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Multitasking by Exploitation of Intracellular Transport Functions: The Many Faces of FcRn

E. Sally Ward* and Raimund J. Ober*[†]

*Department of Immunology, University of Texas Southwestern Medical Center, Dallas, Texas, USA

[†]University of Texas at Dallas, Richardson, Texas, USA

Abstract

The MHC Class I-related receptor, FcRn, transports antibodies of the immunoglobulin G (IgG) class within and across a diverse array of different cell types. Through this transport, FcRn serves multiple roles throughout adult life that extend well beyond its earlier defined function of transcytosing IgGs from mother to offspring. These roles include the maintenance of IgG levels and the delivery of antigen in the form of immune complexes to degradative compartments within cells. Recent studies have led to significant advances in knowledge of the intracellular trafficking of FcRn and (engineered) IgGs at both the molecular and cellular levels. The engineering of FcRn–IgG (or Fc) interactions to generate antibodies of increased longevity represents an area of active interest, particularly in the light of the expanding use of antibodies in therapy. The strict pH dependence of FcRn–IgG interactions, with binding at pH 6 that becomes essentially undetectable as near neutral pH is approached, is essential for efficient transport. The requirement for retention of low affinity at near neutral pH increases the complexity of engineering antibodies for increased half-life. Conversely, engineered IgGs that have gained significant binding for FcRn at this pH can be potent inhibitors of FcRn that lower endogenous IgG levels and have multiple potential uses as therapeutics. In addition, molecular studies of FcRn–IgG interactions indicate that mice have limitations as preclinical models for FcRn function, primarily due to cross-species differences in FcRn-binding specificity.

1. Introduction

The MHC Class I-related receptor, FcRn (n for neonatal), was originally identified as the receptor that transports maternal IgG in mother's milk across the neonatal rodent gut during the suckling period (Brambell, 1970; Rodewald and Abrahamson, 1982; Wallace and Rees, 1980). However, more recent studies have not only shown that this receptor serves to regulate IgG levels and distribution throughout adult life (Ghetie *et al.*, 1996; Israel *et al.*, 1996; Junghans and Anderson, 1996), but also that it has multiple other roles in diverse cell types and tissues (e.g., Akilesh *et al.*, 2008; Dickinson *et al.*, 1999; Kim *et al.*, 2008; Spiekermann *et al.*, 2002; Zhu *et al.*, 2001). FcRn orthologs have been isolated from many species, including mouse, rat, man, sheep, cow, possum, pig, and camel (Adamski *et al.*, 2000; Ahouse *et al.*, 1993; Kacskovics *et al.*, 2000, 2006; Kandil *et al.*, 1995; Mayer *et al.*, 2002; Schnulle and Hurley, 2003; Simister and Mostov, 1989; Story *et al.*, 1994), indicating that this receptor is present in essentially all mammalian species. The multiple functions of

FcRn are dependent on its ability to sort IgG away from lysosomal degradation within cells and release bound cargo during exocytic events at the plasma membrane (Ober *et al.*, 2004a,b; Prabhat *et al.*, 2007). Consequently, this receptor transports IgG within and across cellular barriers for a diverse array of cell types (Antohe *et al.*, 2001; Claypool *et al.*, 2004; Dickinson *et al.*, 1999; Firan *et al.*, 2001; Haymann *et al.*, 2000; McCarthy *et al.*, 2000; Spiekermann *et al.*, 2002; Yoshida *et al.*, 2004). More recently, FcRn has also been shown to control albumin levels (Andersen *et al.*, 2006; Chaudhury *et al.*, 2003). How this receptor behaves at the subcellular level of intracellular trafficking, and what controls its intracellular routing are of fundamental relevance to understanding its function. In addition, given the potential for modulating IgG trafficking pathways and behavior *in vivo*, the earlier report of engineering of antibodies to increase their half-life in mice (Ghetie *et al.*, 1997) has expanded into an area of intense interest in the biopharma industry (Dall'Acqua *et al.*, 2006b; Hinton *et al.*, 2004, 2006; Shields *et al.*, 2001).

In the current review, we will describe the multiple functions of FcRn and the intracellular trafficking pathways of this receptor and its ligand. The modulation of FcRn–ligand interactions for the development of therapeutics will also be discussed, with a particular focus on how the complexity of the pH dependence of FcRn–IgG interactions and cross-species differences in behavior impact this area.

2. FcRn: A Historical Perspective

Neonatal rodents acquire the major portion of their maternal IgG from mothers' milk during the suckling period (Brambell, 1970). An early model for the trafficking of IgG across the neonatal gut was originally proposed in the absence of specific knowledge of the receptor involved (Brambell, 1970): IgGs are taken into enterocytes at the apical surface by receptor-mediated uptake at the acidic pH in the small intestine. These IgGs are then transcytosed across the cells and released at the basolateral membrane which is at near neutral pH. A central feature of this early model was that the unidentified receptor, which was later shown to be FcRn (Rodewald and Abrahamson, 1982; Wallace and Rees, 1980), is a salvage receptor which binds and transports IgG in intact form across cells.

FcRn was subsequently isolated from rodent gut as a heterodimer comprising 12 kDa and 40–45 kDa proteins (Rodewald and Kraehenbuhl, 1984; Simister and Rees, 1985). Significantly, in these early studies, the FcRn–IgG interaction was shown to be highly pH dependent with relatively tight binding at acidic pH (6) and very weak, if not negligible, binding at near neutral pH (Rodewald and Kraehenbuhl, 1984; Simister and Rees, 1985). The cloning of the gene for rat FcRn in 1989 unexpectedly revealed that this receptor comprises an α -chain that is homologous to MHC Class I α -chains, and the 12 kDa component is β 2-microglobulin (β 2m) (Simister and Mostov, 1989). This was followed by the isolation of orthologous FcRn α -chains from mouse and man (Ahouse *et al.*, 1993; Kandil *et al.*, 1995; Story *et al.*, 1994), and subsequently from multiple other species (Adamski *et al.*, 2000; Kacsiovics *et al.*, 2000, 2006; Mayer *et al.*, 2002; Schnulle and Hurley, 2003). Although FcRn orthologs share some similarities, there are cross-species differences at the level of binding specificity that can have functional effects (Ober *et al.*, 2001; Vaccaro *et al.*, 2006), in addition to variations in intracellular trafficking and

subcellular distribution (Claypool *et al.*, 2002; Kuo *et al.*, 2009) (discussed further in Sections 5.5 and 8).

3. FcRn is a Multitasking Receptor

3.1. A role for FcRn in regulating IgG levels

At the time of the isolation of the gene encoding rat FcRn (Simister and Mostov, 1989), the primary function of this receptor was believed to be to deliver maternal IgG to offspring. Although Brambell and colleagues proposed in the 1960s that the cellular processes involved in transporting maternal IgG from mother to young and in regulating IgG levels throughout life might be related (Brambell, 1970; Brambell *et al.*, 1964), data to provide direct support for the involvement of FcRn in both of these processes were absent. However, in the mid-1990s, several observations led to the conclusion that FcRn exploits its capability to transport IgG within and across cells to regulate IgG levels throughout adult life. First, we demonstrated that the same IgG residues (on both CH2 and CH3 domains of the Fc region) are involved in controlling the *in vivo* half-life of Fc fragments and their transport across the neonatal gut (Kim *et al.*, 1994a,b). Second, we observed that mice deficient in $\beta 2m$ that do not express functional FcRn are characterized by abnormally rapid clearance rates of IgG/Fc fragments (Ghetie *et al.*, 1996, see also Israel *et al.*, 1996; Junghans and Anderson, 1996). Third, FcRn expression is not restricted to the gestational or neonatal periods, but can be detected in multiple tissues/cell types throughout adult life (Ghetie *et al.*, 1996). Fourth, an engineered Fc fragment with higher affinity for FcRn at pH 6, but with retention of very low affinity at near neutral pH, was shown to have increased *in vivo* persistence in mice (Ghetie *et al.*, 1997).

The ubiquitous nature of FcRn expression leads to the question as to which cell types are most relevant for the regulation of IgG levels *in vivo*? Distribution studies of IgGs with different binding properties for FcRn indicated that the (micro)vasculature, primarily in skin and muscle with lesser amounts in liver and adipose tissue, contributes to IgG homeostasis (Borvak *et al.*, 1998), consistent with the earlier suggestion that this regulation occurs at diffuse sites throughout the body (Waldmann and Strober, 1969). More recent studies involving bone marrow transfers indicate that FcRn expression in hematopoietic cells such as dendritic cells, monocytes, and macrophages also contributes to the regulation of IgG levels (Akilesh *et al.*, 2007; Qiao *et al.*, 2008). To delineate the role of specific cell types in the maintenance of IgG concentrations *in vivo*, we have generated a mouse strain in which FcRn can be conditionally deleted (Perez-Montoyo *et al.*, 2009). This strain harbors FcRn alleles (exons 5–7) flanked by loxP sites, and in combination with Tie2-Cre mice which express Cre recombinase under the control of the Tie2 promoter in endothelial and hematopoietic cells (Kisanuki *et al.*, 2001) can be used to analyze the impact of site-specific deletion of FcRn in these cells. Analyses of the clearance rates of IgGs in these mice demonstrate that endothelial and hematopoietic cells are the primary sites responsible for FcRn-mediated homeostasis of IgG (Perez-Montoyo *et al.*, 2009).

Although targeted deletion of human FcRn is clearly not possible, the analysis of archived human blood samples from patients with a deficiency in $\beta 2m$ expression has provided a naturally occurring human knockout for FcRn (Wani *et al.*, 2006). These patients have

abnormally low IgG levels. Taken together with correlations between FcRn-binding properties of an IgG and *in vivo* persistence in nonhuman primates (discussed further in Section 7.2), the available data therefore indicate that FcRn is also a major contributor to IgG homeostasis in humans.

3.2. FcRn-mediated transport of IgG across cellular barriers: Opportunities for drug delivery

In addition to the role of FcRn in transporting maternal IgG across the neonatal intestine (Rodewald and Abrahamson, 1982; Wallace and Rees, 1980), the central function of FcRn in transporting IgG across both the mouse yolk sac and human placenta during gestation has been demonstrated (Firan *et al.*, 2001; Medesan *et al.*, 1996). More recently, it has become apparent that FcRn serves to deliver IgGs across cellular barriers throughout life. Extensive analyses of FcRn-mediated trafficking of IgGs and IgG–antigen complexes across epithelial cells in cell lines and in adult mice/nonhuman primates provide insight into these transport processes (Bitonti and Dumont, 2006; Bitonti *et al.*, 2004; Dickinson *et al.*, 1999; Haymann *et al.*, 2000; Kobayashi *et al.*, 2002; Sakagami *et al.*, 2006; Spiekermann *et al.*, 2002; Yoshida *et al.*, 2004). For example, in transgenic mice that are engineered to express mouse FcRn in adult intestinal epithelium, FcRn can transport antigen bound to IgG from the intestinal lumen into the lamina propria to elicit CD4⁺ T cell responses against bacteria (Yoshida *et al.*, 2006). Thus, FcRn can function as a scavenger of luminal antigens in the gut, indicating that it can play an important role in mucosal immunity.

Trans-epithelial transfer offers opportunities for the delivery of therapeutic proteins, and consistent with this, erythropoietin–Fc fusions can be transferred in an FcRn-dependent mode across the lung epithelium of adult mice and nonhuman primates (Bitonti *et al.*, 2004; Spiekermann *et al.*, 2002). Interestingly, a “monomeric” Epo–Fc fusion comprising a single Epo molecule connected to one arm of the dimeric Fc molecule was transported more efficiently than an Epo–Fc dimer containing two Epo molecules per Fc (Bitonti *et al.*, 2004). This enhanced transport was shown to be due in part to an increased affinity for binding of the monomer to FcRn, but in addition, size reduction and/or a change in charge might be contributing properties (Bitonti and Dumont, 2006). Surfaces such as lung epithelium that are bathed in mucus may be particularly susceptible to such effects, indicating that it will be advantageous to design molecules with optimized properties such as charge, size, and minimization of steric hindrance on FcRn binding for a given delivery route. For lung delivery, high potency of the biologic is also important since the volume of the vehicle is, by necessity, relatively low (Wang *et al.*, 2008). Although transport across the intestine avoids this potential limitation, a major challenge is to generate recombinant proteins that are resistant to the hostile proteolytic and acidic environment of this locale. The targeting of FcRn with Fc-fusion proteins to deliver therapeutics *in utero* is also attractive and promise for this approach in a mouse model of the lysosomal storage disease, mucopolysaccharidosis, has been demonstrated (Grubb *et al.*, 2008). Collectively, these studies indicate that the use of FcRn as a drug delivery vehicle has multiple possible applications.

3.3. FcRn can deliver antigen for presentation

The earlier observation that FcRn is expressed in monocyte/macrophages and dendritic cells led to the suggestion that this receptor might play a role in antigen presentation (Zhu *et al.*, 2001). More recent analyses have shown that FcRn can direct immune complexes (ICs) into lysosomes in dendritic cells, which in turn can enhance antigen presentation (Qiao *et al.*, 2008). Although FcRn was originally shown not to be expressed in B cell lines and primary B cells (Akilesh *et al.*, 2007; Ghetie *et al.*, 1996; Zhu *et al.*, 2001), this receptor is present in splenic B cells in mice (Mi *et al.*, 2008; Perez-Montoyo *et al.*, 2009). This extends the expression of FcRn to all major subsets of professional antigen presenting cells (APCs). Taken together with the report that invariant chain, for which the expression is generally restricted to APCs, can associate with FcRn and direct it into lysosomes (Ye *et al.*, 2008), this suggests that the intracellular trafficking pathways in these cells can be modulated to optimize antigen presentation (discussed further in Section 5.7). Interestingly, phagocytosis of opsonized bacteria by human neutrophils is also increased by FcRn expression, leading to the suggestion that the nascent phagocytic cup is acidified to facilitate FcRn–IgG interactions during uptake (Vidarsson *et al.*, 2006). This might provide an explanation for the higher phagocytic activity of monocytes relative to NK cells that do not express FcRn. Taken together, the data therefore indicate that FcRn cannot only enhance phagocytic uptake, but can also redirect antigen complexed with antibodies into degradative compartments that are associated with the loading of antigenic peptides onto MHC Class II molecules within cells.

3.4. Possible functions of FcRn in specialized cell types

FcRn expression in highly specialized cells such as podocytes in the kidney plays an important role in removing IgG from the glomerular basement membrane (Akilesh *et al.*, 2008). Indeed, blocking of FcRn in mice leads to serum-induced nephritis, suggesting that impaired function of this clearance process could predispose toward glomerular disease. This raises questions concerning whether FcRn (dys)function might contribute to the pathology of diseases such as systemic lupus erythematosus, in which IC-mediated kidney damage is common.

FcRn expression has also been demonstrated in multiple ocular tissues, including the cornea, retina, conjunctiva, and the blood–ocular barrier (Kim *et al.*, 2008). The function of FcRn at these sites is currently unknown, but may be related to the immune-privileged status of the eye. Similarly, FcRn is expressed at the blood–brain barrier (BBB) in both the brain microvasculature and the choroid plexus epithelium (Schlachetzki *et al.*, 2002) where it might be important for maintaining low levels of potentially inflammatory antibodies in the CNS. Consistent with this, several studies demonstrate that IgG is transported by FcRn in the brain-to-blood direction (Deane *et al.*, 2005; Zhang and Pardridge, 2001). This directional transport has specific relevance to the clearance of amyloid β peptide (A β) from the brain by A β -specific IgG, which results in a reduction of symptoms of Alzheimer's disease in a mouse model (Deane *et al.*, 2005). Such studies indicate that it could be fruitful to explore this pathway further for A β -directed immunotherapy. However, others have reported that the brain-to-blood exposure ratios for IgG are the same in both wild-type and

FcRn-deficient mice (Wang *et al.*, 2008), indicating that the role of FcRn at this barrier requires further investigation.

4. The Molecular Nature of FcRn–IgG Interactions

4.1. The interaction site for FcRn on IgG

The molecular details of FcRn–IgG interactions have been extensively analyzed. For example, site-directed mutagenesis of recombinant IgG or Fc fragments has been used to identify residues that are involved in the mouse FcRn–IgG interaction for both human and mouse IgG1 (Kim *et al.*, 1994b, 1999; Medesan *et al.*, 1997). These studies have involved a combination of *in vitro* binding analyses and *in vivo* assays in mice, and demonstrate that His310, Ile253, and His435 of IgG play a central role in these interactions (Fig. 4.1). These same residues are involved in the human FcRn–human IgG1 (Firan *et al.*, 2001; Shields *et al.*, 2001) and rat FcRn–IgG (mouse, rat or human) interactions (Martin *et al.*, 2001; Raghavan *et al.*, 1995). Residue 436 (His in mouse IgG1, Tyr in human IgG1) plays a minor role in the binding of IgG to FcRn (Medesan *et al.*, 1997; Shields *et al.*, 2001). The high-resolution X-ray crystallographic structure of rat FcRn complexed with rat IgG2a clearly shows the direct involvement of residues 253, 310, 435, and 436 of IgG in binding (Martin *et al.*, 2001). These four residues are relatively well conserved across species and are located at the CH2–CH3 domain interface of IgG (Deisenhofer, 1981) (Fig. 4.1). The role of the highly conserved His433 of IgG in the interaction across species is more uncertain: in some systems it has been proposed to play a role (Martin *et al.*, 2001; Raghavan *et al.*, 1995; Shields *et al.*, 2001), whereas in others not (Kim *et al.*, 1999; Medesan *et al.*, 1997). Nevertheless, the involvement of several histidines on IgG in complex formation that interact with acidic residues on FcRn provides an explanation for the marked pH dependence of FcRn–IgG binding, with binding at pH 6–6.5 which for most IgGs becomes progressively weaker as pH 7.4 is approached (Popov *et al.*, 1996; Raghavan *et al.*, 1995; Rodewald, 1976; Wallace and Rees, 1980). This pH dependence is essential for FcRn to function as an IgG transporter (see Section 7).

4.2. The interaction site for IgG on FcRn

Structure–function studies in the Bjorkman laboratory have identified FcRn residues that are involved in the rat FcRn–rat IgG2a interaction (Vaughn *et al.*, 1997), and the results of these analyses have been confirmed by the solution of the high-resolution structure of this complex (Martin *et al.*, 2001). To date, structural studies of human FcRn in complex with IgG have not been reported. Although the X-ray crystallographic structure of human FcRn in the absence of ligand indicates that it is structurally similar to rat FcRn, there are also some differences (West and Bjorkman, 2000). Rat FcRn residues that interact with IgG(2a) include Ile1 of β 2m and Glu117, Glu118, Glu132, Trp133, Glu135, and Asp137 of the FcRn α -chain (Fig. 4.2). These amino acids are generally well conserved across species, although some notable exceptions exist. For example, Asp or Glu137 of rodent FcRn is replaced by leucine in human FcRn (Ahouse *et al.*, 1993; Simister and Mostov, 1989; Story *et al.*, 1994) (note that the numbering used throughout this review for human FcRn, which is two residues shorter than mouse/rat FcRn, ignores this two residue deletion and is based on the homology alignment of human and rodent FcRn). This sequence variation accounts, in part at least, for

cross-species differences in binding specificity between rodent and human FcRn (Zhou *et al.*, 2003, 2005) (discussed in Section 8).

The ectodomains of FcRn also bear one or more potential glycosylation sites, raising the question as to whether this might contribute to IgG binding. It has been demonstrated that this is the case for rat FcRn, since carbohydrate attached to an N-linked glycosylation site at residue 128 of the receptor makes contacts with Val348, His433, Asn434, and Lys439 of rat IgG2a (Martin *et al.*, 2001). However, the relevance of an analogous interaction for the mouse FcRn–mouse IgG1 or mouse FcRn–human IgG1 complex is made unlikely by our observation that mutation of His433 or Asn434 individually to alanine in IgG1-derived Fc fragments does not affect activity in mouse FcRn-mediated functions (Kim *et al.*, 1999; Medesan *et al.*, 1997). Furthermore, human FcRn functions effectively in binding to IgG without a potential glycosylation site at residue 128, suggesting that there may be differences at this level between rat and human FcRn. It is, however, interesting to note that differences in glycosylation between human and rat FcRn lead to variations in intracellular trafficking (Kuo *et al.*, 2009) and this is discussed further in Section 5.5.

4.3. The stoichiometry of the FcRn–IgG interaction

The presence of two possible binding sites for FcRn on IgG (or Fc) raises questions concerning the stoichiometry of the interaction. By generating a “hybrid” Fc comprising one CH2–CH3 polypeptide with a defective FcRn interaction site complexed with a wild-type CH2–CH3 polypeptide, two functional sites per Fc (mouse IgG1- or rat IgG2a-derived) have been shown to be essential for full activity *in vivo* in mice (Kim *et al.*, 1994b,c) and *in vitro* transport across rat FcRn-transfected epithelial cells (Tesar *et al.*, 2006). On the other hand, interaction analyses with soluble, recombinant FcRn demonstrated that the stoichiometry can be 2 FcRn:1 IgG or 1:1 (Martin and Bjorkman, 1999; Popov *et al.*, 1996; Sanchez *et al.*, 1999; Schuck *et al.*, 1999). This apparent discrepancy can be resolved by the demonstration that two possible binding sites on IgG (or Fc) are not equivalent (Sanchez *et al.*, 1999; Schuck *et al.*, 1999; Weng *et al.*, 1998), consistent with the concept that binding of FcRn to one site may reduce the affinity for the second site, that is, negative cooperativity (Ghetie and Ward, 1997). Whether this asymmetry is due to steric effects and/or some longer range conformational changes at the CH2–CH3 domain junction is currently unknown. However, the segmental flexibility of the IgG molecule (Nezlin, 1990; Oi *et al.*, 1978), together with the observation that a hinge-less Fc has lower activity in FcRn-mediated functions (Kim *et al.*, 1995), would be consistent with conformational alterations.

5. The Intracellular Trafficking of FcRn

5.1. A model for FcRn trafficking

The pH dependence of FcRn interactions with the majority of naturally occurring IgGs is central to its function (Popov *et al.*, 1996; Raghavan *et al.*, 1995; Rodewald and Kraehenbuhl, 1984; Simister and Rees, 1985; Zhou *et al.*, 2005). Earlier models for how FcRn traffics within cells suggested that in most cell types, IgG is taken up primarily by fluid-phase processes (Brambell *et al.*, 1964; Ghetie and Ward, 1997), since the pH at most cell surfaces is not favorable for binding. However, it is possible that for cells such as those

of epithelial origin, for which Na^+/H^+ exchanger activity results in acidification of the local environment (Hattori *et al.*, 2001), or in the acidic environments of tumors or inflammatory sites (Edlow and Sheldon, 1971; Gerweck and Seetharaman, 1996; Tannock and Rotin, 1989; Ward and Steigbigel, 1978), significant levels of uptake by receptor-mediated processes can also occur. Whatever the route of uptake, entry of IgG into cells is followed by accumulation in early endosomes for which the acidic pH is permissive for binding (Fig. 4.3). If binding of the IgG to FcRn occurs, then the IgG is recycled or transcytosed (Ober *et al.*, 2004b). By contrast, IgGs that do not bind to FcRn enter late endosomes and are subsequently delivered to lysosomes (Ober *et al.*, 2004b). The predictions of this model are consistent with experimental observations: first, IgGs that have reduced affinity for binding to FcRn have shorter *in vivo* half-lives and are transported across cellular barriers less effectively (Firan *et al.*, 2001; Kim *et al.*, 1999; Medesan *et al.*, 1997; Spiekermann *et al.*, 2002). Second, reduced expression of FcRn within cells results in increased degradation of IgG (Ghetie *et al.*, 1996; Israel *et al.*, 1996; Junghans and Anderson, 1996; Roopenian *et al.*, 2003). Third, engineered IgGs that bind to FcRn with increased affinity at near neutral pH are taken into cells by receptor-mediated uptake and not released efficiently at the cell surface following recycling or transcytosis (Vaccaro *et al.*, 2005, 2006).

5.2. Endosomal sorting of IgGs within endothelial cells

Live-cell-fluorescence imaging has been used to analyze several facets of FcRn-mediated trafficking of IgGs in human endothelial cells (Ober *et al.*, 2004a,b; Prabhat *et al.*, 2007; Ram *et al.*, 2008; Gan *et al.*, 2009). For example, the intracellular trafficking of fluorescently labeled IgGs that have different binding properties for FcRn have been compared to address the question as to where and how IgGs are sorted within cells. These IgGs include wild-type human IgG1 and a mutated derivative (H435A, His435 to Ala) that does not bind detectably to human FcRn (Firan *et al.*, 2001; Ober *et al.*, 2004b). Treatment of human FcRn–GFP-transfected endothelial cells with these two IgGs in fluorescently labeled form, followed by live-cell imaging, has led to a dynamic picture as to how IgGs with distinct binding properties for FcRn are sorted within cells. The wild-type IgG1 leaves sorting endosomes in FcRn-positive tubules and vesicles that are also involved in transferrin recycling (Ober *et al.*, 2004b) (Fig. 4.4). Recently, tubulovesicular, FcRn⁺ transport containers (TCs) have been visualized using electron tomography of rat jejunal sections by Bjorkman and colleagues (He *et al.*, 2008) that are most likely analogous to the TCs observed in transfected endothelial cells. By contrast with wild-type IgG1, in endothelial cells the H435A mutant persists in the “vacuole” of sorting endosomes while FcRn-positive tubules and vesicles segregate from these compartments to enter the recycling/transcytotic pathway (Ober *et al.*, 2004b). Ultimately, the H435A mutant can be detected in the lysosomes of these cells, through delivery processes that most likely involve different types of fusion events of late endosomes and lysosomes (Gan *et al.*, 2009; Luzio *et al.*, 2003). Thus, the intracellular trafficking behavior of the two IgGs correlates with their *in vivo* properties: the IgG1 molecule has a long persistence and is transported across cellular barriers, whereas the half-life of the H435A mutant is short and its delivery across cells is at background levels (Firan *et al.*, 2001; Kim *et al.*, 1999).

5.3. Exocytic processes that result in IgG release from endothelial cells

The question as to how IgG molecules are released from cells during exocytosis has also been addressed using total internal fluorescence microscopy (TIRFM) combined with single molecule imaging in live cells (Ober *et al.*, 2004a). It is interesting to note that in most cell types the steady state, cell-surface expression levels of FcRn are low (Antohe *et al.*, 2001; Dickinson *et al.*, 1999; Ghetie *et al.*, 1996; Kristoffersen and Matre, 1996; Ober *et al.*, 2004b; Roberts *et al.*, 1990). This raises the question as to whether FcRn “cohorts” bound IgG to the cell surface during exocytosis or whether bifurcation of FcRn and ligand occurs prior to fusion of exocytic compartments with the plasma membrane. Our live-cell imaging data demonstrated that FcRn is delivered to the plasma membrane during exocytic events (Ober *et al.*, 2004a). Using electron tomographic analyses, clathrin has been shown to be associated with both exo- and endocytic processes involving FcRn (He *et al.*, 2008). This association provides a molecular mechanism by which FcRn can be rapidly retrieved following exocytic fusion, which in turn results in low steady-state levels on the plasma membrane.

In addition to the classic type of full fusion exocytic event, using TIRFM, we observed processes in which IgG was released at the plasma membrane of endothelial cells over relatively long time periods (up to several minutes) in bursts of release, in a process that we named prolonged release (Ober *et al.*, 2004a). Multiple other types of exocytic events were also visualized, suggesting that exocytosis can occur via different processes that fall on a continuum ranging from full fusion to prolonged release. The molecular components that determine the type of exocytic event are currently unknown, but most likely relate to the local concentrations of fusion and fission effectors at the exocytic sites. Importantly, different types of exocytic processes can be observed for an individual cell (Ober *et al.*, 2004a), indicating that these events are not predetermined by the physiological state of the cell. The biological significance of these exocytic pathways remains to be determined, and will need to be preceded by an analysis of the molecular components that regulate, for example, prolonged release versus full fusion.

The implementation of single molecule analyses of FcRn and IgG during exocytosis in endothelial cells has also allowed the behavior of individual IgG and FcRn molecules, rather than bulk populations, to be studied (Ober *et al.*, 2004a). This led to the observation of retrograde movement of IgG and FcRn molecules back to the exocytic site following exocytosis, generating insight into the molecular nature of these processes.

5.4. Imaging FcRn trafficking in three dimensions using multifocal plane microscopy

The observation of multiple different types of exocytic events using TIRFM at the plasma membrane (Ober *et al.*, 2004a) leads to the question as to which intracellular trafficking processes precede different types of release mechanisms? To address this and other questions, we have developed a multifocal plane microscopy (“MUM”) set up that allows simultaneous visualization of multiple planes within the cell combined with TIRFM imaging at the plasma membrane (Prabhat *et al.*, 2004, 2007). This approach has to date given insight into the intracellular events that precede exocytosis: for example, the recycling tubulovesicular TCs that leave sorting endosomes can be categorized into pathways of direct

and indirect recycling processes. In the most direct type of recycling, tubules extend from sorting endosomes and undergo exocytosis while remaining connected (Prabhat *et al.*, 2007). By contrast, for less direct pathways, TCs accumulate in “holding zones” in proximity to the plasma membrane prior to exocytosis.

More recently, we have also used MUM to visualize endocytic events involving FcRn and its IgG ligand (Ram *et al.*, 2008). In these analyses, we have utilized an engineered IgG–FcRn pair of high affinity to enable receptor-mediated uptake at near neutral pH (Vaccaro *et al.*, 2005; Zhou *et al.*, 2005). Reminiscent of the analyses of exocytic processes, these studies demonstrate that endocytic processes can be broadly categorized into two classes: “direct” in which the endocytic TC moves rapidly toward a sorting endosome and fuses and “indirect” in which more circuitous itineraries are taken within the cell prior to endosomal fusion. Collectively, these studies of endo- and exocytosis have implications for understanding the dynamics of FcRn-mediated trafficking and IgG homeostasis, and may relate to the fast and slow recycling processes that have been described for transferrin and its receptor (Sheff *et al.*, 1999).

5.5. FcRn trafficking in polarized epithelial cells

In addition to studies of endothelial cells, much information concerning the intracellular trafficking of FcRn has been gleaned from analyses of Madin–Darby canine kidney (MDCK) cells transfected with human or rat FcRn (Claypool *et al.*, 2004; Tesar *et al.*, 2006), or with rat-derived inner medullary collecting duct (IMCD) cells transfected with rat FcRn (McCarthy *et al.*, 2000). Using these cells as polarized monolayers, the polarity of subcellular trafficking events such as transcytosis and recycling, together with the molecular mechanisms, have been investigated. IMCD cells express rat $\beta 2m$, whereas it is essential to cotransfect human/ rat $\beta 2m$ into (canine) MDCK cells to analyze the trafficking of human/rat FcRn in this heterologous system (Claypool *et al.*, 2002; Praetor and Hunziker, 2002; Tesar *et al.*, 2006; Zhu *et al.*, 2002). Comparison of the distribution of human and rat FcRn in transfected, polarized cells has shown that the distribution of the human receptor is strongly polarized toward the basolateral surface, whereas this bias is reversed for rat FcRn (Claypool *et al.*, 2004; McCarthy *et al.*, 2000). The distribution of human FcRn is also biased toward the basolateral surface of untransfected Caco-2 and T84 cells (both intestinal epithelial cells), indicating that its basolateral bias in MDCK cells is not due to overexpression and/or transfection (Claypool *et al.*, 2004). Indeed, the cross-species difference in basolateral bias has recently been demonstrated to be due to the presence of four potential glycosylation sites in the ectodomains of rat (mouse) FcRn, whereas human FcRn has only one such site (Kuo *et al.*, 2009). Engineering of the three additional glycosylation sites of mouse/ rat FcRn into human FcRn results in increased apical localization in transfected MDCK cells (Kuo *et al.*, 2009), consistent with earlier analyses in which carbohydrate was shown to function as an apical targeting signal (Scheiffele *et al.*, 1995). The relative levels of apical and basolateral localization of FcRn impact the directionality of transcytosis. Specifically, although bidirectional transcytosis of FcRn in both transfected IMCD and MDCK cells occurs, for human FcRn more basolateral to apical transcytosis is observed relative to apical to basolateral transport, whereas this is reversed for rat FcRn (Claypool *et al.*, 2004; Kim *et al.*, 2004; McCarthy *et al.*, 2000; Tesar *et al.*,

2006). Consistent with the redistribution of a “rodentized” variant of human FcRn with four potential glycosylation sites to the apical surface, this FcRn mutant shows the directional bias observed for rodent FcRn, that is, preferential transcytosis of IgG in the apical to basolateral direction (Kuo *et al.*, 2009). However, in the human trophoblast cell line, BeWo, greater transcytosis by endogenous FcRn in the apical to basolateral direction occurs (Leitner *et al.*, 2006), suggesting that there may be fundamental differences in the regulation of transcytosis between different cell types. In the case of BeWo cells, this directionality would be consistent with the role of FcRn in delivering maternal IgG across the placenta (Firan *et al.*, 2001; Story *et al.*, 1994).

5.6. Molecular determinants and effectors of FcRn trafficking

The cytosolic tail motifs of rat FcRn that regulate endocytosis and basolateral targeting have been identified by analyses of mutated FcRn variants in transfected IMCD cells (Newton *et al.*, 2005; Wernick *et al.*, 2005; Wu and Simister, 2001). Both tryptophan (W311; with tryptophan replacing the more common tyrosine in the YXXtheta motif) and dileucine (Leu322Leu323) motifs have been shown to play partially redundant roles in endocytosis (Wu and Simister, 2001). Biochemical studies have shown that the tryptophan motif directly interacts with the μ subunit of AP-2 (Wernick *et al.*, 2005). Taken together with the knowledge that dileucine motifs interact with σ and γ subunits of the adaptor protein AP-2, this has led to the suggestion that two subunits of AP-2 can bind simultaneously to the two cytosolic tail motifs (Wernick *et al.*, 2005). Both tryptophan and dileucine motifs also play a role in basolateral targeting of rat FcRn (Newton *et al.*, 2005). The tryptophan and dileucine motifs are conserved across species that range from camels to humans (Fig. 4.5), suggesting that additional differences such as variations in glycosylation patterns (Kuo *et al.*, 2009) account for cross-species variability in trafficking. However, it is interesting to note that some species (e.g., possum, cows, sheep, dromedaries, pigs, and dogs) have cytosolic tails that are 10 residues shorter than those of other species (e.g., humans, macaques, orangutans, rats, and mice) (Fig. 4.5). In addition, the cytosolic tail of possum has a two-residue insertion, whereas rabbit has a five amino acid deletion (Fig. 4.5). Whether these differences are functionally relevant remains to be tested.

Recent studies have identified a motif in the cytosolic tail of human FcRn encompassing Arg301, Arg302 (Fig. 4.5) that binds to calmodulin (Dickinson *et al.*, 2008). Ablation of this interaction by mutagenesis of FcRn results in reduced transcytosis and decreased stability of this receptor (Dickinson *et al.*, 2008). Since calmodulin binding to FcRn would mask a putative amphipathic α -helix that in other proteins can insert into the membrane and induce or sense curvature (Ford *et al.*, 2002; Lee *et al.*, 2005; McMahon and Gallop, 2005), this might provide a mechanism through which calmodulin can affect endosomal sorting. Together with the knowledge that calmodulin function is highly regulatable, this could constitute an important pathway for the control of FcRn trafficking.

In the context of possible regulators of the intracellular pathways taken by FcRn, several studies indicate that Rab proteins play a role (Tzaban *et al.*, 2009; Ward *et al.*, 2005). These small Ras-like GTPases are known to play regulatory functions in endocytic and exocytic trafficking (Miaczynska and Zerial, 2002; Somsel and Wandinger-Ness, 2000). The activity

of this class of GTPases is controlled by GTP–GDP exchange cycles, and such proteins exist in either membrane-bound or cytosolic forms. In combination with proteins such as soluble NSF attachment protein receptors (SNAREs) that usually exist as transmembrane receptors (Jahn *et al.*, 2003), Rabs are key regulators of fusion events between different membranous compartments (Grosshans *et al.*, 2006; Miaczynska and Zerial, 2002; Somsel and Wandinger-Ness, 2000). Due to the pivotal role that Rabs play in intracellular trafficking, it is therefore of interest to understand which of these proteins are associated with FcRn.

Using fluorescence imaging, we observed that Rab4(a), Rab5(a), and Rab11(a) are all present on FcRn⁺ endosomes (Ward *et al.*, 2005). Rab4 and Rab11 are known to be involved in recycling cargo from sorting endosomes to the plasma membrane (Daro *et al.*, 1996; Green *et al.*, 1997; Sönnichsen *et al.*, 2000; Ullrich *et al.*, 1996; van der Sluijs *et al.*, 1992), whereas Rab5 is an early endosomal marker (Christoforidis *et al.*, 1999; Simonsen *et al.*, 1998). Although FcRn can be sorted into tubulovesicular TCs in Rab4⁺Rab11⁺ or Rab11⁺ compartments, only Rab11 but not Rab4 is associated with FcRn during exocytic events at the plasma membrane (Ward *et al.*, 2005). Rab4 depletion from these TCs occurs via the formation of discrete Rab4⁺ domains that can subsequently separate. The distribution of Rab5, Rab11 and the late endosomal markers Rab7 and Rab 9 (Bucci *et al.*, 2000; Soldati *et al.*, 1995) with tubulovesicular TCs that transport IgG/Fc in the neonatal rodent gut has also been analyzed using electron tomography (He *et al.*, 2008). These studies demonstrate that compartments on the endolysosomal pathway cannot be segregated into groups based on their Rab associations. Rather, there is overlap between the Rabs that are associated with different compartments, consistent with models of “Rab conversion” in which Rabs are gradually lost and replaced by different Rab proteins as endosomes mature (Rink *et al.*, 2005).

Recently, we have analyzed the intracellular trafficking pathways, including Rab GTPases, involved in the constitutive degradation of FcRn in endothelial cells (Gan *et al.*, 2009). Transfer of FcRn from late endosomes to lysosomes occurs via kiss-and-linger-like processes (Bright *et al.*, 2005; Gandhi and Stevens, 2003; Ryan, 2003; Storrie and Desjardins, 1996) that frequently involve tubular extensions, whereas full fusion of late endosomes and lysosomes is rarely observed (Gan *et al.*, 2009). Unexpectedly, in our studies, the “early endosomal” marker Rab5 persists on the limiting membrane of late endosomes until a relatively late stage in maturation. This suggests that (late) endosomes have functional plasticity due to the presence of both Rab5 and Rab7, allowing FcRn to leave these compartments to enter the recycling or lysosomal pathways. Consequently, this prolongs the time window during which FcRn (or other receptors) can be sorted into distinct pathways during endosomal maturation, and might provide a mechanism by which increased fidelity in sorting can be achieved.

Given the potential of FcRn as a drug delivery vehicle, it is of considerable interest to understand the molecular effectors that regulate recycling versus transcytosis in polarized cells. Insight into this has recently been generated by the observation that Rab25, a Rab GTPase that is known to be involved in the transcytosis of IgA by pIgR (Casanova *et al.*, 1999; Wang *et al.*, 2000), also regulates the transcytosis of human FcRn in polarized epithelial (MDCK) cells (Tzaban *et al.*, 2009). By contrast, Rab11a is not involved in

transcytosis but is an important player in recycling to the basolateral, but not apical, membrane of polarized MDCK cells (Tzaban *et al.*, 2009). These observations are consistent with the concept that in epithelial cells, at least, there are endosomal compartments that have functional plasticity, insofar as sorting into both transcytotic and recycling pathways can occur from the same common endosome (Casanova *et al.*, 1999; Thompson *et al.*, 2007; Tzaban *et al.*, 2009; Wang *et al.*, 2000). These studies have significant potential for regulating the directionality of FcRn-mediated transport.

5.7. Effects of ligand valency on intracellular trafficking

To date, the majority of studies of the cell biology of FcRn and its IgG ligand have been carried out using monomeric IgG that has two possible interaction sites for FcRn. Indeed, two active binding sites per IgG or Fc molecule have been shown to be important for activity in FcRn-mediated functions that include transcytosis, recycling, and *in vivo* half-life (Kim *et al.*, 1994b,c; Tesar *et al.*, 2006). It is interesting that a hybrid Fc with only one functional FcRn interaction site is transported more efficiently into lysosomes in rat FcRn-transfected MDCK cells relative to wild-type Fc that has two possible interaction sites (Tesar *et al.*, 2006). Whether this effect is due to a higher off-rate of the Fc from FcRn in endosomes and/or a difference in trafficking induced by FcRn dimerization is currently unknown.

The ligand for FcRn can also be highly multimeric when IgGs form ICs with cognate antigen. It is, therefore, of interest to compare the intracellular trafficking of monomeric IgGs with that of multivalent ICs. It has recently been shown that ICs with the propensity to cross-link FcRn preferentially traffic into lysosomes, thereby enhancing antigen presentation in dendritic cells (Qiao *et al.*, 2008). It remains to be demonstrated whether this trafficking pathway is specific for APCs. This might be the case, since a recent report demonstrated that invariant chain, which is expressed in professional APCs, directs the transport of FcRn into lysosomes (Ye *et al.*, 2008). Such a process results in a pathway for the enhancement of T cell responses by ICs, thereby providing an additional link between humoral and cellular immunity. By contrast, the transport of ICs in intact form across cells such as epithelial barriers (Yoshida *et al.*, 2004) might be enabled by the lack of invariant chain in these cells, at least under steady-state, noninflammatory conditions.

6. Regulation of FcRn Expression

FcRn represents a receptor that is subject to both tissue-specific and developmental regulation. For example, following the suckling period of neonatal rodents, a dramatic decrease in FcRn expression in intestine occurs (Ghetie *et al.*, 1996; Martin *et al.*, 1997). FcRn levels are downregulated by hormones such as corticosteroids and thyroxine that are known to affect gastrointestinal adaptation during the neonatal period (Capano *et al.*, 1994; Martin *et al.*, 1993; Morris and Morris, 1976). The promoter regions for human and rodent FcRn have been analyzed and indicate that the regulation of expression at the transcriptional level is complex with sites for Sp-like transcription factors, AP-1, Ets, or NF-IL6 (Jiang *et al.*, 2004; Kandil *et al.*, 1995; Tiwari and Junghans, 2005). Given the immunological relevance of FcRn, it is plausible that modulation of expression and/or activity by inflammatory (or anti-inflammatory) mediators such as cytokines might occur. In this

context, recent studies have shown that the expression levels of human FcRn in *in vitro* cell lines can be regulated by cytokines such as TNF- α and IFN- γ (Liu *et al.*, 2007b, 2008). Although these cytokines are classically associated with proinflammatory effects, much data supports the concept that they can also be anti-inflammatory (Chu *et al.*, 2000; Cope *et al.*, 1997; Isomaki *et al.*, 2001; Kassiotis and Kollias, 2001; Liu *et al.*, 1998; Willenborg *et al.*, 1999a,b). It is, therefore, interesting that while TNF- α and IL-1 β upregulate the transcription of FcRn through NF κ B binding to intronic sequences of FcRn (Liu *et al.*, 2007b), IFN- γ has the reverse effect by activating JAK/STAT-1 signaling (Liu *et al.*, 2008). Consequently, the factors that control the expression of FcRn and MHC Class I molecules are distinct, since IFN- γ is known to upregulate the levels of the latter. How FcRn expression and function might be modulated by both anti- and proinflammatory cytokines and possibly other immune mediators such as chemokines has broad implications for understanding the factors that regulate inflammatory responses. This area offers multiple possibilities for further exploration.

7. The Complexity of Engineering FcRn–IgG Interactions

7.1. Antibody engineering: From variable to constant regions

Much of antibody engineering over the past two decades has been directed toward the manipulation of antibody variable regions for both targeting and blocking effects (Souriau and Hudson, 2003; Weiner and Carter, 2005). By contrast, the modification of Fc regions to alter their interactions with Fc receptors, particularly to impact FcRn function, is relatively underdeveloped. Fc engineering has obvious implications for the application of therapeutic antibodies (Carter, 2006; Ghetie *et al.*, 1997), and interest in this area is currently expanding (Dall'Acqua *et al.*, 2006a; Hinton *et al.*, 2004; Lazar *et al.*, 2006; Shields *et al.*, 2001; Vaccaro *et al.*, 2005, 2006). Although of considerable importance, recent studies describing the engineering of Fc regions for the enhancement of Fc γ R binding (e.g., Lazar *et al.*, 2006; Shields *et al.*, 2001) fall outside the scope of the current review and will not be discussed further. However, it is important to point out that the sites for FcRn and Fc γ R interactions on IgG are distinct (Duncan *et al.*, 1988; Jefferis *et al.*, 1998; Kim *et al.*, 1994b; Shields *et al.*, 2001), so that in general mutations that impact FcRn binding do not affect function in Fc γ R-dependent assays and vice versa. In the cases where effects on both functionalities are observed (e.g., Shields *et al.*, 2001), this is most likely due to longer range conformational perturbations.

We will first describe how FcRn–IgG interactions can be modified to generate antibodies with altered pharmacokinetics and transport properties, and subsequently discuss how FcRn itself can be targeted to modulate IgG levels *in vivo*. The knowledge that albumin is dependent on FcRn for *in vivo* persistence (Andersen *et al.*, 2006; Chaudhury *et al.*, 2003) can also be exploited by using therapeutic reagents fused to albumin binding peptides or Ig domains with the aim of generating longer lived therapeutics (Dennis *et al.*, 2002; Holt *et al.*, 2008; Nguyen *et al.*, 2006; Stork *et al.*, 2007), but will not be discussed further here.

7.2. Modulating the pharmacokinetic properties of IgG: The importance of pH dependence

The knowledge that FcRn regulates serum IgG levels (Ghetie *et al.*, 1996; Israel *et al.*, 1996; Junghans and Anderson, 1996), together with structure–function studies of FcRn–IgG interactions, presents possibilities for the modulation of the *in vivo* persistence and/or transcellular transport of (therapeutic) antibodies. The approach of “tuning” antibody half-lives by altering FcRn–IgG interactions has obvious relevance to the successful use of therapeutic and diagnostic antibodies. Mouse IgG1-derived Fc fragments that are engineered and selected to have increased affinity for FcRn at pH 6, but with retention of low affinity at near neutral pH, persist for longer in the circulation of mice (Ghetie *et al.*, 1997). This approach has subsequently been used to generate engineered human IgGs that have longer half-lives in primates (Dall'Acqua *et al.*, 2006b; Hinton *et al.*, 2004, 2006) and are transported more efficiently across the *ex vivo* human placenta (Vaccaro *et al.*, 2006).

Although several reports describe a correlation between FcRn-binding properties of engineered IgGs and *in vivo* persistence/transport (Dall'Acqua *et al.*, 2006b; Ghetie *et al.*, 1997; Hinton *et al.*, 2004, 2006; Vaccaro *et al.*, 2006), other studies would appear to contradict this (Datta-Mannan *et al.*, 2007a,b; Gurbaxani and Morrison, 2006; Gurbaxani *et al.*, 2006). This apparently discordant data can be explained in several cases by increased binding of engineered antibodies to FcRn at near neutral pH, which in general occurs as the affinity at pH 6 is improved (Dall'Acqua *et al.*, 2002; Vaccaro *et al.*, 2006). In this context, FcRn–IgG interactions can be distinguished from the majority of other protein–protein interactions by their marked pH dependence. Consequently, there is not a linear relationship between increase in affinity and activity. Gain of significant binding activity at near neutral pH results in reduced release during exocytosis at the plasma membrane and enhanced trafficking of the antibody into lysosomes (Gan *et al.*, 2009). Furthermore, such engineered IgGs accumulate very efficiently in cells since they are taken up by receptor (FcRn)-mediated processes (Mi *et al.*, 2008; Vaccaro *et al.*, 2005, 2006). As the affinity at pH 6 is increased, the concomitant improvement in binding at near neutral pH therefore mitigates the factors such as elevated recycling that lead to longer half-life. The difficulty in separating enhancement in affinities at pH 6 and 7.4 during the engineering of FcRn–IgG interactions therefore limits the increase in *in vivo* persistence that is achievable (discussed in Vaccaro *et al.*, 2006), and this presents a significant challenge in Fc engineering. It is also important to note that some cases of apparent discrepancies between binding data and *in vivo* half-lives could be due to the interaction models for FcRn–IgG complexes that are used and/or the introduction of valency effects induced by immobilization of FcRn on the sensor chip during SPR analyses (Datta-Mannan *et al.*, 2007a; Gurbaxani and Morrison, 2006).

In addition to the detrimental effect of gain of binding at near neutral pH on *in vivo* persistence, shorter lived antibodies can alternatively be generated by engineering IgGs or Fc fragments so that they do not bind detectably to FcRn at any pH (Kim *et al.*, 1994a; Medesan *et al.*, 1997). Such “FcRn-null” antibodies also function poorly in other FcRn-mediated functions such as transport across cellular barriers (Firan *et al.*, 2001; Spiekermann *et al.*, 2002). Although in general not useful in therapeutic settings, FcRn-null antibodies have uses in applications such as tumor imaging where short persistence is desirable to minimize background signal (Kenanova *et al.*, 2005; Olafsen *et al.*, 2006).

7.3. Generation of inhibitors of FcRn function to lower endogenous IgG levels

A prediction of the model shown in Fig. 4.3 is that inhibition of FcRn function will lead to enhanced degradation of IgGs and a reduction in IgG transport. FcRn inhibition can be achieved by injecting relatively large quantities of intravenous immunoglobulin (IVIG) (Akilesh *et al.*, 2004; Hansen and Balthasar, 2002; Jin and Balthasar, 2005). The IgG in these high doses of IVIG competes with endogenous IgG for binding and can reduce pathology in IgG-mediated disease (Akilesh *et al.*, 2004; Hansen and Balthasar, 2002; Jin and Balthasar, 2005; Masson, 1993). IVIG can also be used following the delivery of radiolabeled antitumor antibodies to increase their therapeutic and diagnostic efficacy (Jaggi *et al.*, 2007), resulting in enhancement of whole body clearance of radiolabeled IgG and less nonspecific radiation damage.

In many applications in which IVIG is currently used to enhance the clearance of endogenous IgGs, FcRn blockers that have higher affinity for FcRn relative to endogenous wild-type IgGs could be used at substantially lower doses. For example, anti-FcRn or anti- β 2m antibodies that block Fc/IgG binding to FcRn through variable region binding have been shown to be effective in treating ITP and myasthenia gravis, respectively, in rodent models by lowering the levels of pathogenic IgGs (Getman and Balthasar, 2005; Liu *et al.*, 2007a). We have also generated engineered IgGs ("MST-HN" and "HN") derived from human IgG1 that bind through their Fc regions to FcRn with increased affinity ($\sim$ 200-fold at pH 6 relative to mouse IgG1) and reduced pH dependence (Vaccaro *et al.*, 2005, 2006). These engineered IgGs act as competitive inhibitors with wild-type IgGs for FcRn binding and can enhance the clearance of endogenous IgGs in mice (Vaccaro *et al.*, 2005, 2006) (Fig. 4.6). Such engineered antibodies (Abdegs, for *antibodies that enhance IgG degradation*) have potential uses in modulating endogenous IgG levels. In support of this, a human IgG1 variant (Thr307 to Ala/Glu380 to Ala/Asn434 to Ala) with increased affinity for mouse/human FcRn at both pH 6 and 7.4 has been shown to be effective in treating disease in a serum transfer model of arthritis (Petkova *et al.*, 2006). However, the relatively high doses needed in this study were most likely due to retention of significant pH dependence for binding to FcRn of this antibody, that is, low affinity at near neutral pH (Petkova *et al.*, 2006), which results in poor competitive activity (Vaccaro *et al.*, 2006).

The effects of Abdegs on endogenous IgG levels can be regarded to be "extrinsic," in contrast to "intrinsic" effects that impact the half-life of the engineered IgG itself. In this context, due to the loss of pH-dependent binding to FcRn, both Abdegs and anti-FcRn antibodies (that bind to FcRn through their V regions) have short *in vivo* half-lives (Dall'Acqua *et al.*, 2002; Getman and Balthasar, 2005; Vaccaro *et al.*, 2006). Consistent with this, delivery of Abdegs results in a reduction of serum IgG levels that lasts for several days prior to a rebound of IgG concentrations to their original levels (Vaccaro *et al.*, 2005). The "intrinsic" consequences of an Fc mutation on the *in vivo* half-life of an IgG or Fc fragment itself will impact the "extrinsic" effects of this engineered IgG/Fc on the lowering of endogenous IgG levels, since they will determine the *in vivo* longevity of a potential Abdeg.

Insight at the quantitative level as to how a change in pH dependence of an IgG–FcRn interaction impacts *in vivo* persistence has been obtained by comparing the properties of two engineered human IgG1 molecules, HN and MST-HN (HN, His433 to Lys/Asn434 to Phe; MST-HN, Met252 to Tyr/Ser254 to Thr/Thr256 to Glu/His433 to Lys/Asn434 to Phe) (our unpublished data). These two mutants have similar affinities for mouse FcRn at pH 6, whereas the affinity of the HN mutant is about 10-fold lower at pH 7.2 (Table 4.1). This has allowed the impact of differences in pH dependence on intrinsic (*in vivo* half-life) and extrinsic (lowering of endogenous IgG levels) properties to be assessed in mice. The HN mutant is less effective in lowering endogenous IgG levels than MST-HN, but the HN mutant has a longer *in vivo* persistence (Vaccaro *et al.*, 2005, 2006) (Table 4.1). Thus, there is a trade-off between activity as an FcRn inhibitor and *in vivo* half-life. This indicates that, dependent on the situation, these extrinsic and intrinsic properties need to be counterbalanced to optimize the effect. For example, if a “one-off” rapid clearance of endogenous IgG is needed, then an Abdeg with high affinities for FcRn in the range pH 6–7.4 is expected to be optimal. Conversely, if treatment of an IgG-mediated, chronic disease is required, then a balance between reduced half-life and inhibitory activity needs to be achieved.

Synthetic peptides that block the binding of endogenous IgGs to FcRn in nonhuman primates have also been described (Mezo *et al.*, 2008). One such peptide has been used to make a dimer that is active in reducing serum IgG levels in cynomolgus monkeys (Mezo *et al.*, 2008). The antibody levels rebound in peptide-treated monkeys, consistent with the clearance of the peptide. FcRn-binding peptides, or engineered IgGs/Fc fragments with increased affinity for FcRn, have multiple potential uses and offer an alternative to the use of IVIG which needs to be delivered in relatively high doses for efficacy. In addition, the sources of IVIG are limited and its expense is high. However, in addition to FcRn blockade, IVIG has multiple other possible modes of action that include FcγR-mediated effects (discussed in Clynes, 2007). For example, in mice the monomeric IgG component of IVIG can induce the upregulation of FcγRIIB expression (Bruhns *et al.*, 2003; Samuelsson *et al.*, 2001; Siragam *et al.*, 2005). IVIG treatment can also result in signaling by ICs through the activating receptor, FcγRIII, to inhibit IFN-γ responses or regulate dendritic cell activity in mice (Park-Min *et al.*, 2007; Siragam *et al.*, 2006). Recent studies have shown that monomeric IgGs with sialylated core oligosaccharides, that constitute about 1–2% of IVIG, are responsible for the upregulation of FcγRIIB expression through a mechanism that involves binding to SIGN-R1 (in mice) or DC-SIGN (in humans) (Anthony *et al.*, 2008; Kaneko *et al.*, 2006). For the treatment of IgG-mediated, inflammatory diseases, it is therefore possible that due to the induction of additional anti-inflammatory effects, the use of (engineered) antibodies might be preferable over the use of FcRn-binding peptides that solely target FcRn.

8. Cross-Species Differences in FcRn-Binding Specificity and Implications for Preclinical Models

Despite the similarities of human and mouse FcRn at the sequence level (Ahouse *et al.*, 1993; Story *et al.*, 1994), in addition to the conservation of several key interaction residues

on IgG across species, the binding specificity of human and mouse FcRn are distinct (Ober *et al.*, 2001). For example, mouse FcRn binds promiscuously to IgGs from multiple species, whereas human FcRn is much more selective. Most notably, although human FcRn interacts with relatively low affinity with mouse IgG2b, it does not bind detectably to mouse IgG1, IgG2a, or rat IgGs. This lack of binding provides a molecular explanation for the short *in vivo* persistence of (therapeutic) mouse IgGs in humans (Frodin *et al.*, 1990; Saleh *et al.*, 1992).

Using the earlier crystallographic structure of the rat FcRn–rat IgG2a complex (Martin *et al.*, 2001) as a guide, we have used site-directed mutagenesis combined with interaction analyses to transfer the binding properties of mouse FcRn onto human FcRn (Zhou *et al.*, 2003, 2005). With this approach, several regions of sequence variation are responsible for the specificity differences between mouse and human FcRn: first, residues 132–147, encompassing the nonconserved residue 137 (Leu in human FcRn, Glu in mouse FcRn, Asp in rat FcRn) play a central role (Zhou *et al.*, 2003, 2005). The important role of residue 137, in particular, is consistent with crystallographic and structure–function studies for rat FcRn (Martin and Bjorkman, 2001; Vaughn *et al.*, 1997). Second, residues 79–89 (which in human FcRn encompass a two-residue deletion) have a lesser contribution to the difference in specificity and may modulate the overall orientation of the interaction (Zhou *et al.*, 2005). Residues 79–89 also contain a potential glycosylation site in rodent, but not human, FcRn, leading to the possibility that this might contribute to the cross-species difference in binding properties. However, recent studies (Kuo *et al.*, 2009) have shown that glycosylation at position 87 (numbering based on homology alignment with rodent FcRn) of human FcRn, by mutation of Lys to Asn, does not confer the binding properties of mouse/rat FcRn for mouse IgG1 on the human ortholog (Kuo *et al.*, 2009). It is interesting to note that species such as pigs, sheep, camels, and cows have arginine at position 137 (Kacskovics *et al.*, 2000, 2006; Mayer *et al.*, 2002; Schnulle and Hurley, 2003), whereas dog, rat, and mouse have glutamic/aspartic acid (Ahouse *et al.*, 1993; Kacskovics *et al.*, 2006; Simister and Mostov, 1989) and possum has the same residue as humans (leucine) (Adamski *et al.*, 2000). Given the central role of residue 137 in FcRn–IgG interactions, this leads to the speculation that binding specificities might fall into three or more clades.

In general, the affinities of mouse FcRn for IgGs of multiple different species such as human, rat, mouse, and rabbit are higher than the corresponding human FcRn interactions (Ober *et al.*, 2001). This is of relevance when considering the preclinical analysis of human IgGs in murine models since, for example, the affinity of mouse FcRn for human IgG1 is about 10-fold higher than that of the corresponding human FcRn interaction (Zhou *et al.*, 2005). Consequently, although the mouse FcRn–wild-type human IgG1 interaction retains sufficient pH dependence for this IgG1 to have a relatively long half-life in mice, this is not the case for multiple variants of human IgG(1) that have been engineered to have higher affinity for FcRn (Dall'Acqua *et al.*, 2002; Vaccaro *et al.*, 2006). Specifically, a higher affinity IgG mutant can acquire significant binding to mouse FcRn at near neutral pH while retaining the necessary low affinity for human FcRn to allow efficient recycling in human systems (Vaccaro *et al.*, 2006). Consequently, such IgGs have shortened *in vivo* half-lives and inhibit FcRn function in mice (Dall'Acqua *et al.*, 2002; Vaccaro *et al.*, 2005), whereas

analyses in nonhuman primates (Dall'Acqua *et al.*, 2006b) or the human placental transfer model (Vaccaro *et al.*, 2006) are predictive of longer half-lives in humans. Several engineered IgGs of this class have to date been described (Dall'Acqua *et al.*, 2002, 2006b; Datta-Mannan *et al.*, 2007a,b; Vaccaro *et al.*, 2006), indicating the severe limitations of mice as models. Consequently, there is a need for improved preclinical models that can recapitulate human FcRn function. Although nonhuman primates represent good models, their high cost makes them inaccessible for routine screens. Alternatively, mice that transgenically express human FcRn (Chaudhury *et al.*, 2003; Petkova *et al.*, 2006) are a step toward a suitable preclinical model, but have low endogenous IgG levels due to poor binding of mouse IgGs to human FcRn. Such mice combined with transgenic mice expressing human IgGs (Jakobovits *et al.*, 2007; Scott, 2007) might therefore provide an attractive model.

9. Concluding Remarks

Much has been learnt about FcRn function during the past two decades. Perhaps most importantly, a diverse array of activities at different body sites can be attributed to this multitasking receptor. Furthermore, FcRn impacts both the humoral and cellular arms of the immune response. Consequently, understanding the molecular and cellular mechanisms by which this receptor functions, combined with the engineering of FcRn–IgG interactions, has relevance to fundamental aspects of the immune system in addition to providing possible therapeutic routes for multiple diseases.

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Figure 4.1. Structure (α -carbon trace) of the Fc region of human IgG1 (Deisenhofer, 1981) with the location of the key residues that are involved in binding to mouse or human FcRn indicated. The same residues of mouse IgG1 are also involved in FcRn binding, except that Tyr436 is replaced by histidine. The structure was drawn using Rasmol (courtesy of Roger Sayle, Bioinformatics Research Institute, University of Edinburgh).

Figure 4.2. Structure (α -carbon trace) of rat FcRn with the location of the key residues that are involved in binding to rat IgG2a indicated (Martin *et al.*, 2001). The structure was drawn using Rasmol (courtesy of Roger Sayle, Bioinformatics Research Institute, University of Edinburgh).

Figure 4.3.

Schematic representation of FcRn-mediated recycling of IgG in a polarized cell such as an endothelial cell. IgGs are taken into the cell by fluid phase and enter early endosomes. The pH of the early endosome is permissive for FcRn binding, and binding of the IgG to FcRn results in recycling (or transcytosis, not shown) and salvage from lysosomal degradation. Conversely, unbound IgG enters the lysosome and is degraded.

Figure 4.4.

Individual frames from live-cell imaging of sorting endosomes in human FcRn (hFcRn)-GFP-transfected endothelial (HMEC-1) cells pulse-chased with (A, B) Alexa 546-labeled human IgG1 (hIgG1) or (C, D) Alexa 546-labeled H435A (His435 to Ala) mutant that binds with immeasurably low affinity to human FcRn (Firan *et al.*, 2001). Cells were pulsed with labeled IgG and subsequently chased in medium at 37 °C. Images of live cells were acquired and processed as described in Ober *et al.* (2004b). Arrowheads indicate tubules (FcRn⁺IgG⁺ for A, B, FcRn⁺ only for C, D) and in (A) the tubule separates from the endosome at ~ 20 s. The first frame for each dataset is arbitrarily labeled 0 s, although the frames shown were taken at different times after the start of the chase period. Bar = 1 μm .

Human	RRMRSGLPAPWISLR--GDDTGVLPTPGEAQDADLKDVNVIPATA
Orangutan	RRMRSGLPAPWISLR--GDDTGALLPTPGEAQDADSKDVNVIPATA
Macaque	RRMRSGLPAPWISLR--GDDTGSLPTPGEAQDADSKDINVIPATA
Ovine	RRMRKGLPASWISFR--GEDVGALLPTPGLSKDGES-----
Bovine	RRMRKGLPAPWISFR--GEDVGALLPTPGLSKDGES-----
Dromedary	RR-RKGLPAPWIAFR--GDDIGALLPTPGLSKDAES-----
Swine	RRMRKGLPAPWISFR--GDDVGALLPTPDLAKDAES-----
Canine	RRMRKGLPAPWMSLR--GDDVGALLPTPGVPKDADS-----
Rat	NRMRSGLPAPWLSLS--GDDSGDLLPGGNLPPEAEPQGVNAFPATS
Mouse	GRMRSGLPAPWLSLS--GDDSGDLLPGGNLPPEAEPQGANAFPATS
Possum	SRKRGARPAWIFRRRAGDDVGSLLSAPASAQDSSP-----
Rabbit	RRRR-GLPAPWVFLR--GDDIRTLLP-----QDEGPQDVSAFPATA
Consensus	-R-R---PA-W-----G-D---LL-----

Figure 4.5.

Cytosolic tail sequences of FcRn from different species (Adamski *et al.*, 2000; Ahouse *et al.*, 1993; Kacs Kovics *et al.*, 2000, 2006; Kandil *et al.*, 1995; Mayer *et al.*, 2002; Schnulle and Hurley, 2003; Simister and Mostov, 1989; Story *et al.*, 1994). Identity is indicated by red, and the first residue of the sequence corresponds to residue 301 of mouse/rat FcRn. The consensus sequence is also shown, with the tryptophan (W311) and dileucine (L322, L323) motifs that are important for intracellular trafficking indicated in blue.

Figure 4.6.

Enhancement of clearance of injected wild-type IgG by an Abdeg (Vaccaro *et al.*, 2005). Mice were injected with ^{125}I -labeled wild-type human IgG1, and injected with 500 µg wild-type human IgG1, 200 or 500 µg Abdeg (MST-HN mutant) 72 h later (indicated by arrow). Levels of remaining ^{125}I labeled IgG were determined at the indicated times. Error bars indicate standard deviations. * Indicates that data for these time points for mice treated with 500 or 200 µg Abdeg are significantly different.

Table 4.1

The impact of pH dependence on *in vivo* half-life in mice of engineered variants of human IgG1

Dissociation constant, K_D (nM) ^a			
Human IgG1 (mutant)	pH 6	pH 7.2	β -Phase half-life (h)
Wild type	32	N.D. ^b	250.6±15.3 ^a
MST-HN	1.2	7.4	35.6±1.1
HN	1.5	82	62.8±2.7 ^a

^aDescribed previously in Vaccaro *et al.* (2005, 2006).

^bN.D., not determined because affinity is too low to accurately estimate a dissociation constant.