Thesis for the Master of Science Degree in Molecular Biosciences main field of study in Immunology

FcRn expression, ligands binding properties and its regulation in human immune cells and hepatocytes

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MANUSCRIPT

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ABBREIVATIONS

ADCC	Ab-depenedent cellular cytotoxcity
AEE	Apical early endosome
APC	Antigen presenting cell
ARE	Apical recycling endosome
BEE	Bsolateral early endosome
CDR	Complementarity determining region
C_L	Constant light
C_H	Constant heavy
CMV	Cytomegalovirus
CRE	Common recycling endosome
C-terminus	Carboxy terminus
DC	Dendritic cells
Epo	Erythropoietin
Fab	Fragment antigen binding
Fc	Fragment crystallible
FcRn	Neonatal Fc receptor
H chain	Heavy chain
hFcRn	Human FcRn
hIgG	Human IgG
HLA	Human leukocyte antigen
HSA	Human serum albumin
HSV	Herpes simplex virus
Ig	Immunoglobulin
Ii	Invariant chain
IL	Interleukin
ITP	Immune thrombocytopenia
IVIG	Intravenous Ig therapy
L chain	Light chain
LE	Late endosome
MBL	Mannose binding lectin
MDCK	Madin-Darby canine kidney
MICA	MHC class I-related gene A
mIgG	Mouse IgG
MR	Mannose receptor
MR1	MHC-related protein 1
N-terminal	Amino terminal
pIgR	Polymeric Ig receptor
rFcRn	Rat FcRn
shFcRn	Soluble human FcRn
SpA	Staphylococcal protein A
SPR	Surface plasmon resonance
srFcRn	Soluble rat FcRn
TCR	T-cell receptor
Th	T helper
V_H	Varible heavy

V_L	Variable light
ZAG	Zinc α_2-glycoprotein
WT	Wild type
β_2m	β_2-microglobulin
BGA	Bovine growth hormone
BSA	Bovine serum albumin
CIP	Calf intestinal phosphatase
CV	Column volume
EBV	Epstein Barr virus
E.coli	Escherichia coli
FCS	Foetal calf serum
HRP	Horseradish peroxidase
hβ_2m	Human β_2-microglobulin
ON	Over night
OriP	Origin of replication
PCR	Polymerase chain reaction
RT-PCR	Reverse transcriptase PCR
polyA	Polyadenylation
PS	Penicillin
RPM	Round per minute
RT	Room temperature
SDS-PAGE	Sodium dodecyl polyacrylamide
Skm	Skimmed milk
ELISA	Enzyme-linked immunosorbent assay
TM hFcRn	Transmembrane human FcRn
GAPDH	Glyceraldehyde

1. General Introduction

1.1 Immunoglobulin

The adaptive immune response consists of a variety of cells and molecules, among which lymphocytes and immunoglobulins (Igs) are the key elements in antigen recognition and responses. There are two types of lymphocytes, T cells and B cells. T cells play a pivotal role in regulating the immune response and are also responsible for cellular immunity, while B cells are essential in the effector phase of humoral immunity. After exposure to antigen and mostly with the help of T cells, B cells can differentiate into plasma cells which synthesize antibodies or immunoglobulins that can react with antigen.

Immunoglobulins are a group of closely related glycoproteins composed of 82-96% protein and 4-18% carbohydrate. The basic Ig molecule has a four-chain structure, comprising two identical heavy (H) chains and two identical light (L) chains, linked together by inter-chain disulfide bonds (Fig.1). Intra-chain disulfide bonds are responsible for the formation of loops, leading to the compact, domain-like structure of the molecule. The amino terminal portions of the H and L chains, characterized by a highly variable amino acid composition, are referred to a V_H and V_L, respectively. The constant part of the L chain is designated as C_L, while that the H chain are further divided into three distinct subunits: C_H1, C_H2 and C_H3 (Fig.1). Functionally, the V-regions are involved in antigen binding. The C regions interact to hold the molecule together and are involved in several biological activities.

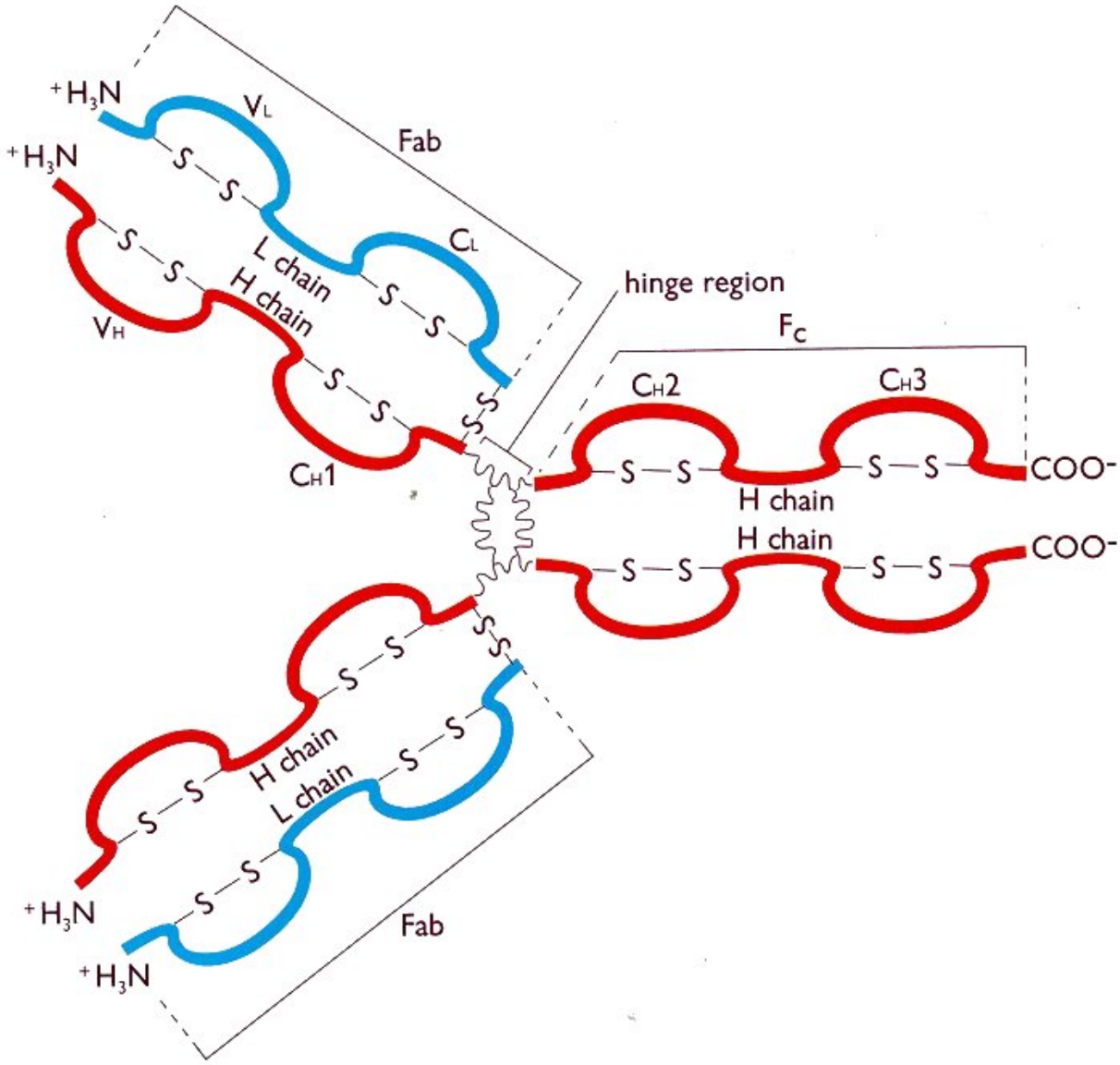

Figure 1. Schematic drawing of the basic structure of the human immunoglobulin molecule. The amino terminal end is characterized by sequence variability (V) in both H and L chains, referred to as the V_H and V_L regions respectively. The rest of the molecule has a relatively constant structure. The C portion of the L chain is termed the C_L region or domain. The constant portion of the H chain is further divided into three structurally discrete regions: C_H1, C_H2 and C_H3. The hinge region is a segment of the H chain, located between the C_H1 and C_H2 domains. Fab: Fragment antigen binding; Fc: Fragment crystallisable. The antibody illustration is taken from Meulenbroek and Zeijlemaker (1996).

The immunoglobulin G (IgG), a major effector molecule of the humoral immune response in man accounts for about 75% of the total Igs in the plasma of healthy individuals. The Igs of the other classes (IgM, IgA, IgD and IgE) each of which has characteristic

properties and functions, constitute the other 25% of the Igs.

Antibodies of the IgG class express their predominant activity during a secondary antibody response. Thus, the appearance of the specific IgG antibodies generally corresponds with the maturation of the antibody response, which is switched on upon repeated contact with antigen. In comparison to antibodies of the IgM class, IgG antibodies have a relatively high affinity and persist in the circulation for a longe time. The five classes of human Igs can be distinguished on the basis of amino acid composition. This is also the basis for antigenic differenes between these molecules and for immunological recognition by specific antibodies.

The polypeptide chains of Igs are encoded by three non-linked cluster of autosomal genes, one cluster coding for H chains of all classes and subclasses, a second one for kappa (k) light chains and a third one for lamda (λ) light chains. These three genes clusters are called the H-, k-and λ gene families respectively. In humans the H gene family is on chromosome 14, the k gene family is on chromosome 2 and the λ gene family is on chromosome 22. Molecular genetic studies have revealed the arrangement of gene segments within the H chain and L chain families. Each H chain is encoded by 4 distinct types of gene segments, designated V_H (variable), D (diversity), J_H (joining) and C_H. The V region of the H chain is encoded by the V_H, D and J_H segments. The L chains are encoded by the 3 gene segments, V_L, J_L and C_L segments.

The C gene segments of the H and L chains encode for the constant regons. Nine immunoglobulin H chain isotypes are found in humans: IgM, IgE, IgG (with subclasses IgG1, IgG2, IgG3 and IgG4) and IgA (with subclasses IgA1 and IgA2).

The C_H gene segments determine the class and/or subclass of the H chain, whereas V_H, D and J_H regions determine the antigen-recognizing part of the Ig molecule. The H and L chains constant genes lie 3' to the V_H, D, J_H, and V_L, J_L genes, respectively. During maturation of progenitor B cells to mature B cells an active H chain exon is formed by V_H, D and J_H, and that of L chain formed by V_L and J_L somatic gene rearrangements (recombined V_HDJ_H and V_LJ_L) which codes for antigen binding variable region of IgG, followed by linkage to a certain C_H gene locus. This is transcribed to mRNA and subsequently translated to an immunoglobulin H chain molecule. The C_H gene closest to the J_H locus, the Cμ gene (IgM), the first isotype gene to be expressed. The other C_H genes can subsequently be expressed by downstream switching mechanisms with simultaneous deletion of the original isotypic C_H genes. The DNA rearrangements that underlie isotype switching and confer their functional diversity on the humoral immune response are directed by cytokines, especially those released

by armed effector CD4 T cells ([1]).

Comparisons of the amino acid sequences of the V regions of Igs show that most of the variability resides in three regions called the hypervariable regions or the complementarity determining regions (CDR1, CDR2 and CDR3; Fig.2). Antibodies with different specificities (i.e. different combining sites) have different complementarity determining regions while antibodies of the exact same specificity have identical complementarity determining regions (*i.e.* CDR is the antibody combining site). Complementarity determining regions are found in both the H and the L chains. The regions between the complementarity determining regions in the V region are called the framework regions. Based on similarities and differences in the framework regions the immunoglobulin H and L chain variable regions can be divided into groups and subgroups. These represent the products of different variable region genes.

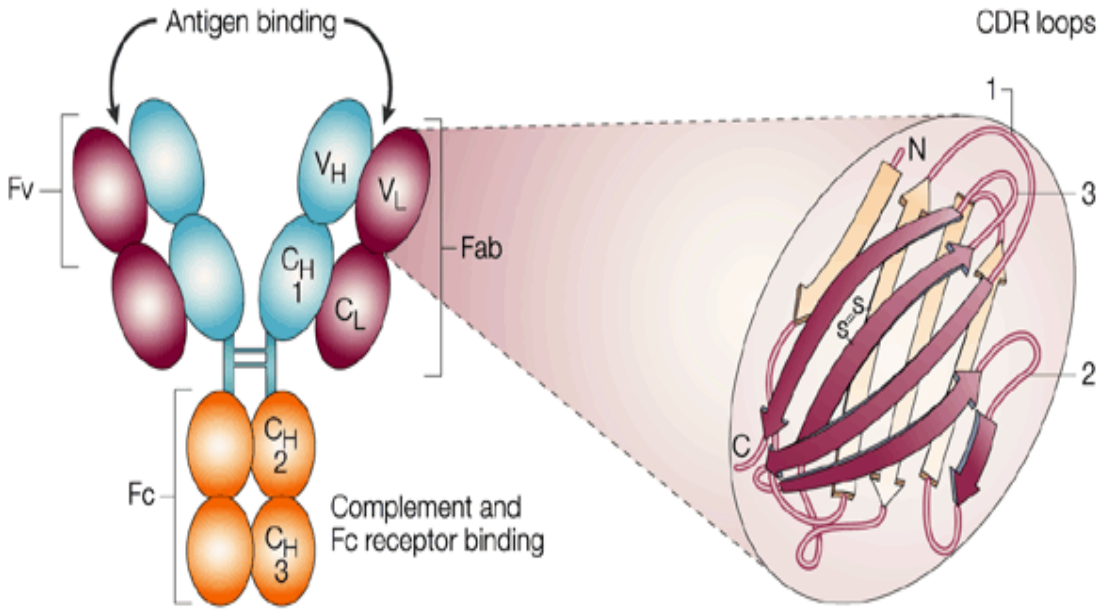

Figure 2. The antibody structure. The variable domains of both H and L chains of the Fabs compose the antigen- binding part of the molecule, termed Fv. Within V domains there are three loops designated CDRs 1, 2 and 3, which confer the highest diversity and define the specificity of antibody binding. The Ig illustration is taken from Brekke and Sandlie (2003).

The most important biological activities of antibodies are related to their effector functions, aimed at inactivation or removal of infectious agents and their products (e.g. bacteria, viruses, toxins). Antibodies of the IgG class exert two major effector functions:

activation of complement and opsonisation. These effector functions, mediated via the fragment crystallible (Fc) region, are induced as a result of interaction of the antibody with its antigen via the variable Fab moiety. As consequence of antigen exposure, IgG is produced for long-term protective immunity. Similar to most other serum proteins, Igs of non-IgG subclasses have a relatively short serum half-lives (1-2 days). In contrast, the half-lives of most IgG antibodies are considerably longer, 6-8 days in mice ([2]) and 22-23 days in humans ([3]). This increases the availability of sufficient specific IgG to fight infection.

1.1.1 Immunoglobulin G Fc region

The Fc region is separated from the antigen binding parts of the IgG molecule by flexible hing region and forms two structural domains, the CH2 and CH3 (Fig.3).

Immunoglobulins are remarkable not only for the diversity of their antigen binding sites but also for their versatility as effector molecules. As the B cell response to an infection gets underway, isotype switching diversifies the functional properties of the antibody Fc region, which contains binding sites for other proteins and cells of the immune system (Fig.3). Fc regions serve two distinct functions: they deliver antibody to anatomical sites that would otherwise be inaccessible and they link bound antigen to molecules or cells that will affect its destruction.

The Fc of IgG is composed of two H chains that each contributes two C domains. The Fc C_H2-C_H interphase is the binding site for a number of proteins that bind to IgG (Fig.3), including protein A from Staphylococcus aureus (SpA) ([4]) and protein G from Streptococcus species (SpG) ([5]) the rheumatoid factor ([6]), the herpes simplex virus IgG binding protein gE-gI ([7]), the mannose binding lectin (MBL; [8]), the mannose receptor ([9]), the major histocompatibility complex (MHC) class I related neonatal Fc receptor (FcRn) ([10]). The complement protein C1q also binds the Fc portion of IgG which play a key role in the recognition of immune complexes ([11]). The binding of C1q to non-aggregated IgG is weak; whereas a thousand fold increased upon the formation of immune complexes is observed ([11]).

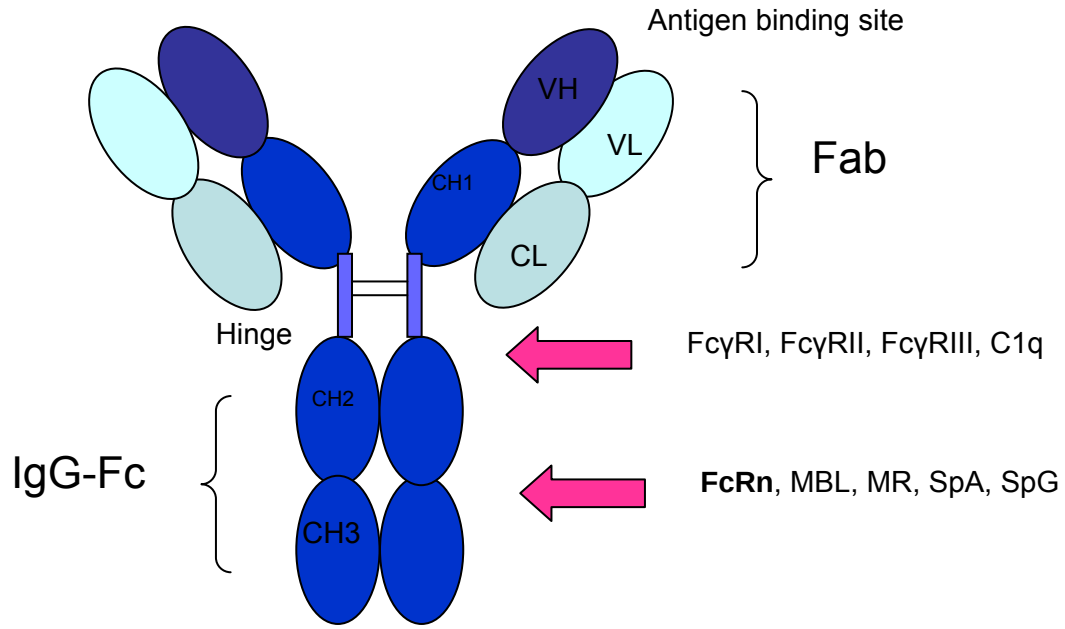

Figure.3. **A schematic representation of a human IgG isotype with its interaction sites**. The two antigen binding sites are at the aminoterminal end of each Fab. Interaction site for FcγRI, FcγRII, FcγRIII and C1q are at the hinge proximal region of IgG-Fc, while the interaction site for FcRn, MBL, MR, SpA and SpG is at the CH2-

1.1.2 Fc receptors

Fc receptors are a family of cell surface molecules that bind the Fc region of the Ig-molecule. Each members of the family recognizes Ig of one isotype or a closely related isotypes through a recognition domain on the heavy chain of the Fc receptor. Different cell types bear Fc receptors for antibodies of different isotypes. The isotype of antibody thus determines which types of cell will be engaged in a given responseIfunction. In humans we have Fc receptors for IgG, IgA, IgE and IgM.

The Fc receptors which include FcγRI, FcγRIIa, FcγRIIb, FcγRIII and FcRn bind to IgG, Fcα/μR to IgA and IgM (pIgR binds dimeric IgA and polymeric IgM), and FcεR to IgE. These Fc receptors play a pivotal role in linking the cellular and humoral immune responses by facilitating the internalization of immune complexes, antigen presentation, antibody dependent cellular cytotoxicity (ADCC), negative regulation of effector functions of Fc receptor bearing cells, regulation of the inflammatory cascade, and autoimmunity ([12, 13, 14, 15]) and Ig transport and homeostasis (discussed below).

The Fc receptors, except FcRn and pIgR, are composed of one or more Ig-like domains, a transmembrane part and a cytosolic tail. Most of the Fc receptors are tightly associated with a

Fc receptor γ-chain dimer. The signalling motif is called either ITAM (activating) or ITIM (inhibitory). FcγRI, FcγRIII, FcεRI and FcαRI are closely associated with a γ-chain dimer.

1.2 Human serum albumin

Human serum albumin (HSA) is 66.5 KDa globular protein, which lacks covalently bonded carbohydrates and lipids and is synthesized in and secreted from liver cells. The primary sequence of HSA shows that the protein is a single polypeptide with 585 residues containing 17 pairs of disulfide bridges and one free cysteine. HSA, as well as serum albumin from other species, has been found to consist of three homologous domains (I-III; Fig.4), probably derived through gene multiplication ([16]) each comprised of two subdomains (A and B) and stabilized by 17 pairs of disulfide bridges.

HSA is the most abundant protein in the blood plasma. Its physiological and pharmacological properties have been extensively studied over several decades. Such studies have revealed that HSA has a high affinity to a very wide range of materials. The important physiological role of the HSA is thought to be in the transport, distribution and metabolism of many endogenous and exogenous ligands (e.g. fatty acids, amino acids, steroids, metals and numerous pharmaceuticals), as well as to maintain the pH and osmotic balance of plasma, needed for proper distribution of body fluids between intravascular compartments and tissues.

It has been known for a long time that the catabolic rate of albumin is directly related to its serum concentration ([17]), but the mechanism has been unknown until recently, when it was shown that FcRn binds HSA and prolongs its lifespan ([18]).

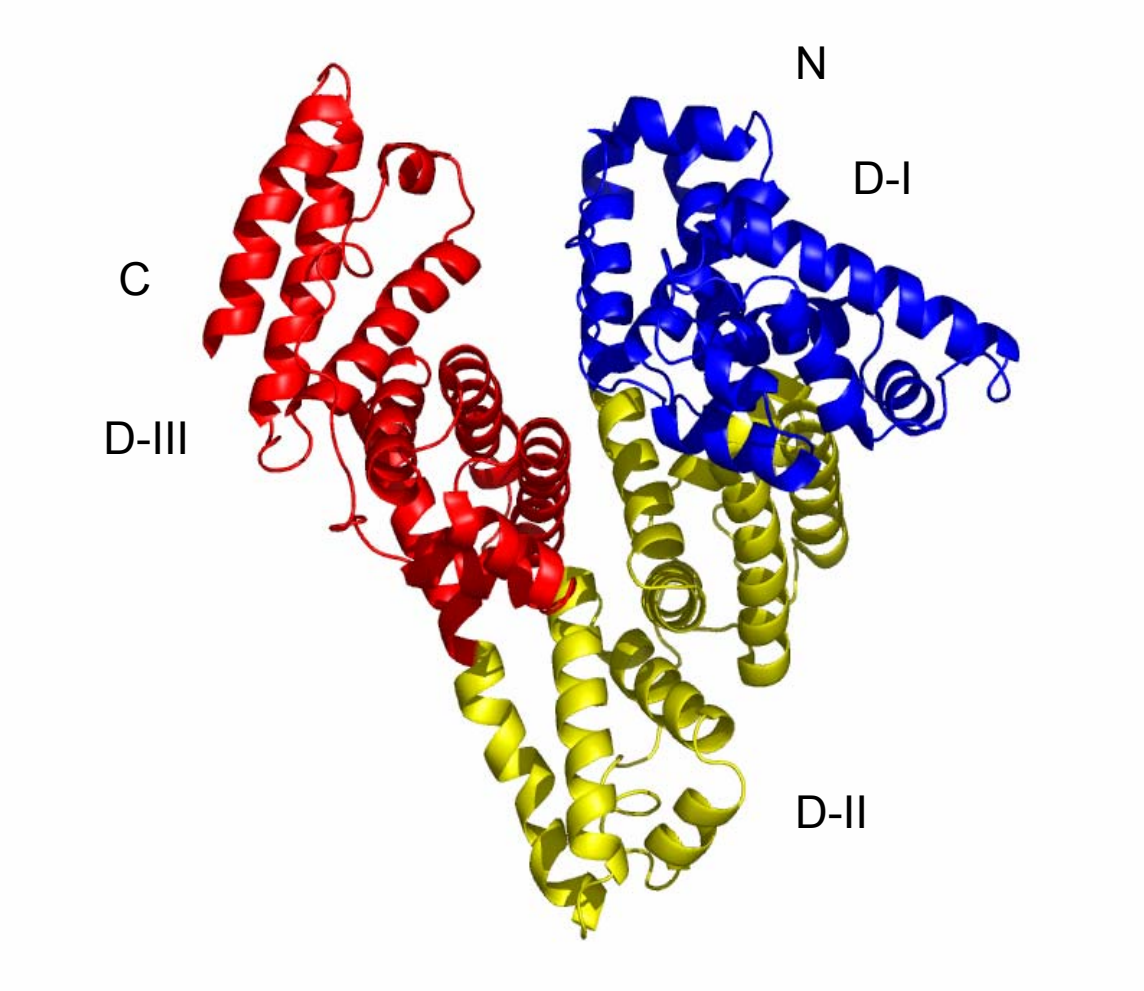

Figure 4. The ribbon structure HSA molecule. HSA consists of three domains, each domain is marked with a different color (blue for domain D-I; yellow D-II; red, D-III). N- and C-termini are marked as N and C, respectively. The figure is made by pymol based on crystal structure. The crystal structure is from Sugio *et al*., Crystal structure of HSA at 2.5 A resolution protein Eng. v12, p.439-446, 1999

1.3 Major Histocompatibility Complex molecules

MHC is a set of molecules displayed on cell surfaces that are responsible for lymphocyte recognition and antigen presentation. The Class I and Class II MHC molecules belong to a group of molecules known as the Ig Supergene Family, which includes Igs, T cell receptors, CD4, CD8, and others. The MHC is encoded by several genes located on short arm of human chromosome 6.

The peptide antigen-presenting MHC molecules are known as classical MHC molecules.

There are also structurally related molecules of both classes that do not function in the presentation of peptide antigens to T cells: these are known as non-classical MHC molecules. The non-classical MHC class II molecules (DM and DO in human) regulate peptide loading onto classical MHC class II molecules. The non-classical MHC class I molecules are more numerous and diverse, and some are important in activating specialized classes of T cells. A notable distinction between classical and non-classical MHC molecules that bears on their different functions is the polymorphism of the classical MHC molecules.

The classical MHC class I molecules are known as human leukocyte antigens A (HLA-A), HLA-B and HLA-C in humans and are characterized by a high level of cell surface expression and a very high degree of polymorphism ([19]). In contrast, the nonclassical class Ib molecules, HLA-E, HLA-F, HLA-G and other MHC class I related molecules in humans, are not significantly polymorphic ([20]). These exert their function at most levels of the immune response, being part of both innate and adaptive immune systems ([21]).

1.4. The MHC class I-related neonatal Fc receptor

1.4.1 Brief overview

Reflecting the central role of IgG in the immune system, mammals have evolved complex mechanisms to transmit immunity from mother to young and to protect IgG from catabolism. Knowledge as to how serum IgG levels are maintained at constant levels in the circulation is of central importance in understanding the regulation of humoral immunity.

Brambell *et al*. ([87]), about 40 years ago, put forward a hypothesis to explain serum IgG homeostasis. This hypothesis proposed that salvage receptors in cells of unknown type acted to bind and recycle pinocytosed IgG molecules back into the serum. He also suggested that these receptors were involved in the transmission of maternal IgG from mother to young. The putative receptors were believed to be saturable, so that any IgG molecules not receptor bound following pinocytosis would suffer degradation. This hypothesis was put forward in the absence of any knowledge of the receptor or cell types involved, but at the conceptual level, provided a satisfactory model by which IgG homeostasis could be maintained.

FcRn was originally identified as the receptor responsible for IgG binding to the intestinal epithelium of neonatal rats ([22]). The gene encoding rat FcRn was first isolated by

Simister and Mostov in 1989 ([23]). Cloning and sequencing of this gene showed that FcRn is a MHC class I homolog and expanded the function of these class molecules beyond their known role in antigen presentation ([23]).

Further, the genes encoding both mouse and human FcRn (hFcRn) alpha chains have been isolated ([24, 25]). The rodent and human genes share homology, with mouse and rat FcRn being highly related and the human form more divergent. The identification of hFcRn in human syncytiotrophoblast ([25, 26, 27]) led to the suggestion that it plays a role in the maternofetal transfer of IgGs. The isolation and characterization of hFcRn provided an important link between studies of FcRn in rodents and humans.

1.4.2 The structure of neonatal Fc receptor

The neonatal Fc receptor (FcRn) is a type I glycoprotein heterodimer that comprises a glycosylated heavy chain (45 kDa for humans and 51 kDa for rodents) in non-covalent association with a soluble light chain consisting of 12 kDa β_2 microglobulin (β_2m) (Fig.5).

Crystallographic structure of rat FcRn shows that like the heavy chain of class I molecules, the heavy chain of FcRn consists of three domains, α_1, α_2 and α_3, followed by a transmembrane region and small cytoplasmic domain ([28]). However, FcRn can not bind peptides owing to rearrangements of its α-helices, and the counterpart of the MHC peptide binding groove in FcRn is filled with side chains. The rearrangement of the FcRn α_2 helix compared to class I molecules is due to a break in the helix introduced by the presence of proline at position 162 resulting in closing of the groove. In class I molecules, the two spanning α-helices are separated by a groove ([29]). In all crystal structures of class I molecules solved sofar; the peptide-binding groove is occupied by either a mixture of endogenous peptides or by a single defined peptide. Owing to an overall repositioning of the α_1 helix and bending of the C-terminal portion of the α_2 helix, the FcRn helices are considerably closer together. Near the middle of the FcRn platform, the α_2 helix moves over to fill up the space between the helices. Surface representations of the tops of the FcRn and the class I α_1 and α_2 domains show that there is no continuous groove in FcRn. This correlates with biochemical results showing that soluble FcRn does not contain endogenous peptides ([30]). The two FcRn immunoglobulin-like domains α3 and β_2m, superimpose closely upon the corresponding domains of class I molecules. Many of the heavy chain/ β_2m contacts are conserved between FcRn and class I molecules. Compared to class I structures, the FcRn heavy chain makes

additional contacts to β_2m, in that the loop between β-strands 1 and 2 of α_1 domain dips downwards to contact β_2m.

In contrast to extracellular domains, the transmembrane region and cytoplasmic tails of FcRn and MHC class I are not closely related ([23]). There is divergence in the cytoplasmic regions consistent with the different functional activities of the two types of proteins. Studies of rat FcRn cytoplasmic domain show presence of endocytosis signals ([31]). Tryptophan at position 311 and dileucine motif are necessary components of endocytosis signals. Leucine at position 322 and 323, together with aspartic acid at position 317 and/or 318 are parts of a typical dileucine-based endocytosis signal ([31]). In polarized cell models, FcRn in the plasma membrane is predominantly at basolateral surface. This distribution depends on signals that overlap endocytosis signal. The tryptophan-based basolateral targeting, which requires the aspartate pair of the dileucine based-signal, and endocytosis signals are distinct but overlapping ([32]).

The amino acids sequence analysis of mouse and rat heavy chains share 91% identity ([24]) while the hFcRn heavy chain shares 65% amino acids identity with its rat homologue ([25]). In spite of its similarity to MHC class I, the FcRn heavy chain is encoded outside the MHC, on chromosome 7 in mice and chromosome 19 in humans. FcRn heavy chains are non.covalently associated with β_2m, the class I light chain.

The β_2m which is required for both surface expression and function of FcRn ([33]) is encoded on chromosome 15 in humans. Indications that an association of the FcRn heavy chain with β_2m is important for the assembly of a functional receptor come from β_2m-knockout mice ([34]), which showed defects in several functions associated with FcRn. Newborn β_2m-deficient pups show lower IgG serum level at birth and accumulate less IgG before weaning than normal littermates ([34]). Further, adult mice lacking β_2m have a higher IgG turnover, resulting in lower serum IgG levels ([35, 36, 37]). Expression of the hFcRn α-chain alone or in combination with β_2m in human melanoma FO-1 cells (FO-1) showed that β_2m is important for cell surface expression of FcRn and that, in the absence of β_2m, the receptor is retained in the endoplasmic reticulum ([38]). In the absence of β_2m, IgG binding is decreased compared with that of native FcRn. Thus, assembly of the FcRn heavy chain with β_2m is important for both transport of FcRn from the endoplasmic reticulum to the cell surface and efficient pH-dependent IgG binding ([38]).

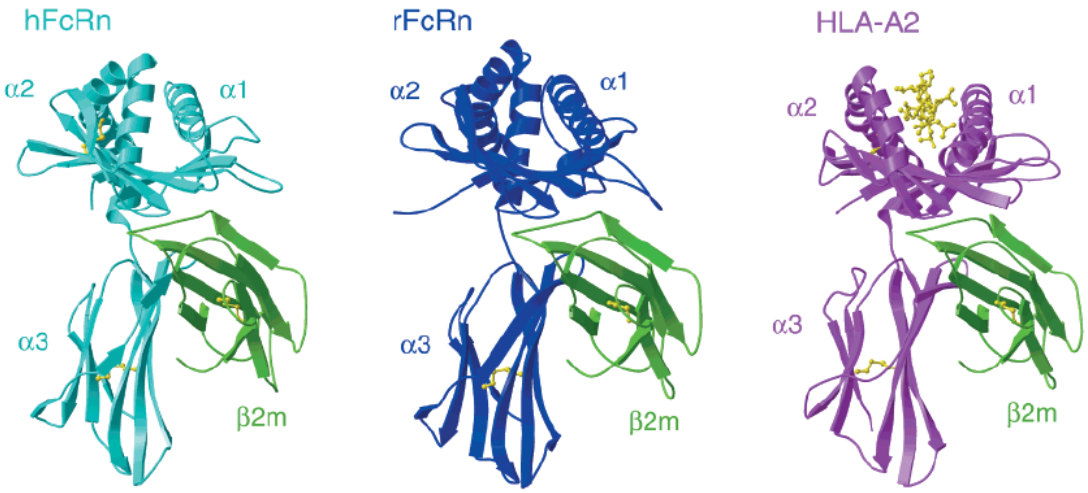

Figure 5. Ribbon structural diagrams of hFcRn, rFcRn, and the class I MHC molecule (HLA-A2). The illustration is taken from West and Bjorkman (2000).

1.4.3 FcRn interacts with its ligands in a pH-dependent manner

Studies have indicated that the distinguishing feature of FcRn interaction with its ligands is its strict pH dependence, with binding at acidic pH (6-6.5), and undetectable interaction at slightly basic pH (7.4) ([39]). No conformational change is observed between the structures of FcRn at pH 6.5 and pH 8 that could account for the differences in affinity for IgG ([40]). Rather, the pH-dependent FcRn-ligand binding are mediated by electrostatic interactions attributed to conserved amino acid residues located at the C_H2-C_H3 domain of IgG-Fc (Table 1). This involves mainly the imidazole groups on H310 and H435 (Fig. 6A) of IgG which are positively charged and facilitate interaction with negatively charged residues in the FcRn α_2-domain, whereas at physiological pH 7.4, the side chains are neutral. The main FcRn α_2-domain residues involved are E117, E118, D137 and E135 (Fig. 6B).

Table 1. Variations of IgG sequences in the region involved in the binding of FcRn

		Amino Acid Sequence at Position:														
		252	**253**	**254**	**255**	**256**	**257**	**307**	**308**	**309**	**310**	**311**	**433**	**434**	**435**	**436**
mouse	IgG1	Thr	Ile	Thr	Leu	Thr	Pro	Pro	Ile	Met	His	Gln	His	Asn	His	His
	IgG2a	Met	Ile	Ser	Leu	Thr	Pro	Pro	Ile	Gln	His	Gln	His	Asn	His	His
	IgG2b	Met	Ile	Ser	Leu	Thr	Pro	Pro	Ile	Gln	His	Gln	Lys	Asn	Tyr	Tyr
	IgG3	Met	Ile	Ser	Leu	Thr	Pro	Pro	Ile	Gln	His	Gln	His	Asn	His	His
rat	IgG1	Thr	Ile	Thr	Leu	Thr	Pro	Pro	Ile	Leu	His	Gln	His	Asn	His	His
	IgGa	Thr	Ile	Thr	Leu	Thr	Pro	Pro	Ile	Val	His	Arg	His	Asn	His	His
	IgG2b	Leu	Ile	Ser	Gln	Asn	Ala	Pro	Ile	Gln	His	Gln	His	Asn	His	His
	IgG2c	Met	Ile	Thr	Leu	Thr	Pro	His	Ile	Gln	His	Gln	His	Asn	His	His
human	IgG1	Met	Ile	Ser	Arg	Thr	Pro	Thr	Val	Leu	His	Gln	His	Asn	His	Tyr
	IgG2	Met	Ile	Ser	Arg	Thr	Pro	Thr	Val	Val	His	Gln	His	Asn	His	Tyr
	IgG3*	Met	Ile	Ser	Arg	Thr	Pro	Thr	Val	Leu	His	Gln	His	Asn	Arg	Phe
	IgG4	Met	Ile	Ser	Arg	Thr	Pro	Thr	Val	Leu	His	Gln	His	Asn	His	Tyr

*Allotype containing Arg instead of His at Position 435

The table is taken from Gethie and Ward (2000)

Reports show that FcRn also binds HSA in a pH dependent manner ([18]). Studies in FcRn-deficient mice have shown that the half-life and the steady-state concentration of albumin were decreased relative to wild-type mice. The hypothesis that FcRn binds both IgG and HSA is also evidenced by a β_2m gene mutation that underlies the hypercatabolism and reduced serum levels of HSA and IgG in the two siblings with familial hypercatabolic hypoproteinemia ([41]). This experiment of nature confirms that FcRn binds both HSA and IgG.

FcRn binds both HSA and IgG simultaneously in a pH dependent manner ([42]). The pH dependence of the FcRn-albumin interaction suggests that, like IgG, albumin binding to the receptor might also be mediated through titratable histidine residues. Andersen *et al*. ([43]) compared the sequence of the FcRn α_2-domain from eleven different species, and identified histidine residues that were conserved in all (H166) or seven (H161) of these species (Fig.7), and showed that the conserved H166 (Fig. 8) is a key player in the FcRn-albumin interaction. The corresponding interacting residue on HSA is probably negatively charged and surface exposed on domain III. The experiments measuring binding of FcRn to the three recombinant albumin domains establish that albumin D-III alone is both necessary and sufficient for binding to FcRn ([42]). Not only is D-III the only domain of the three to bind immobilized

shFcRn, but D-III binding is equimolar to HSA binding.

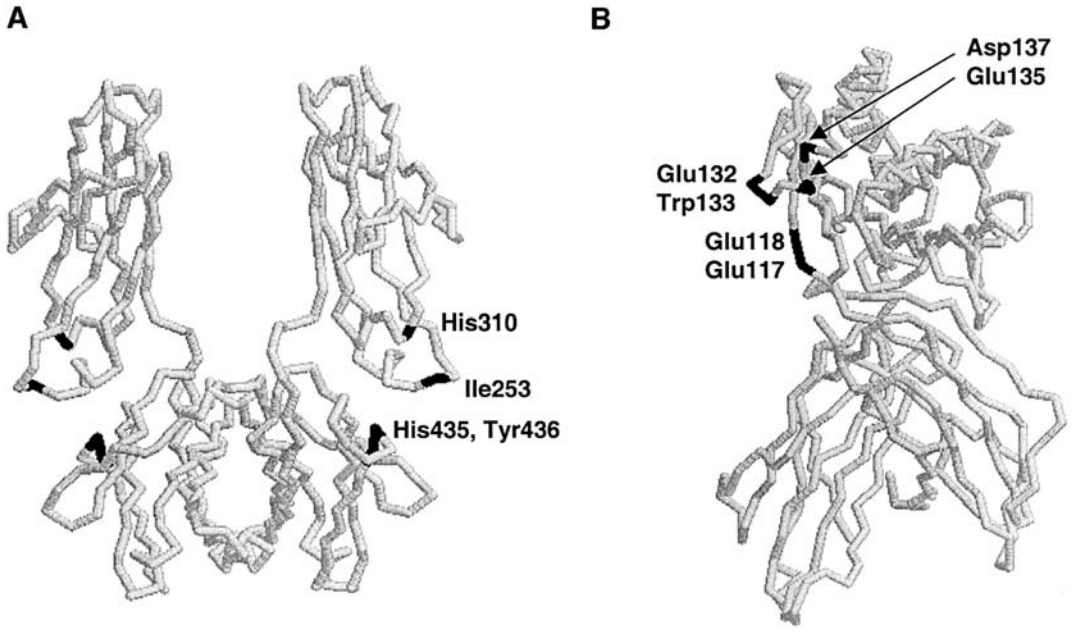

Figure 6. (A) pH sensitive histidine residues in the Fc CH2-CH3 interphase of IgG important in the FcRn interaction. (B) Shows FcRn α2-domain involved in binding to IgG. Figure is taken from Annu.Rev.Immunol.2000.18.739-766.

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                                •          ⇊          ⇊ ↓                    ∇  ∇
rat          88 GTFTLQGLLGCELAPDNSSLPTAVFALNGEEFMRFNPRTGNWSGEWPETDIVGNLWMKQPEAARKESEFLLTSCPERLLGHLERGRQNLEWK 179
mouse        88 GTYTLQGLLGCELASDNSSVPTAVFALNGEEFMKFNPRIGNWTGEWPETEIVANLWMKQPDAARKESEFLLNSCPERLLGHLERGRRNLEWK 179
human        86 GPYTLQGLLGCELGPDNTSVPTAKFALNGEEFMNFDLKQGTWGGDWPEALAISQRWQQQDKAANKELTFLLFSCPHRLREHLERGRGNLEWK 177
macaque      86 GPYTLQGLLGCELSPDNTSVPTAKFALNGEEFMNFDLKQGTWGGDWPEALAISQRWQQQDKAANKELTFLLFSCPHRLREHLERGRGNLEWK 177
orangutan    86 GPYTLQGLLGCELGPDNTSVPTAKFALNGEEFMNFDLKQGTWGGDWPEALAVSQRWQQQDKAANKELTFLLFSCPHRLREHLERGRGNLEWK 177
pig          86 GPFTLQGLLGCELGPDNVSVPVATFALNGEEFMKFDTKLGTWDGEWPEARTIGSKWMQEPDAVNKEKTFLLYSCPHRLLGHLERGRGNLEWK 177
camel        86 DSYTLQGLLGCELGPDNVSVPMAKYALNGEEFMEFDPKLGIWDGDWPEARTIGIKWMKHPEAVNKEKTFLLYSCPHRLLGHLERGRGNLEWK 177
sheep        85 GPFTLQGLLGCELGPDNVSVPVAKFALNGEEFMMFDPKLGIWDGDWPESRTVSIQWTKQPEAVNKEKTFLLYSCPHRLLGHLERGRGNLEWK 176
cattle       85 GPFTMQGLLGCELGPDNVSVPVAKFALNGEEFMMFDPKLGIWDGDWPESRTVSIKWTQQPEAVNKEKTFLLYSCPHRLLGHLERGRGNLEWK 176
dog          86 GPYTLQGLLGCELGPDNTSVPVAKFALNGEDFMTFDPKLGTWNGDWPETETVSKRWMQQAGAVSKERTFLLYSCPQRLLGHLERGRGNLEWK 177
possum       84 GAHTFQGLVGCQLNPDNSSQHTARYALDGLDLLTFDPVSREWFGDTAEALNVKKGWANESQRAEKDAEFLLTTCPQKLKSHLQKGQGNFHWK 175
                ...*:***:**:* .** *   * :**:* ::: *:    * *: .*:  :  * :.  . *:  *** :**.:*  **::*: *:.**
```

Figure 7. **Alignment of predicted α_2-domain amino acid sequences from eleven FcRn heavy chain**. Sequences (rat, mouse, human, macaque, orang-utan, pig, camel, sheep, cattle, dog and possum). Amino acid residues that are identical in all sequences are indicated by (*), conserved substitutions are indicated by (:), semi-conservative substitutions are indicated by (.). The potential N-linked glycosylation site is highlightened by filled circle (•). Amino acid residues involved in IgG binding are indicated by downward arrow and partially or fully conserved histidine residues (hFcRn heavy chain; H161 and H166A) are indicated by open triangle (Δ). The alignment is taken from **Andersen *et al*., (2006).**

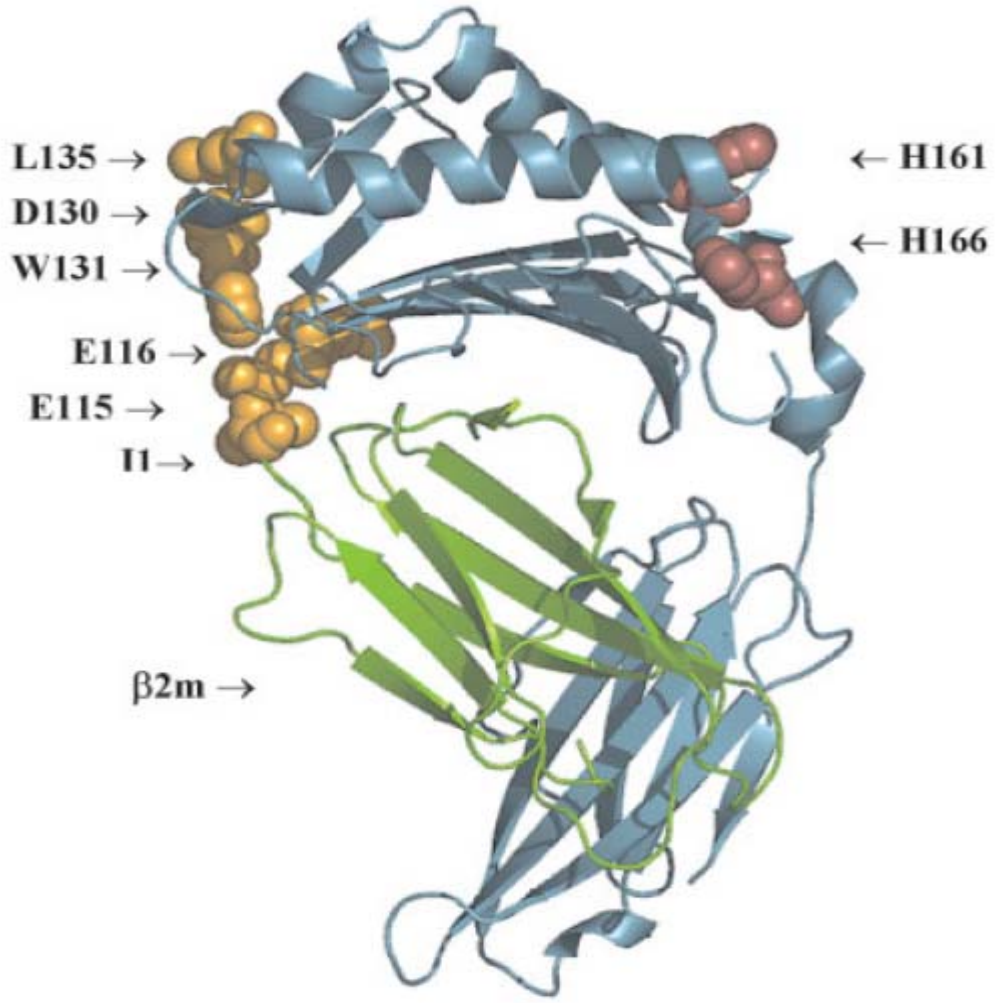

Figure 8. Localization of relevant hFcRn α2-domain residues. A ribbon diagram of the crystal structure of shFcRn, the heavy chain is displayed in blue, β2m in green. The IgG interacting residues (E115, E116, D130, W131 and L135) are marked as yellow balls and the two α2-domain located histidine residues, H161 and H166, are marked in purple. The figure are taken from **Andersen *et al*., (2006).**

1.4.4 The stoichiometry of the FcRn-ligand interaction

To understand FcRn functions, it is important to explore the structural bases of pH-dependent IgG-FcRn interaction. In this context, studies have been carried out and different models forwarded to show the stoichiometry of the interaction between IgG and FcRn.

The stoichiometry of the interaction between soluble FcRn and Fc has been reported as either 2:1 for rat FcRn ([44]) or 1:1 for mouse FcRn. The 1:1 interaction indicate that binding of

FcRn to one CH2-CH3 domain interface site precludes an FcRn:Fc interaction at the second site ([45]). Further crystallographic studies show that rat FcRn can interact with the Fc portion of IgG in a repeating array in which FcRn dimers are bridged by Fc fragme nts to create an "oligomeric ribbon" with a 2n:n stoichiometry. Analysis of complexes formed in solution between soluble rat or mouse FcRn and Fc showed that both forms of FcRn produce 2:1 receptor-ligand complexes, but that alterations of the carbohydrate moieties on mouse FcRn can result in an apparent stoichiometry of 1:1 ([46]).

The crystal structure of hFcRn has been determined and compared to the previously described structure of rat FcRn and to the structures of MHC and MHC-related proteins. Human FcRn is structurally similar to the rat receptor but does not form receptor dimers in the crystals as observed in crystals of rat FcRn ([47]). The interaction between hFcRn and IgG was characterized by determining the binding stoichiometry using equilibrium gel filtration and by deriving binding affinities for the different human IgG subclasses using a surface plasmon resonance assay ([47]). Like rat and mouse FcRn, hFcRn interacts with IgG with a 2:1 receptor:ligand stoichiometry. The binding of hFcRn to the four human IgG subclasses shows subclass and allotype variations but no clear subclass affinity differences that correlate with serum half-lives ([47]). Further evidences are required for elucidation of the disparities among these different models explaining the stoichiometry of interaction between FcRn and IgG.

1.4.5 Preferential binding of hFcRn with IgG

Although mice are widely used in the pre-clinical testing of antibodies, human and mouse FcRn have significant differences in binding specificity. It is established that FcRn is the receptor that salvages from degradation and regulates the serum half-life of IgG in mice. Reports indicate that the human homologue of mouse FcRn serves similar functions in humans. Mouse IgG is promiscuous in binding specificity and binds to all IgG of species analysed, including human, mouse, rat, guinea pig, bovine, sheep, and rat IgG. In contrast, hFcRn is surprisingly stringent, and only interact well with human, rabbit and guinea pig IgGs ([48]).

Human FcRn does not bind detectably to mouse IgG1, IgG2a, and shows a very weak interaction with mouse IgG2b. The high selectivity of the hFcRn-IgG interaction therefore gives a molecular explanation for the observation that mouse IgG is cleared rapidly from the

human circulation ([49, 50]).

1.4.6 Multiple functions of FcRn

1.4.6.1 Transfer of immunity (passive immunization)

Passive acquisition of antibody is important to the newborn prior to the development of a fully functional immune system. Transfer of maternal IgG to the fetus or infant humoral immunity to antigens encountered by the mother. Transmission of IgG is mediated by the neonatal Fc receptor.

In newborn rodents, FcRn on the apical side of intestinal enterocytes bind to maternal IgG in ingested milk, escort the IgG across the gut epithelium, then release it into the blood stream from the basolateral surface in the process of transcytosis ([51], Fig.9a). The pH difference between the apical (pH 6.0-6.5) and basolateral (pH 7.0-7.5) sides of intestinal epithelial cells ensures efficient unidirectional transport of IgG.

Although the acidic pH at the apical surface of intestinal epithelial cells permits cell surface FcRn to bind IgG, FcRn can also function in IgG transport when there is no net pH gradient. FcRn transports IgG across the syncytiotrophoblastic cells of the human placenta (reviwed in [51]) in this way. For this function, it is believed that IgG in the blood (pH 7.4) enters cells in a receptor-independent manner via fluid phase endocytosis, after which it is delivered to acidic endosomes where it binds to FcRn (Fig.9a). Upon delivery of FcRn-IgG complexes to the cell surface, the slightly basic pH of the blood causes IgG release into the circulation.

An ex vivo placental model has been used to analyze the maternofetal transfer of a recombinant, humanized (IgG1) antibody in which His435 has been mutated to alanine (H435A). In vitro binding studies using surface plasmon resonance indicate that the mutation ablates binding of the antibody to recombinant mouse and human FcRn ([52]). Relative to the wild-type antibody, the H435A mutant is deficient in transfer across the placenta. Significantly, the mutation does not affect binding to Fc gamma RIII, an FcR that has been suggested in earlier studies to mediate the transfer of maternal IgG ([53]). The analyses demonstrate that binding of an IgG to FcRn is a prerequisite for transport across the perfused placenta ([52]). FcRn therefore plays a central role in the maternofetal delivery of IgG and this has implications for the use of protein engineering to improve the properties of therapeutic

antibodies ([52]).

The placental barrier between maternal and fetal circulatory systems, across which IgG must pass, consists of two cell layers and an intervening stroma ([54]). The first of these cell layers is the epithelial syncytiotrophoblast, which completely covers chorionic villi and constitutes the point of direct fetal contact with circulating maternal blood. Once across the syncytiotrophoblast, IgG appears to transit the villus interstitium via bulk fluid flow ([55, 56]). How IgG crosses the fetal villus capillary endothelial cell layer is not known.

1.4.6.2 Maintenance of IgG and HSA homeostasis

In addition to mediating the transfer of maternal IgG, FcRn is also important in regulating the amount of IgG in serum. Because a high concentration of IgG bathes most cells, it is readily internalized by non-specific mechanisms of fluid-phase endocytosis. Unlike most other protein solutes internalized by fluid phase endocytosis, however, IgG is rescued from degradation in the lysosome by FcRn ([57]). FcRn binds to IgG and recycles it back to the plasma membrane and into circulation. Once FcRn binding is saturated, the non-receptor bound IgG is delivered along with other fluid phase cargo to the lysosomes, where it is then degraded (Fig.9b). Therefore, IgG level in serum are governed by the saturable nature of the intracellular FcRn-IgG interaction ([51]). As early as in 1966, a mechanism identical to that proposed by Brambell for protecting IgG from degradation was applied to albumin as well. The inverse relationship between serum albumin concentration and its half life suggested that albumin would be protected from a catabolic fate by a receptor-mediated mechanism much like that proposed for IgG. Recently, this was confirmed when it was shown that hFcRn binds not only hIgG but HSA as well ([18]). The FcRn-mediated recycling has been shown to save as much albumin from degradation as the liver produces ([58]). It follows that FcRn diverts not only IgG but albumin as well from a degradative fate by similar pH-dependent mechanisms, prolonging the lifespan of both molecules. It has been suggested that endothelial cells of small arterioles and capillaries involve in these functions ([59]). It is possible that the organ separated from blood by fenestrated or discontinuous endothelium may participate in similar function but requires investigation.

1.4.6.3 Bidirectional transmission of IgG across mucosal barriers

Humoral immunity as mediated by IgG plays an important role in mucosal tissues as a defense against pathogens. Within the gastrointestinal, respiratory and genitourinary tracts immunoglobulins access to mucosal secretions must occur in the context of a barrier imposed by epithelial cells that separates the host from the external environment.

The FcRn is involved in the bidirectional transmission of IgG/immune complex across mucosal barriers ([60, 61]) (Fig.9C). The FcRn can then transport the IgG/antigen complex back across the intestinal barrier into the lamina propria for processing by dendritic cells and presentation to CD4(+) T cells for subsequent immune activation ([62]). This mechanism has been associated with defense against an epithelium-associated pathogen Citrobacter rodentium ([62]). In addition, neonatal mice have been shown to be protected against the luminal parasite Heligmosomoides polygyrus by IgG delivered directly in milk or via FcRn from the neonatal serum into the intestinal lumen to exert its protective effect ([63]). Thus, FcRn through its ability to bind and release IgG, integrates luminal antigen encounters with systemic immune compartments and, as such, provides essential host defense at the mucosal surfaces.

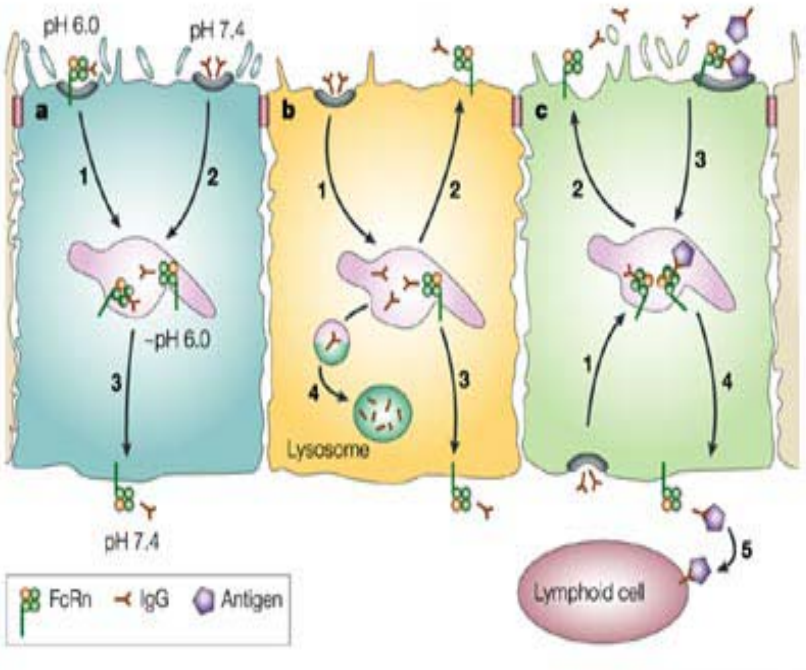

Figure 9. Mechanisms of FcRn functions: (**a**) **IgG transport**. At acidic pH (6.0-6.5) IgG will bind to FcRn on the surface of the cell (step 1). Where the pH outside the cell is neutral, IgG is internalised by fluid face endocytosis (step 2) and then FcRn binding occurs in the acid intracellular compartment. FcRn-IgG complex are transported to basolateral surface (step 3), where the pH is basic. (**b) Regulation of IgG homeostasis**. In endothelial cells IgG is taken up by fluid phase endocytosis and delivered to endosome (step 1), where it interacts with FcRn. Ligand bound to receptor is either recycled to apical plasma membrane where it returned to blood (step 2) or transported to and released at basolateral pole of the cell (step 3). When IgG concentration is high, unbound IgG is delivered to lysosomal degradative pathwayalong (step 4). (**c) FcRn-IgG in immune activation and tolerance.** IgG is taken in at basolateral phase of the cell (step 1) and transported by FcRn to the apical pole of the cell, where it is released at the surfaceto the (step 2). FcRn-IgG-antigen complexes internalized by fluid phase endocytosis or through their interaction with FcRn (step 3) transported to basolateral pole of the cell (step 4), delivering immune complexes to the lamina propria for subsequent induction of immune activation or tolerance (step 5). The illustration is taken from Rojas and Apodaca (2002)

1.4.6.4 Roles of FcRn in immune cells.

Studies have shown that FcRn is functionally expressed by monocytes, macrophages, dendritic cells and monocytic cell lines such as THP-1 cells ([64]) but established cell lines derived from B-lymphocyte, T-lymphocyte, and NK cell lineages failed to express FcRn heavy chain ([64]). FcRn is also expressed in polymorphonuclear neutrophils ([65]).

Recent study shows that FcRn is highly expressed in freshly isolated human and mouse neutrophils (PMNs) and IgG-mediated phagocytosis by these cells was facilitated by FcRn ([65]). It was found that FcRn enhances phagocytosis in a pH-dependent manner. IgG-opsonized bacteria were inefficiently phagocytosed by neutrophils from β_2m knock-out or FcRn α-chain knock-out mice, which both lack expression of FcRn. Similarly, low phagocytic activity was also observed with mutated IgG (H435A), which is incapable of binding to FcRn, while retaining normal binding to classical leukocyte Fcγ receptor. They observed FcRn translocation to nascent phagosomes, where FcRn facilitates IgG-mediated bacterial phagocytosis through signalling motifs found within the cytoplasmic tail. These results point to a novel role for FcRn in phagocyte biology ([65]).

1.5 Relevance of FcRn in therapeutics

The FcRn plays an essential role in extending the half-life (t(1/2)) of IgG antibodies and IgG-Fc-based therapeutics in the circulation. The role of FcRn in the regulation IgG homeostasis suggests that the modulation of IgG binding affinities for FcRn might be an effective approach for the treatment of IgG-mediated disease.

Several studies have demonstrated a correlation between the binding affinity of IgGs to FcRn and their serum half-lives in mice, including engineered antibody fragments with longer serum half-lives ([66]). Studies also extended this correlation to human IgG2 antibody variants in primates ([67]). Further, several human IgG1 mutants with increased binding affinity to hFcRn at pH 6.0 were generated that retained pH-dependent release. A pharmacokinetics study in rhesus monkeys of the IgG1 variants indicated that its serum half-life was approximately 2.5-fold longer than the wild-type antibody ([68]). Antigen binding was unaffected by the Fc mutations, while several effector functions appeared to be minimally altered. These properties suggest that engineered antibodies with longer serum half-lives may prove to be effective therapeutics in humans ([68])

Engineering the Fc region of a hIgG to generate a mutated antibody that modulates the concentrations of endogenous IgGs *in vivo* has been shown. An IgG whose Fc region was engineered to bind with higher affinity and reduced pH dependence to FcRn potently inhibits FcRn-IgG interactions and induces a rapid decrease of IgG levels in mice ([69]). Such FcRn blockers (or 'Abdegs,' for antibodies that enhance IgG degradation) may have uses in reducing IgG levels in antibody-mediated diseases and in inducing the rapid clearance of IgG-toxin or IgG-drug complexes. The Abdeg design is to alter the level of endogenous, unmanipulated IgGs by enhancing their clearance rates.

FcRn blockade by intravenous Ig (IVIG) significantly increases the catabolism of serum IgG in mice ([70]). Studies on the therapeutic effects of an anti-rat FcRn mAb, 1G3, in two rat models of myasthenia gravis resulted in dose-dependent amelioration of the disease symptoms after passive experimental autoimmune myasthenia gravis was induced by administration of an anti-acetylcholine receptor (AChR) mAb ([71]). The effect of 1G3 was also studied in an active model of experimental autoimmune myasthenia gravis in which rats were immunized with AChR. Treatment with 1G3 significantly reduced the severity of the disease symptoms as well as the levels of total IgG and anti-AChR IgG relative to untreated animals. These shows that FcRn blockade may be an effective way to treat antibody mediated autoimmune

diseases ([71])

In imaging, it is desirable to have a short half-life and, although this can be achieved by using Fab fragments, it is now also possible to engineer complete antibodies with single amino acid substitution (e.g. Ile253 to Ala) ([72, 73]), which have reduced serum persistence.

Since it was recently discovered that HSA binds to FcRn and extends its lifespan, there may be focus on the possibility of constructing albumin coupled drugs, as these will have FcRn binding properties and a prolonged *in vivo* half-life.

Retargeting T lymphocytes to tumor cells for destruction by recombinant bispecific antibodies (e.g. single chain dibody) have been reported. However, therapeutic efficacy is hampered by a short serum half-life of these small molecules having molecule masses of 50-60 kDa. Thus, improvement of the pharmacokinetic properties of small bispecific antibody formats is required to enhance efficacy *in vivo*. Taking advantage of long half-life conferred by FcRn, recombinant bispecific antibody-albumin fusion proteins were generated and fusion to HSA strongly increases circulation time for biological activity and pharmacokinetic properties ([74]). Rapid targeting, excellent tumor deposition and retention, coupled with high tumor to blood ratios may make albumin.Fab fusion an exceptional molecule for imaging and cancer therapy ([75]).

As FcRn targeting drug, delivery of erythropoietin (Epo) conjugated to the Fc domain of IgG1 in mouse and non-human primates has been reported ([76, 77]). FcRn-dependent absorption was most efficient when the Epo-Fc fusion protein was deposited predominantly in the upper and central airways of the lung, where epithelial expression of FcRn was most prominently detected. The bioavailability of the EpoFc monomer when delivered through the lung was approximately equal to that reported for unconjugated Epo delivered s.c. in humans (Bitonti *et al*., 2004). This points to a functional FcRn-dependent transport pathway in the lung that can be used for the delivery of therapeutic proteins.

PROJECT DESCRIPTION

1. Background

The MHC class I related neonatal Fc receptor, FcRn was first described functionally in rodents as the receptor that transfers IgG in the maternal milk from the intestine of neonate rodents to the blood stream ([39, 78]). The human form of FcRn was identified in the placental syncytiotrophoblast, showing that this receptor plays a role in passive immunization of the fetus by maternofetal transport of IgG ([27, 81, 82, 25]).

Subsequent studies have shown FcRn mRNA in many tissues of adult rats, mice and humans, and FcRn is present in several adult tissues and in cell lines. In addition to mediating the transfer of maternal IgG, FcRn is also important in regulating the amount of hIgG in serum by prolonging its half-life. Reports show that FcRn also binds HSA in a pH dependent manner ([18]).

A hallmark of FcRn interaction with its ligands is its strict pH dependence. The binding occurs at the cell surface or in the intracellular vesicles where the pH is 6.0-6.5, while IgG is released at pH 7.0-7.5 ([39, 88]).

2. Methods: Cell culture, RT-PCR, ELISA, Western immunoblotting and flow cytometry

3. The aims of the study are to investigate:

1. Expression of FcRn in human immune cells and hepatocytes.
2. Ligand binding properties of soluble and membrane-bound FcRn.
3. Effects of anti-inflammatory substances on FcRn expression.

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MANUSCRIPT

FcRn EXPRESSION, LIGANDS BINDING PROPERTIES AND ITS REGULATION IN HUMAN IMMUNE CELLS AND HEPATOCYTES

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ABSTRACT

Expression and diverse functions of MHC class I related neonatal Fc receptor in different tissues is continually reported. To contribute to the understanding of how the receptor functions according to cell type, we investigated the expression and ligands binding properties of FcRn in human immune cells and hepatocytes. Here, we report that heterodimeric FcRn is expressed in these cells as evidenced by RT-PCR, Western immunoblottting and flow cytometry. The receptor expression is shown to be predominantly intracellular as compared to that on the surface. Our report on human hepatocytes and monocytic K-562 cells is for the first time. In addition, the human hepatic cell lines were shown to express FcγRII but no evidence of FcγRI and FcγRIII. Our pH dependent cell binding assays have demonstrated increased binding of hIgG and HSA to membrane-bound hFcRn at acidic pH in these cells and the pH dependent binding of IgG is Fc mediated. The increased binding of IgG and HSA at acidic pH is completely mediated by FcRn as shown by anti-FcRn inhibition and not affected by isotype goat IgG. It was found that IgG and HSA bind to the membrane-FcRn independently of each other and this is the first report of pH dependent HSA binding to a membrane-bound hFcRn. Moreover, we have shown that anti-inflammatory substances such as both naturally occurring and synthetic glucocorticoids, and L-thyroxine clearly downregulate the FcRn expression in human monocytic U-937 and hepatic cell lines.

2. INTRODUCTION

IgG and HSA are the most abundant of the serum proteins and their long half-life is due to FcRn binding properties.

The FcRn was first described functionally in rodents as the receptor that transfers IgG in the maternal milk from the intestine of neonate rodents to the blood stream ([1, 2]). Subsequently, the isolation and characterization of rat FcRn from the yolk sac endoderm led to the proposal by Roberts *et al* ([3]) that this Fc receptor was involved in the maternofetal transfer of IgGs. The human form of FcRn was identified in the placental syncytiotrophoblast, suggesting that this receptor plays a role in passive immunization of the fetus by maternofetal transport of IgG ([4, 5, 6, 7]). Subsequent studies have shown FcRn mRNA in many tissues of adult rats, mice and humans, and FcRn is present in several adult tissues and in cell lines.

FcRn is a MHC class I related molecule that comprises a glycosylated heavy chain (45 kDa for humans and 51 kDa for rodents) in non-covalent association with β_2m (12 kDa). Assembly of the FcRn heavy chain with β_2m is important for both transport of FcRn from the endoplasmic reticulum to the cell surface and functions ([8, 9]).

In addition to mediating the transfer of maternal IgG, FcRn is also important in regulating the amount of hIgG in serum by prolonging its half-life. The rate of IgG turnover increases as the amount of IgG in the serum rises ([10]). Turnover occurs in endothelial cells and involves a saturable process that is FcRn mediated ([11]). This has been demonstrated in β2m deficient mice that have abnormally low IgG half life in comparison to control animals ([12]).

The FcRn is also involved in the bidirectional transmission of IgG/immune complex across mucosal barriers ([13, 14]). The FcRn can then transport the IgG/antigen complex back across the intestinal barrier into the lamina propria for processing by dendritic cells and presentation to CD4(+) T cells for subsequent immune activation ([15]). This mechanism has been associated with defense against an epithelium-associated pathogen Citrobacter rodentium ([15]). In addition, neonatal mice have been shown to be protected against the luminal parasite Heligmosomoides polygyrus by IgG delivered directly in milk or via FcRn from the neonatal serum into the intestinal lumen to exert its protective effect ([16]). Thus, FcRn integrates luminal antigen encounters with systemic immune compartments and, as such, provides essential host defense at the mucosal surfaces.

A distinguishing feature of FcRn interaction with IgG is its pH dependence. The binding occurs at the cell surface or in the intracellular vesicles where the pH is 6.0-6.5, while IgG is

released at pH 7.0-7.5 ([1, 17]). IgG molecules that do not bind to FcRn are delivered to the default lysosomal pathway and degraded ([18]). The interaction site for hFcRn on IgG has been mapped and shown to encompass conserved residues at the C_H2-C_H3 domain interphase, and these residues include Ile253, His310 and His435 ([19, 20, 21, 22, 23]). The IgG histidine residues regulate the pH dependent interaction, resulting in stronger binding at acidic pH relative to neutral pH. In addition, it has been shown that the interaction between hFcRn and IgG is highly stringent as hFcRn only shows binding to a limited number of species. In contrast, rodent FcRn does not show this stringency ([24]). It has been shown that residue Leu137 of hFcRn is responsible for this specificity ([25]).

Reports show that FcRn also binds HSA in a pH dependent manner ([26]). Studies in FcRn-deficient mice have shown that the half-life and the steady-state concentration of albumin were decreased relative to wild-type mice. The FcRn-mediated recycling has been shown to save as much albumin from degradation as the liver produces ([27]). It follows that FcRn diverts not only IgG, but albumin as well from a degradative fate by similar pH-dependent mechanisms, prolonging the lifespan of both molecules.

FcRn binds both HSA and IgG simultaneously ([28]). A recent report has shown that the conserved H166 residue in the α_2-domain of hFcRn heavy chain has a central role in facilitating the pH-dependent binding to HSA ([29]). The corresponding interacting residue on HSA is probably negatively charged and surface exposed on domain III ([28]). Significantly, H166 is conserved in eleven species investigated, and this strongly suggests that FcRn utilizes a common mechanism for maintaining albumin concentration at high levels in all these species ([29]). The IgG interacting residues on FcRn α_2-domain are E115, E116, D130, W131 and L135.

Recently, studies have shown that FcRn is also functionally expressed by monocytes, macrophages, DCs, PMNs and monocytic cell lines such as THP-1 cells ([30]) but not in B-lymphocyte, T-lymphocyte, and NK cell lineages ([30, 31]). The functional role in immune cells is far from fully understood, but recent data show that FcRn enhances phagocytosis in a pH-dependent manner. IgG-opsonized bacteria were inefficiently phagocytosed by neutrophils from β_2m and or FcRn heavy chain deficient mice, similarly, low phagocytic activity was observed with mutated IgG (H435A), which is incapable of binding to FcRn, while retaining normal binding to the classical leukocyte Fcγ receptor. In addition, FcRn translocation to phagosomes was shown to be facilitated through signalling motifs found within the FcRn cytoplasmic tail. These results point to a novel role for FcRn in phagocyte biology ([31]).

Even though the binding of IgG to FcRn in immune cells has been described, there is no report on HSA binding and its functional consequences.

The presence of polymerized albumin receptor on Hep-G2 cell has been reported, and purified hepatitis B virus attach to the cells via this receptor. Synthetic peptides analogous to part of its surface protein were used to study polymerized albumin-dependent attachment of the virus to Hep-G2 cells. Antibodies raised against the peptide were used to inhibit the hepatitis B virus attachment to HepG2 cells. Polymerized albumin has specific saturable receptor on HepG2 cells with two classes of binding sites ([40]).

Further studies suggest that polymeric human serum albumin may facilitate the attachment of the virus during the infectious process. Hepatocytes showed binding activity for both polymeric and monomeric albumin from different species. The receptor-ligand interaction was temperature and pH dependent ([42]). These findings suggest that human hepatocytes display species-non-specific albumin binding sites, which are glycoproteins ([42]).

FcRn has been shown to be functionally expressed on the surface of adult rat hepatocytes and hypothesized to mediate transport of IgG from serum to bile ([13])**.** However, other report shows that serum to bile transport of IgG was unaffected in mice functionally deleted for FcRn ([32]). Accordingly, the hypothesis is rejected that FcRn functions as sorting receptor in liver. In contrast, FcRn in hepatocytes has been implicated in protecting IgG from the catabolism that accompanies endocytic activity these cells ([32]). Eventhough the expression of FcRn is reported in rat hepatocytes no data from human studies exist. In addition, there are disparities about FcRn functions in hepatocytes as related to IgG and HSA homeostasis. Taken together, the expression of FcRn in immune cells and hepatocytes, and its functional importance in relation to IgG and HSA homeostasis, and IgG-mediated phagocytosis demand further investigations.

In the present study, we investigated the expression and functional binding of IgG and HSA to human immune cells (monocytic U-937, THP-1, K-562) and freshly isolated peripheral blood mononuclear cells (PBMC). We found that both ligands showed increased binding at acidic pH to membrane-bound FcRn, and the receptor-ligand interactions were blocked by excess amount of IgG, HSA, protein G or polyclonal anti-FcRn antibodies. This is the first report demonstrating HSA binding in a pH dependent fashion to a membrane-bound FcRn.

In addition, we report that FcRn is expressed in human hepatic cell lines (Hep3B and HepG2). We show that the receptor is functionally expressed as revealed by increased binding

to both IgG and HSA at acidic pH. Assessments of other Fcγ receptors show that FcγRII (CD32) is expressed but not FcγRI (CD64) and FcγRIII (CD16). We also demonstrate that treatment of anti-inflammatory substances clearly downregulate FcRn expression in both monocytic and hepatic cell lines.

ABBREVIATIONS

Ab	Antibody
AChR	Anti-acetylcholine receptor
BCA	Bicinchoninic acid
C-terminus	Carboxy terminus
DC	Dendritic cells
Epo	Erythropoietin
Fab	Fragment antigen binding
Fc	Fragment crystallible
FcRn	Neonatal Fc receptor
FITC	Fluorescein isothiocyanate
hFcRn	Human FcRn
hIgG	Human IgG
HLA	Human leukocyte antigen
HSA	Human serum albumin
HSV	Herpes simplex virus
Hep	Hepatic
Ig	Immunoglobulin
ITP	Immune thrombocytopenia
IVIG	Intravenous Ig therapy
MDCK	Madin-Darby canine kidney
mIgG	Mouse IgG
N-terminal	Amino terminal
rFcRn	Rat FcRn
shFcRn	Soluble human FcRn
SPR	Surface plasmon resonance
WT	Wild type
BSA	Bovine serum albumin
β_2m	β_2-microglobulin
CIP	Calf intestinal phosphatase
E.coli	Escherichia coli
FCS	Foetal calf serum
HRP	Horseradish peroxidase
hβ_2m	Human β_2-microglobulin
ON	Over night
OriP	Origin of replication
PCR	Polymerase chain reaction
RT-PCR	Reverse transcriptase PCR
PS	Penicillin
PBMCs	Peripheral blood mononuclear cells
RPM	Round per minute
RT	Room temperature
SDS-PAGE	Sodium dodecyl polyacrylamide
Skm	Skimmed milk
ELISA	Enzyme-linked immunosorbent assay
TM hFcRn	Transmembrane human FcRn
GAPDH	Glyceraldehyde phosphate dehydrogenase

3. Materials and methods

3.1 General methods

3.1.1Vectors

The eukaryotic vector pcDNA3 (Figure 1A) was originally from Invitrogen (Grand Island, NY). It was modified with a gene encoding glutathione S-transferase (GST) inserted into the polylinker on restriction sites XhoI and ApaI giving rise to pcDNA3-GST vector. As such it was a gift from F. E. Johansen (The National Hospital, Oslo, Norway). The vector was further modified with cDNA encoding extracellular part of human FcRn (hFcRn) heavy chain inserted into the polylinker at EcoRI and XhoI restriction sites and cDNA encoding human β_2-microglobulin (hβ_2m) in the restriction site NruI resulting in the pcDNA3-FcRn-GST.Pcmv-beta2m-polyA vector (Figure 1B; Berntzen et al, 2005). The vectors pEGFP-N1, pECFP-N1, pERFP-N1 and pEYFP-N1 (BD Biosciences Clontech, Qume Drive, San Jose, CA, USA) were gifts from Oddmund Bakke (IMBV, University of Oslo, Norway). A schematic representation of pEGFP-N1 is shown in figure 2.

A. **B.**

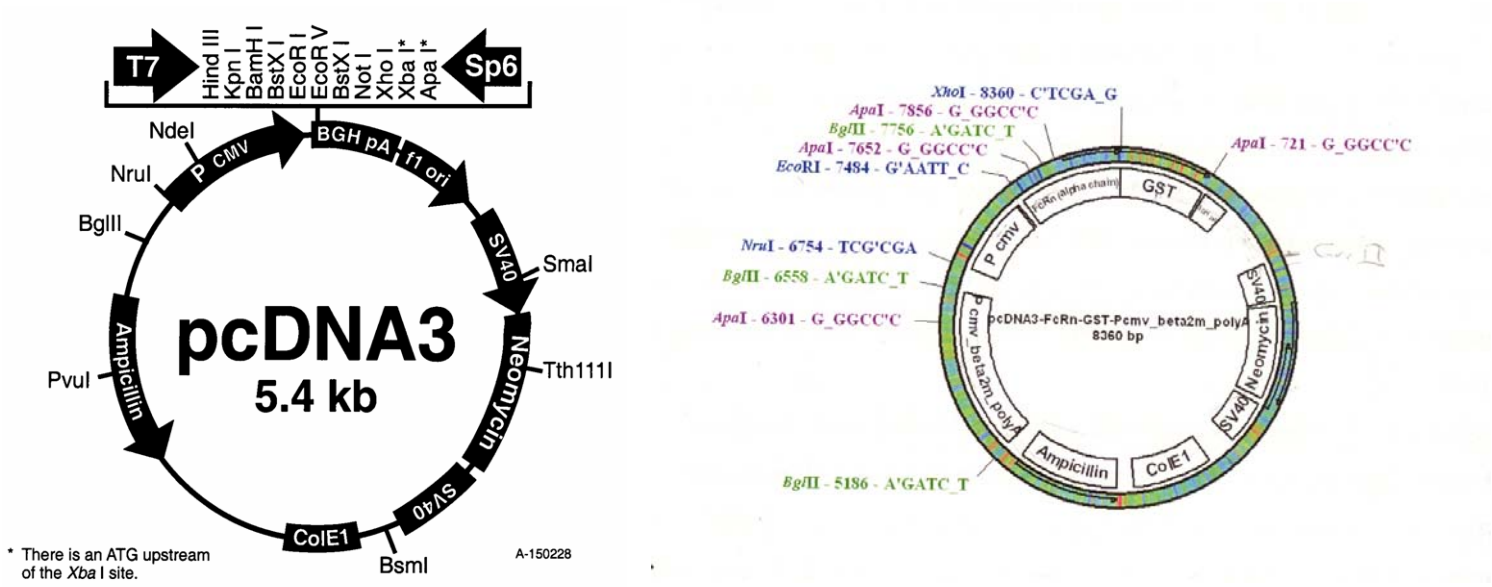


Figure 1. Plasmid maps

A. The original vector map for pcDNA3 as it appears in the vector catalogue available at: http://www.invitrogen.com. **B.** The map shows pcDNA3-FcRn-GST.Pcmv-beta2m-polyA with the sequence encoding the extracellular part of hFcRn heavy chain in the polylinker between the restriction sites EcoRI and XhoI, and hβ_2m in the NruI site. CMV: promoter for cytomegalovirus, BGH pA: polyadenylation signal from the bovine growth hormone gene, SV40ori: origin of replication from SV40 virus, SV40: the promoter from SV40 virus, ColE1: origin, Ampicillin: resistance gene for replication in bacteria, Neomycin: resistance gene for selection of stable expression in mammalian cells, T7: the T7 promoter, Sp6: the Sp6 promoter. The complete vector sequence for pcDNA3 is available at: http://www.invitrogen.com. The illustration of the pcDNA3-GST vector was performed using the programme pDRAW32 available at: http://www.acaclone.com. Restriction enzymes shown in blue are unique restriction sites.

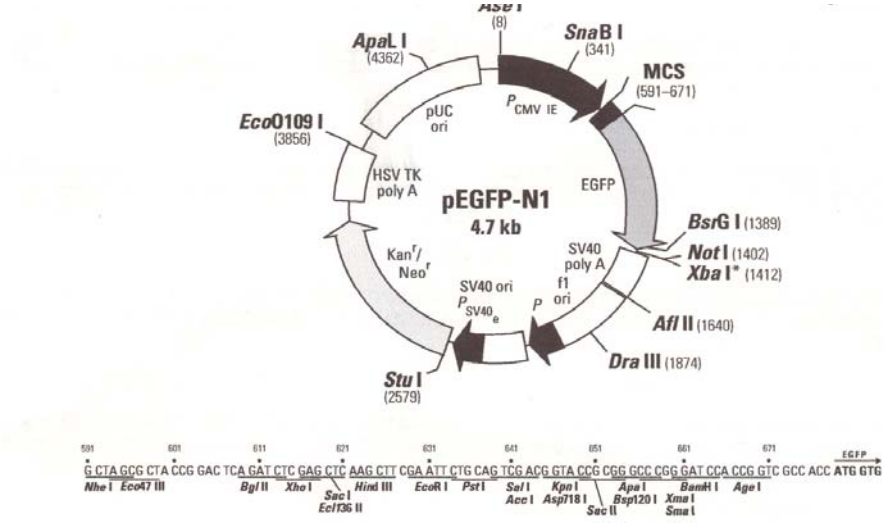


Figure 2. Plasmid map

The original vector map for pEGFP-N1 as it appears in the vector catalogue available at: www.bdbiosciences.com. CMV IE: Human cytomegalovirus immediate early promoter MCS: multiple cloning sites, EGFP: Enhanced green fluorescent protein gene, SV40 polyA: Early mRNA polyadenylation signal, f1 ori: single-strand DNA origin, P: SV40 early promoter, SV40 ori: SV40 origin of replication, Kan /Neo: Kanamycin/neomycin resistance gene, HSV TK: Herpes simplex virus tyymidine kinase polyadenylation signal, pUC: plasmid replication origin.

3.1.2 The Wizard™ Plus Minipreps DNA Purification System

The Wizard™ Plus Minipreps DNA Purification System from Promega (Madison WI, USA) was routinely used for small-scale purification of plasmid DNA. Plasmid DNA was purified using reagents and anion exchange resin columns supplied with the kit. The purification was done as described in the protocol. The minipreps were eluted in distilled H_2O (dH_2O), and their DNA concentration and purity were determined by spectrophotometric measurement ($A_{280/260nm}$) on a GeneQuant spectrophotometer (Amersham Pharmacia Biotech, UK). The integrity of plasmid constructs were confirmed by restriction analysis, agarose gel electrophoresis and verified by sequencing by GATC (Gmbh, Konstanz, Germany).

3.1.3 The Wizard™ Plus Midipreps DNA Purification System

Prior to transfection of the plasmid constructs, the plasmids were isolated using the The Wizard™Plus Midipreps DNA Purification System (Promega). Plasmid DNA was purified essentially as described in the kit protocol.

3.1.4 DNA precipitation

The *see*DNA™ co-precipitant for RNA and DNA kit (Amersham Pharmacia Biotech) was used for nucleic acid precipitation of restriction digests and ligation reactions prior to transformation. The precipitation was done as described in the protocol, using the reagents supplied in the kit, except from ethanol. Briefly, 2 µl of uniform *see*DNA suspension and 0.1 volumes of 3 M sodium acetate, pH 5.2, were added to the DNA and mixed briefly. Then 2 volumes of 95% ethanol was added, vortexed 5-10 sec. and incubated at room temperature (RT) for 2 min. The sample was centrifuged at 14000 xg for 5 min, supernatant (SN) was removed and a pink pellet was observed. The pellet was rinsed in 500 µl 70% (v/v) ethanol, followed by a brief vortex, and centrifuged as

above. The pink pellet was resuspended in dH_2O.

3.1.5 Restriction enzyme digestion of DNA

Restriction enzyme digestion of DNA was performed according to the specific conditions required for the individual restriction enzymes (e.g. buffer, BSA and temperature). The reactions were incubated for at least two hours at the recommended temperature. All restriction enzymes and buffers were from New England BioLabs (NEB, Beverly, MA).

3.1.6 Bacterial transformation

3.1.6.1 Transformation by electroporation

Bacterial transformations were performed with electrocompetent XL1-blue *Escherichia Coli* (*E. coli*) cells (Stratagene, La jolla, CA) *E. coli* cells (Stratagene), essentially as described in Molecular Cloning (Sambrook *et al*., 2001) and BIO-Rad Genepulser™ electroporation manual. Briefly, 50 µl cells were thawed on ice. A portion of 2 µl SeeDNA precipitated (1.1.4) ligation mixture was added, and the cells were incubated on ice. The transformation mixture was then pulsed in an ice-cold 0.1cm gap electroporation BTX Disposable Cuvette P/N (BTX Inc., Hawthorne, NY). The Gene-pulser™ apparatus (BTX Inc.) was used with the following settings: 25 µF, 200 Ω and 1.30 kV. After electroporation, 1 ml of 2 xYT medium was added to the cuvette and transferred to an Eppendorf tube (Eppendorf AG, Hamburg, Germany) for incubation at 37°C for 1 hour (h). The cells were plated on agar plates containing 100 mg/ml ampicillin and incubated over night (ON) at 37°C. The resulting colonies were screened.

3.1.6.2 Transformation of $CaCl_2$ competent $Top10^F$ E.coli cells

The $CaCl_2$ competent $Top10^F$ *E.coli* cells were thawed on ice. 300 µl cells were added to each tube with *See*DNA precipitated (1.1.4) ligation mixture and incubated on ice for 45 min. The cells were then exposed to heat-shock by incubating at 42ºC for 2 min. 1 ml 1x LB medium was added to the cells and further incubated at 37ºC for 60 min. The cells, then centrifuged at 4000 rpm for 4 min at RT. The supernatant was discarded and the pellet was resuspended in the remaining medium and plated on agar plates containing 100 mg/ml ampicillin and incubated over night (ON) at 37°C. The resulting colonies were screened.

3.1.7 SDS-PAGE

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to general protocol. Briefly, gel apparatus was assembled and the Criterion XT™ Precast Gel (4-12% Bis-Tris or 4-15% Tris-HCl; Bio-Rad Laboratories, Alfred Nobel Drive, Hercules, CA) was placed in the tank that was filled with Running buffer (144 g/l glycine, 36.3 g/l and 10% SDS or XT MOPS running buffer, 1x; PIERCE). The comb was removed and the wells in the stacking gel were rinsed with running buffer. Protein samples and molecular weight standard were mixed with loading buffer (Tris-HCl, SDS, DTT, glycerol and bromophenol blue) and applied to the wells and electrophoresed for 1 h and 40 min at 100v.

3.1.8 Agarose gel electrophoresis

Depending on the size of the DNA fragments to be separated, 1-2 % agarose gels were prepared. Analytic as well as preparative gels containing agarose (Sigma, St. Louis, MO) were dissolved in 50 µl 1x TAE buffer (40 mM Tris-acetate, 1 mM EDTA) and 1 µg/ml ethidium bromide. 6 x SB was added to each sample before electrophoresis. Gel

electrophoresis was carried out in 1x TAE buffer at 90-100 V/250 mA. DNA was visualized by exposure to UV-light and compared with lamda HindIII or phix174 size markers (New England Bio Labs).

3.1.9 Purification of PCR products

Products from the polymerase chain reactions (PCR) were purified using the QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany), which is based on adsorption of DNA to silica particles in the presence of high salt concentration. The purification was performed according to the QIAGEN manual with using the reagents supplied with the kit.

3.1.10 Extraction of DNA from agarose gel

DNA fragments were separated by agarose gel electrophoresis and purified using the QIAquick Gel Extraction Kit (QIAGEN), which is based on solubilization of agarose and selective adsorption of DNA to silica particles in the presence of high salt concentration. The purification was performed according to the QIAGEN manual using the reagents supplied with the kit.

3.1.11 Modification of DNA ends

Calf intestinal phosphatase (CIP) (Finnzymes, Espoo, Finland) was used to remove phosphate residues from the 5´ protruding ends of the linearized vector in order to reduce the background due to religation of the vector. 0.5 units CIP/pmol DNA ends were added to restriction enzyme digestion mixture, which include a specific CIP buffer (Finnzymes), for at least 1 h. The reaction was inactivated by heating to 75°C for 10 min in the presence of the 5 mM EDTA (pH 8.0).

3.1.12 Ligation of DNA fragments

Appropriate amounts of insert and vector fragments were added to 2 µl of 10x ligation buffer (Roche Diagnostics, GmbH, Mannheim, Germany) and 1 µl of T4 Ligase (Roche Diagnostics). The ligation reaction volume was adjusted to 20 µl with dH_2O. The ligation reaction was allowed to occur ON at RT or at 20°C, before heat inactivation at 65°C for 20 min. All ligations were performed as described above.

3.2 Cells

The adherent human hepatocarcinoma cell lines Hep-3B and Hep-G2 (Gift from Trond Berg; IMBV, University of Oslo. Norway) were maintained in Dulbecco modified eagle medium (DMEM) (BioWhittaker, Belgium), supplemented with 4 mM L-glutamine, 100 U/ml penicillin (PS; Integro, The Netherlands), 0.1 mM nonessential aminoacids (NEAA), and 10 % foetal calf serum (FCS; Integro). The human monocytic cell lines U937, THP-1 and K-562 (American Type Cultur Collection, Manassas, VA, USA) were maintained in RPMI 1640 (BioWhittaker), supplemented with 100 U/ml PS and 10 % FCS. All reagents were from Gibco (BRL, Paisley, Scotland). Madin-Darby Canine Kidney (MDCK) cells stably transfected with $h\beta_2m$ was established by Julie Dee Qian (IMBV, University of Oslo, Norway) and maintained in Dulbecco modified eagle medium (DMEM), supplemented with 4 mM L-glutamine, 100 U/ml penicillin, and 10 % FCS. Hybridoma cell line expressing anti-FcRn mouse monoclonal antibody 1G3 was obtained from ATCC (American Type Cultur Collection, Manassas, VA, USA) and cultured according to the supplier's protocol. Briefly, cells were plated at a seeding density of 120,000 cells/cm^2 in a 75-cm^2 flask, and cultures were maintained in a humidified atmosphere (5% CO2-95% air) at 37ºC with HL-1 medium (BioWhittaker, Walkersville, MD, USA) supplemented with 4 mM L-glutamine (Invitrogen), 1 mM sodium pyruvate (Invitrogen), and 1% FCS (Invitrogen), and the cells were further cultured to confluency. The supernatant was harvested and IgG was purified (1.12)

3.3 Cell lysate preparation

Total cell lysate was made by using CelLytic M cell lysis Reagent (Sigma, Saint Louis, USA) together with protease inhibitor cocktail (Sigma) as described in the general protocol. Briefly, approximately 8-10 x 10^6 cells were washed by centrifugation at 1200 rpm for 5 min. Pelleted cells were resuspended in 700 µl CelLytic M reagent with 30 µl protease inhibitor cocktail and incubated on a shaker for 15 min. The lysed cells were centrifuged for 15 min at 4000 rpm to remove cellular debris. Then, supernatant collected was stored at -70ºC until use.

3.4 BCA Protein Assay (Quantification of total protein in cell lysates)

The total protein in lysate (1.3) was quantified using the BCA™ protein assay Kit (PIERCE, Meridian Road Rockford, USA) according to the manufacturer's protocol. Briefly, BCA™ Working Reagent was prepared by mixing Reagent A with Reagent B in a 50:1 ratio, and 25 µl duplicates of diluted lysates were incubated with 200 µl Working Reagent at 37 ºC for 30 min. After cooling the plate to room temperature (RT), the absorbance was measured at 570 nm on a VICTOR™ 1420 Multilabel counter (PerkinElmer Life Sciences, Welleslay, MA, USA). Standard curves were prepared from a series of dilutions of bovine serum albumin (BSA) ranging from 20-2000 µg/ml.

3.5 Total RNA isolation

The total RNA was harvested using the method described by Absolutely RNA Miniprep Kit (Stratagene). Briefly, 600 µl lysis buffer was added to 5-6 x 10^6 pelleted cells, mixed and vortexed. The lysate was filtered by passing through filter spin cup that were seated in receptacle tube. The filtrate retained was mixed with equal volume of 70% ethanol, and 700 µl of the mixture transferred to RNA-binding spin cups seated in receptacle tubes. The spin cups retained and washed with low salt wash buffer and treated with 5 µl of Rnase-free DNaseI mixed with 50 µl DNase digestion buffer by incubating

at 37ºC for 15 min. The spin cups were washed with high and low salt wash buffer in succession. The RNA eluted using elution buffer. The extracted total RNA was quantified by ND-1000 spectrophotometer at 260 nm (NanoDrop Technologies, Wilmington, DE, USA).

3.6 cDNA synthesis

For cDNA synthesis 2 µg of total RNA was subjected to reverse transcription (RT) reaction using Superscript II reverse transcriptase (Invitrogen), and anchored Oligo (dT)20 primer (Invitogen). The samples were denatured at 95ºC for 5 min in order to remove secondary structure of RNA prior to addition of RTase (Invitrogen), followed by further by incubations at 25ºC for 10 min, 42ºC for 60 min, 70ºC for 5 min and finally 4ºC for 2 min. The yielded first-stranded cDNA was stored at -70ºC until use.

3.7 Polymerase chain reaction (PCR) for amplication of hFcRn transcripts

Aliquots of cDNA were subjected to PCR reaction using primers (Table 1) specific to the transmembrane hFcRn (TM-hFcRn; FcRnforwnew and FcRnrevnew) and hβ_2m ($\beta_2$mforw2 and $\beta_2$mrev). These were designed based on published sequences of TM-hFcRn (1098 bp, GeneBank, NM 004107) and hβ_2m (360 bp, GeneBank, NM 004048). All primers were from Medprobe (Eurogenetic, San Diego, USA). The melting temperatures were estimated using the formula Tm= 2(A+T) + 4(G+C). Each PCR reaction was performed in a final volume of 50 µl with 0.5 µl of high fidelity Phusion DNA polymerase (Finnzymes, Finland) 0.02 µM of each primer using Thermal cycler (Eppendorf Mastercycler® Gradient). PCR reaction procedures commenced with a denaturation at 95ºC for 5 min followed by amplification with a total of 35 cycles at 95ºC for 2 min, 68ºC for 2 min and 72ºC 10 min. The samples from each PCR products were electrophoresed on 2% agarose gel and were examined with respect to phix174 DNA ladder (1.1.7). Negative controls were samples reaction omitting either or both cDNA and

primers were also prepared. The housekeeping gene for glyceraldehyde-3- phosphate dehydrogenase (GAPDH) was amplified as described (M. Sporstol, 2007).

3.8 Cloning of sequence encoding TM-hFcRn and hβ_2m

The amplified TM-hFcRn cDNA was digested (1.1.5) with XhoI (NEB) and EcoRI (NEB), purified (1.1.10) and cloned as a 1098 bp fragment into the polylinker of pcDNA3-FcRn-GST.Pcmv-beta2m-polyA. This gave rise to pcDNA3-(TM-hFcRn)-GST.Pcmv-beta2m-polyA vector. The PCR product for hβ_2m was digested (1.1.5) with restriction enzymes HindIII and XbaI, purified (1.1.10) and cloned as a 360 bp fragment into pcDNA3-GST on HindIII (NEB) and XbaI (NEB) sites. This gave rise to the pcDNA3-hβ_2m vector.

3.9 Subcloning of the sequence encoding TM-hFcRn into fluorescent vectors

The vector pcDNA3-(TM-hFcRn)-GST.Pcmv-beta2m-polyA (1.8) was used as a template for amplication by PCR of TM-hFcRn cDNA (1.7) with primers GFPforw and NewFPVrev (Table 2). The PCR product was digested using XhoI and EcoRI (1.1.5) and subcloned in N-terminal fusion of the genes of the fluorescent proteins in pEGFP-N1, pECFP-N1, pERFP-N1 and pEYFP-N1). This gave rise to pTM-hFcRn-EGFP-N1, pTM-hFcRn-ECFP-N1, pTM-hFcRn-ERPP-N1 and pTM-hFcRn-EYFP-N1 vectors.

Table 1. Primers used for amplication of TM-hFcRn and hβ_2m:

Primer	Sequence
FcRnforwnew:	5’ ATT**GAATTC**[a]ATGGGGGTCCCGCGGCCTCAG 3’
FcRnrevnew:	5’ ATT**CTCGAG**TCA[b]GGCGGTGGCTGGAATCACATTTAC 3’
$\beta_2$mforw2:	5′-TCC **AAG CTT**GGC GGG-3′
$\beta_2$mrev:	5′-GTT **CTT GAA**CCT CCA-3′

a: The restriction sites are bold.

b: Stop codon is underlined

Table 2. Primers used for construction of the pFcRn-EGFP-N1, pFcRn-ECFP-N1, pFcRn-ERFP-N1 and pFcRn-EYFP-N1 vectors:

Primer	Sequence
GFPforw:	5’ ATT**CTCGAG**[a]ATGGGGGTCCCGCG 3’
NewFPVrev:	5’ATT**GAATTC**CGGCGGTGGCGTGGAATCACATTTAC 3’

a: The restriction sites are bold

3.10 Transient transfection of MDCK cells expressing hβ$_2$m with fluorescent protein vectors

All constructs were transiently transfected using the LIPO-FECTAMINE™ method (Invitrogen). The day before transfection, the cells were trypsinized (BioWhittaker) and counted. 4x 10^5 MDCK cells per ml were plated in a 12 wells plate with microscope cover slip placed in the center. The day after, the cells were 70-80% confluent. A portion of 10 μg DNA was added to 500 μl medium without serum (OPTI-MEM) for each bottle to be transfected. 20 μl LF2000 Reagent (Invitrogen) was diluted into 500 μl medium without serum and incubated for 5 min at RT. Diluted LF2000 was combined with the diluted DNA and incubated at RT for 20 min to allow DNA-LF2000 to form. The complexes were added directly to each 12 wells plate and mixed with the cells before incubation at 37°C in a CO_2 incubator. After 24 h cells washed, fixed, Hoechst (Sigma) stained (for nuclear staining) and mounted on microscope slides for examination with Confocal microscope.

3.11 Anti-hFcRn production

Recombinant soluble human FcRn (shFcRn) or the hFcRn heavy chain only (Andersen *et al,* 2007, document submitted) was used for goat immunization. The immunization and antisera collection were made in collaboration with T. E. Michaelsen (Norwegian Public Health Institute, Norway). Briefly, goats were immunized subcutaneously with 50 μg purified shFcRn or only the hFcRn heavy chain in 500 μl PBS pH 7.3 mixed with 750 μl Freund Complete Adjuvant (Difco, Detroit USA). On day 11 after immunization a booster dose (50 μg) was given with Freund Incomplete Adjuvant (Difco, Detroit, USA), which was repeated 24 days later. After additional four weeks, the goats were bled regularly every second week. Two additional booster doses (50 μg) with Freund Incomplete Adjuvant were given six and fourteen weeks after the third immunization. The collected and pooled anti-FcRn goat sera were filtered prior to

purification.

3.12 Anti-hFcRn purification

Purification of polyclonal anti-hFcRn antibody from goat sera or mouse hybridoma cell culture supernatant (mAb 1G3) was performed using HiTrap protein G HP, 5 ml column (GE Healthcare Bio-Sciences AB Uppsala, Sweden) or a hFcRn heavy chain coupled affinity matrix (Andersen et al, unpublished). The protein G purification was performed as described in the manual. The column was connected to AKTA™ chromatography system (Bio-Rad, BioLogic LP). The column was cleaned with elution buffer (0.1 M glycine-Hcl pH 2.7) and then equilibrated by washing with 10 column volumes of binding buffer (20 mM sodium phosphate pH 7.0). Equal volume of filtered (0.45 μm; Numbrecht, Germany) sample and binding buffer were mixed and loaded to the system. The flow through was collected. The column was washed with 5-10 column volume of binding buffer or until no material appears in the effluent. The wash fractions were collected. The sample was eluted with 2 to 5 column volumes of elution buffer (0.1 M glycine-HCl) in collection tubes containing neutralizing buffer (1M Tris-Hcl, pH 9.0). The column was re-equilibrated with binding buffer.

The purity of the eluates was analysed on SDS-PAGE (1.1.7) and upconcentrated using Amicon Centricon Plus-20 (YM-100) Centrifugal Filter Devices (Millipore Corporation Bedford, MA, USA) for concentration and purification. The final concentration was estimated using ND-1000 spectrophotometer at 280 nm (NanoDrop Technologies, Wilmington, DE, USA). Antibodies (~3 mg/ml) were biotinylated using 1 mg/ml biotin N-hydroxysuccinimide ester (Sigma), and incubated on rotator for 4 h at RT followed by 4°C ON. The mixture was then spun through a centricon YM-10 (Millipore) five times to remove un-conjugated NHS-biotin (J.T. Andersen, IMBV, UIO, Norway).

3.13 Anti-hFcRn analysis by ELISA

The 96-Wells plate (Nunc, Denmark) was coated with 2 μg/ml of shFcRn (Andersen

et al, 2007 submitted) or 2 µg/ml of hβ$_2$m (Abcam, Cambridge, UK) and stored at 4º ON. As a blocking solution, 2% dry skim milk (Accumedia Manufacturers Inc.Lansing, MI, USA) /1x PBS (Skm/PBS) was added to the coated wells at RT for 1h. The purified polyclonal anti-hFcRn (1.12) diluted (1:100, 1:1000, 1:10000) in 2% Skm/ PBS was added to the wells and incubated at RT for 1 h. The wells were washed four times with 1x PBS supplemented with Tween 20 (PBST). Wells were then incubated for 1 h at RT with a horseradish peroxidase (HRP) conjugated protein G (1:3000; Calbiochem, San Diego, CA, USA). Wells were washed four times with PBST before 100 µl of the substrate 2,2'-azino-bis 3-etylbenzthiazoline-6-sulfonic acid (ABTS; Sigma) in 2 mM citric acid, pH 4, diammonium salt (Sigma) containng 0,2% H_2O_2 was added (ABTS/ H_2O_2). The colour reaction was measured by absorbance at 405 nm on a Sunrise TECAN spectrophotometer (TECAN Maennedorf, Switzerland). The schematic representation is shown in figure 3.

3.14 Detection of intact hFcRn in cell lysate by ELISA

The 96-Wells plate (Amersham Pharmacia Biotech) was coated with 50-100 µg/ml described in (1.13).

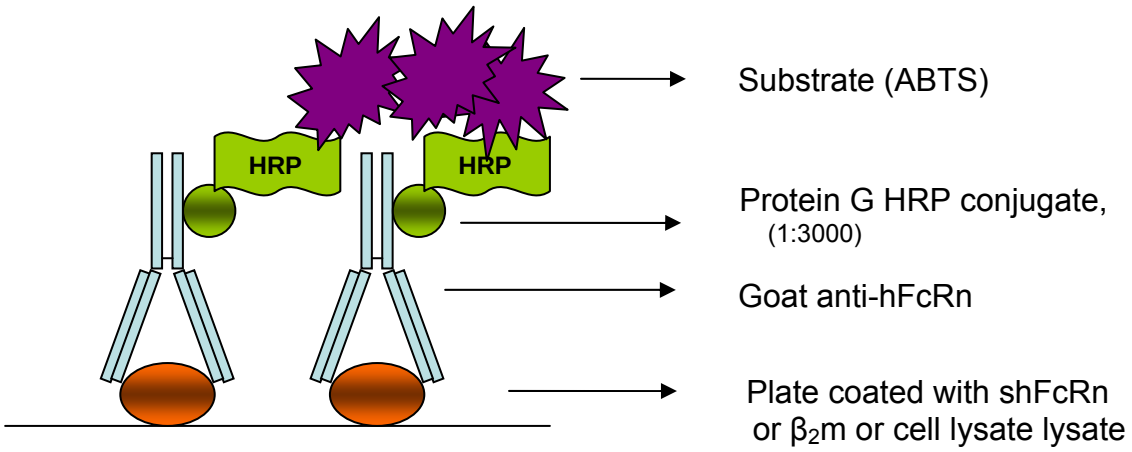


Figure 3. Detection of hFcRn binding by anti-hFcRn. Wells were coated with shFcRn, hβ$_2$m or total protein in lysate at 4°C ON, then washed and incubated with anti-hFcRn antibody. Detection was using a HRP- conjugated protein G before substrate was added.

3.15 Western immunoblotting dectetion of FcRn expression

Normalized cell lysate (1.4) with 40-50 µg total protein was reduced in 2 µl of dithiohreitol (DTT; Sigma) electrophoresed through a 12% SDS-PAGE (1.1.7). Proteins were blotted from the gels onto a Polyvinylidene fluoride (PVDF) membrane (Millipore Corporation Bedford, USA) in Tris/glycine buffer (25 mM Tris and 192 mM glycine, 20% methanol, pH 8.3) at 25V for 35 min. The membrane was blocked in 4% Skm /PBS for 1 h at RT. After blocking, membrane was washed in Skm/PBS three times for 5 min on shaking and probed with polyclonal goat anti-hFcRn (1.12) diluted 1:1000 in 4% Skm/PBS, and anti-hβ_2m from rabbit (1:5000; Sigma) at RT for 1 h. Then, membrane was washed five times for 5 min with PBST. The HRP-conjugated mouse monoclonal antibody (1:5000; Sigma) or the HRP-conjugated anti-rabbit antibody diluted 1:5000 (from donkey; Amersham Pharmacia Biotech) were added and incubated at RT for 1 h. The membrane was washed five times for 5 min before the membrane was treated with SuperSignal West pico Lumino/Enhancer and stable peroxidase solutions (PIERCE). The treated membrane was detected on Kodak film using Optimax x-ray film processor (Protec medizintechnik GmbH co. KG, Germany).

3.16 pH dependent IgG-FcRn binding assay on lysates

100 µl hIgG coupled sepharose (Amersham Biosciences) was placed in eppendorf tubes and calibrated with 1ml sodium/potassium phosphate buffer with pH 5.5 (buffer 5.5) or pH 7.4 (buffer 7.4) on rotation for 10 min, centrifuged at 10, 000 rpm for 5 min and repeated three times. 400-600 µg of total protein in lysate (1.3) and 1ml buffer with pH 5.5 or pH 7.4 was mixed and pH was checked with pH-indicator strips (Merck KGaA, Darmstadt, Germany). Lysates were then added to the sepharose-hIgG and incubated in buffer with 5.5 or 7.4 ON at 4ºC. The mixtures were washed three times for 10min on rotation with centrifugation in between in one ml buffer with pH 5.5 or pH 7.4. The bound proteins were eluted with sodium/potassium phosphate with pH 8. Western immunoblotting analysis was made on eluted fractions as described (1.15).

3.17 Isolation of peripheral blood mononuclear cells (PBMC)

Blood was collected in tubes with heparin from a volunteer. Peripheral blood mononuclear cells (PBMC) were isolated by means of a density gradient centrifugation technique in which Lymphoprep™ (NYCOMED PHARMA AS, Oslo Norway) was used as a gradient medium. Briefly, 10 ml of 1x PBS pipetted to each of the centrifuge tube containing 10 ml blood. 5 ml of blood/PBS poured down to 5 ml Lymphoprep placed at the bottom of the tube and centrifuged at 2000 rpm for 20 min at 20ºC without brake.
The PBMC located in the interphase collected using a Pasteur pipette and transferred to centrifuge tube. Equal volume of PBS was added and centrifuged at 1700 rpm for 5 min at 4ºC with brake three times. Then, the cells were counted using a hemocytometer.

3.18 Assessment of expression of FcγRI, FcγRII and FcγRIII by Flow cymetry

For each staining, 2 x 10^6 cells were washed three times with 1x PBS, and added to 96-wells plates (Nunc). Cells were blocked with 2% bovine serum albumin (BSA; Sigma) in PBS (BSA/PBS) for 1h, washed by centrifugation at 800 rpm three times for 5 min with PBS/BSA. The cells were stained with Fluorescein Isothiocyanate (FITC) conjugated mouse anti-human CD64 antibody (Serotec Ltd, Oxford, UK), anti-human CD32 antibody (BD Pharmingen, Franklin lakes, NJ, USA) and detected by goat anti-mouse FITC (CALTAG Laboratories, Burlingame, California, USA), and with biotinylated mouse anti-CD16 monoclonal antibody (Abcam) followed by streptavidin FITC (DakoCytomation Ltd., cambridgeshire, UK). Finally, the stained cells were fixed by treating with 3 % paraformaldeyde (PFA; Fluka) for 30 min.

3.19 Analysis of surface and intracellular expression of FcRn by Flow cytometry

For each staining, approximately 2 x 10^6 cells were washed three times with 1x PBS, and added to 96-wells plates (Nunc). Cells were blocked with 2% BSA/PBS for 1 h, washed by centrifugation at 800 rpm three times for 5 min with PBS/BSA. Cells were

incubated for 1 h with biotinylated polyclonal anti-hFcRn, isotype control goat IgG (Gift from T. M. Michalesen) diluted 1:100 in 2% BSA/PBS or the 1G3 monoclonal anti-FcRn (1.12) diluted 1:50 in 2% BSA/PBS. The cells were washed and centrifugated at 800 rpm three times for 5min with 2% PBS/BSA. Cells incubated for 1 h with streptavidin-FITC or anti-mouse IgG $F(ab')_2$ FITC produced in goat (Sigma) diluted 1:200 in 2% BSA/PBS, washed by centrifugation at 800 rpm three times for 5 min with 2% PBS/BSA. All staining was conducted at 4ºC and also were fixed with 3% PFA for 30 min. For intracellular staining, the cells were permeabilized by treatment with 0.5% saponin (Sigma) for 30 min and stained as above.

3.20. Surface plasmon resonance analyses

Surface plasmon resonance experiments were carried out using a Biacore 3000 (Biacore AB, Uppsala, Sweden). Flow cells of CM5 sensor chips were coupled with shFcRn (~800 RU) using amine coupling chemistry all as described in the protocol provided by the manufacturer. The coupling was performed by injecting 10 μg/ml of the protein in 10 mM sodium acetate, pH 5.0 (Biacore AB). For all binding experiments the phosphate buffer (67 mM phosphate buffer, 0.15 M NaCl, 0.005 % Tween20) at pH 5.5 was used as running buffer as well as dilution buffer. Injection of 15 μg/ml FITC conjugated human IgG (hIgG) (Sigma) or 268 μg/ml FITC-conjugated HSA (Sigma) was performed at 10 μl/min at 25°C.

3.21 Analysis of pH dependent FcRn-IgG/HSA binding by Flow cytometry.

For each staining, 2 x 10^6 cells were washed with 1x PBS three times and added to 96-wells plates (Nunc) and fixed by treating with 3% PFA for 30 min. The cells were washed by centrifugation at 800 rpm with buffer pH 5.5 or 7.4. Then, the cells were incubated for 1 h with blocking solution (0.5% Skm milk in buffer pH 5.5 or 7.4) and pH was checked using pH-indicator strips (Merck). The cells washed further with

corresponding buffers and then incubated with hIgG-FITC (10-100 µg/ml; Sigma) or HSA-FITC (15-100 µg/ml; Sigma), and mF(ab')2 fragment-FITC (Sigma) or mIgG2a-FITC (Sigma) were used as controls for 1 h. Double staining was carried out using hIgG-FITC (10-100 µg/ml) and HSA-FITC (15-100 µg/ml) at the same time. Inhibition analysis with polyclonal anti-hFcRn (1.12), unconjugated hIgG (Aventis Behring GmbH, Marburg, Germany), HSA (Sigma) or protein G from Streptococcus species (Fluka, Buchs, Switzerland) was performed. The cells were then washed and analysed with Flow cytometry using buffer pH 5.5 or 7.4.

3.22 Flow cytometric analysis of the effect of anti-inflammatory drugs on FcRn expression

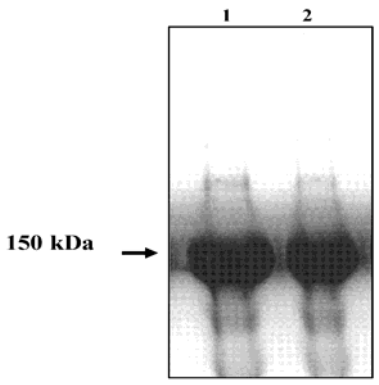
Approximately, 6 x 10^5-7 x 10^5 cells were treated with 10 nM-1000 nM of corticosterone, prednisolone, beclomethasone dipropionate, and thyroxine (all from Sigma) in corresponding growth media. After 3 days, 2 x 10^6 cells were stained as described (1.19) and analysed by Flow cytometry.

4. RESULTS

4.1 Generation and functional testing of hFcRn preparations

To produce an hFcRn specific antiserum, goats were selected for immunization due to previous studies that clearly demonstrate highly stringent binding specificity of hFcRn with no detectable binding to goat/sheep IgG Fc ([24]). In addition, goat IgG subclasses have low or no binding affinity for other human Fcγ-receptors. Immunizations were made with intact shFcRn and isolated hFcRn heavy chains. Goat antisera were produced and purified as described in *materials and methods*. Protein G purified preparations were analysed by non-reducing SDS-PAGE (Fig. 1A). As expected, the goat IgG migrated as bands corresponding to ~150 kDa. To test the functional integrity of purified anti-hFcRn preparations, an ELISA was performed with coating of shFcRn followed by serial dilutions of the preparations. Figure 1 shows that antibodies from goat immunized with intact shFcRn reacted towards both intact shFcRn (G2448; Fig. 1B) and the hβ_2m subunit (G2448; Fig. 1C). In contrast, those with the hFcRn heavy chain only reacted with intact shFcRn (G3231; Fig.1D) but not hβ_2m (G3231; Fig.1E). The antibody preparations were further purified on FcRn heavy chain coupled affinity matrix.

A.



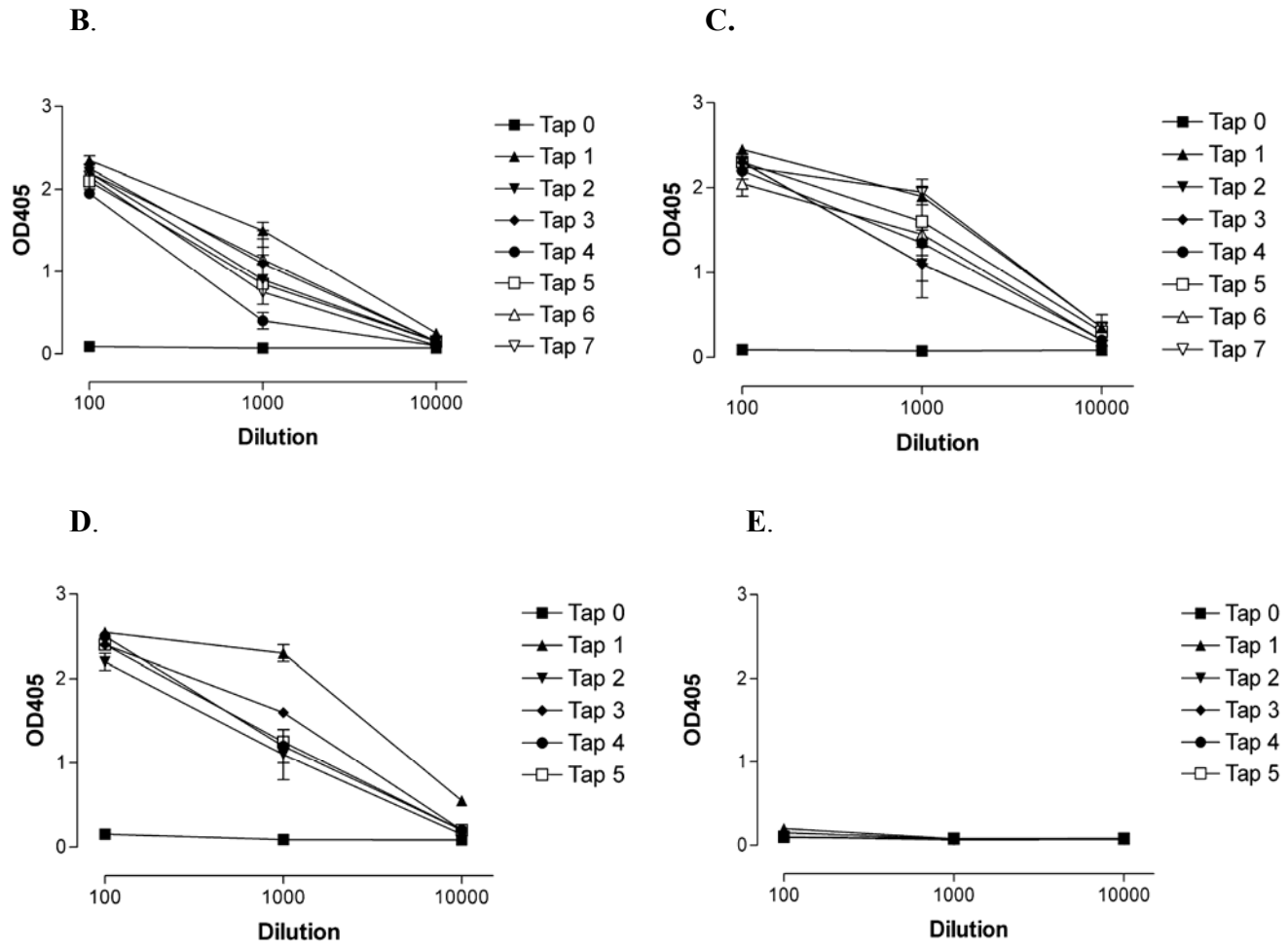


Figure 1. Analyses of anti-FcRn antibody preparations. (A) Non-reducing 12% SDS-PAGE analysis of antibody fractions eluted from the protein G column. Lane 1 corresponds to G2248. Lane 2 corresponds to G3231. The molecular size is indicated by an arrow. ELISA on antibody preparations from goat immunized with intact shFcRn tested against shFcRn (B) and hβ2m (C). ELISA on antibody preparations from goat immunized with only the hFcRn heavy chain tested against shFcRn (D) and hβ2m (E). Tap 0 samples are taken from pre-immunized goats. Tap 1-7 samples are taken post immunizations. Each sample was diluted 1:100, 1:1000 and 1:10000.

4.2 Expression of FcRn and Fcγ receptors on human monocytic cell lines

To determine the expression profile of FcRn in human immune cells, we examined three human monocytic cell lines (U-937, THP-1, and K-562). Flow cytometric analyses were performed using the purified polyclonal anti-FcRn preparation (G3231). The histograms in figure 2 reveal the expression of hFcRn on the cell surface of all three cell lines (Fig. 2A, C and E). In addition, the total expression was determined after saponin treatment (Fig. 2B, D and F). The FcRn expression was also confirmed using a commercial available anti-FcRn monoclonal (1G3) antibody (data not shown). These results are in agreement with previous reports showing FcRn expression in monocytic U-937 and THP-1 cell lines ([30, 31]). Our data for the first timeshow FcRn expression on the K-562 cell line.

To explore functional binding of IgG to surface expressed hFcRn, the expression profiles of classical Fcγ receptors have to be assessed. The monocytic cell lines were stained with specific antibodies and analyses by flow cytometry. The data obtained show expression of FcγRI (Fig. 3A, D and G) and FcγRII (Fig. 3B, E and H), but no evidence for FcγRIII expression (Figure 3C, F and I).

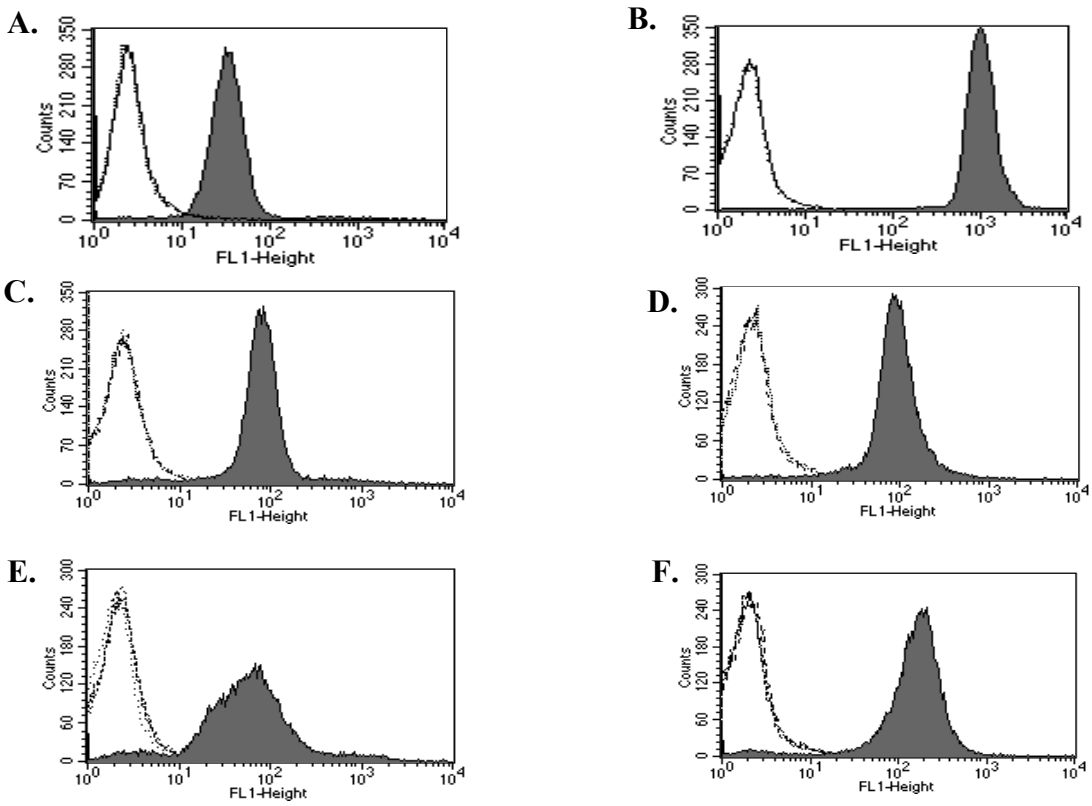


Figure 2. Expression and cellular distribution of FcRn in human monocytic cell lines. Cells were stained with biotinylated anti-FcRn followed by streptavdin-FITC and analysed by flow cytometry. U-937 cells surface stained (A) and total (B). THP-1 cells surface stained (C) and total (D). K-562 cells surface stained (E) and total (F). FcRn expression is shown as gray filled histograms, streptavidin-FITC and isotype (biotinylated goat IgG) controls as unfilled histograms. Histograms are given with ordinate indicating number of cells and abscissa indicating the fluorescence intensity.

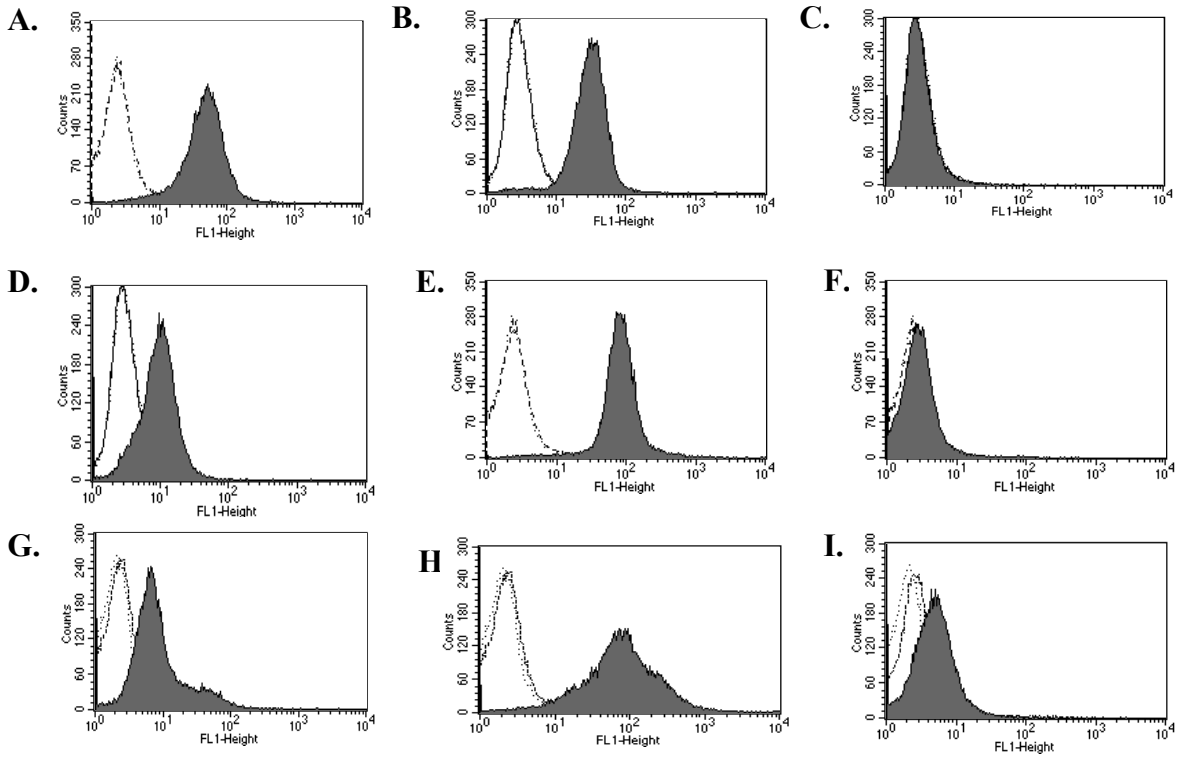


Figure 3. Expression of Fcγ receptors. Cells were stained with monoclonal antibodies towards each Fcγ receptor as described in *material and methods*. U-937 cells surface stained for FcγRI, FcγRII and FcγRIII (A, B and C). THP-1 cells surface stained for FcγRI, FcγRII and FcγRIII (E, F and G). K-562 cells surface stained for FcγRI, FcγRII and FcγRIII (G, H and I). Fcγ receptors are shown as gray filled histograms and controls (isotype and secondary antibodies) as unfilled histograms. Histograms are given with ordinate indicating number of cells and abscissa indicating the fluorescence intensity.

4.3 pH dependent binding of IgG and HSA to human monocytic cell lines

Initially, the functional integrity of FITC conjugated hIgG and HSA were tested by surface plasmon resonance. Recombinant shFcRn was immobilized on CM5 sensor chips by amine coupling. Representative sensorgrams show that IgG-FITC and HSA-FITC bind FcRn in a pH dependent manner, binding at pH 6.0 and release at pH 7.4 (Appendix A).

To investigate the functional binding of these ligands, we continued using the U-937

cell line. A pH dependent cell binding assay was established where fixed cells were stained with IgG-FITC or HSA-FITC at pH 5.5 and pH 7.4 followed by flow cytometry , all as described in *material and methods*. Figures 4A-B show the binding of the ligands expressed as mean fluorescence intensity (MFI) at pH 5.5 and pH 7.4. Increased binding of IgG-FITC (Fig. 4A) and HSA-FITC (Fig. 4B) were observed at acidic pH compared to pH 7.4, which supports functional binding to FcRn. In contrast, the Fc removed $F(ab')_2$ fragment and mIgG2a did not bind (Fig. 4A). Furthermore, labelled hIgG pre-incubated with *Streptococcus* protein G showed reduced binding (Fig. 4A). These data are in agreement with an IgG Fc-mediated interaction and a stringent discrimination of mIgG as reported by others ([25, 24]).

To assess the contribution of FcRn in the increased binding of IgG and HSA shown at acidic pH, inhibition studies were performed with pre-incubation of cells with unlabelled hIgG and HSA. Figures 5A-B clearly show decreased binding of IgG-FITC and HSA-FITC in the presence of IgG and HSA at pH 5.5. Furthermore, the polyclonal anti-FcRn has been shown to block the interactions sites of IgG and HSA on hFcRn (Andersen *et al*., unpublished). Importantly, we here demonstrate that pre-incubation of cells with the anti-FcRn preparation dramatically decreased binding of IgG as well as HSA (Fig.5). Thus, the data presented strongly support functional and pH dependent binding to hFcRn. In addition, this is the first report showing pH dependent binding of HSA to membrane-bound FcRn.

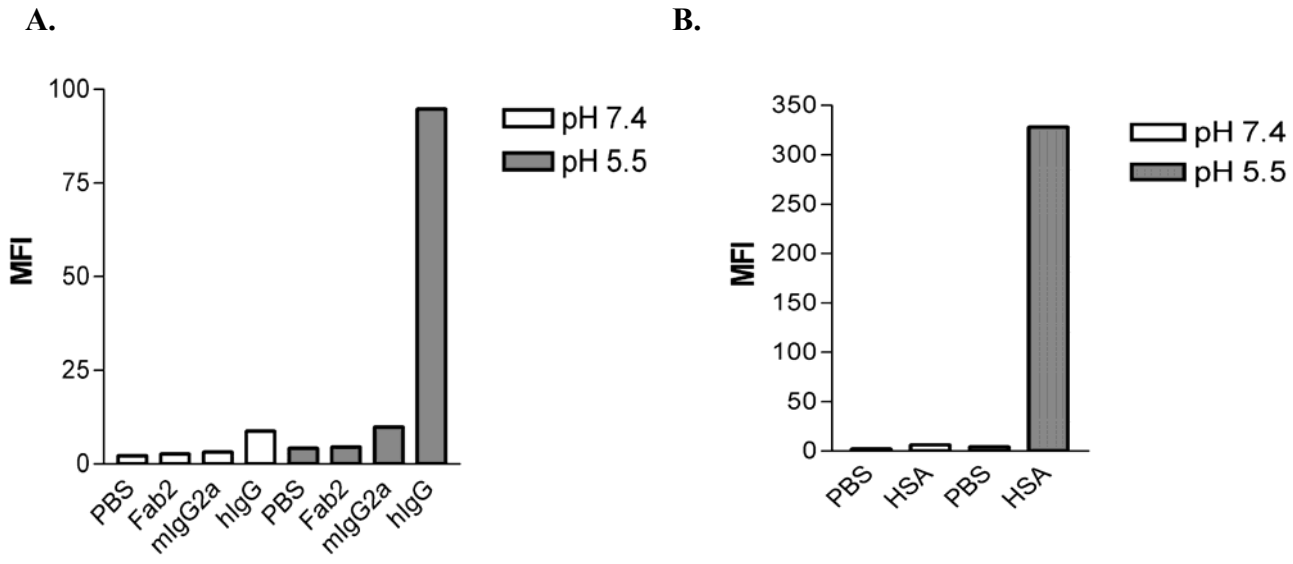


Figure 4. FcRn ligands binding to monocytic U-937 cells. (A) Binding of hIgG, F(ab')2 and mIgG2a to U-937 cells at pH 5.5 and at pH 7.4. (B) Binding of HSA at pH 5.5 and pH 7.4. The binding is expressed as mean fluorescence intensity (MFI). The experiments were repeated with similar results.

To determine whether hIgG and HSA bind to membrane-bound hFcRn independently of each other, we functionally stained cells with IgG-FITC and HSA-FITC simultaneously at acidic pH. Figure 6 shows that simultaneous staining gave an additive binding affect compared with individual staining, which may indicate that both ligands bind simultaneously to hFcRn. These data are in agreement with biochemical studies performed on recombinant soluble molecules ([26, 28, 29]). Taken together, the results suggest that both IgG and HSA bind membrane-bound hFcRn and that the increased binding at acidic pH is completely mediated by FcRn.

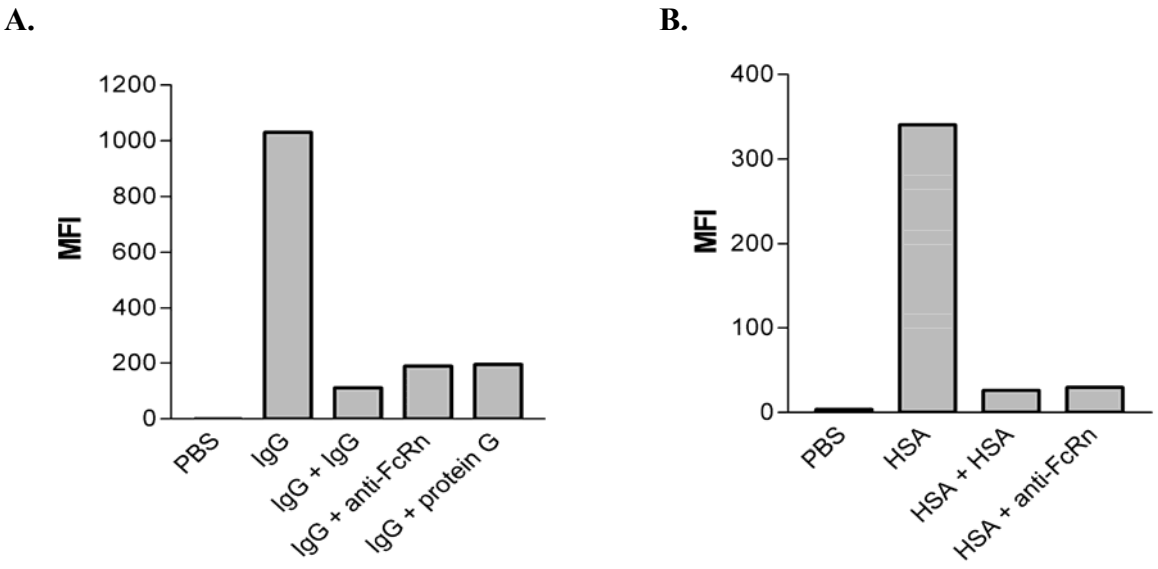


Figure 5. hFcRn-ligand inhibition analysis on U-937 cells. (A) Pre-incubation of U-937 cells with unlabelled hIgG, polyclonal anti-FcRn and protein G blocked the hIgG-FITC binding. (B) Pre-incubation of U-937 cells with unlabelled HSA and polyclonal anti-FcRn Ab blocked the HSA-FITC binding. The binding is expressed as mean fluorescence intensity (MFI). The experiments were repeated with similar results.

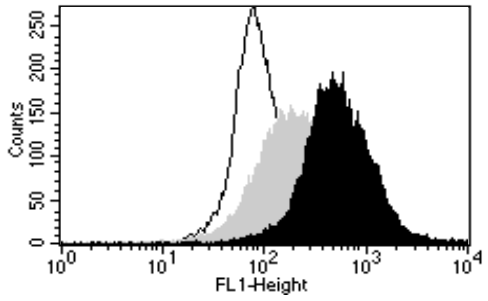


Figure 6. **The hIgG and HSA bind to membrane bound hFcRn independently of each other.** U-937 cells were surface stained with HSA-FITC and hIgG-FITC simultaneously. HSA-FITC, hIgG-FITC and together are shown as white, gray and black filled histograms, respectively. Histograms are given with ordinate indicating number of cells and abscissa indicating the fluorescence intensity.

4.4 Expression of FcRn and Fcγ receptors on peripheral blood mononuclear cells

To extend the observations with human monocytic cell lines to primary immune cells, we isolated PBMCs and performed flow cytometry to determine FcRn expression. We found that these cells express FcRn as showed both on cell surface (Fig.7A) and total (Fig.7B). In addition, the expression profiles of classical Fcγ receptors were assessed. The PBMCs were stained with monoclonal antibodies and analysed by flow cytometry. The data obtained show expression of FcγRI (Fig. 8A) and FcγRII (Fig. 8B) but no evidence for FcγRIII expression (Fig. 8C).

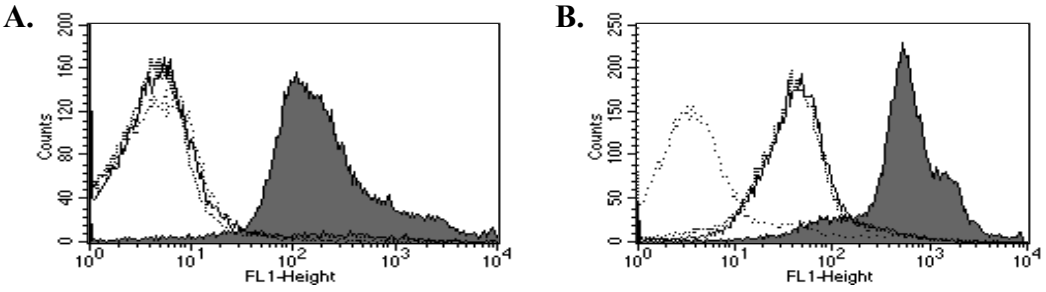


Figure 7. Expression and cellular distribution of FcRn in PBMCs. Cells were stained with biotinylated anti-FcRn followed by streptavdin-FITC and analysed by flow cytometry. PBMCs surface stained (A) and total (B). FcRn expression is shown as gray filled histograms, streptavidin-FITC and isotype (biotinylated goat IgG) controls as unfilled histograms. Histograms are given with ordinate indicating number of cells and abscissa indicating the fluorescence intensity.

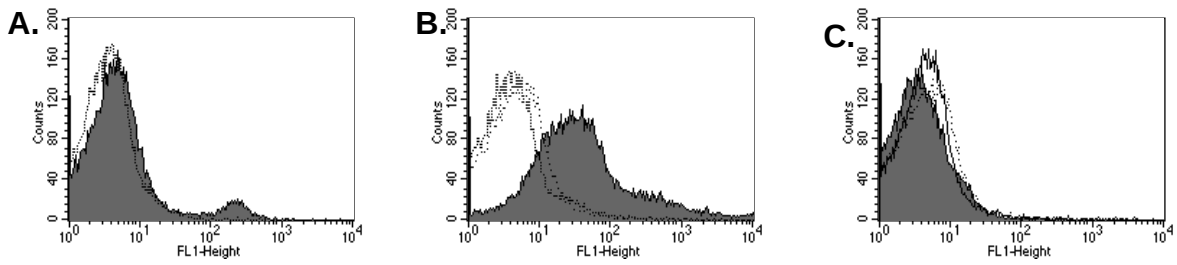


Figure 8. Expression of Fcγ receptors. Cells were stained with monoclonal antibodies towards each Fcγ receptor as described in *material and methods*. PBMCs surface stained for FcγRI, FcγRII and FcγRIII (A, B and C). Fcγ receptors are shown as gray filled histograms and controls (isotype and secondary antibodies) as unfilled histograms. Histograms are given with ordinate indicating number of cells and abscissa indicating the fluorescence intensity.

To investigate the functional binding of IgG and HSA, the pH dependent cell binding assay was used. Figures 9A and 9C show binding of the ligands expressed as mean fluorescence intensity (MFI) at pH 5.5 and pH 7.4. Increased binding of IgG-FITC (Fig. 9A) and HSA-FITC (Fig. 9C) was observed at acidic pH compared to pH 7.4. In addition, labelled hIgG pre-incubated with *Streptococcus* protein G reduced IgG-FITC binding, clearly demonstrating Fc mediated binding at pH 5.5 (Fig. 9B). Furthermore, pre-incubation of PBMCs with the anti-FcRn preparation shows great impact on the pH dependent binding of both IgG (Fig. 9A) and HSA (Fig. 9C). Importantly, irrelevant goat IgG did not affect binding of either ligands.

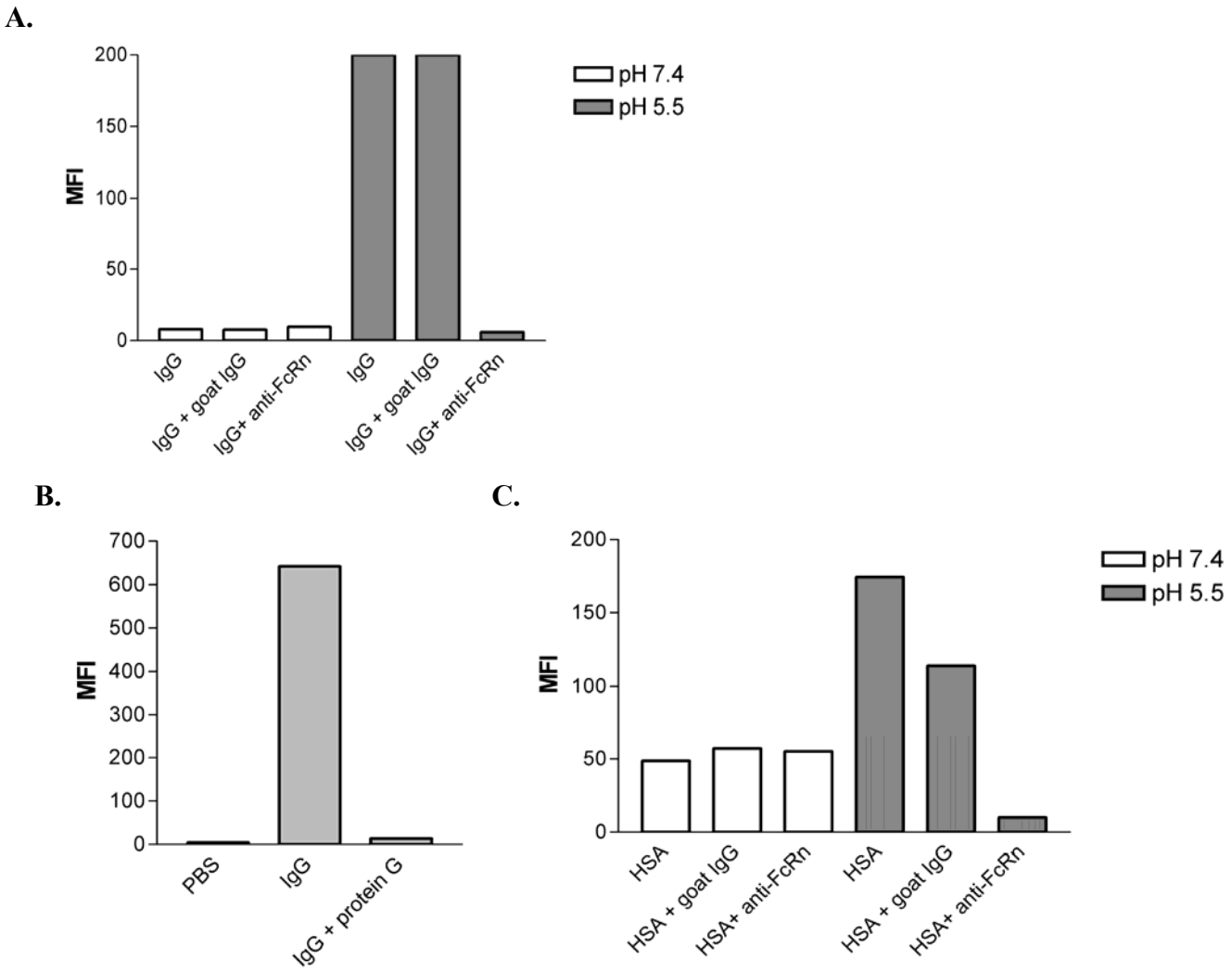


Figure 9. hFcRn-ligand inhibition analysis on PBMCs. (A) Binding of IgG-FITC alone or in the presence of goat IgG or polyclonal anti-FcRn followed by hIgG-FITC at pH 7.4 and pH 5.5. (B) Binding of IgG-FITC and IgG-FITC pre-incubated with *Streptococcus* protein G. (C) Binding of HSA-FITC alone or in the presence of goat IgG or polyclonal anti-FcRn followed by HSA-FITC at pH 7.4 and pH 5.5. The binding is expressed as mean fluorescence intensity (MFI).

4.5 Human hepatocytes express FcRn mRNA

Expression and diverse functions of FcRn in different tissues is continually reported. To contribute to the understanding of how the receptor functions according to cell type, we investigated the expression of FcRn in human hepatocytes.

To investigate whether human hepatocytic cell lines (Hep-3B and Hep-G2) express specific mRNA for the hFcRn heavy chain, RT-PCR was performed. The monocytic U-937 cell line was used as a positive control in addition to the housekeeping gene GAPDH that was used as an internal control in all reactions. First, reverse transcriptions were done on total RNA from the cells using anchored Oligo (dT)20 primer which enable synthesis of full-length cDNA, followed by PCR with primers specific for heavy chain. The PCR products were run on an agarose gel, and as shown in figure 10A, fragments were obtained from both hepatic cell lines and they migrated as expected bands of 1098 bp. To determine whether the PCR products were indeed amplified hFcRn heavy chain, the bands were excised and purified for sequencing. The PCR products sequenced (Appendix B) were identical to the previously determined cDNA sequence of hFcRn (GeneBank, NM 004107; [7]). Furthermore, the mRNA for β_2m was reverse transcribed and PCR amplified (Fig. 10B). The amplified fragments migrated as bands corresponding to the expected size of 360 bp. These results showed that hFcRn transcripts were expressed in human hepatocytes.

The hFcRn heavy chain cDNA in pcDNA3-(TM-hFcRn)-GST.Pcmv-beta2m-polyA vector was subcloned into fluorescent protein vectors. To show whether the constructs are functional, MDCK cell line stably expressing hβ_2m transiently transfected with vectors encoding fluorescent proteins in N-terminal fusion with hFcRn heavy chain cDNA. Analysis of these cells by confocal microscopy showed FcRn expression and its distribution in edosomal compartments of the cells (Appendix C).

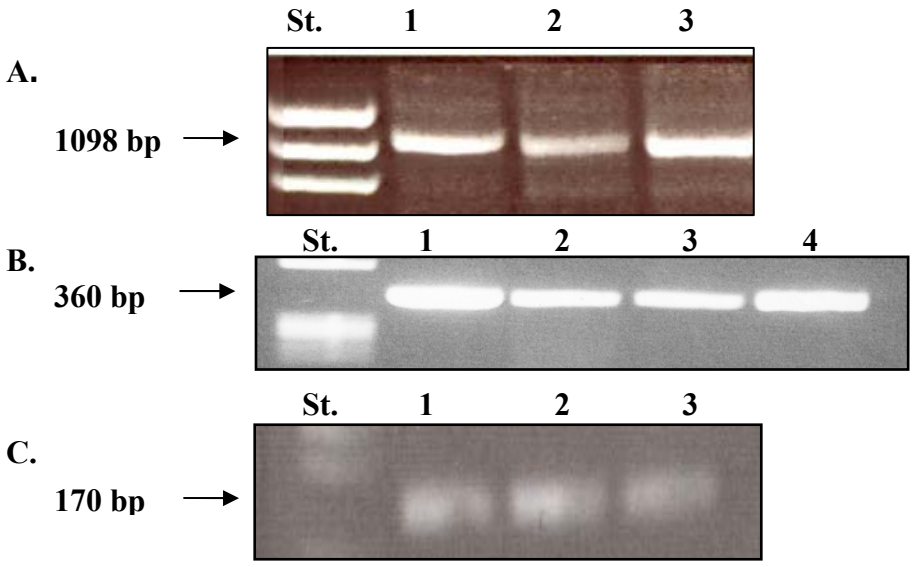


Figure 10. Expression of the hFcRn heavy chain and hβ2m mRNA. RNA was reverse transcribed and PCRs were performed as described *in material and methods.* (A) The hFcRn heavy chain PCR products amplified from U-937 (Lane 1), Hep3B (Lane 2) and HepG2 (Lane 3). (B) The hβ2m PCR products from U-937 (Lane 1), Hep-3B (Lane 2), Hep-G2 (Lane 3) and the pCDNA3-hβ2m vector (Lane 4). (C) The GAPDH PCR products amplified from U-937 (Lane 1), Hep-3B (Lane 2) and Hep-G2 (Lane 3). Amplified PCR products were run on 1 % agarose gel and stained with ethidium bromide. St. is standard molecular weight phixX174 DNA marker. The molecular sizes are indicated by arrows.

4.6 Human hepatocytes express FcRn

Western immunoblotting was carried out on total lysates using the polyclonal anti-hFcRn preparation. Figure 11A shows detection of bands corresponding to ~45 kDa in lysates from all cell lines. In addition, bands of ~12 kDa corresponding to $h\beta_2m$ were detected. The protein bands are consistent with the calculated molecular weight of hFcRn heavy chain and $h\beta_2m$. ELISA analyses on total lysates also support the expression of both proteins (data not shown). Furthermore, Western immunoblotting of total protein isolated from human liver shows detectable expression of the both the hFcRn heavy chain as well as the $h\beta_2m$ subunit (Andersen *et al*; data not shown).

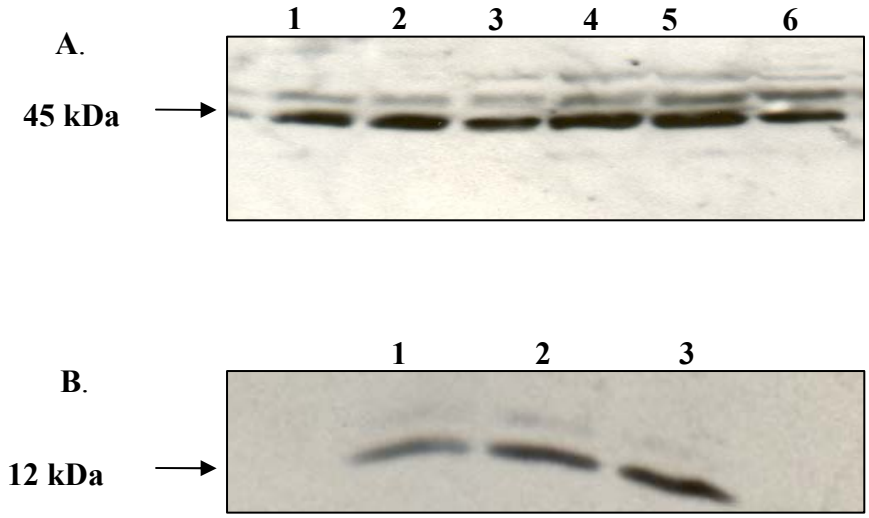


Figure 11. Expression of hFcRn. (A) Detection of hFcRn heavy chain in protein lysates from U-937 (Lane 1 and 2), Hep-3B (Lane 3 and 4) and Hep-G2 (Lane 5 and 6) cells by western immunoblotting. (B) Detection of hβ2m in protein lysates from U-937 (Lane 1), Hep-3B (Lane 2) and Hep-G2 (Lane 3) by western immunoblotting. Similar experiments repeated with identical results.

Furthermore, flow cytometric analyses were performed to demonstrate surface expression of hFcRn using the goat anti-FcRn preparation. Figure 12 shows histograms for surface staining of Hep-3B (Fig. 12A) and Hep-G2 (Fig. 12B), and total hFcRn expression was verified after saponin treatment of Hep-3B (Fig. 12C) and Hep-G2 (Fig. 12D). The presence of a shoulder seen for both hepatic cell lines at surface was detected in all experiments. The results clearly reveal the presence of the transmembrane hFcRn. Taken together, both FcRn subunits were shown to be expressed in the hepatic cell lines ensuring the expression of a complete heterodimeric transmembrane FcRn.

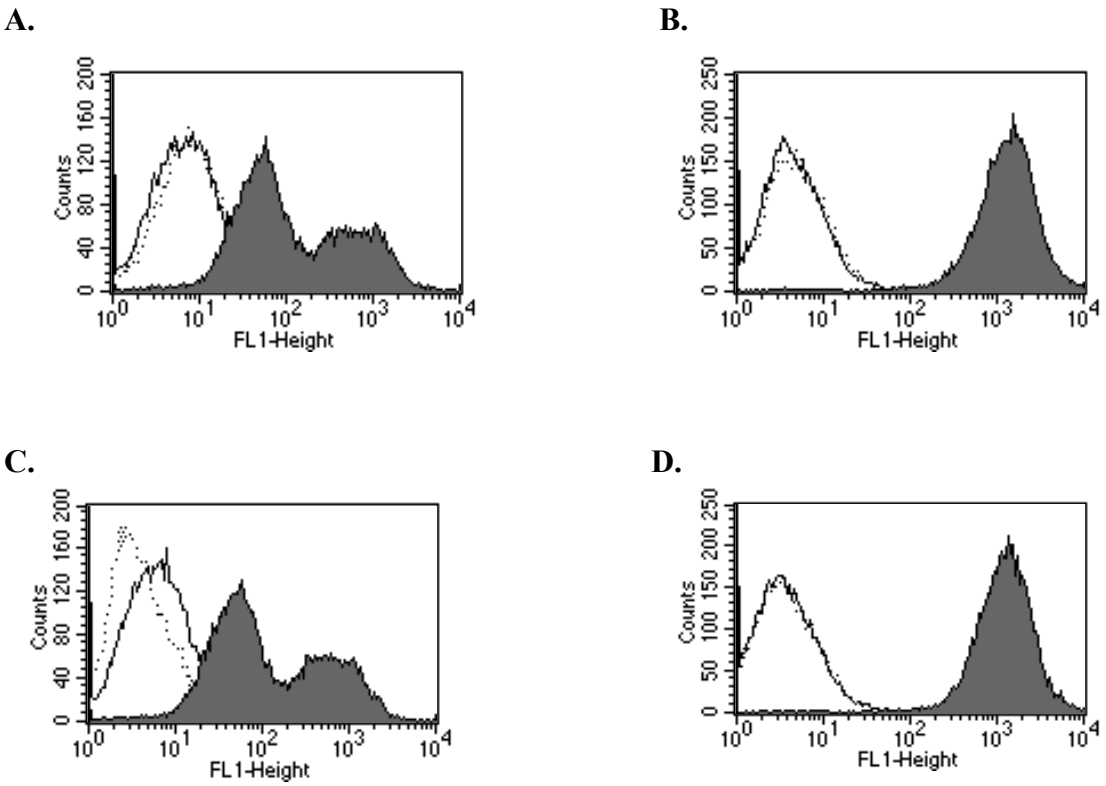


Figure 12. Expression and cellular distribution of FcRn in human hepatocytes. Cells were stained with biotinylated anti-FcRn followed by streptavdin-FITC and analysed by flow cytometry. Hep-3B cells surface stained (A) and total (B). Hep-G2 cells surface stained (C) and total (D). FcRn expression is shown as gray filled histograms, streptavidin-FITC and isotype (biotinylated goat IgG) controls as gray lined histograms. Histograms are given with ordinate indicating number of cells and abscissa indicating the fluorescence intensity.

4.7 Functional FcRn is expressed in human hepatocytes

To investigate the functionality of FcRn expressed in human hepatocytes, a pH-dependent binding assay was established. Total protein lysates were immunoprecipitated using hIgG covalently coupled to Sepharose 4B. Binding experiments were performed at pH 5.5 as well as at pH 8.0, all as described in *material and methods*. Eluted fractions were applied to reducing SDS-PAGE analysis followed by Western immunoblotting.

Figure 13 shows that hFcRn binds IgG at pH 5.5 but not at pH 8.0. The major bands correspond to ~ 45 kDa, which is in agreement with the expected size of the hFcRn heavy chain. In addition, hβ_2m was co-eluted and detected as a band of ~12 kDa. Taken together, the data show that hFcRn is expressed by the hepatocytes as a heterodimeric molecule and is to binds IgG at pH 5.5 but not at pH 8. The results confirm the results from are similar with previous work that describes pH dependent binding of IgG to a receptor in rat neonatal enterocytes ([45]).

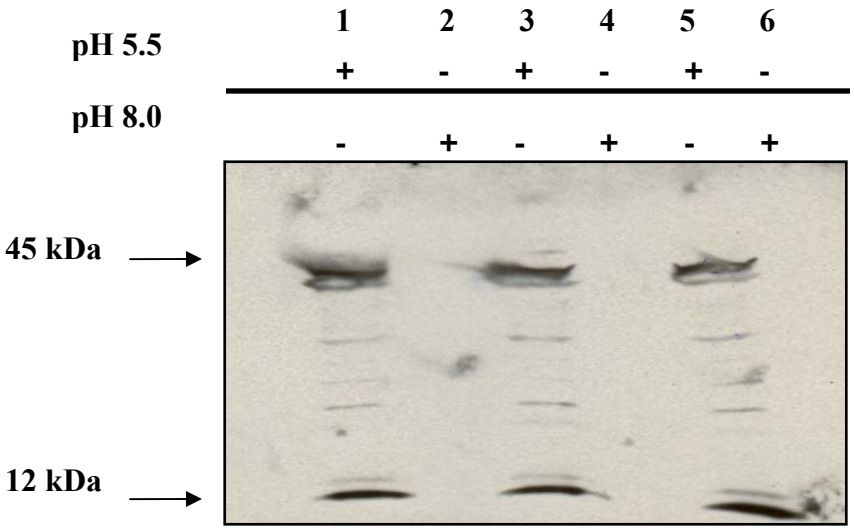


Figure 13. Detection of pH-dependent FcRn binding to hIgG. 400-600 μg of total proteins were incubated with human IgG coupled Sepharose at 4ºC at pH 5.5 or pH 8.0 as described in *materials and methods*. The eluted fractions were subjected to 12 % SDS-PAGE under reducing conditions. U-937 fractions from experiments performed at pH 5.5 (lane 1) and pH 8.0 (lane 2). Hep-3B fractions from experiments performed at pH 5.5 (lane 3) and pH 8.0 (lane 4). Hep-G2 fractions from experiments performed at pH 5.5 (lane 5) and pH 8.0 (lane 6). Proteins were probed with goat anti-hFcRn heavy chain and rabbit anti-hβ2m antibodies followed by HRP-conjugated secondary antibodies. The location and size of the hFcRn heavy chain and hβ2m are indicated by arrows.

4.8 Membrane-bound hFcRn binds both hIgG and HSA in a pH dependent manner

To explore IgG binding to surface expressed hFcRn, the expression profiles of classical Fcγ receptors had to be considered. Both hepatic cell lines were stained and data obtained showed expression of the low affinity binding FcγRII (CD32) but no evidence of FcγRI (CD64) and FcγRIII (CD16) expression (Fig.14).

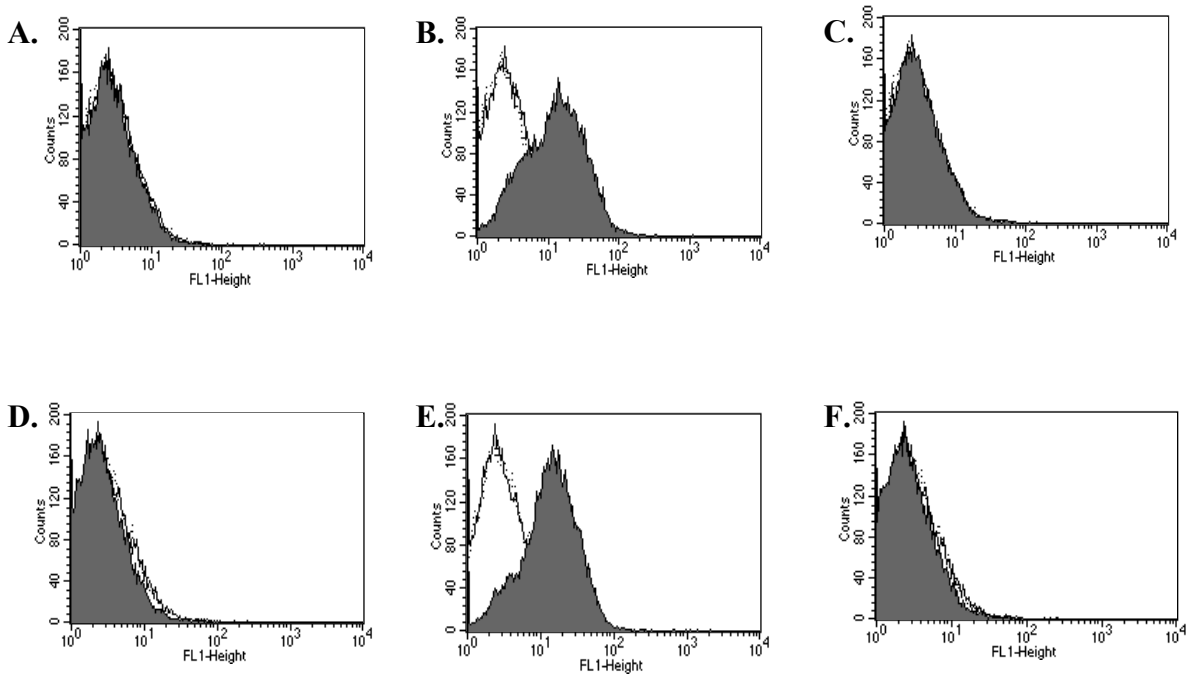


Figure 14. Expression of Fcγ receptors. Cells were stained with monoclonal antibodies towards each Fcγ receptor as described in *material and methods*. Hep-3B cells surface stained for for FcγRI, FcγRII and FcγRIII (A, B and C). Hep-G2 cells surface stained for FcγRI, FcγRII and FcγRIII (D, E and F). Fcγ receptors are shown as gray filled histograms and controls (isotype and secondary antibodies) as unfilled histograms. Histograms are given with ordinate indicating number of cells and abscissa indicating the fluorescence intensity.

To investigate whether the membrane-bound FcRn binds IgG in a pH dependent manner, cells were stained using hIgG-FITC followed by flow cytometry analyses. We found that Hep-3B showed increased binding of hIgG at pH 5.5 as compared to pH 7.4 (Fig.15A-B).

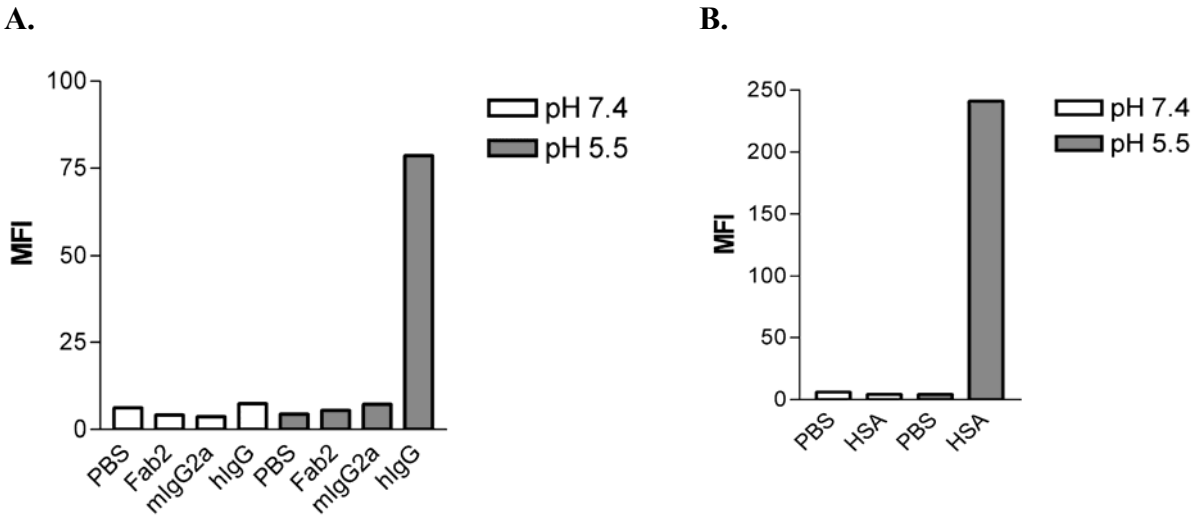


Figure 15. FcRn ligands binding to Hep3B cells. (A) Binding of hIgG-FITC, Fab2 and mIgG2a (A) to Hep3B cells at pH 5.5 and at pH 7.4. (B) Binding of HSA at pH 5.5 and pH 7.4. The binding is expressed as mean fluorescence intensity (MFI). The experiments were repeated with similar results.

To confirm selective ligand binding, cells were pre-incubated with unlabelled hIgG and HSA. The results clearly demonstrate competitive binding by showing decreased binding of FITC conjugated ligands to Hep-3B (Fig.16A-B). Furthermore, cells were pre-blocked with polyclonal anti-FcRn followed by staining with IgG-FITC and HSA-FITC. In addition, labelled hIgG pre-incubated with *Streptococcus* protein G resulted in reduced IgG-FITC binding, clearly demonstrating Fc mediating binding at pH 5.5 (Fig. 16A). Figure 15A-B shows that targeting of hFcRn with anti-FcRn inhibits functional binding of the ligands. Thus, the increased binding shown at acidic pH is related to specific FcRn binding. Similar results were obtained from experiments performed on the Hep-G2 cell line (data not shown).

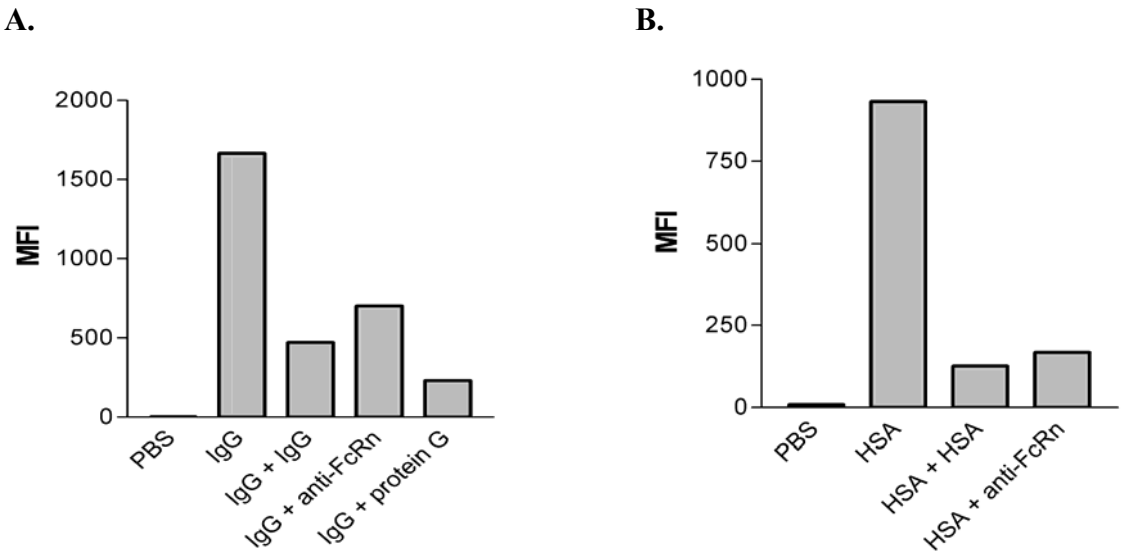


Figure 16. **hFcRn-ligand inhibition analysis on Hep-3B cells.** (A) Hep-3B cells stained with IgG-FITC alone or stained after pre-incubation with polyclonal anti-FcRn or in presence of protein G. (B) Hep-3B cells stained with HSA-FITC alone or stained after pre-incubation with polyclonal anti-FcRn. The binding is expressed as mean fluorescence intensity (MFI). The experiments were repeated with similar results.

To determine whether IgG and HSA bind to membrane-bound hFcRn simultaneously, cells were stained with either IgG-FITC or HSA-FITC alone or simultaneously at pH 5.5. Figure 17 shows that double staining of Hep-3B resulted in an additive binding effect compared with individual staining.

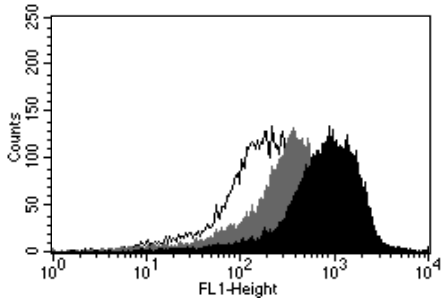


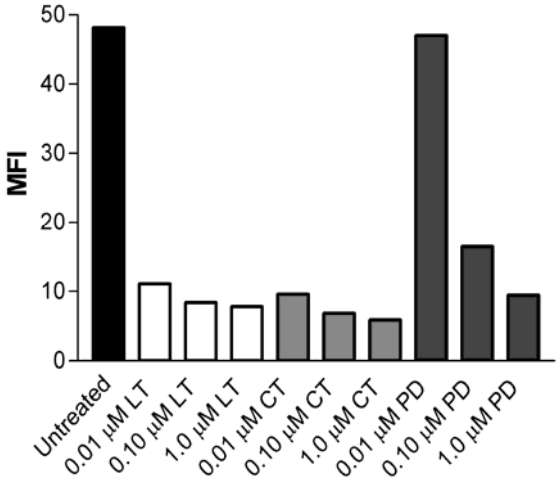
Figure 17. **The hIgG and HSA bind to membrane bound hFcRn independently of each other**. Hep-3B cells were surface stained with HSA-FITC and hIgG-FITC simultaneously. HSA-FITC, hIgG-FITC and together are shown as white, gray and black filled histograms, respectively. Histograms are given with ordinate indicating number of cells and abscissa indicating the fluorescence intensity.

4.9 Anti-inflammatory substances modulate the expression of the hFcRn

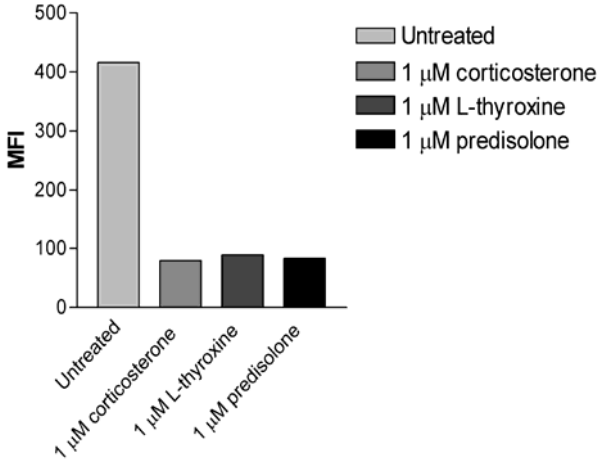
Previous data have shown down regulation of rat FcRn three weeks postnatally ([43, 44]). The molecular mechanism is not fully understood, but exposure to exogenous glucocorticoides and thyroxine hormone causes inhibition of transepitelial transport of orally administered IgG. These classes of substances are widely used as anti-inflammatory agents in first-line therapy of persistent asthma, inflammatory and immune diseases ([48]). To assess whether glucocorticoides and L-thyroxine influence IgG transport by regulation of hFcRn expression, the monocytic U-937 cell line in addition to the hepatic cell lines (Hep-3B and Hep-G2) were treated with different naturally occurring hormones (corticosterone and L-thyroxine) as well as a synthetic version (prednisolone). Cells were given 0.010-1 μM of each substance and three days post treatment, and the hFcRn expression was assessed by flow cytometric analyses. The results clearly demonstrate decreased receptor surface expression on both U-937 (Fig. 18A) and Hep-3B (Fig. 18B) in a concentration dependent manner for all substances given, compared to untreated cells. In addition, two other clinically used anti-inflammatory synthetic versions (dexamethasone and beclomethasone dipropionate) were tested and shown to

downregulate hFcRn in a similar manner (data not shown). Similar results were also obtained with the Hep-G2 cell line (data not shown). Furthermore, the effect on total hFcRn expression was investigated using 1 μM of the given substance. Three days post treatment cells were permeablized followed by staining of hFcRn. The data obtained show a dramatic downregulation of receptor expression in U-937 (Fig. 18C) and Hep-3B (Fig. 18D) for all substances tested. These data may contribute to the clinical understanding of anti-inflammatory treatment, especially in regard to critical regulation of IgG and HSA homeostasis.

A.



B.



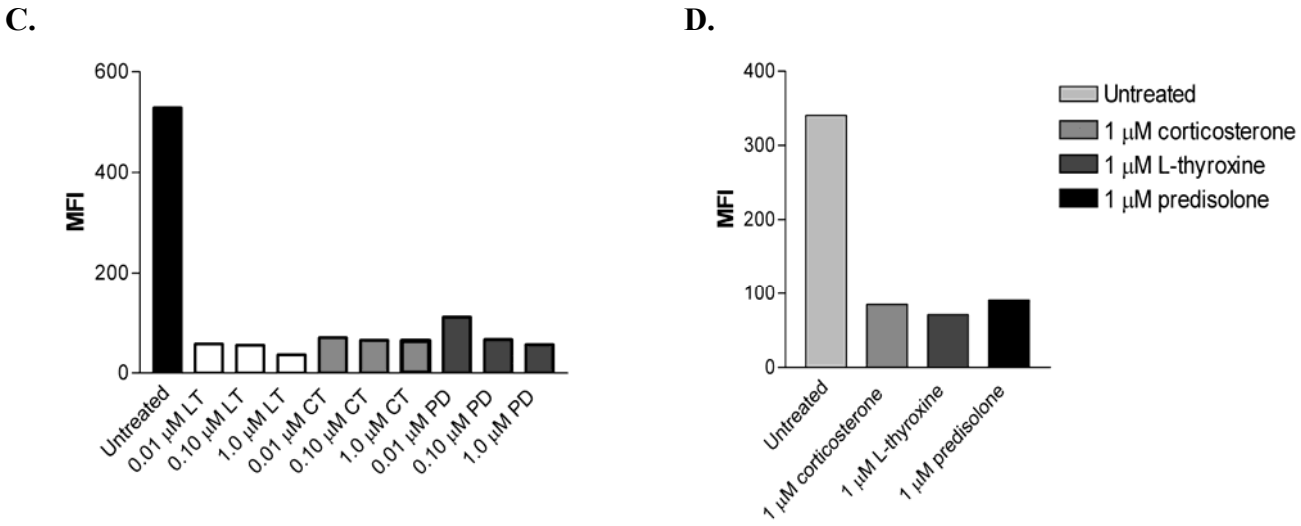


Figure 18. Effects of anti-inflammatory substances on FcRn expression. (A) FcRn surface expression in U-937 cells after treating with 0.01 μM, 0.1 μM, and 1.0 μM of L-thyroxine (LT), corticosterone (CT) or predisolone (PD). (B) Total FcRn expression in U-937 cells after treating with 1 μM L-thyroxine, corticosterone and predisolone. (C) FcRn surface expression in Hep-3B cells after treating with 0.01 μM, 0.1 μM, and 1.0 μM of L-thyroxine (LT), corticosterone (CT) and predisolone (PD). (D) Total FcRn expression in Hep-3B cells after treating with 1 μM L-thyroxine, corticosterone and predisolone. The FcRn expression is shown as mean fluorescence intensity (MFI) three days post treatment.

5. Discussion

Originally, FcRn was characterized functionally as the receptor that transfers IgG in the maternal milk from the intestine of neonatal rats to the blood stream. This is paralleled by transfer of IgG to fetus by transplacental transport in humans. Subsequent studies have revealed expression of the receptor and important functions beyond neonatal/fetal life in adult mammals including human. In this context, there is no report on FcRn expression and functions in human hepatocytes and limited data in immune cells. In this study, we have shown FcRn expression in three human monocytic U-937, THP-1 and K-562 cells, and PBMCs. Our studies, for the first time demonstrate that FcRn is expressed by human hepatic Hep-3B and Hep-G2 cells. We also found that the hepatic cells express FcγRII. Whether it is isoform A or B and its function remains be described.

It has been described that FcRn binds IgG in the slightly acidic pH of the intestinal lumen of neonatal rodent, and that IgG is released into the blood stream where the pH is basic (pH 7.4) ([44]). In our investigations, we found that binding of human monocytic cells and hepatocytes to hIgG and HSA display complete pH-dependence, showing increased binding at pH 5.5. In this study, we demonstrate that pre-incubation of cells with the polyclonal anti-FcRn preparation from goat blocked the binding of IgG as well as HSA observed at pH 5.5. This is important, because goat IgGs does not bind to human Fcγ receptors and hFcRn shows no binding to goat IgGs. Furthermore, irrelevant goat IgG did not affect the binding. Therefore, it can be concluded that the increased binding of hIgG and HSA to membrane-bound hFcRn is only due to FcRn. In light of these observations, the possibility of FcRn blockade by anti-FcRn has important therapeutic implications in humans. FcRn blockade by intravenous Ig (IVIG) significantly increases the catabolism of serum IgG in mice ([45]). Studies on the therapeutic effects of an anti-rat FcRn mAb, 1G3, in two rat models of myasthenia gravis resulted in dose-dependent amelioration of the disease symptoms after passive experimental disease was induced by administration of an anti-acetylcholine receptor (AChR) mAb ([46]). The effect of 1G3 was also studied in an active model of experimental autoimmune myasthenia gravis in which rats were

immunized with AChR, and again treatment with 1G3 significantly reduced the severity of the disease symptoms as well as the levels of total IgG and anti-AChR IgG relative to untreated animals ([46]).

In addition, we report for the first time the pH dependent binding of HSA to membrane bound FcRn. Simultaneous staining of both ligands resulted in an additive binding effect compared with individual staining, which may indicate that both ligands bind independently to hFcRn. These data are in agreement with data obtained on biochemical studies performed on recombinant molecules ([26, 28, 29]).

In our studies, FcRn was found both intracellularly and on the cell surface of the monocytic cells, PBMCs and hepatocytes. The expression of FcRn on the surface may indicate a function of FcRn under physiological condition which involves delivery of IgG and HSA from the intracellular to extracellular milieu as protection from lysosomal degradation. Alternatively, the role of the surface FcRn may be in receptor mediated endocytosis, where FcRn may bind its ligands at acidic pH and the FcRn-ligand complexes delivered to acidic endosomal compartments in the cell. This is possible for FcRn expressed on the surface of immune cells and might have a role in pathological conditions such as tissue inflammation ([33]) and tumor infiltration ([34, 35]), where acidic extracellular conditions are created by alterations in tissue metabolism. The extracellular pH within solid tumors has been observed to be below physiological levels, ranging from 5.6-7.7 ([34]) which includes the pH optimum of FcRn binding. Macrophages are recruited in the earliest phases of inflammatory condition such as inflammatory bowel disease ([36]) and they widely infiltrate to solid tumor tissues ([37]).

Where the pH outside the cell is neutral, IgG and HSA are initially internalized by endocytosis and FcRn binding occurs at low pH in intracellular compartments. Unbound IgG and HSA are delivered to the default lysosomal pathway and degraded. In contrast, the bound IgG and HSA to FcRn are protected and recycled back into surrounding tissue fluid. The fact that most of the receptor is found intracellularly, and that the steady state level of surface FcRn is very low suggests rapid recycling or that most of FcRn remain inside vesicles. Both immune cells and hepatocytes harbour Fcγ receptors for immune complexes, and these may deliver IgG to acidic compartments. Regarding albumin

uptake by receptor mediated mechanism, little is known about albumin receptors on such cells. None is characterized on immune cells, and a receptor for glutaraldehyde treated albumin is implicated in hepatitis B virus infection ([38, 39, 40, 41]).

FcRn was shown to be functionally expressed on the surface of adult rat hepatocytes and hypothesized to mediate transport of IgG from serum to bile ([13]). Rather, FcRn in hepatocytes has been implicated in protecting IgG from catabolism ([32]). Here, we confirm that the expression of FcRn by hepatocytes both on the surface and intracellularly may indicate a role in protection of IgG and HSA from catabolism and the maintenance of IgG and HSA levels in the blood. Therefore, the expression of FcRn on the cell surface of hepatocytes may reflect highly active sorting of IgG and HSA by FcRn from the endocytic pathway to the cell surface. Human hepatocytes have similar plasma fluid exposure as that of vascular endothelial cells which are the cells suggested protecting IgG from degradation ([11]). Histologically, human hepatocytes are in direct contact with huge amount of plasma fluid for the reason that separation from the blood stream is by discontinuous or fenestrated sinusoidal capillary that lack basement membrane. The human liver has two blood supplies and at any one time, as much as 10% of all the blood in the body is present in the liver. This anatomical context points to hepatocytes as the place where major catabolism of plasma proteins like IgG and albumin can occur.

Interestingly, both hepatocytes and hepatitis B virus bind albumin. Hepatitis B virus is known to infect hepatocytes and the infectivity of the virus is facilitated by the presence of surface antigen associated albumin binding protein. A receptor for glutaraldehyde treated albumin is found on hepatocytes ([38, 39, 40, 41]), and liver the only place where albumin biosynthesis takes place. Specific albumin receptors exist and are characterized as glycoproteins ([42]), however, its/their exact molecular identity remains to be elucidated.

Previous data on neonatal rats have shown downregulation of rat FcRn three weeks postnatal life ([44]). Exogenous glucocorticoids and thyroxine hormone have shown inhibition of transepithelial transport of orally administered IgG ([43]). Whether these effects are due to decreased FcRn expression has not thoroughly been investigated.

To address the question whether glucocorticoids, and L-thyroxine could influence

the hFcRn expression, the monocytic U-937 cells in addition to the hepatic Hep-3B and Hep-G2 cells were treated with different naturally occurring hormones (corticosterone and L-thyroxine) as well as a synthetic ones such as prednisolone, dexamethasone and beclomethasone dipropionate. The results clearly demonstrate decreased receptor expression on human U-937, Hep-3B and Hep-G2 cells in a concentration dependent manner for substances tested, compared to the untreated cells. In the present study, the sensitivity of the substances used and the time that needs to produce the effect were not considered. However, the results correlate well with clinical and experimental observations. Patients on glucocorticoid therapy often have decreased serum IgG concentration ([49]). This clinical observation has not been addressed in relation to factors that maintain IgG serum levels. Different factors may contribute to this observation but under the condition that FcRn expression is diminished, it is possible that IgG is neither transported nor protected from degradation. This is consistent with the previous report that hydrocortisone treatment results in hypogammaglobulinemia. The ability of glucocorticoids to decrease FcRn expression at the transcriptional level has been suggested ([43]) even though the exact mechanism is not understood.

Regarding HSA, there is no report on the correlation of glucocorticoids therapy and decreased serum concentration. But, we postulate that identical mechanism may operate given that FcRn binds in its function both to IgG and HSA in a similar fashion.

The levels of sensitivity, time period for action required, and the exact mechanisms involved in downregulation of FcRn expression and its correlation to reduced serum concentration of IgG and HSA need further investigation.

Such data may contribute to the clinical understanding of anti-inflammatory treatments, especially with regard to the regulation of IgG and HSA homeostasis.

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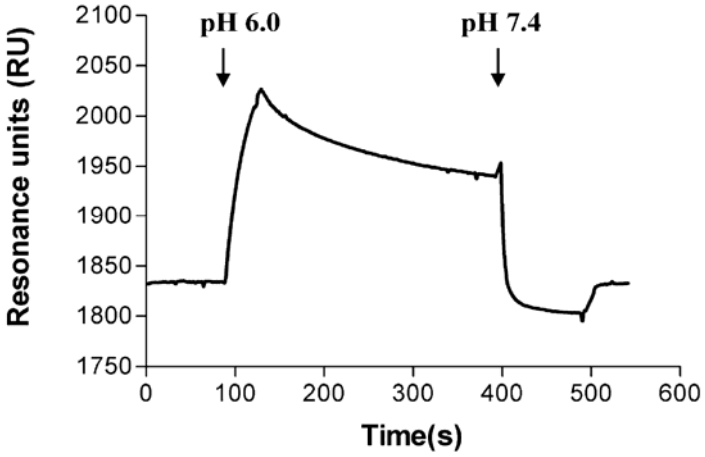
Appendix A.

The functional integrity of FITC conjugated hIgG and HSA were tested by surface plasmon resonance.

Recombinant shFcRn was immobilized on a CM5 sensor chip by amine coupling. IgG-FITC and HSA-FITC were injected at pH 6.0 followed by injection of a buffer with pH 7.4. Representative sensorgrams show that IgG-FITC (1) and HSA-FITC (2) bind FcRn in a pH dependent manner, binding at pH 6.0 and release at pH 7.4.

1.



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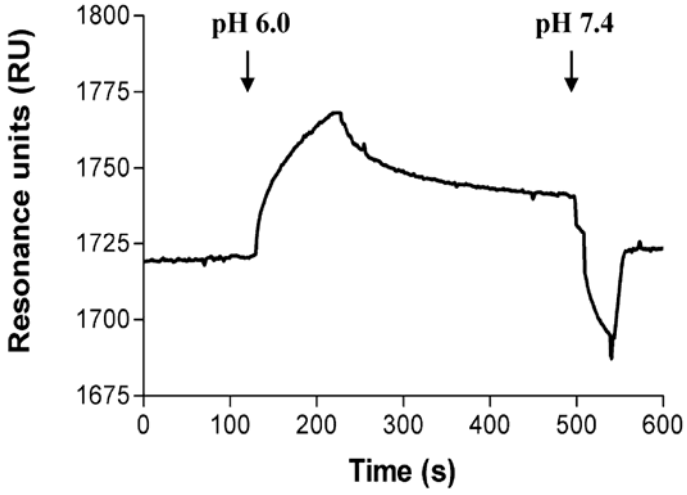

Appendix B.

Sequence of transmembrane (TM) human FcRn isolated from the hepatic HepG2 cell line:

Template: pcDNA3-TM hFcRn-GST-Pcmv-β_2m-polyA
Primer: FcRn primer-2926 and GST-rev (custom designed by GATC)
Total length: 1098 nucleotides

GATC Gmbh, Germany, performed the sequencing reactions and the results are given below with CLUSTAL W (1.83) multiple sequence alignment.
In the alignment: "Human" stands for the published cDNA sequence of hFcRn (GeneBank, NM 004107). The isolated TM hFcRn cDNA from HepG2 cells is aligned. The sequences are 100% identical. The start codons (ATG) are shown in bold and the stop codons (TGA) are underlined.

```
Human       ATGGGGGTCCCGCGGCCTCAGCCCTGGGCGCTGGGGCTCC 40
HepG2       ATGGGGGTCCCGCGGCCTCAGCCCTGGGCGCTGGGGCTCC 40
            ****************************************
Human       TGCTCTTTCTCCTTCCTGGGAGCCTGGGCGCAGAAAGCCA 80
HepG2       TGCTCTTTCTCCTTCCTGGGAGCCTGGGCGCAGAAAGCCA 80
            ****************************************
Human       CCTCTCCCTCCTGTACCACCTTACCGCGGTGTCCTCGCCT 120
HepG2       CCTCTCCCTCCTGTACCACCTTACCGCGGTGTCCTCGCCT 120
            ****************************************
Human       GCCCCGGGGACTCCTGCCTTCTGGGTGTCCGGCTGGCTGG 160
HepG2       GCCCCGGGGACTCCTGCCTTCTGGGTGTCCGGCTGGCTGG 160
            ****************************************
Human       GCCCGCAGCAGTACCTGAGCTACAATAGCCTGCGGGGCGA 200
HepG2       GCCCGCAGCAGTACCTGAGCTACAATAGCCTGCGGGGCGA 200
            ****************************************
Human       GGCGGAGCCCTGTGGAGCTTGGGTCTGGGAAAACCAGGTG 240
HepG2       GGCGGAGCCCTGTGGAGCTTGGGTCTGGGAAAACCAGGTG 240
            ****************************************
Human       TCCTGGTATTGGGAGAAAGAGACCACAGATCTGAGGATCA 280
HepG2       TCCTGGTATTGGGAGAAAGAGACCACAGATCTGAGGATCA 280
            ****************************************
Human       AGGAGAAGCTCTTTCTGGAAGCTTTCAAAGCTTTGGGGGG 320
HepG2       AGGAGAAGCTCTTTCTGGAAGCTTTCAAAGCTTTGGGGGG 320
            ****************************************
Human       AAAAGGTCCCTACACTCTGCAGGGCCTGCTGGGCTGTGAA 360
HepG2       AAAAGGTCCCTACACTCTGCAGGGCCTGCTGGGCTGTGAA 360
            ****************************************
Human       CTGGGCCCTGACAACACCTCGGTGCCCACCGCCAAGTTCG 400
HepG2       CTGGGCCCTGACAACACCTCGGTGCCCACCGCCAAGTTCG 400
            ****************************************
Human       CCTGAACGGCGAGGAGTTCCATGAATTTCGACCTCAAGCA 440
HepG2       CCTGAACGGCGAGGAGTTCCATGAATTTCGACCTCAAGCA 440
            ****************************************
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Human       GGGCACCTGGGGTGGGGACTGGCCCGAGGCCCTGGCTATC 480
HepG2       GGGCACCTGGGGTGGGGACTGGCCCGAGGCCCTGGCTATC 480
            ****************************************
Human       AGTCAGCGGTGGCAGCAGCAGGACAAGGCGGCCAACAAGG 520
HepG2       AGTCAGCGGTGGCAGCAGCAGGACAAGGCGGCCAACAAGG 520
            ****************************************
Human       AGCTCACCTTCCTGCTATTCTCCTGCCCGCACCGCCTGCG 560
HepG2       AGCTCACCTTCCTGCTATTCTCCTGCCCGCACCGCCTGCG 560
            ****************************************
Human       GGAGCACCTGGAGAGGGGCCGCGGAAACCTGGAGTGGAAG 600
HepG2       GGAGCACCTGGAGAGGGGCCGCGGAAACCTGGAGTGGAAG 600
            ****************************************
Human       GAGCCCCCCTCCATGCGCCTGAAGGCCCGACCCAGCAGCC 640
HepG2       GAGCCCCCCTCCATGCGCCTGAAGGCCCGACCCAGCAGCC 640
            ****************************************
Human       CTGGCTTTTCCGTGCTTACCTGCAGCGCCTTCTCCTTCTA 680
HepG2       CTGGCTTTTCCGTGCTTACCTGCAGCGCCTTCTCCTTCTA 680
            ****************************************
Human       CCCTCCGGAGCTGCAACTTCGGTTCCTGCGGAATGGGCTG 720
HepG2       CCCTCCGGAGCTGCAACTTCGGTTCCTGCGGAATGGGCTG 720
            ****************************************
Human       GCCGCTGGCACCGGCCAGGGTGACTTCGGCCCCAACAGTG 760
HepG2       GCCGCTGGCACCGGCCAGGGTGACTTCGGCCCCAACAGTG 760
            ****************************************
Human       ACGGATCCTTCCACGCCTCGTCGTCACTAACAGTCAAAAG 800
HepG2       ACGGATCCTTCCACGCCTCGTCGTCACTAACAGTCAAAAG 800
            ****************************************
Human       TGGCGATGAGCACCACTACTGCTGCATTGTGCAGCACGCG 840
HepG2       TGGCGATGAGCACCACTACTGCTGCATTGTGCAGCACGCG 840
            ****************************************
Human       GGGCTGGCGCAGCCCCTCAGGGTGGAGCTGGAATCTCCAG 880
HepG2       GGGCTGGCGCAGCCCCTCAGGGTGGAGCTGGAATCTCCAG 880
            ****************************************
Human       CCAAGTCCTCCGTGCTCGTGGTGGGAATCGTCATCGGTGT 920
HepG2       CCAAGTCCTCCGTGCTCGTGGTGGGAATCGTCATCGGTGT 920
            ****************************************
Human       CTTGCTACTCACGGCAGCGGCTGTAGGAGGAGCTCTGTTG 960
HepG2       CTTGCTACTCACGGCAGCGGCTGTAGGAGGAGCTCTGTTG 960
            ****************************************
Human       TGGAGAAGGATGAGGAGTGGGCTGCCAGCCCCTTGGATCT 1000
HepG2       TGGAGAAGGATGAGGAGTGGGCTGCCAGCCCCTTGGATCT 1000
            ****************************************
Human       CCCTTCGTGGAGACGACACCGGGGTCCTCCTGCCCACCCC 1040
HepG2       CCCTTCGTGGAGACGACACCGGGGTCCTCCTGCCCACCCC 1040
            ****************************************
Human       AGGGGAGGCCCAGGATGCTGATTTGAAGGATGTAAATGTG 1080
HepG2       AGGGGAGGCCCAGGATGCTGATTTGAAGGATGTAAATGTG 1080
            ****************************************
Human       ATTCCAGCCACCGCCTGA 1098
HepG2       ATTCCAGCCACCGCCTGA 1098
            ******************
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Appendix C.

Transient transfection of MDCK cells expressing hβ_2m with fluorescent protein vectors.

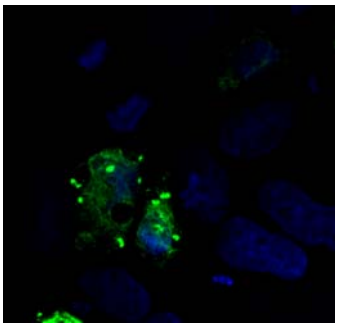
The MDCK cell line stably expressing hβ_2m was transiently transfected with vectors encoding fluorescent proteins in N-terminal fusion with the hFcRn heavy chain cDNA. The cells were examined with confocal microscope.

1. pEGFP-N1-TM hFcRn is transmembrane human FcRn in N-terminal fusion with green fluorescent protein (GFP) gene in pEGFP-N1 vector. Deep green staining shows FcRn distribution in edosomal compartments.
2. pREP-N1-TM hFcRn is transmembrane human FcRn in N-terminal fusion with red fluorescent protein (RFP) gene in pRFP-N1 vector. Deep red staining shows FcRn distribution in endosomal compartments.

Nucleus/DNA was stained blue with Hoechst

1.

pEGFP-N1- TM hFcRn



2.

pRFP-N1-TM hFcRn