant region. This provides a matched case in which all differences are target independent and driven solely by the variable region. Despite the similarity between the 2 candidates, in Phase 1 clinical trials ustekinumab had a median half-life of 21 days, comparable to most clinical mAbs, while the half-life of briakinumab was only 8 d.28 To evaluate the causes for this large discrepancy in PK, we ran a wide range of early developability screening assays, revealing a strong increase in self- and cross-interaction tendency for briakinumab. This developability profile translates into increased clearance rates in both huFcRn transgenic and FcRn knockout mice, demonstrating an
FcRn-independent mechanism for clearance driven by antibody nonspecificity.

Results

Application of early stage developability assays

Briakinumab and ustekinumab were expressed recombinantly as IgG1 isotypes using published variable region sequences. To isolate any differences to the variable regions, both antibodies were expressed with a common Fc region. To further understand the relative contributions of FcRn affinity and other biophysical properties to PK properties, ustekinumab and briakinumab were subjected to a panel of early stage screening assays. These included 3 cross-interaction related assays (cross interaction chromatography (CIC), baculovirus ELISA, and polyspecificity reagent (PSR) binding assay), as well as one self-interaction related assay (affinity capture self-interaction nanoparticle spectroscopy (AC-SINS)). The CIC plots for the 2 antibodies are shown in Fig. 1 and assay results are summarized in Table 1. Briakinumab has a longer retention time on the CIC column (10.3 vs. Eight.5 min), scores higher on the BVP assay (7.9 vs. Three.9), and has a higher PSR median fluorescence intensity (1938 vs. 271); taken together, these data indicate a significantly increased tendency for nonspecific interactions for briakinumab relative to ustekinumab. Additionally, briakinumab displays a higher tendency for self-interactions, with a greater wave length shift in the AC-SINS assay (31.1 vs. One.6 nm). Compared to the antibody sets used in previous studies, ustekinumab scores close to baseline in all 4 assays, while briakinumab scores above the predictive threshold for poor developability in all assays.

FcRn affinity measurement via biolayer interferometry

In addition to the early stage developability assays, we assessed the affinity of both antibodies toward FcRn as well as other antigens via BLI on a ForteBio Octet HTX system. For all assays, the antigen (FcRn) was biotinylated and loaded onto streptavidin tips, and then dipped in solution containing the test antibody for association. To mimic the physiological process, association was measured in pH 6.0 antibody solution while dissociation was measured in pH 7.3 buffer. As a reference, we additionally measured the affinity of adalimumab, a marketed antibody.

All three antibodies displayed similar association rates, while briakinumab showed a higher maximum response and decrease in dissociation rate, leading to an overall apparent increase in apparent affinity (Fig. 2A, Table 2). This trend was similar when both association and dissociation were measured in either pH 6.0 or pH 7.3 buffer (Fig. S1). A previous report suggested that this increase in apparent affinity may be due to charge driven interactions between the variable region of briakinumab and FcRn, so we additionally assessed the affinity of F(ab’)2 constructs. In concordance with the previous finding, briakinumab displayed binding to FcRn regardless of the buffer pH, while neither of the other antibodies displayed any appreciable binding (Fig. 2B; Fig. S2 A-C ). Additionally, binding was measured in a high salt buffer, where charge-charge interactions are partially shielded. Response levels were weakened, suggesting that charge does indeed play some role in this binding interaction (Fig. S2D-F).

To further explore the nature of this interaction, we next assessed the specificity of this interaction by evaluating binding to a polyclonal antibody mixture, similar to the CIC assay, as well as hen egg lysozyme (HEL) and blank SA tips. While again there was little signal for ustekinumab and adalimumab, there was significant signal for briakinumab when assayed against polyclonal IgG (Fig. 2C), HEL (Fig. S4), as well as SA (Fig. 2D). This binding was mostly independent of pH, but again was damped in the presence of high salt (Figs. S3-5). Qualitative similarity of the briakinumab/FcRn interaction (Fig. 2A) to the mAb’s interaction with completely irrelevant proteins (Figs. 2C, D, and Fig. S4) predicts that, in the complex in vivo environment, it is highly unlikely that FcRn binding could drive tissue adsorption over and above the numerous interactions that will occur with cell and tissue surfaces.

Measurement of pharmacokinetics in FcRn knockout mice

Neither ustekinumab nor briakinumab binds to the mouse variant of IL-12/23, allowing for evaluation of PK parameters in the absence of TMDD. Previous work has shown significantly different PK parameters for ustekinumab and briakinumab in human FcRn transgenic mice. This study used clinical preparations of the 2 antibodies with similar, but not identical, constant regions, and these results are reproduced in Fig. 3A (the results depicted highlight the early time points for more direct comparison to the data in Fig. 3B.) In order to assess whether differential clearance occurs in the complete absence of FcRn, both antibodies were administered via tail vein injection into FcRn knockout (KO) mice. As expected, both antibodies showed accelerated clearance rates compared to wild type mice (Fig. 3B, Table 3), presumably due to nonspecific tissue

Table 1. Early stage self- and cross-interaction assays.

|                     | Briakinumab | Ustekinumab | Cutoff threshold |
|---------------------|-------------|-------------|-----------------|
| AC-SINS Δλmax (nm)  | 31.1        | 1.6         | 5.0             |
| BVP Score           | 7.9         | 3.9         | 5.0             |
| CIC Retention time (min) | 10.3     | 8.5         | 10.0            |
| PSR MFI             | 1938        | 271         | 500             |

Figure 1. Cross-interaction chromatography. Chromatograms for ustekinumab (dashed line) and briakinumab (solid line). Dotted line at 8.6 minutes represents the retention time of the reference antibody, adalimumab.
Comparing the 2, briakinumab cleared at a faster rate than ustekinumab (207.0 ± 25.0 mL/day/kg vs 124.3 ± 31.1 mL/day/kg). This difference can mainly be attributed to a shorter terminal half-life (9.7 ± 0.3 hr vs 12.2 ± 0.5 hr). Both of these differences are statistically significant (p = 0.02 and p = 0.002, respectively).

**Discussion**

Previously, we have shown a correlation between high self- and cross-interaction measures and clearance rates in mice. While this earlier study did not explicitly determine the influence of FcRn affinity on clearance rates, all antibodies were expressed with a common constant region and any variation in behavior was driven entirely by variable region differences. This implied the phenotype was FcRn independent, but we were unable to definitively conclude this because variable region mediated differences in FcRn affinity were still formally possible.

In this case study, we demonstrate that 2 antibodies, which bind to the identical antigen and are expressed with the same Fc region, show significantly different development profiles. In all self- and cross-interaction assays, ustekinumab performs similarly to another well-behaved clinical antibody, while briakinumab displays strong signs of poor developability, scoring above thresholds for poor properties in all assays. In binding experiments with FcRn, association rates were similar between the 2 antibodies, while the dissociation rate for briakinumab was slower. This apparent decrease in off rate may be explained by the nonspecific nature of the antibody, as there was comparable binding of briakinumab to polyclonal goat antibodies, HEL, and unloaded SA tips. These issues translate to PK studies, where the 2 antibodies have significantly different clearance rates both in the presence and absence of FcRn, suggesting that nonspecificity drives the accelerated clearance of briakinumab.

In a previous analysis of these 2 antibodies, it was suggested that a positively charged patch in the variable region of briakinumab led to an increased affinity for FcRn via slower dissociation, measured by retention time on an agarose column coated with FcRn. No negative control experiment was reported determining retention time on an uncoated column to gauge the contribution of nonspecific interactions with the streptavidin sepharose itself (e.g., as reported here in Fig. 2D). Given the poor developability profile of briakinumab and its extended retention time on the highly analogous CIC assay, it appears likely that the longer retention time is largely due to increased nonspecific interactions with the column rather than specific FcRn interactions. This is further supported by appreciable binding to a wide variety of antigens in BLI

![Figure 2. BLI binding experiments. Octet sensorgrams of binding experiments between human IgG1 and FcRn (A) or human F(ab')2 and FcRn (B), goat polyclonal IgG (C), or blank SA tips (D). For all plots antibodies are referenced as follows: ustekinumab (dashed lines), briakinumab (solid lines), and adalimumab (dotted lines). In all experiments, association was measured at pH 6.0 and dissociation at pH 7.3.](image-url)

| Table 2. Affinity measurements by BLI. |
|-------------------------------------|
| k_{on} (M^{-1}s^{-1}) at pH 6.0 | 1.8 × 10^{3} | 1.4 × 10^{3} | 1.2 × 10^{3} |
| k_{off} (s^{-1}) at pH 7.3 | 0.5 × 10^{-1} | 0.1 × 10^{-1} | 0.1 × 10^{-1} |
| Effective K_{D} (\mu M) | 0.27 | 0.72 | 0.80 |

*Note: Effective K_{D} is defined as \( \frac{k_{on}}{k_{off}} \) where the on rate is measured at pH 6.0 and the off rate is measured at pH 7.3.*

| Table 3. Pharmacokinetic parameters in FcRn KO mice. |
|---------------------------------|
| Terminal Half-life (hr) | 9.8 ± 0.3 | 13.8 ± 0.5 |
| AUC (\mu g·day/L) | 241.8 ± 2.2 | 399.3 ± 10.1 |
| Clearance (mL/day/kg) | 207.1 ± 2.4 | 125.4 ± 31.9 |

The phenotype was FcRn independent, but we were unable to definitively conclude this because variable region mediated differences in FcRn affinity were still formally possible.
Figure 3. Pharmacokinetic analysis. (A) Data reproduction of pharmacokinetic study in hFcRn transgenic mice from Schoch et al. Only early time points are displayed to highlight initial distribution kinetics of ustekinumab (gray, dashed line) versus briakinumab (black, solid line). (B) Pharmacokinetic study in FcRn KO mice. Serum concentration levels for single-bolus intravenous dosing of ustekinumab (gray, dashed line) and briakinumab (black, solid line) were assessed in 3 mice each. Each time point represents the average serum concentration for the 3 mice, and these averages were fit for each antibody using a 2 phase non-compartmental decay curve. Error bars represent standard error.

experiments, including SA, the coating for the beads used in FcRn affinity chromatography. While the affinity column could be useful for evaluating differences in FcRn affinity, negative controls are important in order to differentiate specific binding from other nonspecific interactions, which may be dominant, as in this case. Misattribution of the result of increased FcRn-column retention time to a specific protein/protein interaction could lead to attempts to engineer out that specific interaction, when in fact a more general polyreactivity was the root cause for increased column retention.

Looking at the cause of nonspecificity in briakinumab, the charge dependence of this phenotype was consistent with our BLI experiments, and is consistent with previous findings that positively charged patches can lead to increased nonspecific binding to cells and other proteins. These results are also consistent with a similar study using the IgG4 isotype, in which a higher pI led to a shortened half-life and higher clearance rates regardless of presence of FcRn. Studies on larger sets of antibodies suggest that the correlation between pI and clearance rates is not generalizable; however, a localized patch of positive charge may be sufficient to increase interactions with the negatively charged surface of cell membranes. Nonspecificity driven clearance is not solely charge dependent, and other factors, including hydrophobicity, have been implicated in driving accelerated clearance rates.

The exact mechanism that drives accelerated clearance in vivo in the absence of FcRn remains to be fully evaluated, but some recent studies can provide clues. Datta-Mannan et al. recently demonstrated increased uptake and catabolism in cell culture assays using nonspecific mAbs, indicating an increased net rate of fluid phase endocytosis/pinocytosis and likely sequestration from recycling salvage pathways. Additionally, when these mAbs were administered to mice, they rapidly accumulated in the liver, likely a result of nonspecific tissue binding and cell uptake. Computational efforts to create a physiologically based PK model for IgG biodistribution and clearance have also pointed to the modest effect of changing FcRn affinity, suggesting that endocytic uptake parameters can have a more dramatic effect on observed half-lives. In cases where TMDD and nonspecific binding were both present, these phenotypes dominated and masked any PK improvements from a known Fc region point mutation that improves FcRn-related recycling.

Our findings, together with previous reports, support 3 major factors that can affect mAb clearance rates: TMDD, nonspecific binding, and FcRn affinity. TMDD effects may be ameliorated by engineering pH-dependent binding, and nonspecific binding can be modulated by antibody engineering. The somewhat limited success of engineering efforts to improve antibody clearance PK by modulating FcRn affinity may be in many instances attributable to dominant nonspecific tissue clearance mechanisms that operate independently of FcRn recycling, as demonstrated for the antibodies in this report. Eliminating sticky binders early in the library-screening portion of the discovery process helps forestall more arduous repairs of individual clones later in development.

Materials and methods

Antibody production and purification

Adalimumab, briakinumab and ustekinumab were expressed recombinantly as IgG1 isotypes using published variable region sequences in transient HEK 293 cells. These antibodies were purified by MabSelect SuRe resin (GE Healthcare Life Sciences, Pittsburgh, PA). To obtain F(ab’)2 constructs, antibodies were digested using FabRICATOR (Ides) enzyme (Genovis, Cambridge, MA) following the manufacturer’s instructions. The Fc was removed by MabSelect SuRe resin and the F(ab’)2 was purified by either LambdaFabSelect resin (GE Healthcare Life Sciences, Pittsburgh, PA) or CaptureSelect IgG-CH1 affinity matrix (Thermo Fisher Scientific, Grand Island, NY). All the samples were then buffer exchanged into phosphate buffered saline (1x PBS, 150 mM NaCl, pH 7.3). The purity and molecular weight of each full length IgG and F(ab’)2 were confirmed by size-exclusion chromatography and liquid chromatography-mass spectrometry.

Developability assays

All early stage developability assays (CIC, BVP, PSR binding, AC-SINS) were performed as previously described.24,25,32,33,45
**IgG binding experiment**

To examine if full-length IgGs bind to FcRn in a pH-dependent manner, the FcRn binding experiment was performed using a ForteBio Octet HTX. Recombinant human FcRn (Novoprotein, Summit, NJ) was biotinylated using EZ-Link sulfo-NHS-LC-Biotin (Thermo scientific, Rockford, IL) at 0.5 mg/ml in PBS (pH 7.3). A molar ratio of 1:7 of FcRn to sulfo-NHS-LC-Biotin was mixed and incubated overnight at 4°C. After incubation, excess labeling reagent was removed through buffer exchanging into PBS (pH 7.3) using a 40 kDa Zeba spin desalting column (Thermo scientific, Rockford, IL). The biotinylated-FcRn was loaded to streptavidin (SA) sensors (18-5021, ForteBio, Menlo Park, CA) for 360 s to maximize loading response (~6 nm). b-FcRn loaded SA sensors were dipped into PBS (pH 6.0) for 60 s to establish a baseline, followed by exposures to each IgG solution (200 nM in PBS, pH 6.0) for 180 s for association and then dissociation in PBS (pH 7.3) for 180 s. The IgG binding experiments were also performed under a single pH condition (either pH 6.0 or 7.3) for all baseline, association and dissociation steps.

**F(ab′)2 binding experiments**

To examine if F(ab′)2 binds to FcRn, HEL, goat polyclonal antibodies, and SA, the F(ab′)2 binding experiments were performed using a ForteBio Octet HTX. The goat polyclonal antibodies and HEL were biotinylated in a similar fashion to FcRn. In a F(ab′)2 binding experiment, the biotinylated proteins were loaded onto SA sensors with maximum loading. These sensors with b-FcRn, b-goat polyclonal antibodies, b-HEL or blank SA sensors were first dipped into PBS (pH 6.0) for 60 s to establish a baseline, and then exposed to each F(ab′)2 solution (600 nM in PBS, pH 6.0) for 180 s for association and dissociation in PBS (pH 7.3) for 180 s. The F(ab′2) binding experiments with FcRn, goat polyclonal IgG, HEL and bare SA sensors were repeated in a high salt PBS (1x PBS, 450 mM NaCl) using the same protocol. In addition, experiments with a single pH condition (either pH 6.0 or 7.3) were performed in both PBS and high salt PBS for all the biotinylated proteins.

**Determination of clearance in mice**

Antibody PK data were determined in FcRn− mice (B6.129×1-Fcgrtm1Dcr/Dcr1, Jackson Laboratory stock #003982) using a single bolus intravenous dose. Each antibody was dosed via tail vein injection at 5 mg/kg into 3 mice. Serum samples were collected immediately after injection and at 0.25, 0.5, 1, 3, 5, 8, 24, 48, and 72 hours post injection. Antibody concentration was quantified by ELISA using Meso Scale Discovery (MSD). Measurements were quantified by normalization to a standard curve for each antibody, and PK profiles were fit in Graphpad Prism using a 2 phase non-compartmental model. Fits for the 3 mice in each group were averaged to obtain a single PK curve for each antibody, from which total clearance rate and standard error were calculated.

**Statistical analysis**

All statistical analysis was completed with the assistance of Graphpad Prism v. Six.0. All errors reported are standard error and individual PK parameters were compared using a Students t-test.

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

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