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Review

# Targeting FcRn for the modulation of antibody dynamics☆

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ABSTRACT

The MHC class I-related receptor, FcRn, is a multitasking protein that transports its IgG ligand within and across cells of diverse origins. The role of this receptor as a global regulator of IgG homeostasis and transport, combined with knowledge of the molecular details of FcRn–IgG interactions, has led to opportunities to modulate the in vivo dynamics of antibodies and their antigens through protein engineering. Consequently, the generation of half-life extended antibodies has shown a rapid expansion over the past decade. Further, FcRn itself can be targeted by inhibitors to induce decreased levels of circulating IgGs, which could have applications in multiple clinical settings. The engineering of antibody–antigen interactions to reduce antibody-mediated buffering of soluble ligand has also developed into an active area of investigation, leading to novel antibody platforms designed to result in more effective antigen clearance. Similarly, the target-mediated elimination of antibodies by internalizing, membrane bound antigens (receptors) can be decreased using novel engineering approaches. These strategies, combined with subcellular trafficking analyses of antibody/antigen/FcRn behavior in cells to predict in vivo behavior, have considerable promise for the production of next generation therapeutics and diagnostics.



## 1. Introduction

The function of the MHC class I-related receptor, FcRn, as a transporter of antibody molecules of the immunoglobulin G (IgG) class within and across multiple cell types is well documented (Roopenian and Akilesh, 2007; Ward and Ober, 2009). Through this transport function, FcRn serves not only to transport antibodies across cellular barriers (Bitonti et al., 2004; Claypool et al., 2004; Dickinson et al., 1999; Firan et al., 2001; McCarthy et al., 2000; Spiekermann et al., 2002), but also to regulate the levels of antibodies in the body (Ghetie et al., 1996; Israel et al., 1996; Junghans and Anderson, 1996). In addition, FcRn binds and transports albumin (Chaudhury et al., 2003). The ability of FcRn to serve as a homeostatic regulator of IgG and albumin is essential for normal health, in addition to having implications for protein engineering directed toward modulating the pharmacokinetics of therapeutics and the dynamic behavior of endogenous antibodies. In the current review we will discuss the molecular and cellular aspects of FcRn activity, with a particular focus on its role in IgG homeostasis. How this knowledge can be exploited to modulate IgG dynamics in vivo will also be presented.

## 2. The molecular players of FcRn–IgG interactions

The residues at the $CH_2$–$CH_3$ domain interface of an IgG that mediate FcRn binding are well defined through site-directed mutagenesis and X-ray crystallographic analyses (Firan et al., 2001; Kim et al., 1994, 1999; Martin et al., 2001; Medesan et al., 1997; Raghavan et al., 1995; Shields et al., 2001). These studies have led to the identification of three IgG residues that are critical for binding (Ile253, His310 and His435). The residues are located at the $CH_2$–$CH_3$ domain interface of the IgG molecule and are either invariant (Ile253, His310) or present in the majority (His435) of

*Abbreviations:* Abdeg, antibodies that enhance IgG degradation; CDR, complementarity determining region; Epo, erythropoietin; HMEC, human microvasculature-derived endothelial cells; ITP, idiopathic thrombocytopenic purpura; IC, immune complex; IL-6, interleukin-6; IL-6R, interleukin-6 receptor; LDL, low density lipoprotein; LDLR, low density lipoprotein receptor; MDCK, Madin–Darby canine kidney; PET, positron emission tomography; PCSK9, proprotein convertase substilisin kexin type 9; p*I*, isoelectric point; TC, transport carrier; VH, variable heavy; VL, variable light.

☆ This article belongs to Therapeutic Antibodies.

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human, rat and mouse IgGs (Deisenhofer, 1981; Kabat et al., 1991). By contrast with the high conservation of residues 253, 310 and 435, residue 436 (His in mouse IgG1, Tyr in human IgG1) shows higher variability across species and plays a more minor role in FcRn–IgG interactions (Medesan et al., 1997; Shields et al., 2001). Although His433 has been reported to contribute to FcR–IgG binding in several studies (Martin et al., 2001; Raghavan et al., 1995), other analyses have indicated that it does not (Kim et al., 1999; Medesan et al., 1997; Oganesyan et al., 2014).

The histidine residues on IgG confer the pH dependence of FcRn–IgG complex formation which is central to the functional activity of this receptor (Kim et al., 1994; Raghavan et al., 1995). Specifically, most naturally occurring IgGs bind with relatively high affinity to FcRn at pH 5.5–6.0, with the interaction becoming progressively weaker as near neutral pH is approached (Popov et al., 1996; Raghavan et al., 1995; Rodewald, 1976; Simister and Rees, 1985; Wallace and Rees, 1980; West and Bjorkman, 2000; Zhou et al., 2005). Consequently, the binding of most IgG isotypes with FcRn at physiological pH (7.3–7.4) is either negligible or of too low affinity to determine an accurate equilibrium dissociation constant. The IgG histidines interact with acidic residues on FcRn such as Glu117 in rodent FcRn or human FcRn [note that human FcRn is two residues shorter than rodent FcRn (Ahouse et al., 1993; Simister and Mostov, 1989; Story et al., 1994). The numbering system used for human FcRn in this review is based on homology alignment]. The X-ray crystallographic structures of rat FcRn–rat IgG2a (Fc) and uncomplexed human FcRn were reported many years ago (Burmeister et al., 1994; Martin et al., 2001; West and Bjorkman, 2000), whereas a co-crystal structure of human FcRn with human IgG1 (Fc) was only recently described (Oganesyan et al., 2014). This latter study presented the tripartite complex of human FcRn with human serum albumin and an engineered human IgG1-derived Fc fragment with increased affinity for FcRn ['YTE' (Dall'Acqua et al., 2002, 2006), discussed further below]. Importantly, the structure demonstrates that through interactions with Glu117 on human FcRn, His310 on IgG is the major player in mediating the pH-dependence of the interaction. This analysis is also consistent with biophysical studies indicating that the albumin and IgG recognition sites on FcRn do not overlap (Andersen et al., 2006; Sand et al., 2014).

## 3. Cross-species differences in FcRn binding

Variations in residues encompassing the FcRn–IgG interaction site across species lead to differences in binding behavior that bear direct relevance to the use of mice as a preclinical model (Martin et al., 2001; Ober et al., 2001; Oganesyan et al., 2014). To date, affinities of FcRn for multimeric immune complexes (ICs) have not been reported due to the complexity of analyzing the binding of heterogenous ICs to this receptor, resulting in the availability of data only for 'monomeric' IgGs (i.e. an IgG homodimer). Specifically, mouse FcRn has substantially higher affinities than human FcRn for binding to IgGs (Ober et al., 2001). For example, human FcRn binds very weakly, if at all, to most mouse IgG isotypes. In addition, the interaction of human IgG1 with human FcRn is about 15-fold lower in affinity compared with binding to mouse FcRn (370 nM vs. 24 nM at pH 6.0) (Zhou et al., 2005). This can impact the interpretation of preclinical data obtained using engineered (human) antibodies in mice (Dall'Acqua et al., 2002, 2006; Vaccaro et al., 2006). These cross-species differences have motivated the development of human FcRn transgenic mice that provide valuable models for the analyses of therapeutic antibodies (Haraya et al., 2014; Petkova et al., 2006; Proetzel and Roopenian, 2014; Zalevsky et al., 2010).

The molecular basis for the variations in binding characteristics between mouse and human FcRn has been elucidated by systematic replacement of human FcRn residues with the corresponding amino acids from mouse FcRn (Zhou et al., 2003, 2005). This has resulted in the identification of two specific regions of FcRn that confer the distinct binding behavior: first, residues 132–147, including the non-conserved residue 137 [Glu in mouse FcRn; Leu in human FcRn (Ahouse et al., 1993; Story et al., 1994)]; second, residues 79–89, which in human FcRn is two amino acids shorter than in mouse FcRn (Ahouse et al., 1993; Story et al., 1994). Residue 137 of FcRn plays a dominant role in the cross-species difference (Zhou et al., 2003). Although this residue is Asp or Glu in rats, mice and dogs (Ahouse et al., 1993; Kacskovics et al., 2006; Simister and Mostov, 1989) and Leu in humans or possums (Adamski et al., 2000; Story et al., 1994), multiple other species such as camels, cows, pigs and sheep contain Arg at this position (Kacskovics et al., 2000, 2006; Mayer et al., 2002; Schnulle and Hurley, 2003). Given the crucial role of residue 137 for the complexes characterized to date (Martin and Bjorkman, 1999; Oganesyan et al., 2014; Vaughn et al., 1997), it is therefore probable that the molecular nature of IgG interactions with Arg137-containing FcRn molecules will be distinct to those for rodent, canine, possum or human FcRn.

## 4. The sites of FcRn expression

The expression of FcRn was originally believed to be restricted to sites involved in the delivery of maternal IgG during gestation (humans, rodents) and from mothers milk during the neonatal period (rodents) (Rodewald and Abrahamson, 1982; Rodewald and Kraehenbuhl, 1984; Simister and Rees, 1985; Wallace and Rees, 1980). Indeed, the initial identification of this receptor in the neonatal rodent gut led to its designation as the neonatal Fc receptor, or FcR*n*. However, the expression of this receptor in multiple tissues and cell types throughout life is now well defined (Rath et al., 2013; Roopenian and Akilesh, 2007; Ward and Ober, 2009). In addition to the presence of FcRn in endothelial, epithelial and the majority of hematopoietic cells (Akilesh et al., 2007; Borvak et al., 1998; Dickinson et al., 1999; Mi et al., 2008; Qiao et al., 2008; Zhu et al., 2001), this receptor is expressed in specialized cell types such as podocytes, the blood brain barrier and ocular tissues including the cornea, retina and conjunctiva (Akilesh et al., 2008; Schlachetzki et al., 2002; Zhang and Pardridge, 2001).

The ubiquitous expression of FcRn throughout the body has prompted studies directed toward defining the functional sites at which FcRn maintains IgG levels (Akilesh et al., 2007; Perez-Montoyo et al., 2009; Qiao et al., 2008). The use of adoptive transfers of hematopoietic cells from FcRn-deficient mice into wild type mice (and vice versa) indicates that these cells contribute to the regulation of IgG homeostasis (Akilesh et al., 2007; Qiao et al., 2008). Mice harboring a floxed FcRn allele that enables site-specific deletion following appropriate intercrossing with Cre-expressing strains have also been generated (Perez-Montoyo et al., 2009). The production of a mouse strain lacking FcRn expression in both hematopoietic and endothelial cells has resulted in the demonstration that, collectively, these cells are the primary sites at which IgG levels are regulated in vivo (Perez-Montoyo et al., 2009).

Beyond a role in IgG homeostasis, multiple studies have demonstrated that FcRn is a multitasking receptor with diverse functional roles related to its ability to transport antibodies within and across cells (Rath et al., 2013; Roopenian and Akilesh, 2007; Ward and Ober, 2009). In particular, the transport function of FcRn in epithelial cells has been extensively analyzed (Bitonti et al., 2004; Claypool et al., 2004; Dickinson et al., 1999; Firan et al., 2001; Ladinsky et al., 2012; McCarthy et al., 2000; Mi et al., 2008; Spiekermann et al., 2002). FcRn can also deliver IgGs across mucosal surfaces to confer protective immunity (Ko et al., 2014; Li et al., 2011; Yoshida et al., 2006a,b). Further, FcRn expression in antigen

presenting cells contributes to phagocytosis and antigen presentation, including cross-presentation, resulting in enhancement of both CD4+ and CD8+ T cell responses (Baker et al., 2011, 2013; Cervenak et al., 2011; Qiao et al., 2008; Vidarsson et al., 2006). The activity in cross-presentation enhances CD8+ T cell responses against tumors (Baker et al., 2013). These functions for FcRn have been reviewed elsewhere and will not be discussed further here (Baker et al., 2014; Roopenian and Akilesh, 2007; Ward and Ober, 2009).

## 5. Subcellular trafficking behavior of FcRn and IgG ligand

### 5.1. Trafficking analyses in endothelial cells

The intracellular trafficking pathways taken by FcRn and its IgG ligand have been investigated in transfected human microvasculature-derived endothelial cells (HMEC-1 cells) using fluorescence microscopy (Gan et al., 2009; Ober et al., 2004a,b; Prabhat et al., 2007; Ram et al., 2008; Weflen et al., 2013). The interaction of the majority of IgG isotypes characterized to date with FcRn at the surface of most cell types is not permissive due to the near neutral pH of the extracellular environment. Thus, the consensus view is that IgG enters cells by fluid phase pinocytosis or any other cellular processes that engulf medium from the extracellular milieu. However, due to the technical limitations of imaging in the presence of high levels of (fluorescently labeled) IgG, this initial step of the pathway has not been amenable to microscopy analyses. The elucidation of subsequent steps using microscopy indicates that IgG is bound to FcRn in early (sorting) endosomes and subsequently sorted into tubulovesicular transport carriers (TCs) (Ober et al., 2004b). These TCs have also been observed using electron tomography of rat jejunal sections isolated from rats following intubation with nanogold labeled Fc fragments (He et al., 2008; Ladinsky et al., 2012). By contrast with salvaged IgG, antibody molecules that do not bind to FcRn due to low affinity or saturation of FcRn interaction sites fail to segregate into TCs and enter the degradative, lysosomal pathway (Ober et al., 2004b).

Recent studies using a combination of localized photoactivation and multifocal plane microscopy, involving the simultaneous imaging of several different focal planes in live cells (Prabhat et al., 2004, 2007), have been used to investigate the subcellular trafficking pathways taken by FcRn within endothelial cells (Gan et al., 2013). These analyses have shown that following segregation from sorting (early) endosomes, FcRn-positive TCs can take several different pathways (Fig. 1): first, TCs can traffick to the plasma membrane and undergo exocytosis (exocytic pathway). Second, they can be transferred to another sorting endosome (interendosomal transfer). Third, TCs can leave a sorting endosome and return to the same endosome in what appears to be a futile process (looping). Despite these different intracellular pathways, quantitative recycling assays reveal that the majority of IgG is recycled from the cells (Vaccaro et al., 2006; Ward et al., 2003). This is consistent with earlier analyses demonstrating that TCs can follow direct or indirect routes inside cells prior to being exocytosed (Prabhat et al., 2007). During exocytic release of IgG, FcRn-positive TCs fuse with the plasma membrane to release their cargo (Ober et al., 2004a; Prabhat et al., 2007). However, the low steady state level of FcRn on the plasma membrane indicates that this receptor is rapidly re-internalized following exocytosis.

It is well documented that multivalent ICs enter lysosomes following uptake into hematopoietic cells that are involved in antigen presentation and express both FcRn and the 'classical' FcγRs (Delamarre et al., 2005; Qiao et al., 2008; Trombetta and Mellman, 2005). However, until recently the fate of such ICs in FcRn-expressing cells that do not normally express FcγRs was unexplored. By contrast with monomeric IgGs, microscopy studies in HMEC-1 cells have revealed that multivalent ICs are excluded from tubular TCs as these compartments segregate from sorting endosomes, resulting in lysosomal delivery of both FcRn and its bound cargo (Weflen et al., 2013). Interestingly, deletion or replacement of the cytosolic tail of FcRn does not inhibit this lysosomal delivery pathway, suggesting that the physical nature of the complex is the primary driving force for exclusion from recycling TCs.

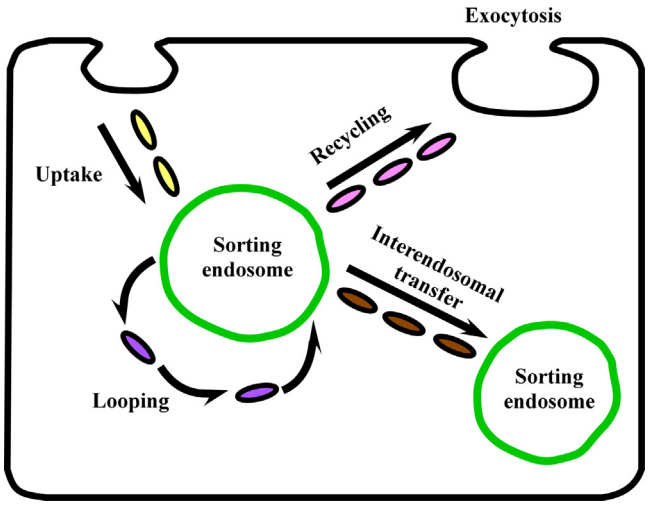


**Fig. 1.** Schematic representation of the different subcellular trafficking pathways taken by FcRn-positive TCs in endothelial cells. For further details, see Gan et al. (2013).

Although the principles of FcRn-mediating sorting of IgGs away from lysosomal degradation in endosomal compartments are likely to be similar across distinct cell types, the homeostatic role of hematopoietic cells (Akilesh et al., 2007; Perez-Montoyo et al., 2009; Qiao et al., 2008) indicates that it will also be instructive to analyze trafficking processes in these cells. For example, FcRn expression levels differ across cell types (Akilesh et al., 2007; Perez-Montoyo et al., 2009; Zhu et al., 2001). In addition, the endolysosomal and recycling pathways are expected to vary between endothelial and hematopoietic cells, given their distinct activities in antigen presentation and other cellular functions.

### 5.2. Transcytosis across epithelial barriers

FcRn can also transport antibodies bidirectionally across polarized cellular barriers such as epithelial cells, and the subcellular trafficking pathways have been extensively studied in intestinal epithelial cells and FcRn-transfected Madin–Darby canine kidney (MDCK) cells (Claypool et al., 2002, 2004; Dickinson et al., 1999; Kuo et al., 2009; Ladinsky et al., 2012; Mi et al., 2008; Tzaban et al., 2009). This transport function is essential for FcRn-mediated delivery of antibodies to multiple sites in the body, including the mucosal surfaces for protective immunity (Ko et al., 2014; Li et al., 2011; Yoshida et al., 2006a,b). On the other hand, FcRn-mediated transcytosis of IgG-opsonized human immunodeficiency virus type I has been reported to facilitate viral transmission by enhancing the delivery of this virus across epithelial barriers (Gupta et al., 2014).

FcRn-mediated transport can be exploited for the delivery of exogenous (therapeutic) antibodies or other biologics. For example, Fc–erythropoietin (Epo) fusion constructs are transported from the lungs into the bloodstream following inhalation (Bitonti et al., 2004; Spiekermann et al., 2002). More recently, Fc-coated insulin-loaded nanoparticles have been shown to be transferred across the gut following oral delivery (Pridgen et al., 2013). A potential

limitation of this approach is that the nanoparticles may accumulate and follow the lysosomal trafficking pathway in nontarget cell populations (Pridgen et al., 2013). Nevertheless, this strategy has considerable promise for the delivery of relatively high effective doses of biologic through the oral route.

## 6. Engineering FcRn–IgG interactions to modulate in vivo pharmacokinetics and transport

### 6.1. Half-life extension

Over the past decade or so, the use of Fc engineering to generate antibodies with increased in vivo half-lives and transport across cellular barriers has developed into a major area of interest for the production of 'second' generation therapeutic antibodies (Dall'Acqua et al., 2006; Hinton et al., 2004, 2006; Vaccaro et al., 2006; Yeung et al., 2009; Zalevsky et al., 2010). In 1997, it was reported that the mutagenesis of residues surrounding the FcRn–IgG interaction site followed by phage display and selection could be used to isolate a mouse IgG1-derived Fc fragment with ~3.5-fold increased binding affinity for mouse FcRn at pH 6.0 and up to ~1.6-fold increased persistence in mice (Ghetie et al., 1997) (Table 1). This was followed by a study in 2002 in which similar approaches resulted in the generation of human IgG1-derived Fc fragments with higher affinity for mouse and human FcRn (Dall'Acqua et al., 2002). Significantly, the selection for maintenance of pH-dependent binding (high affinity at around pH 6–6.5, very low affinity at pH 7.2–7.4) was not as stringent as for the earlier study, resulting in the isolation of mutated Fc fragments with significant binding to mouse FcRn at near neutral pH (Dall'Acqua et al., 2002). This loss of pH dependence resulted in shorter in vivo half lives in mice, despite higher affinity binding to FcRn at acidic pH. This study therefore provided a clear demonstration of the need for efficient release of ligand from FcRn following recycling and exocytosis at the plasma membrane. Nevertheless, due to the cross-species differences in FcRn-binding specificities (Ober et al., 2001), one of the mutants (Met252 to Tyr, Ser254 to Thr, Thr256 to Glu, or 'YTE') isolated in this study (Dall'Acqua et al., 2002) showed negligible binding to human FcRn at near neutral pH combined with enhanced affinity for this receptor at acidic, endosomal pH (~6.0). Consistent with these binding properties, the YTE mutant has an extended half-life in cynomolgus monkeys (almost 4-fold; 21.2 vs. 5.7 days) and humans (~3.7-fold; 69.5 vs. 18.9 days for 0.3 mg/kg dose) and forms the basis of the YTE platform that is currently in clinical trials for several different indications (Dall'Acqua et al., 2006; Robbie et al., 2013) (Table 1).

Additional half-life extending mutations for human IgGs have been described that result in 1.5–5-fold increases in persistence over the wild type parent in non-human primates or human FcRn transgenic mice (Borrok et al., 2015; Datta-Mannan et al., 2012a; Hinton et al., 2004, 2006; Monnet et al., 2014; Petkova et al., 2006; Wilson and Taura, 2013; Yeung et al., 2009; Zalevsky et al., 2010) (Table 1). These mutations confer increased binding to FcRn at pH 6.0 with retention of low affinity interactions at near neutral pH. A recent structural study of the YTE mutant (Fc fragment) in complex with human FcRn revealed the molecular basis for the increased affinity, and has also been used to rationalize the affinity improvement for several other FcRn-enhanced antibodies (Borrok et al., 2015; Oganesyan et al., 2014) (Fig. 2). Significantly, in preclinical studies in human FcRn transgenic mice, the M428L/N434S ('LS') mutations confer increased efficacy in tumor models (Zalevsky et al., 2010). Thus, engineering antibodies for half-life extension offers considerable promise to enable reduced, lower frequency dosing. Half-life extending mutations are also expected to increase

**Table 1**
Properties of half-life extended antibodies.

| Mutations | IgG species (isotype) | FcRn species | Fold increase in affinity at pH 6.0[a] | Species/strains for half-life analysis | Fold increase of half-life | References |
|---|---|---|---|---|---|---|
| T252L/T254S/T256F | Mouse IgG1 | Mouse | ~3.5 | Mice | ~1.6 | Ghetie et al. (1997) |
| M252Y/S254T/T256E | Human IgG1 | Cynomolgus monkey | ~9 | Cynomolgus monkey | ~3.7 | Dall'Acqua et al. (2006) |
| | Human IgG1 | Human | ~11 | Human | ~3.5[e] | Dall'Acqua et al. (2006) and Robbie et al. (2013) |
| | Human IgG1 | Human | ~1.9 | Human FcRn transgenic mice | ~2.7 | Monnet et al. (2014) |
| | Human IgG4 | Human | ~4.7[b] | Human FcRn transgenic mice | ~1.7 | Wilson and Taura (2013) |
| M252Y/S254T/T256E/S228P | Human IgG4 | Human | ~4.7[b] | Human FcRn transgenic mice | ~2.9 | |
| Y31-M252Y/S254T/T256E | Human IgG1 | Human | ~25.5 | Human FcRn transgenic mice | 5 | Borrok et al. (2015) |
| | Human IgG1 | Human | ~25.5 | Cynomolgus monkey | ~1.8 | |
| T250Q/M428L | Human IgG2 | Human/rhesus monkey | ~28[c] | Rhesus monkey | ~1.8 | Hinton et al. (2004) |
| | Human IgG1 | Human/rhesus monkey | ~29[c] (human)/ 37[c] (rhesus) | Rhesus monkey | ~2.5 | Hinton et al. (2006) |
| | Human IgG4 | Human | 49[c] | – | – | |
| | Human IgG4 | Human | ~10.7–115[d] | Cynomolgus monkey | ~0.86–2.6 | Datta-Mannan et al. (2012a) |
| N434A | Human IgG1 | Human | 1.6[c] | Human FcRn transgenic mice | ~1.6 | Petkova et al. (2006) |
| | Human IgG1 | Human | ~3 | Cynomolgus monkey | ~2.3 | Yeung et al. (2009) |
| T307/E380A/N434A | Human IgG1 | Human | 3.3[c] | Human FcRn transgenic mice | ~1.5 | Petkova et al. (2006) |
| M428L/N434S | Human IgG1 | Human | 11 | Cynomolgus monkey | 3.2 | Zalevsky et al. (2010) |
| V308P | Human IgG4 | Cynomolgus monkey | ~43.3–390[d] | Cynomolgus monkey | ~1.9–3.2 | Datta-Mannan et al. (2012a) |
| N315D/A330V/N361D/A378V/ N434Y | Human IgG1 | Human | 7.4 | Human FcRn transgenic mice | 2.3 | Monnet et al. (2014) |
| E294D/T307P/N434Y | | | 5.2 | | 2.8 | |
| V259I/N315D/N434Y | | | 6.1 | | 2.3 | |
| T307A/N315D/A330V/E382V/ N389T/N434Y | | | 4.2 | | 2.3 | |

[a] Determined by surface plasmon resonance unless otherwise noted.
[b] Determined by ELISA-based assay.
[c] Determined by cell-based assay.
[d] Measured for five different antibodies with varying antigen specificities.
[e] For injected dose of 0.3 mg/kg.

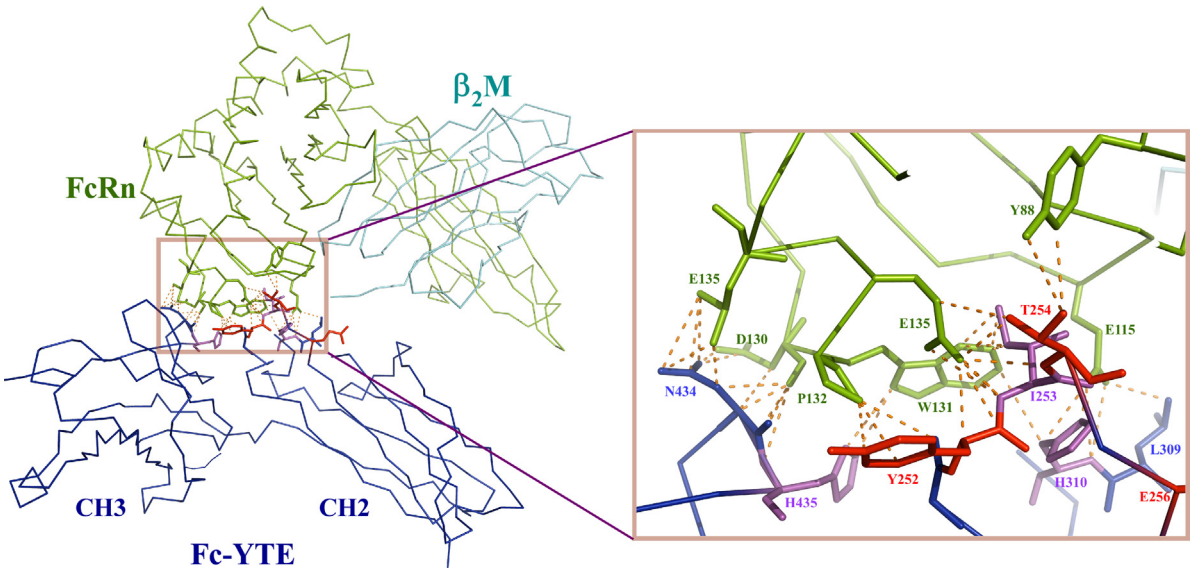


**Fig. 2.** Three dimensional structure of the FcRn:Fc-YTE complex (Oganesyan et al., 2014). The X-ray crystallographic structure of the FcRn:Fc-YTE complex is represented as a ribbon diagram with the Cα chains of FcRn, $\beta_2$-microglobulin and Fc-YTE colored in green, cyan and blue, respectively. The inset displays an enlarged view of the residues at the interface of the FcRn:Fc-YTE interaction. Salt bridges and hydrogen bonds between the indicated residues of FcRn and Fc-YTE are identified with a cutoff distance of 3.6 Å and are displayed using dotted yellow lines. Fc-YTE residues (Ile253, His310 and His435) playing a crucial role in binding to FcRn are shown in purple and the altered residues that constitute the YTE mutant (Tyr252, Thr254 and Glu256) resulting in affinity enhancement to FcRn at acidic pH are shown in red.

FcRn-mediated transport across cellular barriers, and evidence to support this has been obtained using an ex vivo placental explant model (Vaccaro et al., 2006). In addition, a recent study has demonstrated that an anti-human immunodeficiency virus antibody with increased mucosal delivery through enhanced FcRn binding provides increased protection against viral infection in non-human primates (Ko et al., 2014).

The question arises as to what is the maximum achievable increase in persistence (and transport) using Fc engineering, particularly since retention of low affinity at near neutral pH is essential for antibody recycling and exocytic release from FcRn-expressing cells? Several studies have demonstrated that, in general, affinity improvements for FcRn–IgG interactions at pH 6.0 are accompanied by increases in affinity at near neutral pH (Borrok et al., 2015; Dall'Acqua et al., 2002; Yeung et al., 2009). For example, Lowman and colleagues determined the affinities of a panel of engineered human IgG1 mutants for binding to human FcRn and showed that they are around 100-fold lower at near neutral pH than at pH 6.0 (Yeung et al., 2009). Comparison of the half-lives of two human IgG1 mutants with analogous affinities ($K_D$ ~1 nM) for mouse FcRn at pH 6.0, but 10-fold differences ($K_D$s of 7.2 and 82 nM) at near neutral pH, has demonstrated the negative effect of increased binding at this higher pH on in vivo persistence (Ward and Ober, 2009). In this context, a recent study defined the threshold affinity for human FcRn at pH 7.4 that mitigates the half-life extending effect of affinity increases at pH 6.0 (Borrok et al., 2015). Based on pharmacokinetic studies in human FcRn transgenic mice, this threshold is between ~400 and ~900 nM. Given the counterbalancing effects of increased binding at pH 6.0 and pH 7.3–7.4 on in vivo persistence, the concomitant increase in binding strength at pH 6.0 and pH 7.4 therefore imposes a limit on the half-life extensions that can be achieved.

### 6.2. Naturally occurring antibodies with reduced pH dependent binding to FcRn

Although His435 is present in the majority of mouse and human IgG isotypes, this residue is replaced by Arg in a subset of human IgG3 allotypes (e.g. g3m16) and Tyr in mouse IgG2b (Kabat et al., 1991). The important contribution of His435 to FcRn interactions raises the question as to how these substitutions might affect binding. Studies in which His435 in human IgG1 was mutated to Arg, and reciprocally, Arg435 in human IgG3 mutated to His, demonstrate that the His-containing antibodies have higher affinities for FcRn at pH 6.0, longer in vivo half-lives and improved transport across the placental barrier (Einarsdottir et al., 2014; Kim et al., 1999; Stapleton et al., 2011). Consistent with the p*K*a of the Arg side chain, Arg-containing allotypes of IgG3 retain significant binding to FcRn at near neutral pH, which also contributes to the lower activity in functions dependent on FcRn-mediated transport. Similarly, relative to mouse IgG1 or IgG2a, mouse IgG2b binds with increased affinity to mouse or rat FcRn at pH ~7 (Raghavan et al., 1995; Zhou et al., 2003). These three mouse isotypes have a similar affinity for mouse FcRn at pH 6.0, whereas the reduced pH dependence of IgG2b contributes to shorter in vivo persistence (Pollock et al., 1990; Vieira and Rajewsky, 1988).

### 6.3. Does FcRn play a role in controlling bioavailability?

Although the majority of therapeutic antibodies are delivered intravenously, several are delivered via the subcutaneous route (Wang et al., 2008). Subcutaneous delivery offers the major advantage that antibodies can be self-administered, prompting interest in understanding the processes through which subcutaneously delivered therapeutics, including antibodies, enter the circulation. As a corollary, knowledge of these processes may inform antibody engineering strategies to increase the effective therapeutic dose.

Subcutaneous bioavailability of a therapeutic is defined as the amount of delivered dose entering the circulation. This is determined by the extent of catabolism at the injection site and the efficiency of systemic absorption via the lymphatics and across the blood vessels (Wang et al., 2008). In FcRn-deficient mice, the subcutaneous bioavailability of a mouse IgG1 antibody was reported to be several-fold lower than that in wild type mice, implicating a role for FcRn in either local protection against catabolism, transcytotic delivery across cellular barriers or both (Wang et al., 2008). However, more recent studies directed toward determining whether affinity enhancement of an IgG for FcRn increases

its subcutaneous bioavailability have generated discrepant results (Datta-Mannan et al., 2012b; Deng et al., 2012). In one report, a mutated mouse IgG2a (N434H) with 13-fold increased affinity for mouse FcRn at pH 6.0 and retention of almost negligible binding at pH 7.4 had improved bioavailability relative to its wild type counterpart in mice (Deng et al., 2012). By contrast, analyses in cynomolgus monkeys did not demonstrate increased bioavailability for IgG4 molecules containing the half-life extending mutations, T250Q/M428L (Datta-Mannan et al., 2012b). Given the importance of optimizing IgG delivery through parenteral routes, additional work needs to be carried out to define a possible role for FcRn.

### 6.4. Do the characteristics of FcRn–IgG interactions correlate with in vivo behavior?

It is established that loss of binding of an antibody for FcRn at acidic, endosomal pH results in decreased in vivo persistence and inefficient transport across cellular barriers (Kim et al., 1999; Medesan et al., 1997; Spiekermann et al., 2002). However, for engineered antibodies that retain, or have increased affinity at this pH, there are discrepancies concerning whether the biophysical nature of an FcRn–IgG interaction correlates with its in vivo half-life/pharmacokinetic properties (Dall'Acqua et al., 2002; Datta-Mannan et al., 2007; Deng et al., 2010; Hinton et al., 2006; Suzuki et al., 2010; Yeung et al., 2009). Inconsistencies between binding properties and in vivo clearance may in part be due to variations in the binding assays used (e.g. immobilized FcRn vs. IgG) and the different experimental approaches and models employed to determine interaction affinities/kinetics by surface plasmon resonance (Datta-Mannan et al., 2007; Datta-Mannan and Wroblewski, 2014; Gurbaxani et al., 2006; Neuber et al., 2014; Wang et al., 2011a; West and Bjorkman, 2000; Yeung et al., 2009; Zhou et al., 2005). Multiple studies also demonstrate the negative effect of FcRn binding at near neutral pH on in vivo persistence (Borrok et al., 2015; Dall'Acqua et al., 2002; Datta-Mannan et al., 2007; Vaccaro et al., 2006; Yeung et al., 2009), and variations in assays to assess this may lead to apparently conflicting data. However, even if there is concordance concerning interaction behavior, this does not uniformly correlate with in vivo pharmacokinetics (Datta-Mannan and Wroblewski, 2014). There are several additional factors that may contribute to this lack of correlation: first, for antibodies that bind negligibly to FcRn at near neutral pH, uptake into the majority of cells is dependent on fluid phase pinocytosis. Significantly, studies of Igawa and colleagues in mice and cynomolgus monkeys (Igawa et al., 2010b) have indicated that this can be affected by the isoelectric point (p*I*) of the antibody since the surface of cells is negatively charged. Hence the higher the p*I*, the greater the internalization of antibody into cells and possibility of lysosomal degradation. Specifically, the substitution of both complementarity determining region (CDR) and framework residues with acidic amino acids in an anti-interleukin 6 receptor (IL-6R) antibody lowered the p*I*, resulting in increased in vivo persistence (Igawa et al., 2010b). Further, the choice of (light chain) framework for a humanized anti-hepatitis C glycoprotein antibody modulates the p*I*, conferring distinct pharmacokinetic properties in rats (Li et al., 2014). Second, for some antibodies, the endosomal environment (salt concentration, temperature, etc.) may result in different binding properties to those observed when carrying out interaction analyses in vitro, typically at 25 °C. Third, the degradation or modification of antibodies during storage can lead to loss of binding affinity for FcRn. For example, methionine oxidation of two residues, Met252 and Met428, in proximity to the FcRn–(human) IgG1 interaction site can lead to decreased in vivo half-life (Bertolotti-Ciarlet et al., 2009; Gao et al., 2015; Wang et al., 2011b). Fourth, when targeting membrane-bound receptors, or multivalent soluble ligands, receptor-antibody internalization and degradation or IC-mediated internalization by FcγRs, respectively, may contribute to clearance (McKeage and Perry, 2002; Rowinsky et al., 2004; Suzuki et al., 2010; Tabrizi et al., 2006). Studies demonstrating that FcRn can contribute to IC trafficking into lysosomes following FcγR-mediated internalization into endosomes (Baker et al., 2011; Qiao et al., 2008), indicate that this latter pathway of enhanced clearance also has some FcRn dependency. The effects of 'antigen sinks', whether soluble or membrane bound, can become particularly relevant when high levels of antigen are being targeted.

Given the possible sources of inconsistencies between FcRn binding characteristics and in vivo properties, the question arises as to how can in vitro properties be reliably translated to in vivo behavior? One possibility that may overcome the first three limitations described above is to use in vitro subcellular trafficking analyses in FcRn-expressing cells as an additional correlate for in vivo behavior. Given the variability in binding constants from one laboratory to the next, it may be also be instructive to develop a standardized protocol for FcRn–IgG interaction studies as discussed in Datta-Mannan and Wroblewski (2014), Neuber et al. (2014), and Yeung et al. (2009). This becomes particularly important in light of the Food and Drug Administration's requirement to report FcRn interaction properties for antibodies of potential therapeutic value.

In the context of the molecular nature of FcRn–IgG interactions, the Fab arms of antibody have been reported to affect binding to FcRn (Wang et al., 2011a) although this is discordant with the observations of others (Neuber et al., 2014). Interestingly, the recent use of hydrogen/deuterium exchange mass spectrometry to analyze human FcRn/human IgG1 complexes provides support for the involvement of Fab residues in binding to FcRn or a conformational link between the Fc and Fab fragments following complex formation (Jensen et al., 2015). Similarly, the protein moiety on Fc-fusions or the interaction of soluble ligand (TNF-α) with cognate antibody can modulate FcRn binding (Suzuki et al., 2010). Consequently, in addition to the possible enhancement of clearance by antigen sinks discussed above, the concentration of soluble ligand levels can affect pharmacokinetic behavior through altering FcRn interactions.

## 7. Modulating IgG dynamics by FcRn inhibition

By contrast with half-life extension of a therapeutic antibody, the inhibition of FcRn reduces antibody levels in the body. Several classes of FcRn inhibitors have been developed: peptides or small molecules (Low and Mezo, 2009; Mezo et al., 2008; Wang et al., 2013), anti-FcRn antibodies that bind through their V-regions (Getman and Balthasar, 2005) and Fc-engineered antibodies called Abdegs [for 'antibodies that enhance IgG degradation' (Vaccaro et al., 2005); Fig. 3]. All of these inhibitors recognize FcRn with increased affinity in the pH range 6.0–7.4 and competitively block IgG binding. Consequently, they are efficiently internalized into FcRn-expressing cells through FcRn-mediated endocytosis, compete with wild type IgGs for FcRn binding in endosomes and are not released during exocytic events at the cell surface (Vaccaro et al., 2005). The inhibitors have been demonstrated to reduce endogenous IgG levels in mice, and their efficacy in clearing pathogenic, autoreactive antibodies and ameliorating disease in mouse models of idiopathic thrombocytopenic purpura (ITP), myasthenia gravis, arthritis and multiple sclerosis has been demonstrated (Challa et al., 2013; Getman and Balthasar, 2005; Liu et al., 2007; Patel et al., 2011).

A potential drawback of the use of FcRn inhibitors is that they exhibit relatively short in vivo half-lives due to their increased binding to FcRn at near neutral pH (Dall'Acqua et al., 2002; Getman and Balthasar, 2005; Vaccaro et al., 2006). Inhibitors based on Fc-engineering have been shown to follow the constitutive

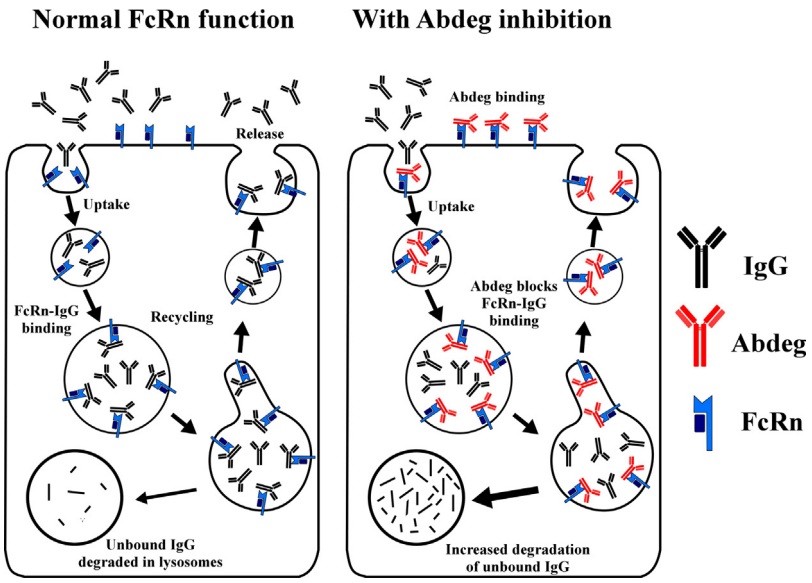


**Fig. 3.** Schematic representation of the effect of FcRn inhibition by an Abdeg on the subcellular trafficking of IgG. In the absence of inhibition, FcRn:IgG complexes are efficiently recycled and IgG is exocytosed (left panel). In the presence of an Abdeg, which binds to FcRn with increased affinity at pH 6.0–7.4, the interaction of wild type IgG with FcRn in endosomes is blocked (right panel). Consequently, increased levels of IgG enter lysosomes and are degraded.

degradation pathway for FcRn and traffick into lysosomes (Gan et al., 2009). Thus, for the treatment of chronic diseases frequent dosing may be necessary, although such agents could have utility in ameliorating disease flares. In this context, half-life extended antibodies which retain very low affinity binding to FcRn at near neutral pH also have a competitive advantage over their wild type counterparts (Petkova et al., 2006). However, for effective competition with FcRn there is a need to use higher doses of antibodies of this class compared with shorter-lived inhibitors that accumulate more efficiently in cells through FcRn-mediated binding at the cell surface (Petkova et al., 2006).

There are multiple examples of situations where it may be desirable to induce a rapid, one-time clearance of antibodies in vivo, including the elimination of radiolabeled, circulating antibody during tumor imaging and of antibody:toxin complexes. Due to their high specificity, antibodies represent attractive agents for use in diagnostic imaging. However, their prolonged in vivo persistence results in high levels of background and can also induce off-target tissue damage (Goldstein et al., 2013; Jaggi et al., 2007; Wu, 2014). The delivery of an Fc-engineered inhibitor (Abdeg) following the loading of tumor with radiolabeled antibody results in significant improvements in contrast during positron emission tomography [PET (Swiercz et al., 2014)]. As such, Abdegs or other FcRn inhibitors have promise for use in PET and other whole body imaging modalities involving diagnostic antibodies. Similarly, FcRn blockade could have applications in reducing dose limiting toxicities following the loading of tumor with radiolabeled antibodies during radioimmunotherapy (Goldenberg et al., 2012; Palanca-Wessels and Press, 2010).

## 8. Targeting antibody variable regions to alter antigen dynamics

### 8.1. Generation of recycling antibodies

The advantages of half-life extension with respect to the dosing of a therapeutic antibody are clear. However, if an antibody is being used to target a soluble ligand, such as a cytokine or other inflammatory mediator, the antibody itself can prolong the in vivo persistence of its antigen by 'buffering' (Davda and Hansen, 2010; Finkelman et al., 1993; Junghans and Anderson, 1996; O'Hear and Foote, 2005; Phelan et al., 2008; Rehlaender and Cho, 1998). This effect can result in substantial increases in antigen concentration. Further, a related problem occurs if the concentration of antigen is high since stoichiometric amounts of antibody are required. For example, complement C5 is typically present at high levels in the patient population indicated for treatment, necessitating the use of very large doses of the anti-C5 antibody, eculizumab (Zareba, 2007).

Strategies to overcome the limitations related to antibody buffering and the need to use stoichiometric antibody doses have recently been explored in several different studies (Chaparro-Riggers et al., 2012; Devanaboyina et al., 2013; Igawa et al., 2010a, 2014). The rationale used in these analyses is to engineer the V regions of antibodies to endow them with relatively tight binding to antigen at near neutral pH and release at endosomal, acidic pH. Antibodies with these interaction properties are expected to release antigen in acidic endosomal compartments following internalization of antibody–antigen complexes into cells (Fig. 4). Consequently, antigen enters the lysosomal pathway whereas the antibody is recycled for re-use. The use of these 'recycling' antibodies (Igawa et al., 2014) not only results in reductions in the buffering effect, but also enables lower doses of antibody to be effective.

Antibodies with substantially higher affinity at near neutral pH than at pH 5.5–6.0 are rarely isolated from immunized animals (Igawa et al., 2014). The use of antibody engineering approaches is therefore necessary to generate effective recycling or 'acid-switched' antibodies. It is well known that the p*K*a of histidine is appropriate for introducing pH dependence in the physiological pH range into protein–protein interactions. Typically, the engineering of pH-dependent antigen binding has involved the use of histidine scanning, whereby the residues in the CDRs of the antibody are systematically replaced with histidines (Chaparro-Riggers et al., 2012; Devanaboyina et al., 2013; Igawa et al., 2010a). As an alternative approach, CDR residues of a single domain VHH antibody have been mutated to histidine or retained as wild type amino acids to generate a combinatorial histidine library, followed by the use of phage display (Murtaugh et al., 2011). Computational design can also be implemented to generate pH-dependent protein–protein interactions (Sarkar et al., 2002; Strauch et al., 2014).

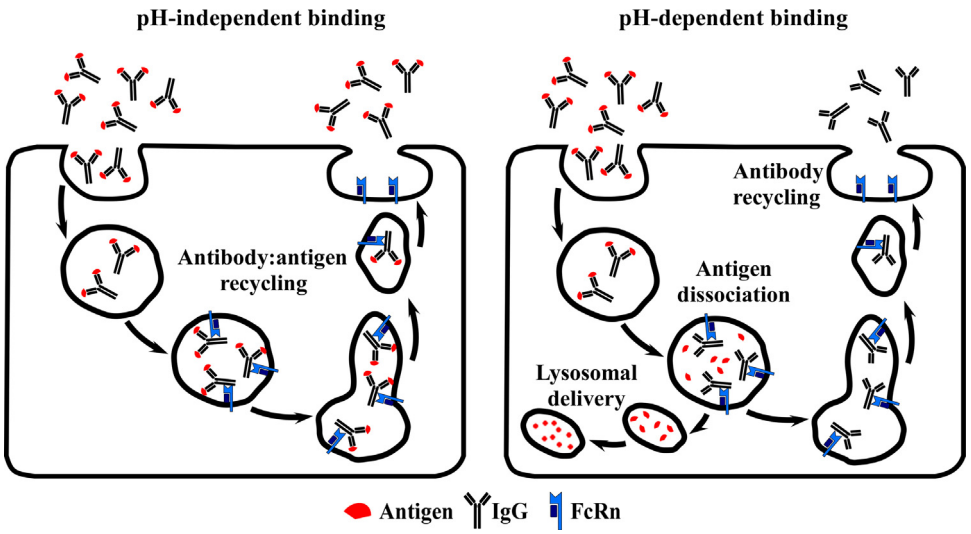


**Fig. 4.** Schematic representation showing how the pH dependent binding of an antibody to a soluble antigen (higher affinity at pH 7.4 than at pH 6.0) increases antigen degradation. Antigen in complex with the pH-independent antibody is taken up into the cell and sorted to recycling TCs through binding to FcRn (left panel). The antigen:pH-independent antibody complex is exocytosed at the cell membrane. By contrast, pH-dependent antibodies dissociate from antigen in early endosomes, resulting in lysosomal delivery of the antigen (right panel). The pH-dependent antibody is recycled by FcRn for re-use.

The efficacy of acid-switching in inducing increased antigen clearance rates in mouse and/or cynomolgus monkey models has been demonstrated for antibodies specific for the cytokine IL-6 and soluble IL-6R (Devanaboyina et al., 2013; Igawa et al., 2010a). In addition, the regulator of plasma levels of low density lipoprotein (LDL), proprotein convertase substilisin kexin type 9 (PCSK9), has been targeted (Chaparro-Riggers et al., 2012). PCSK9 binds to the LDL receptor (LDLR) and redirects the receptor from the recycling pathway into lysosomes. This results in lower LDLR levels with consequent increases in plasma LDL. pH-independent antibodies specific for PCSK9 are rapidly consumed via the lysosomal pathway following delivery, whereas their in vivo persistence and efficacy can be increased by conversion to pH-dependent variants.

The advantages of pH-dependent antigen binding can also be exploited for antibodies specific for membrane bound receptors (Igawa et al., 2010a). Antibody binding to membrane-associated target across the pH range 6–7.4 usually results in biphasic clearance kinetics due to receptor-mediated internalization and trafficking into lysosomes (target-mediated clearance) followed by a slower elimination phase of antibody that is not receptor-bound (McKeage and Perry, 2002; Rowinsky et al., 2004; Tabrizi et al., 2006). This effect is exacerbated for receptors that are expressed at high levels. The introduction of pH-dependent binding into the antibody, with higher affinity at near neutral pH compared with acidic endosomal pH, can ameliorate target-mediated clearance by enabling the dissociation of antibody from internalized receptor in endosomes followed by FcRn-mediated recycling of the free antibody (Chaparro-Riggers et al., 2012; Igawa et al., 2010a). For example, a pH-dependent recycling antibody specific for IL-6R (which can exist in both soluble and membrane bound form) has superior properties with respect to both antibody persistence and the lowering of the inflammatory mediators associated with IL-6:IL-6R signaling compared with its pH-independent counterpart (Igawa et al., 2010a).

Fluorescence microscopy studies have given insight into the subcellular trafficking behavior of an engineered recycling antibody for antibody:IL-6 complexes (Devanaboyina et al., 2013). A highly pH-dependent variant of the parent antibody was generated by systematically replacing CDR3 residues of the heavy and light chain variable (VH and VL) domains with His, resulting in a mutant containing an Asp to His substitution in CDR3 of the heavy chain with the desired properties. In the presence of this antibody, IL-6 accumulates in the vacuole of sorting endosomes, ultimately trafficking into lysosomes, whereas the antibody associates with FcRn on the endosomal limiting membrane and is recycled in tubulovesicular TCs (Fig. 5) (Devanaboyina et al., 2013). By contrast, IL-6 molecules associated with antibodies that do not show pH-dependent antigen binding are recycled as tripartite FcRn:IgG:IL-6 complexes (Devanaboyina et al., 2013). Consistent with the subcellular trafficking behavior, IL-6 has a shorter in vivo persistence in the presence of the pH-dependent antibody compared with that observed for pH-independent antibody complexes.

A persistent challenge for the generation of recycling antibodies is to engineer or design mutations that result in very low affinity for antigen at pH 5.5–6.0 whilst retaining high affinity at physiological pH. The requisite affinity at near neutral pH will depend on the nature of the antigen. Further, although the off-rate of the antibody–antigen interaction at pH 6.0 has been suggested to be a more important factor than affinity in improving the pharmacokinetic profile of an anti-PCSK9 antibody (Chaparro-Riggers et al., 2012), the generality of this suggestion merits further investigation. Consequently, there remains a need at both the level of experimental and modeling studies to better understand the design principles for recycling antibodies of high efficacy. Further, it may be possible to exploit knowledge concerning differences between the endosomal and extracellular environment other than pH, such as $Ca^{2+}$ concentrations, to generate antibodies that rapidly dissociate from their antigen within early endosomes (Igawa et al., 2014).

### 8.2. Sweeping antibodies

Receptor-mediated endocytosis of ligand followed by lysosomal degradation is a well-defined subcellular trafficking pathway. Compared with fluid phase uptake, receptor-mediated internalization is a highly efficient process for the accumulation of ligand within cells. This has prompted the design of engineered antibodies that, through binding to FcRn with moderate affinity at near neutral pH, efficiently accumulate in cells with their associated antigen (Igawa et al., 2013, 2014). These antibodies, combined with pH-dependent modulation of their variable region/antigen interaction, can be extremely effective in clearing soluble antigens. Antibodies of this class have appropriately been designated ‘sweeping’ antibodies and are substantially more potent than conventional antibodies in mediating antigen removal (Igawa et al., 2013, 2014).

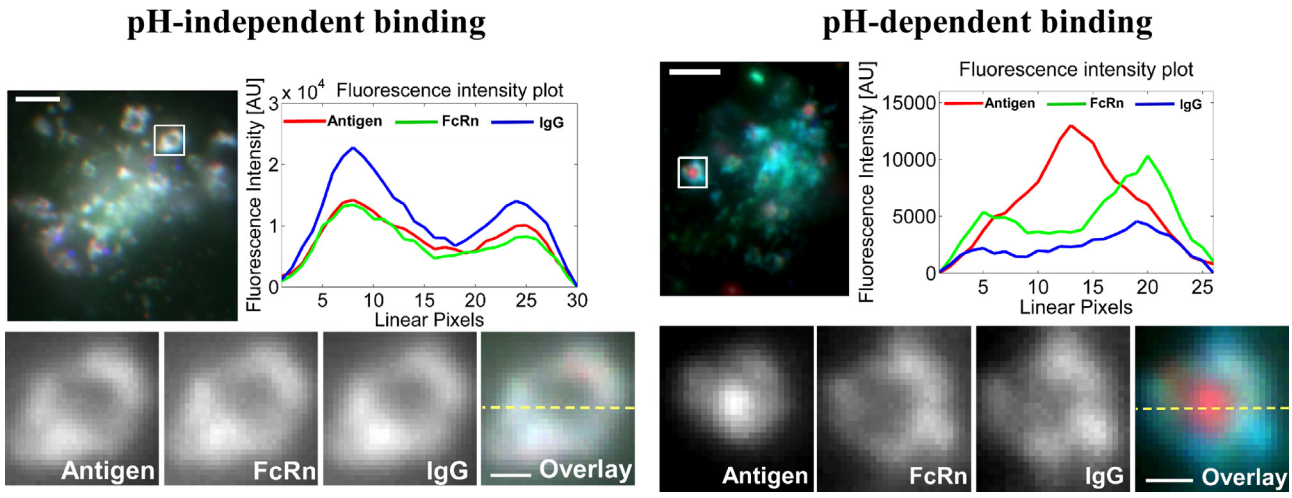


**Fig. 5.** Fluorescence microscopy analyses of the endosomal trafficking behavior of antigen (IL-6) in the presence of antibodies that differ in their pH dependence for binding to antigen. Panels present representative images of endothelial cells (HMEC-1) co-transfected with FcRn-GFP and human $\beta_2$-microglobulin. Following transfection, cells were pulsed with antigen:pH-independent antibody complexes (left panels) or antigen:pH-dependent antibody complexes (right panels). Whole cell images are shown in the upper panels, with expansions of cropped regions to present individual endosomes. For the pH-independent antibody, antigen (IL-6), FcRn and IgG are colocalized on the limiting membrane of the endosome (lower left panels). By contrast, antigen dissociates into the endosomal vacuole in the presence of the pH-dependent antibody (lower right panels). In the expanded images, individual fluorophores are represented in black and white whereas for the overlays, antigen (Alexa 555), FcRn (GFP) and IgG (Alexa 647) are pseudocolored red, green and blue respectively. Background-subtracted fluorescence intensities along the dotted lines in the overlays are shown in the fluorescence intensity plots. The scale bars for the complete cell images and the cropped endosomes represent 4 and 0.5 μm, respectively.

As expected, there is a counterbalance between increased cellular uptake due to higher affinity at neutral pH and in vivo persistence. Nevertheless, appropriate tuning of the FcRn-binding properties of sweeping antibodies should be possible to enable optimization of the extent and duration of antigen clearance.

## 9. Future directions

Major progress has been made in engineering both the Fc regions and variable domains of antibodies for half-life extension and pH-dependent binding, with consequent improvements in efficacy in multiple systems. To facilitate further development, an improved understanding of the cell biology of different FcRn-expressing cells is desirable. For example, knowledge concerning fluid phase uptake processes, recycling rates, the endosomal environment and FcRn endocytic rates is limited. Further, variations in these characteristics across cell types, such as hematopoietic vs. endothelial cells, are unexplored. A more complete understanding of these properties could lead to the design of more effective therapeutics.

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