11
The Diverse Roles of FcRn: Implications for Antibody Engineering

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11.1
Introduction

Over the last 15 years it has become evident that the MHC Class I-related receptor, FcRn, serves multiple functions through its ability to transport immunoglobulin G (IgG) within and across cells (reviewed in [1–3]). FcRn is expressed throughout life in a diverse array of tissues and cell types (e.g., [4–11]). As an IgG transporter, this receptor not only maintains constant IgG concentrations in the body [4, 12, 13], but also delivers antibodies of this class across cellular barriers [5, 14–21]. In this chapter we first give a brief overview of the early studies of FcRn followed by the molecular details of FcRn–IgG interactions and a description of FcRn behavior at the level of intracellular trafficking. Finally, we discuss how FcRn–IgG interactions can be engineered to alter both the pharmacokinetic properties of an IgG and the concentrations of endogenous IgGs. In addition, although more recent reports have demonstrated that FcRn binds and maintains the homeostasis of albumin [22, 23], this topic will not be discussed in detail here. Instead, the reader is referred to Chapter 10.

11.2
FcRn: Early Characterization and Diverse Expression Patterns

The role of FcRn as its namesake, the neonatal Fc receptor, in transporting maternal IgG from mother to young across the neonatal intestine was characterized in the 1980s [24–27]. These studies included the seminal work of Simister and Mostov describing the cloning of the rat FcRn α-chain gene and the identification of this polypeptide as a member of the MHC Class I receptor family [27]. Subsequent analyses have resulted in the isolation of FcRn from human placenta [28, 29] and the demonstration that it is an essential player in the transport of maternal IgG from mother to young during the third trimester of pregnancy [15].

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FcRn α–chain genes have been isolated from many different species [27, 29–36], indicating that the protein is broadly expressed in mammals. Although FcRn genes show some conservation across species, there are differences in both putative interaction residues with IgG and regions involved in trafficking. The functional impact of these differences for IgG interactions have been investigated for mouse/rat and human FcRn (discussed below), but in many cases, the way these variations impact on activity is unknown.

11.3 The Molecular Details of FcRn–IgG Interactions

A combination of mutagenesis and structural studies has been used to map the interaction site of FcRn on IgG in rodents and humans [37–41]. IgG residues Ile253, His310, His435 are centrally involved in the interactions [37–41]. These residues are conserved across most human and mouse/rat IgG isotypes and are located at the CH2–CH3 domain interface [42] (Figure 11.1). His436 (present in most mouse IgG isotypes) or Tyr436 (present in most human IgG isotypes) play a lesser but significant role in binding to FcRn [38, 43]. Multiple studies have shown that the FcRn binding site on IgG does not overlap with the interaction sites for the classical FcγRs and complement C1q [37, 43–45]. The distinct interaction sites have implications for the engineering of antibodies that are discussed further below.

Mutagenesis and structural studies have also been used to identify the residues of rat FcRn that are involved in IgG binding [41, 46]. These residues include Ile1 of β2-microglobulin and Glu117, Glu118, Glu132, Trp133, Glu135 and Asp137 of the FcRn α-chain. The interaction of acidic residues of FcRn with histidines on IgG represents the primary contributor to the pH dependence of the interaction,

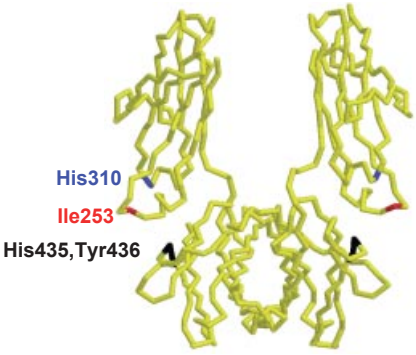

Figure 11.1 Structure (α-carbon trace) of the Fc region of human IgG1 [42] with the location of the key residues that are involved in binding to mouse or human FcRn indicated [15, 39, 43] (drawn using Rasmol, courtesy of Roger Sayle). The same residues of mouse IgG1 are also involved in FcRn binding [37, 38] except that Tyr436 is replaced by histidines in most mouse IgG isotypes.

with binding at acidic pH that for most IgG isotypes becomes undetectable at near neutral pH [25, 40, 47, 48].

Importantly, human and mouse FcRn exhibit marked differences in binding specificity [49]. Although mouse FcRn binds to IgGs from a wide array of species, human FcRn is much more selective [49]. For example, mouse IgGs bind very poorly, if at all, to human FcRn. Conversely, mouse FcRn binds to human IgG1 with higher affinity relative to the corresponding human FcRn-human IgG1 interaction. This cross-species difference can confound the interpretation of data when (engineered) human IgGs are tested preclinically in mice [50] (discussed further below). Site-directed mutagenesis of human FcRn has been used to determine the molecular basis for the difference in binding between human and mouse FcRn [51, 52]. These studies have shown that an amino acid difference at residue 137 (Leu in human FcRn, Glu or Asp in mouse or rat FcRn, respectively) is the major contributor, with other regions playing more minor roles.

FcRn has two possible binding sites on IgG, and these sites are not equivalent [53–55]. This has led to the concept that binding of FcRn to one site reduces the affinity of the second site [53, 56] through either steric effects or longer range conformational changes. This raises the question as to whether two functional sites per IgG molecule are necessary for activity in FcRn-mediated functions. This has been addressed by generating hybrid Fc fragments comprising one wild-type Ig heavy chain associated with a mutated heavy chain that does not bind to FcRn [37, 57, 58]. Such hybrid molecules are poorly delivered across cellular barriers [37, 58] and have reduced *in vivo* half-lives [57]. Whether this lower activity is due to effects on FcRn trafficking (through lack of dimerization of the receptor) or reduced avidity for interactions with membrane-bound FcRn, or a combination of both, is currently unknown.

11.4 FcRn Is Expressed Ubiquitously throughout the Body Where It Serves Multiple Functions

In addition to the overexpression of FcRn in the neonatal gut and the human placenta, this receptor is expressed in endothelial, epithelial and many, but not all, hematopoietic cells throughout life (e.g., [4–11, 18]). Although FcRn is expressed in all "professional" antigen presenting cells (dendritic cells, macrophages and B cells), it is not detectable in T cells [7, 59]. FcRn is also present in more specialized cells/sites such as corneal epithelium/endothelium and podocytes in the kidney [10, 11, 60]. This raises the question as to which functions it might serve at these different cellular/body sites.

To identify which sites are important for the control of IgG levels, we have generated mice containing a "floxed" FcRn allele that can be conditionally deleted in different cell subsets by crossing with appropriate Cre recombinase expressing strains [59]. To date, we have characterized mice that lack FcRn in all endothelial and hematopoietic cells by using a Cre recombinase strain in which expression is

driven by the Tie2 promoter [61]. These studies, combined with others using bone marrow transfers [8, 9], have shown that hematopoietic and endothelial cells constitute the primary sites for the regulation of IgG persistence *in vivo* [59].

In addition to an important contribution of hematopoietic cells for the control of IgG concentrations and pharmacokinetics [8, 9], expression of FcRn in antigen presenting cells has been shown to play a role in presentation to cognate T cells [9, 62]. Specifically, presentation of antigen derived from immune complexes is more efficient in FcRn-sufficient dendritic cells relative to their knockout counterparts [9]. This has been demonstrated to be due to enhanced, FcRn-dependent trafficking of such complexes into lysosomes in dendritic cells. In addition, invariant chain drives FcRn into lysosomes in antigen presenting cells [63]. This invariant chain enhancement of lysosomal expression could provide an explanation for the differences in fate of immune complexes across cell types: in invariant chain expressing antigen presenting cells, immune complexes enter lysosomes [9], whereas in epithelial cells these complexes are transcytosed [21].

Recent studies have also explored the role of FcRn expression in the kidney [10, 60]. FcRn is present in proximal tubular epithelial cells, podocytes and renal vascular endothelia [10, 60]. Studies in FcRn α-chain deficient mice have demonstrated that FcRn in podocytes is involved in removing IgG from the glomerular basement membrane, thereby enhancing removal of IgG from the kidneys and reducing glomerular damage [10]. Interestingly, using the approach of transplanting kidneys from wild-type mice into FcRn-/- recipients, renal FcRn has been shown to be a key player in the maintenance of serum albumin levels while assisting in the elimination of IgG [60]. This indicates that by analogy with *in vitro* studies using rat FcRn [58], rodent FcRn handles albumin and IgG differently. How this is achieved is poorly understood but is an area that deserves attention.

The function of FcRn expression at the blood–brain barrier (BBB) and other immune privileged sites, such as the eye, has attracted significant interest [11, 64–67]. In some studies, FcRn has been reported to enhance the egress of IgG from the brain [65, 66], whereas others have shown that FcRn plays no role in the disposition of IgG at this site [67]. Defining whether FcRn is involved, and can even be modulated, in IgG transport at the BBB has obvious implications for drug delivery and the treatment of neurological diseases.

11.5
The Cell Biology of FcRn and Its Intracellular Transport of IgG

Over the past decade, studies have been directed towards defining how IgG cargo behaves at the level of subcellular trafficking in both endothelial and epithelial cells (e.g., [14, 20, 58, 68–72]). A model for FcRn-mediated trafficking of IgGs is shown in Figure 11.2. Recent studies have given insight into the intracellular sorting, recycling and exocytic processes that result in FcRn-mediated recycling and in some cases, transcytosis, of IgG. For example, live cell imaging of endothelial cells has demonstrated that wild-type IgGs are recycled from sorting endo-

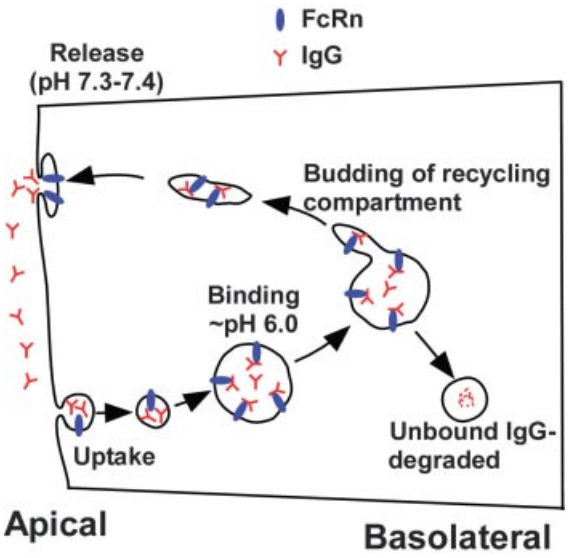

Figure 11.2 Model for FcRn-mediated trafficking of IgG in polarized endothelial cells. IgG is taken up into the cells by fluid phase pinocytosis and enters early, acidic endosomes in which it can bind to FcRn. Bound IgG is recycled (or transcytosed; not shown) and released at the cell surface due to the change in pH. Unbound IgG enters lysosomes and is degraded.

somes (i.e., large endosomal structures of around 1–2 μm) into the recycling pathway [68]. By contrast, IgGs that are mutated so that they no longer bind to FcRn are not sorted. By default, these IgGs remain in the vacuole of the sorting endosome as it matures to form a late endosome. The contents of these late endosomes are ultimately delivered to lysosomes where they are degraded.

Recycling of IgG by FcRn is followed by exocytic release that can involve complete fusion of the FcRn positive compartment with the plasma membrane and release of IgG [20, 69]. Alternatively, other types of exocytic processes can occur, ranging from partial fusion to prolonged release in which IgG is released in fusion events that resemble kiss-and-run or kiss-and-linger interactions similar to those described for neurotransmitter release at synaptic junctions [20, 73–75]. The visualization of distinct types of fusion events at the plasma membrane using total internal reflection fluorescence microscopy (TIRFM; [76]), without understanding which intracellular pathways preceded them, led to the development of a multifocal imaging modality in our laboratory [69, 77]. This multifocal plane microscopy (MUM) involves the simultaneous collection of fluorescent signal from multiple focal planes within a cell in addition to the use of TIRFM. This allows rapidly moving tubulovesicular transport containers (TCs) to be tracked as they move, for example, from sorting endosomes to exocytic sites at the plasma membrane. Similarly, the analysis of endocytic processes followed by intracellular tracking of vesicular TCs on the early endosomal pathway is possible [70]. The tubulovesicular

TCs observed in these analyses are analogous to those described by Bjorkman and colleagues using electron tomography to study rat jejunal sections [78].

The use of MUM has resulted in the identification of different pathways for both recycling/exocytosis and endocytosis [69, 70]. In the most direct pathway of endosomal recycling and exocytosis, tubules can extend from sorting endosomes and fuse with the plasma membrane while still attached to the originating compartment [69]. Alternatively, recycling compartments can take more indirect itineraries within the cell before they undergo exocytosis (Figure 11.3). By analogy, FcRn is endocytosed from the plasma membrane and enters sorting endosomes via direct and indirect pathways [70]. This raises the question as to what determines the pathways that are taken? Understanding these processes in molecular terms could open up avenues for modulating antibody behavior *in vivo*.

11.6
The Molecular Determinants of FcRn Trafficking

Studies in several laboratories have analyzed the determinants of FcRn that regulate intracellular trafficking [80–83]. Both dileucine and tyrosine based motifs are involved in controlling endocytosis, transcytosis and basolateral targeting [80, 82]. In addition, biochemical studies have demonstrated that the cytosolic tail of FcRn

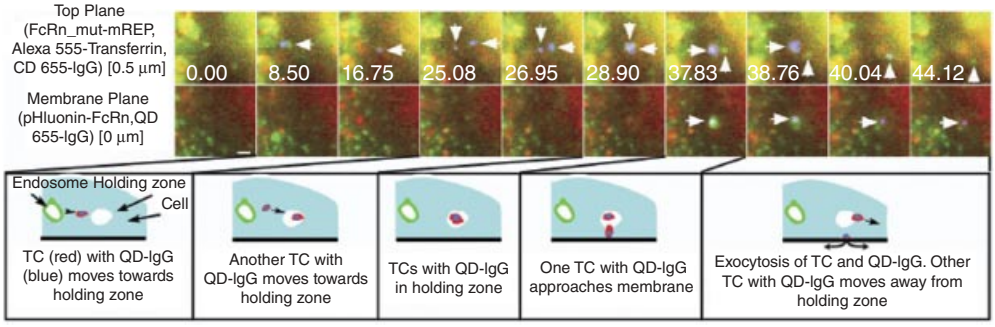

Figure 11.3 An example of the use of multifocal plane microscopy (MUM) to analyze the trafficking pathways taken by IgG and FcRn following sorting in endosomes. Endothelial (HMEC-1) cells were cotransfected with pHluorin-FcRn and FcRn-mRFP (a mutated, high affinity variant of FcRn [52] was used) and incubated in medium (pH 7.3) with quantum dot (QD-655)-human IgG1 mutant (MST-HN [79]) complexes and Alexa 555-labeled transferrin before and during imaging. FcRn and transferrin are indicated by green and QD-IgG complexes by red. The events of interest are highlighted in blue. An FcRn/transferrin-positive TC with IgG (leftward arrow) leaves a sorting endosome at 8.50 s. Later, another TC containing QD-IgG (downward arrows) enters the same region of the cell that we have designated a "holding zone" (28.90 s). One of the TCs exocytoses (38.76 s), releasing IgG (rightward arrows) on the membrane plane. The second TC (upward arrows) moves away from the holding zone in the top plane. Further details are described in Prabhat *et al* [69]. Bar = 1 µm.

associates with the μ chain of clathrin [81], indicating that endocytic uptake occurs through clathrin-coated pits. This is also consistent with more recent electron tomography studies demonstrating the presence of clathrin at both endocytic and exocytic sites [78]. The molecular basis for the dominance of apical to basolateral transcytosis of IgG by rat FcRn, which is reversed for human FcRn, has also been investigated [83]. Interestingly, this difference can be accounted for by variations in the numbers of *N*-linked glycans on the ectodomain (i.e., exposed to endosomal lumen) of FcRn: in humans there is one glycosylation site, whereas rat/mouse FcRn has four sites [27, 29, 30]. Generation of a mutated human FcRn with four glycans results in a redistribution of the rodentized variant to the apical surface and reversal of the predominant direction of transport to that observed for rodent FcRn, that is, apical to basolateral delivery [83].

Towards the goal of defining which molecular effectors determine the trafficking pathways taken by FcRn and its bound ligand, several studies have investigated possible associations, and even direct involvement, of different Rab GTPases in intracellular trafficking [71, 72, 84]. Rab GTPases represent effector proteins that play a major role in regulating the trafficking behavior of proteins on the pathways that include secretion and endocytosis. For example, Rab4, Rab5, Rab11 and Rab25 are involved in regulating endocytic recycling and transcytosis in different cell types (e.g., [85–91]). In endothelial cells, we have shown that although both Rab4a and Rab11a are present on "leaving" FcRn+ TCs as they segregate from sorting endosomes, only Rab11a approaches the plasma membrane during exocytic events [71]. This suggests that Rab11a is an important player in exocytosis, whereas Rab4a functions at an earlier point in the recycling pathway. In polarized epithelial cells, the role of Rab11a and Rab25 in recycling and transcytosis has also been investigated [72]. These studies have shown that Rab25 and the actin motor myosin Vb play an important role in transcytosis. By contrast, Rab11a does not affect transcytosis but is involved in the regulation of recycling at the basolateral membrane. Unraveling the role of different Rab GTPases in the regulation of FcRn and IgG trafficking could have important implications for the modulation of intracellular trafficking pathways which in turn could affect IgG distribution and transport.

11.7 Engineering IgG–FcRn Interactions

The identification of FcRn as the receptor that regulates IgG levels and transport suggests ways of modulating the *in vivo* half-life and delivery of IgG across cellular barriers. Increasing the persistence becomes particularly relevant for therapeutic antibodies [92–96], where production and delivery costs represent a major financial burden and clinical overhead. In 1997 we showed that the engineering of a mouse Fc fragment for increased affinity for FcRn at pH 6.0 generated an antibody fragment with increased *in vivo* half-life relative to its parent [92]. Multiple subsequent reports have described similar engineering for human IgGs with consequent increased persistence in non-human primates or mice that transgenically express

human FcRn [93–95, 97, 98]. Through these studies the importance of the pH dependence of FcRn–IgG interactions has become apparent: antibodies that gain significant binding at near neutral pH (by contrast with their parent wild-type antibodies that do not bind detectably) have reduced *in vivo* half-lives [50, 98, 99]. Antibodies of this class accumulate to high levels in cells due to FcRn-mediated uptake and inefficient release during exocytosis [79]. Microscopy studies demonstrate that these antibodies follow the constitutive degradation pathway of FcRn into lysosomes [100].

The effects of alterations in the pH dependence of FcRn–IgG interactions has led to controversy concerning possible correlations between changes in affinity and *in vivo* persistence [101–103]. Some of these controversies can be explained if the gain of binding at near neutral pH is taken into consideration. For example, the comparison of the half-lives of two human IgG1 molecules that have the same affinity for murine FcRn at pH 6.0, but 10-fold differences at near neutral pH, exemplifies how increased affinity at near neutral pH reduces persistence [1]. Similarly, a mutated antibody (N434A) that has 4-fold increased affinity for human FcRn at pH 6.0 while retaining low affinity at pH 7.4 has a longer half life in non-human primates (cynomolgus monkeys) than a mutant (N434W) with 80-fold enhancement at pH 6.0 but significant binding at pH 7.4 [98]. Since increases in affinity at pH 6.0 are generally accompanied by parallel increases at near neutral pH [98, 99], the intrinsic nature of FcRn–IgG interactions will most likely impose an upper limit to the half-life extension that can be achieved.

The cross-species differences for FcRn binding properties between man and mouse have major implications for the use of mice as preclinical models [50]. This becomes particularly relevant when engineering antibodies for increased *in vivo* persistence. Specifically, the higher affinity of mouse FcRn for (human) IgGs relative to human FcRn can result in significant binding of an "affinity enhanced" antibody for mouse FcRn at near neutral pH. By contrast, this antibody could retain very low affinity for human FcRn at this pH as a consequence of cross-species differences. Thus, although such an engineered antibody is predicted to have increased longevity in humans (or non-human primates), it will have a shorter half-life than its parent wild-type molecule in mice. An example of this effect has been described for a mutated human IgG1 that transports better than its wild-type parent across the human *ex vivo* placenta (indicating improved transport and longevity), but has a half life of around 63 hours ($t_{1/2}$ = ~250 hours for wild-type) in mice [50]. The difference in binding specificity across species has motivated the development of engineered "humanized" mice that transgenically express human FcRn and are deleted for endogenous FcRn [97, 104]. These mice provide valuable preclinical tools for the analysis of human antibodies, and have recently been used to demonstrate the increased efficacy of an antitumor antibody with extended half-life [96].

Interestingly, recent studies have also shown that the isoelectric point (pI) of an antibody can impact its *in vivo* persistence [105]. Specifically, IgG molecules with high pI are eliminated more rapidly than their counterparts with lower pI. It has been suggested that the effect of pI is due to the repulsion of more negatively

charged proteins (with lower pI) from the cell surface of pinocytic/endocytic cells that are involved in IgG degradation. This mechanism therefore represents an FcRn-independent pathway through which the persistence of an antibody can be modulated. However, antibodies with lower pI are expected to have fundamentally different properties *in vivo* relative to those engineered for half-life extension through modification of FcRn binding: antibodies with lower pI would be predicted to be transported across cellular barriers less efficiently relative to their analogs with higher pI. By contrast, engineering for increased FcRn binding with maintenance of low affinity at near neutral pH results in improved transcellular delivery [50].

11.8 Inhibitors of FcRn Function

Antibodies that have gained significant binding for FcRn at near neutral pH are highly efficient inhibitors of FcRn [79]. Specifically, they are taken into FcRn-expressing cells by receptor-mediated endocytosis and compete (due to their higher affinity at acidic pH) with wild-type IgGs for binding to receptors in endosomes. We showed that these antibodies can be used to lower the levels of endogenous IgGs *in vivo* [79], and more recently, that they can be used to treat arthritis in a serum transfer model [106]. Other inhibitors of FcRn, including synthetic dimeric peptides, have also been described [107]. In addition, antibodies that bind through their variable regions to FcRn can block immune thrombocytopenia and myasthenia gravis in rodent models of disease [108, 109]. As such, these FcRn inhibitors have significant potential for the treatment of IgG-mediated autoimmunity. It is also possible that they will have uses in other indications such as the induction of rapid clearance of toxin-antibody complexes and blockade of transport of pathogenic antibodies across the placenta during pregnancy.

Intravenous gammaglobulin (IVIG) is used in high doses for a wide array of indications, including the therapy of antibody-mediated autoimmunity (e.g., [110, 111]). Despite its widespread use, the mechanism of action of this reagent remains a matter of debate. In some studies, evidence in support of FcRn blockade has been presented [112–115], whereas others have argued that effects on FcRn do not contribute to anti-inflammatory sequelae that result from the induction of FcγRIIb upregulation by sialylated IgG molecules [116–118]. These mechanisms are not mutually exclusive, and it is clear that at the high doses of IVIG used (approaching the whole body load of endogenous IgG), competition for FcRn binding will occur. The combination of recombinant, sialylated Abdegs that have both properties of FcγRIIb upregulation and potent inhibition could therefore represent an attractive approach for the treatment of inflammatory, IgG-mediated disease. However, several studies in mouse models of autoimmunity have indicated that FcRn-inhibition alone is sufficient to ameliorate disease [106, 108, 109, 119], suggesting that the combined effects of sialylation and FcRn blockade might not be necessary in all therapeutic situations.

11.9
Engineering Mice with Altered FcRn Function

In addition to the generation of mice that are deleted for FcRn, the realization that transgenic overexpression of FcRn might be useful for the production of high levels of antibodies motivated the development of mice overexpressing bovine FcRn [120]. Consistent with the increased activity of FcRn, these mice have abnormally high levels of serum IgG in the steady state. Following immunization, they show substantial increases in immunogen-specific antibody levels, in addition to greater numbers of antigen specific B cells and plasma cells. Perhaps surprisingly, these mice do not show indications of antibody-mediated autoimmunity or renal damage. Consequently such mice provide powerful tools for the efficient production of antibodies.

11.10
Concluding Remarks

The multiple activities of FcRn, emanating from its ability to transport IgG within and across cells of many different types, have become apparent during the past decade. This reveals new opportunities for targeting FcRn activity that can be exploited to generate "new generation" therapeutics. In the future, we anticipate that an improved understanding of the molecular determinants of the intracellular trafficking of FcRn and IgG will lead to an ability to modulate FcRn-mediated transport of IgGs for therapeutic benefit.

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