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REVIEW ARTICLE:

Application of FcRn Binding Assays To Guide mAb Development

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Running Title: **FcRn Binding Assays and mAb Development**

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ABSRACT

Monoclonal antibodies (mAbs) represent an important class of therapeutic modalities. In order to optimize their pharmaceutical properties, studies have focused on improving mAb pharmacokinetic/pharmacodynamics (PK/PD) profiles by modulating their interactions with the neonatal Fc receptor (FcRn). The influence of both the chemical and physical properties of IgGs has been examined in the context of FcRn interactions. In this regard, a variety of FcRn binding assays and tools have been developed and used to characterize the interaction with IgGs. However, a predictive relationship between the FcRn binding interaction of IgGs *in vitro* and their pharmacokinetics *in vivo* broadly across mAbs remains elusive. Many studies have increasingly suggested that the interplay between the characteristics of the mAb and the nature of its target can influence disposition and elimination. Thus, it is beginning to become increasingly evident that along with FcRn interactions the consideration of the non-FcRn based biological processes active in mAb disposition should be integrated into mAb development/optimization. Herein, we describe how the pharmacokinetics of mAbs can be modulated through FcRn interactions and provide perspectives on interpreting the receptor binding parameters with other mechanisms involved in antibody disposition to aid in guiding mAb development.

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INTRODUCTION:

Over the last two decades, human or humanized monoclonal antibodies (mAbs) have been widely successful as medicinal options in numerous disease indications including cancer, inflammation, bone, cardiovascular and autoimmune conditions (Chan and Carter, 2010; Weiner et al., 2010). Over 30 antibodies have received FDA approval and hundreds more are currently in clinical development (Tabrizi and Roskos, 2007; Reichert, 2010). The compelling efficacy of these biological agents along with advances in protein engineering and directed evolution strategies has spurred a tremendous effort to optimize their pharmaceutical properties (Roopenian and Akilesh, 2007; Presta, 2008). Tuning the potency/affinity of these agents towards their targets in combination with efforts to optimize their pharmacokinetic/pharmacodynamics (PK/PD) relationships can lead to molecules having improved safety and efficacy profiles. In addition, reducing the dose and/or dose frequency improves patient convenience and increases compliance yielding better therapeutic outcomes. A number of studies have focused on modulating the interaction of mAbs with the neonatal Fc receptor (FcRn) in an effort to advance improvements in these areas (Dall'Acqua et al., 2002; Hinton et al., 2004; Dall'Acqua et al., 2006; Hinton et al., 2006; Datta-Mannan et al., 2007a; Datta-Mannan et al., 2007b; Yeung et al., 2009; Deng et al., 2010; Datta-Mannan et al., 2012a; Datta-Mannan et al., 2012b).

FcRn was first identified in the early to mid-1990's as a protective receptor for IgGs and was proposed to be responsible for the relatively long persistence of IgGs in circulation compared with other endogenous proteins (Ghetie et al., 1996; Ghetie and Ward, 1997). With the increase in the development of IgG-based therapeutics, improving the interaction of mAbs with FcRn was

a logical approach aimed at modulating their PK/PD properties. Mechanistically, it has been proposed that altering the properties of the IgG:FcRn interaction may influence the intracellular trafficking/partitioning of IgGs resulting in changes of IgG peripheral clearance and circulating half-life *in vivo* (Goebl et al., 2008; Ober et al., 2004a; Ober et al., 2004b; Prabhat et al., 2004; Ward et al., 2003; Ward et al., 2005). In this regard, many preclinical studies have focused on understanding aspects of the IgG:FcRn interaction, which can serve as the basis of a rational strategy to guide/inform mAb development. A variety of FcRn binding assays and tools have been developed and used to characterize the interaction with IgGs. However, the ability to define a predictive relationship between the FcRn binding interaction of IgGs *in vitro* and their pharmacokinetics *in vivo* broadly across mAbs remains elusive (Gurbaxani et al., 2006; Suzuki et al., 2010; Datta-Mannan et al., 2012a). Many studies have increasingly demonstrated that an interplay between the characteristics of the antibody (i.e., biophysical properties, antigen affinity, glycosylation, proteolytic stability) and its therapeutic target (membrane-bound or soluble antigen, antigen load) can influence disposition and elimination (Igawa et al., 2010b; Khawli et al., 2010; Yeung et al., 2010; Wang et al., 2011a; Wang et al., 2011b; Bumbaca et al., 2012; Chaparro-Riggers et al., 2012). From this, it has become apparent that the complexity of biological processes involved in mAb disposition must be considered, along with the molecule-to-molecule nature of these factors, to rationally guide antibody engineering and development strategies. In this review, we will discuss how the pharmacokinetics of mAbs can be affected by modulation of their interactions with FcRn and attempt to put this binding interaction parameter into perspective with other mechanisms involved in antibody disposition to aid in guiding mAb development.

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THE RECEPTOR INTERACTION ASSAYS

FcRn plays an essential role in the regulation of IgG concentrations in circulation by protecting/salvaging IgG from lysosomal degradation and recycling molecules taken into tissue cells back into the blood (Ghetie et al., 1996; Ghetie and Ward, 1997; Ward et al., 2003). A cornerstone of this interaction is the tightly regulated, pH-dependent manner with high affinity binding occurring at an acidic pH (pH ~6) and weak to no binding interactions as the pH is raised to neutral (pH 7.4) (Ober et al., 2004a; Ober et al., 2004b; Prabhat et al., 2004; Goebl et al., 2008). It is largely presumed that after fluid-phase uptake, IgG binding by the receptor occurs in the acidic (pH ~6) microenvironment of the endosomes within cells. Following the interaction at acidic pH, FcRn recycles IgG back into circulation upon exposure to physiological pH (pH ~7.4) (Ward et al., 2003; Ober et al., 2004a; Ober et al., 2004b; Prabhat et al., 2004; Ward et al., 2005; Goebl et al., 2008). IgG that is not bound to FcRn within endosomes undergoes proteolytic degradation in lysosomes (Ward et al., 2003; Ward et al., 2005). Thus, the proportion of IgG processed through the recycling versus degradative pathways is believed to be important in determining the clearance and half-life of an IgG in the circulation.

Given the understanding around the mechanism, FcRn:IgG binding assays have focused on the strict pH-dependent nature of the interaction, attempting to optimize the binding affinities at acidic (pH ~5 to 6) and neutral pH (pH ~7.4). Characterizing both aspects of the FcRn:IgG interaction are required to aid in making connections to the potential influence on mAb pharmacokinetics (discussed further in Interpretation of FcRn Binding Assays). A number of techniques and tools have been developed to characterize both the pH-dependency of the interaction and binding affinity including, cell-based systems, immunoassays, solution based-approaches and surface plasmon resonance (SPR). The following section discusses these tools,

the parameters of the FcRn:IgG interaction that these approaches have been predominantly used to characterize/measure and the advantages/limitations of each approach.

SPR has been the most broadly-used format for measuring FcRn:IgG interactions. The SPR formats have included covalent coupling of either FcRn (FcRn down method) or IgG (IgG down method) to the surface of a sensor chip and flowing the unconjugated species over the chip surface (Raghavan et al., 1995; Ghetie et al., 1997; Dall'Acqua et al., 2006; Datta-Mannan et al., 2007a; Yeung et al., 2009). This approach allows characterization of the interaction at acidic (generally pH ~6) and basic (pH ~7.4 to 8) pH conditions. Both assay formats yield a number of quantitative parameters including the rates of association (k_{on}) and dissociation (k_{off}), as well as, the binding affinity (K_D) of the interaction. The inherent nature of the 2:1 FcRn:IgG binding stoichiometry of the interaction has been proposed to influence the degree of avidity observed in the two SPR assay formats. As a result, a number of different K_D values have been reported for human IgG binding to both human and cynomolgus monkey at pH 6 depending on the method applied. This has added challenge to both cross-lab comparisons and the systematic correlation of *in vitro*-to-*in vivo* outcomes. For example, in our and several other labs (Raghavan et al., 1995; Ghetie et al., 1997; Datta-Mannan et al., 2007a; Wang et al., 2011a), the K_D of the FcRn:IgG interaction was measured in a bivalent BIAcore assay (FcRn down), in which antibodies were injected over a FcRn-coated sensor chip. Using this format, the binding affinity of wild-type human IgGs for FcRn is generally in the range of ~100-200 nM at pH 6. However, when flowing soluble FcRn over an antibody-coupled chip (IgG down) monovalent K_d values of ~1-2 μM have been reported for similar IgGs (Dall'Acqua et al., 2006; Yeung et al., 2009). While it could be argued that the bivalent assay may better reflect how membrane-anchored FcRn interacts with antibodies in nature, the choice of the FcRn binding assay format

fundamentally relates to the nature of the question around which the interaction is being investigated. For example, the bivalent FcRn down format may provide a larger window for studying the dissociation of the receptor:antibody complex at neutral pH. A clearer understanding of differences in this parameter may allow a deeper understanding of the consequences for antibody pharmacokinetics (Wang et al., 2011a). Conversely, the low apparent binding affinity in the monovalent IgG down assay (even at acidic pH) would likely make this dissociation parameter technically difficult to accurately measure at more unfavorable, alkaline pH. Along these lines, Yeung *et al*. have proposed the monovalent FcRn K_D derived from the IgG down assay is a more consistent parameter for comparing the extent of affinity improvement across different antibody variants since it is less sensitive to the IgG's inherent affinity compared to the bivalent format (Yeung et al., 2009). It is worth noting, that given the FcRn down assay better recapitulates the intrinsic avidity component in the native FcRn:IgG interaction within cells, this format may allow a better dissection of the role of IgG affinity versus avidity for FcRn and the relationship of these parameters with antibody clearance. From a pragmatic perspective, the inherent advantages/limitations of the two assay formats with regards to affinity versus avidity should be considered when evaluating data from each assay format. While the data from the two SPR assay formats each provide useful information, comparison of data across the approaches can also lead to ambiguity and difficulty in quantitative analyses between studies due to the inherent caveats of each approach. Thus, applying consistency in the comparison of FcRn binding data within a single assay format for multiple IgGs is an important consideration for making mechanistic connections with their pharmacokinetics.

Irrespective of the SPR assay format applied, binding data at pH 6 and 7.4 represent key parameters that clearly relate to *in vivo* pharmacokinetic outcomes. For example, a number of

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investigators have reported that binding to the receptor at neutral pH is negatively correlated with IgG clearance and half-life *in vivo*, regardless of the antibody binding affinity for FcRn at pH 6 (Ghetie et al., 1997; Datta-Mannan et al., 2007a; Yeung et al., 2009). Similarly, studies in FcRn knockout mice or with IgGs that have mutations in the Fc region that clearly knockout FcRn binding at acidic pH also display poor pharmacokinetic properties (Ghetie et al., 1996; Christianson et al., 1997; West and Bjorkman, 2000; Deng et al., 2010). Given the tight connection to poor kinetic outcomes *in vivo*, SPR assay formats provide a rapid screen for eliminating antibodies that show no binding to the receptor at pH 6 or do interact with FcRn at neutral pH. Additional assay formats, including immunoassays and cell-based approaches, have also yielded similar information in regard to these negative FcRn interaction properties (Hinton et al., 2004; Datta-Mannan et al., 2007a; Mathur et al., 2013). A theoretical advantage of the SPR assay format, aside from determination of binding affinity, is the ability to mine the data to discern quantitative subtleties in the rates of association and dissociation. However, a broad predictive relationship between any of the FcRn interaction parameters (k_{on}, k_{off} and K_D) *in vitro* with antibody pharmacokinetics *in vivo* has remained elusive.

In addition to the direct FcRn binding assays, a few approaches have also been used to probe the pH-dependent dissociation parameters, which have been proposed to better recapitulate the transition of pH conditions the receptor:IgG complex is exposed to at various points during intracellular trafficking. Immunoassays and solution based interaction strategies have been applied to monitor the extent of pre-formed FcRn:IgG complex dissociation after exposure to an increasing pH gradient (Datta-Mannan et al., 2007a;Datta-Mannan et al., 2007b). These assays were useful for understanding the rapid clearance of a set of antibodies that bound to FcRn at pH 6 yet showed no direct binding to FcRn at neutral pH (Datta-Mannan et al., 2007a;Datta-Mannan

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et al., 2007b). In this case, after formation of the receptor:IgG complexes the IgGs showed an inability to separate from the receptor when exposed to basic pH (Datta-Mannan et al., 2007a;Datta-Mannan et al., 2007b). The rapid *in vivo* peripheral clearance of the IgGs was mechanistically attributed to the lack of dissociation of the receptor complex when exposed to physiological conditions (Datta-Mannan et al., 2007a;Datta-Mannan et al., 2007b). In such a circumstance, the IgG is postulated to be trapped in a pathway of futile recycling. With the complex incapable of dissociation at the cell surface, the *in vivo* outcome is similar to that observed for the negative affect of antibodies directly associating with FcRn at neutral pH. It is worth noting that although these are less well-studied parameters than the direct binding interactions by SPR (and other methods), this more detailed characterization may provide useful insight into aspects of receptor complex binding equilibrium, which may more closely represent processes that exist during initial cellular internalization, endosomal sorting and release into the circulation.

While the methods discussed above leverage the soluble form of FcRn to probe interactions with antibodies, there are also a few examples of cell-based receptor assays used for studying interactions with IgGs. Generally, the cell-based assays have been used to either qualitatively describe FcRn-mediated antibody intracellular trafficking or quantitate receptor:IgG interaction parameters. The qualitative assays have been valuable for better delineating the mechanism of action of the receptor and its influence on IgG disposition (Goebl et al., 2008; Ober et al., 2004a; Ober et al., 2004b; Prabhat et al., 2004; Ward et al., 2003; Ward et al., 2005) however due to the descriptive nature of these results, the qualitative assays are often times difficult to use to make comparisons around receptor interactions across a large number of IgG molecules. Thus, quantitative cell-based binding assays are frequently used to measure receptor:IgG interactions.

However, some of these assays use engineered cell lines with FcRn constitutively expressed on the membrane surface for studying receptor:IgG interactions (Hinton et al., 2004; Hinton et al., 2006; Mathur et al., 2013). Similar to the studies with soluble FcRn, the quantitative cell-based assays have been used to examine the relative FcRn binding affinity and the pH-dependency of interaction in the context of mutations within the Fc region IgGs, various biochemical/biophysical properties of IgGs (ie influence of IgG aggregation, methionine oxidation), the effect of IgG subclass and other molecular attributes of IgGs (i.e., effect of Fab region) on FcRn (Hinton et al., 2004; Hinton et al., 2006; Mathur et al., 2013). Although there are no published data directly comparing an antibody molecule(s) in quantitative cell assays with the soluble receptor formats, data from both would suggest the conclusions around the facets of IgG parameters that affect FcRn binding are similar; however, understanding the sensitivity difference/similarities of the two approaches is currently a gap. It is theoretically plausible that compared with the assays that utilize soluble FcRn, the quantitative cell-based approaches may be advantageous in that the manner FcRn is presented is closer to its physiological or native state. Thus, these methods may also lend to a more complete understanding of IgG interactions with FcRn in the context of other IgG interactions, which may occur with glycans and/or other components of the cellular membrane due to their stereochemical nature or charge distribution properties. However, it should be noted that since the validated quantitative cell assays measure FcRn interactions with antibodies at the plasma membrane surface they do not fully recapitulate intracellular endosomal trafficking that IgGs undergo physiologically when interacting with the receptor.

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APPLICATIONS AND INTERPRETATIONS OF FcRn BINDING ASSAYS TOWARDS UNDERSTANDING MAB PHARMACOKINETICS

It is clear that FcRn binding assays have been useful in providing information around the properties of the IgG:FcRn interaction that have significant negative consequence to mAb PK properties *in vivo* (Ghetie et al., 1997; Datta-Mannan et al., 2007a; Yeung et al., 2009). As previously mentioned, what is less understood are the influences of subtleties in this interaction on *in vivo* behavior. Since these 'aberrant' receptor binding properties have only been reported in the context of mutations to the Fc region of IgGs, it is really in the environment of antibody engineering/optimization that the pharmaceutical industry has attempted to apply binding assays and develop a better understanding of how these *in vitro* interactions relate to IgG pharmacokinetics *in vivo* (Dall'Acqua et al., 2002; Hinton et al., 2004; Dall'Acqua et al., 2006; Hinton et al., 2006; Datta-Mannan et al., 2007a; Datta-Mannan et al., 2007b; Yeung et al., 2009; Deng et al., 2010; Yeung et al., 2010). However, in isolation the characterization of the IgG:FcRn interaction often provides limited insight into the desired *in vivo* properties of a therapeutic antibody (Datta-Mannan et al., 2012a). It has been established that the biophysical and chemical properties of IgGs including deamidation, oxidation, charge distribution and post-translational modifications influence *in vivo* behavior whether related or unrelated to their FcRn interaction (Igawa et al., 2010a; Khawli et al., 2010; Yeung et al., 2010; Wang et al., 2011a; Wang et al., 2011b). The complexity and interaction of these multiple factors influencing the *in vivo* behavior of therapeutic antibodies must be understood in order to facilitate rational protein engineering strategies. In addition, the implications of these studies are broad and extend into IgG expression/purification control strategies along with additional CMC considerations including handling and storage of mAb therapeutics. With this perspective in mind, we will

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discuss characteristics of antibody structure, the biological target and the nature of mAb:antigen interaction as factors that need to be considered in an integrative manner along with FcRn binding to better modulate mAb PK/PD properties.

While the utility of FcRn binding assays towards the development of Fc variant mAbs with improved pharmacokinetics is incontrovertible, there are discrepancies in the *in vitro* FcRn binding affinity–pharmacokinetic relationship that make the predictive quantitative translation across antibodies and their target antigens somewhat tenuous. Along these lines, retrospective analyses of several humanized mAbs having similar human FcRn binding properties demonstrated that they had ~1.5- to ~2-fold differences in their elimination half-lives in humans suggesting a lack of a direct quantitative correlation with FcRn binding (Suzuki et al., 2010). Similarly, Gurbaxani *et al.* were unable to directly correlate FcRn binding affinity to the pharmacokinetics of a number of IgGs in mice (Gurbaxani et al., 2006). There are certainly a number of preclinical reports that have shown that specific Fc variants (T250Q/M428L, V308P, M428L, M252Y/S254T/T256E, M428L/N434S, N434A, N434H) that improve IgG affinity for FcRn at pH 6 can result in ~2- to ~4-fold longer *in vivo* elimination phase half-life in either cynomolgus or rhesus monkeys (Dall'acqua et al., 2002; Dall'acqua et al., 2006; Datta-Mannan et al., 2007a; Datta-Mannan et al., 2007b; Deng et al., 2010; Hinton et al., 2004; Hinton et al., 2006; Yeung et al., 2009; Yeung et al., 2010). This general relationship also has been validated more recently in humans with the YTE set of mutations constructed on the Fc of anti-respiratory syncytial virus (RSV) (Robbie et al., 2013). However, the quantitative correlation of FcRn affinity improvement with *in vivo* pharmacokinetic parameters also has been ambiguous in primate studies of engineered mAbs (Hinton et al., 2004; Yeung et al., 2009; Deng et al., 2010; Datta-Mannan et al., 2012a). Deng and coworkers, showed an anti-tumor necrosis factor-α mAb

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Fc variant (N434H) with ~3-fold higher FcRn affinity at pH 6 had similar pharmacokinetics in cynomolgus monkeys when compared to a lower receptor affinity variant constructed on the same mAb backbone (N434A) (Deng et al., 2010); although these findings may have been compromised by anti-drug antibodies (ADA). In another set of examples, Fc mutations (M252Y/S254T/T256E) that increased receptor binding at pH 6 by ~10-fold have shown ~3- to 4-fold improvements in mAb half-life in monkeys (Dall'Acqua et al., 2006), whereas, Fc mutations (T250Q/M428L) which display ~30 fold-increases in FcRn binding have shown only ~2.5-fold improvements in half-life (Hinton et al., 2004; Hinton et al., 2006). As discussed above, one of the challenges in establishing a systematic understanding of the FcRn affinity-pharmacokinetic relationship are the different methodologies/technologies that have been used to measure IgG:FcRn interactions which thereby makes a direct comparison of binding properties between studies challenging. Even in cases where these attributes are well understood, there is still a quantitative and qualitative discrepancy in the *in vitro–in vivo* relation indicating that there are likely additional factors or properties of the mAbs along with their FcRn affinity that influence the *in vivo* pharmacokinetics (Gurbaxani et al., 2006; Suzuki et al., 2010; Datta-Mannan et al., 2012a; Gurbaxani et al., 2013).

Indeed, there are a number of inconsistent reports regarding the relationship between *in vitro* FcRn binding data and mAb pharmacokinetics that are not readily explained by the simple set of FcRn interaction parameters at acidic and basic pH. It is entirely possible that in some instances the observed *in vitro* FcRn affinity differences may have been due to assay artifacts such as Fab interactions with the receptor and/or highly charged or hydrophobic regions within the IgG facilitating non-Fc attributable FcRn binding. It is difficult to readily discern the contributions of receptor interaction artifacts on *in vitro* to *in vivo* inconsistencies within the literature given the

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number of isolated reports. However, it seems reasonable to suggest that, in isolation, measurement of the absolute affinity of the IgG-FcRn interaction provides little insight into *in vivo* pharmacokinetic properties.

In a recent study, a systematic assessment of 15 mAbs demonstrated that *a priori* prediction of pharmacokinetic properties, based solely on the FcRn *in vitro* affinity-*in vivo* pharmacokinetic relationship, was not possible (Datta-Mannan et al., 2012a). Thus, it is logical to conclude that in some cases the relative contribution of other factors affecting IgG disposition *in vivo* are critical to consider in regard to interpreting this property to further mAb development. The most obvious is the case of specific target-mediated mAb disposition. In these instances, the preferential mAb-binding interaction *in vivo* is to its particular target antigen (i.e., receptor or membrane-associated antigen) at the surface of cell membranes. Since the mAb binds antigen at the cell surface and the interaction is typically of high affinity (pM to low nM), the mAbs disposition will be governed through this specific cellular interaction as opposed to the generic FcRn salvage mechanism. At non-saturating concentrations, the target-mediated internalization of the mAb from the peripheral circulation is the major pathway of mAb elimination that minimizes the contribution of the FcRn pathway. As the mAb concentrations increase, target-mediated disposition becomes saturated unmasking the contribution of FcRn-to-mAb disposition. In addition, the inherent properties of antigen (such as its turnover, concentration, expression pattern, tissue distribution) will be a major dictator of mAb disposition overriding the influence of FcRn-mediated salvage. These phenomena were observed in a baboon pharmacokinetic study of a humanized anti-CD4 antibody with a single Fc substitution (N434H) designed to improve its binding to FcRn relative to its wild-type mAb equivalent (Zheng et al., 2011). The authors contended that at non-saturating concentrations, CD4 receptor-mediated internalization

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was the major elimination pathway for the variant mAb (Zheng et al., 2011). As the antibody concentration increased, the CD-4 receptors became saturated and an ~2-fold slower clearance of the variant IgG was observed compared to its wild-type counterpart (Zheng et al., 2011). The slower clearance of the variant mAb was attributed to its enhanced FcRn interactions, which became a greater part of the total mAb elimination following antigen saturation. Additionally, for mAbs that target soluble antigens it is entirely possible that the mAb binding affinity for FcRn is different when they are bound to antigen. In some instances it may be possible that the complex has a reduced FcRn affinity which leads to target-mediated mAb elimination. This was hypothesized in a recent study for a PCSK9 mAb (Chaparro-Riggers et al., 2012). If the soluble antigen is present in high concentrations in the blood and/or has a high rate of turnover, such as in the case of PCSK9, clearance of the complex via reduced FcRn binding will be a major contributor to the mAb elimination (Chaparro-Riggers et al., 2012). Thus, in some instances consideration of measuring the FcRn binding affinity of mAb:antigen complex may provide insight into the translation to *in vivo* observations. Taken together, these examples illustrate that a firm understanding of the biology of the target is critical to understanding how *in vitro* FcRn binding data can be used to guide mAb development.

While the interplay of target-mediated mAb clearance mechanisms contributes to some of the pharmacokinetic discrepancies observed with the FcRn binding assays in the aforementioned examples, a number of studies with Fc-engineered mAbs have employed *in vivo* systems with low/no endogenous antigen or mAb doses that saturated target (Dall'Acqua et al., 2002; Hinton et al., 2004; Yeung et al., 2009; Datta-Mannan et al., 2012a). Antibodies directed towards low expression soluble targets or given at high doses are more likely to show improved pharmacokinetic properties with Fc engineering, as in the case of the engineered anti-RSV mAb

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(Dall'Acqua et al., 2006). Despite this, recent reports also have identified other non-FcRn clearance pathways and mAb biochemical characteristics that can significantly impact mAb disposition (Igawa et al., 2010b; Khawli et al., 2010; Yeung et al., 2010; Wang et al., 2011a; Wang et al., 2011b) . These studies have suggested that factors including the pI/charge patches, stability/aggregation potential, post-translational modifications and the characteristics of the Fab region can all have significant impact on antibody disposition. These molecular characteristics may in themselves influence mAb structure so as to alter the 'true' interaction with FcRn *in vivo*, or may contribute to non-specific cell surface binding that alters the rate of endocytosis and the relative proportion of FcRn mediated recycling. Given the multiplicity of factors that influence mAb disposition *in vivo* the interpretation of FcRn binding data in isolation can be inappropriate to guide mAb development. Thus, in addition to FcRn-binding assays being used to guide mAb development, additional non-FcRn determinants and antibody characteristics will need to be considered for integrated data-driven decision making. Even with this approach, it is likely that *a priori* prediction of the pharmacokinetic outcomes will remain difficult. Despite the gap for using FcRn binding assays/data in a manner to prospectively predict mAb pharmacokinetic outcomes, the assays have been useful for retrospective analyses in probing factors that affect mAb clearance. For example, in one study a close association between the in vitro FcRn dissociation at neutral pH of mAbs with the same Fc and different Fab regions and their in vivo pharmacokinetics was useful for delineating the Fab domain effects on FcRn binding (Wang et al., 2011a). In another study, FcRn binding assays were useful for demonstrating that methionine oxidation in the Fc region can reduce FcRn binding and lead to an increased peripheral clearance of mAbs (Wang et al., 2011b). Thus profiling the FcRn binding properties of mAbs with various conformational and/or physiochemical changes in conjunction with mAb

pharmacokinetics can be an useful approach for establishing the contribution of receptor interactions with these parameters to mAb disposition *in vivo*. These parameters can then be monitored especially during the manufacture and storage of mAbs to develop criterion/ranges for receptor binding values that trend with affecting mAb disposition.

LEVERAGING FcRn BINDING ASSAYS IN AN INTEGRATED MANNER

In the context of mAb's exhibiting significant target-mediated disposition or non-optimal biophysical characteristics (as discussed above), FcRn may not be a major driving force determining overall disposition parameters. As such, modulating the IgG:FcRn interaction may yield little to no benefit from a PK/PD perspective without considering the impact of these specific or non-specific pathways. Optimizing the potential benefit from improving the FcRn interaction likely requires integration with engineering aimed at altering target-receptor interactions and improving biochemical/biophysical properties.

Towards this concept, recent reports have utilized combined engineering approaches to modulate both the target and FcRn interactions yielding antibodies with improved in vivo PK/PD properties. For example, an anti-IL6 receptor (IL-6R) antibody was designed to rapidly dissociate from both soluble/shed and membrane bound IL-6R within the acidic environment of the endosome (pH 6.0) while maintaining its binding affinity to IL-6R in plasma or at the cell surface (pH 7.4) (Igawa et al., 2010a). Two distinct engineering opportunities were demonstrated in this example (Igawa et al., 2010a; Igawa et al., 2013). In the context of the cell-surface receptor interaction, altered cellular trafficking, allowed the mAb to be released from the receptor and subsequently associate with FcRn and be recycled versus degraded (Igawa et al., 2010a). Once the balance of pH-dependent association with the receptor was resolved,

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engineering to improve FcRn binding yielded improved recycling and, in turn, the PK/PD properties of the molecule (Igawa et al., 2010a; Igawa et al., 2013). Similarly, the pH-sensitive engineered IL-6R mAb was able to 'unload/dump' soluble/shed receptor that became associated with the mAb resulting in the recycling of naked antibody capable of re-binding to cell surface IL6R prolonging its functional activity (Igawa et al., 2010a). The validation of such an approach also was demonstrated for a mAb engineered for pH-sensitive binding to PCSK9, a soluble protein with high turnover in rodents and primates. In this case, the authors proposed that the manner/epitope in which this particular mAb bound antigen reduced its ability to be salvaged by FcRn when bound to its target (Chaparro-Riggers et al., 2012). Engineering for pH-sensitive antigen binding served to indirectly leverage the FcRn pathway, resulting in 'productive' recycling of non-complexed mAb unmasking the true benefit of optimization of the FcRn salvage pathway (Chaparro-Riggers et al., 2012). Taken together, these examples illustrate the advantages that an integrated mAb engineering approach may have in yielding the next generation of improved antibody therapeutics.

SUMMARY AND FUTURE PERSPECTIVES

Based on a preponderance of the literature it is clear that FcRn is critical to maintenance of IgG homeostasis, but it is also evident that an engineering strategy based exclusively on modulating the interaction of IgG with FcRn may be insufficient to lead to improved PK/PD properties of therapeutic antibodies. In addition to this interaction, it is important to understand the interplay between aspects of non-FcRn clearance pathways, such as those mediated by a mAbs' target, the nature of the target itself (i.e., soluble, membrane bound), as well as, the biophysical characteristics of the IgG in a holistic manner to determine the balance between

these and FcRn in affecting the molecules' disposition. Recent studies demonstrating that combined engineering approaches addressing the interaction characteristics of the Fc and Fab can be successfully leveraged to provide unique benefit from a PK/PD perspective may be the next path to improved therapeutic properties of this class of molecules. Modeling and simulation also has provided insight as to properties required to improve mAb pharmacokinetics, but these efforts need additional integration of the kinetics of membrane/receptor turnover, cellular pathways and the influence of the intracellular environment on mAb-receptor interactions. Whether all factors can be appropriately accounted for to prospectively guide engineering strategies requires additional studies attempting to refine the parameters most relevant to accurate *in vitro-in vivo* correlation and prediction.

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