ABSTRACT

Title of Document: **FCRN MEDIATED MUCOSAL IMMUNITY AND SUBUNIT VACCINE DELIVERY**

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FcRn, the neonatal Fc receptor, is an MHC class I related molecule, functions as an IgG protector and transporter. Binding of IgG by FcRn exclusively occurrs at acidic pH, in correlation with the fact that FcRn mainly resides in acidic endosomes. Herein, we found an association of FcRn with invariant chain (Ii). The interaction was initiated within the endoplasmic reticulum by Ii binding to either the FcRn heavy chain alone or heavy chain-beta-2-microglobulin complex and appeared to be maintained throughout the endocytic pathway. The CLIP in Ii was not required for FcRn-Ii association. The interaction was detected in IFN-γ-treated THP-1, epithelial and endothelial cells, and immature mouse DCs. A truncated FcRn without the cytoplasmic tail was unable to traffic to early endosomes; however, its location in early endosomes was restored by Ii expression. FcRn was detected in the late endosome/lysosome only in the presence of Ii or upon exposure to IFN-γ. In immature human or mouse DCs, FcRn was barely detected in the late

endosome/lysosome in the absence of Ii. Taken together, the intracellular trafficking of FcRn is regulated by its intrinsic sorting information and/or Ii chain.

Vaccine strategies to prevent invasive mucosal pathogens are being sought due to the fact that 90% of infectious diseases are initiated at mucosal surfaces. However, our ability to deliver an mucosal vaccine antigen for induction of the protective immunity is limited. FcRn mediates the transport of IgG across polarized epithelial cells. Taking advantage of this unique transfer pathway, I sought to delivery of antigens across mucosal barrier using IgG Fc fused proteins. It was demonstrated that intranasal immunization with a model antigen herpes simplex virus type-2 (HSV-2) glycoprotein gD fused with an IgG Fc fragment combination with CpG ODN adjuvant resulted in a complete protection of wild type, but not FcRn knockout mice that were intravaginally challenged with virulent HSV-2 186. The immunization induced efficient mucosal and systemic antibody as well as long lasting memory immune responses. These results are the first to demonstrate that the FcRn-IgG transcellular pathway may represent a novel mucosal vaccine delivery path against mucosal infections.

FCRN MEDIATED MUCOSAL IMMUNITY AND VACCINE DELIVERY

By

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Dedication

This dissertation is dedicated to my parents and elder sister, my wife and my son Zackery. Thank you so much for your support and patience. I love you all very much!

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Adaptor Protein	AP
Antibody dependent cell mediated cytotoxity	ADCC
antigen presenting cell	APC
B-lymphocyte-induced maturation protein 1	Blimp-1
B cell receptor	BCR
β_2-microglobulin	β_2m
bone marrow derived dendritic cell	BMDC
carboxyfluorescein succinimidyl ester	CFSE
caspase-recruting-domains	CARD
cervical lymph nodes	CLN
class II-associated invariant chain peptide	CLIP
cluster of differentiation	CD
cytotoxic T lymphocyte	CTL
dendritic cell	DC
diacylglycerol	DAG
dulbecco's modification of eagle's medium	DMEM
early endosomal Ag-1	EEA1
endo-N-acetylglucosaminidase	Endo H
endoplasmic reticulum	ER
enzyme Linked Immunosorbent Assay	ELISA
enzyme Linked Immunosorbent spot Assay	ELISPOT
Fc gamma receptor	FcγR

fluorescein isothiocyanate	FITC
follicular T helper cell	Tfh
germinal center	GC
helper $CD4^+$T cell	Th
Heavy chain	HC
horseradish peroxidase	HRP
human immunodeficiency virus	HIV
idiopathic thrombocytopenic purpura	ITP
immunoglobulin	Ig
immunoreceptor tyrosine-based activation motif	ITAM
immunoreceptor tyrosine-based inhibitory activation motif	ITIM
induced bronchial associated lymphoid tissues	iBALT
inductible nitric oxide synthase	iNOS
inositol-1,4,5-trisphosphate	IP3
interleukin	IL
interferon gamma	IFN-γ
intestinal epithelial cell	IEC
intravenous immunogloblulin	IVIG
invariant chain	Ii
isolated lymphoid follicles	ILFs
laboratory of genetics and physiology 2	LGP-2
lamina propria lymphocytes	LPLs
leucine reach repeat	LRR

lipopolysaccharide	LPS
long lived plasma cells	LLPC
lysosome-associated membrane glycoprotein-1	LAMP-1
macrophage	MΦ
major histocompatibility complex	MHC
Mannose-6- phosphate receptor	MPR
mediastinal lymph nodes	MLNs
melanoma differentiation associated gene 5	MDA-5
memory B cells	MBC
Microfold cell	M cell
mucosa-associated lymphoid tissue	MALT
nasopharynx-associated lymphoid tissue	NALT
nature kill cell	NK
nature kill T cell	NKT
neonatal Fc receptor	FcRn
Nuclear factor-kappa B	NF-κB
Nuclear factor activated T cell	NFAT
nucleotide-binding oligomerization domain	NOD
nucleotide-binding oligomerization domain-like receptors	NLRs
the paired box 5	PAX-5
pattern recognition receptor	PRR
pathogen associated molecular pattern	PAMP
peanut agglutinin	PNA

peripheral blood mononuclear cell	PBMC
peyer's patches	PPs
phosphatidlyinositol-3,4,5-trisphosphate	PIP3
protein kinase C	PKC
protein kinase R	PKR
reactive oxygen species	ROS
recombination-activating gene	RAG
retinoic acid-inducible gene-I	RIG-I
retinoic acid-inducible gene-I (RIG-I)-like receptors	RLRs
reverse transcription-PCR	RT-PCR
single stranded RNA	ssRNA
systemic lupus erythematosus	SLE
T cell receptor	TCR
toll-like receptor	TLR
T regulatory cell	Treg
trans-golgi network	TGN
Transporter of antigen processing	TAP
tumor necrosis factor	TNF
vesicular stomatitis virus	VSV

Chapter 1: General Introduction and Specific Aims

IMMUNITY

In biology, immunity describes the ability of the body combating pathogens and other unwanted biological invaders. Mammalian immunity can be classified into two main arms: innate immunity and adaptive immunity. Innate immunity is evolutionally conserved, preexists and mediates immediate responses to invading pathogens, regardless of antigen specificity. By contrast, adaptive immunity, evolved exclusively in vertebrates, is generated by the somatic recombination of germ line-encoded genes, priming and responding to pathogens in antigen specific ways. Innate and adaptive immunity were thought as operating separately in the past, but it is now apparent that innate and adaptive immunity cooperate with each other to maximally control microbial infections. Innate immunity is known to initiate, shape and influence the effectiveness of adaptive immunity, whereas adaptive immunity can facilitate innate cells to better clear the invading pathogens.

Innate Immunity

Innate immunity stands as the first line of defense against pathogen invasion. The major functions of innate immunity are (i) to mount immediate responses to detrimental microorganisms, either to limit the replication, colonization of pathogens in situ or alert neighbor cells to enter the anti-microbial states while the adaptive immunity is still in the process of being activated and expanded and (ii) to optimize

the conditions for inducing the most effective adaptive immune responses against the encountered pathogens. Overall, innate immunity is composed of a complicated range of organs, tissues, cells and molecules, such as poloarized epithelium formed physiological barriers, constitutively secreted mucus and anti-microbial molecules such as defensins, complement proteins, lysozymes and acute phase proteins, the release of reactive oxygen species and lytic granules, phagocytes underlying the epithelium, and nature killer cells infiltrated from the circulation.

Phagocytes such as macrophages and dendritic cells can recognize microbe directly through pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRR) (1). Pathogen associated molecular patterns are conserved molecular signatures among microbes of a given class, which are not produced by host (2). This lays the foundation for host innate immune system to distinguish self from invading microbes (2, 3). The best known examples of PRRs are Toll-like receptors (TLRs), which was first discovered in the *Drosophila* model in which a mutation of a receptor called "Toll" increased susceptible to fungal infection due to deficiencies in the induction of anti-fungi peptides (4). Subsequently, the human homologue of *Drosophila* Toll protein, human TLR4, was cloned and demonstrated to induce the production of inflammatory cytokines as well as the expression of co-stimulatory molecules (5). To date, a wide variety of PRRs has been identified and characterized: in human, 10 different TLRs have been found, and mouse has 12 of TLRs (6). TLRs are expressed either on the cell surface (TLR2, 4. and 5) or in endosomes/lysosomes (TLR3, 7 and 9), recognizing a wide range of PAMPs including lipid, lipoproteins, glycoproteins, glycans and nucleic acids derived from bacteria, virus, fungi and parasites (6).

Upon recognition of those PAMPS, TLRs will trigger signal cascades, inducing anti-microbial activities and inflammation (7).

Besides TLRs, another two groups of microbial detection system recently have emerged: retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs) and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs). RLRs are RNA helicase family receptors, specializing in the recognition of viral RNA replicating-intermediates in the cytoplasm and inducing the production of anti-viral type I interferons (8). In the RLR family, three receptors have been found: RIG-I, melanoma differentiation associated gene 5 (MDA5) and laboratory of genetics and physiology 2 (LGP2) (8, 9). It has been proposed that RIG-I is involved in the recognition of single stranded RNA (ssRNA) virus, such as influenza A virus, VSV, Japanese encephalitis virus and paramyxoviruses. RIG-I was found to specifically recognize ssRNA containing 5'-triphosphate moiety. Some ssRNA viruses, such as influenza and VSV, bear such unique structure in their genomes (9, 10). By contrast, host self RNA does not have such a moiety: 5'-triphosphate is either removed or masked by a cap structure. This is another example of discriminating between self and nonself employed by host innate immune system (8). MDA5 was demonstrated to mainly recognize double stranded RNAs either directly from virus or replicating intermediates of viral genomes. It has beem reported that MDA5 was required for the recognition of picornaviruses and Poly I:C (11). LGP2 has been suggested to serve as negative regulators of RIG-I and MDA5 (6, 12).

The NLR family senses the presence of PAMPs and endogenous "danger signals" within cells (13). NLRs are composed of three domains: N-terminal protein interaction domain, a central nucleotide-binding domain and a C-terminal Leucine-Rich Repeat (LRR)(14, 15). NLRs comprise of a large family of cytosolic PRRs, thus far at least 23 human and 34 murine NLRs have been found (8, 16). Some of them have been well characterized, such as NOD1, NOD2 and NALP3 (NATCH-LRR-PYD-Containing Protein 3) (15, 17). NOD1 and NOD2 can detect intracellular bacterial components, while NALP3 can be in a complex with adaptor proteins, including apoptosis-associated speck-like protein containing a CARD domain (ASC) and CARDINAL to form a multi-protein complex called "inflammasome" in response to PAMPs, or danger signals, such as heat-shock proteins, monouric acids and alum, which in turn recruits and activates caspase-1 (18-20). Caspase-1 can cleave the inactive pro-IL-1 and other IL-1 family cytokine precursor (IL-18 and IL-33), and convert them into bioactive inflammatory cytokines (18).

Innate immunity plays crucial roles in the initiation and guidance of adaptive immunity. Dendritic cells (DCs) serving as professional antigen presenting cells express a wide variety of PRRs (21-23). Innate signals activated through PRRs-PAMPs recognition controls the activation of DCs, indicated by up-regulation of costimulatory molecules, CD80 and CD86 (23-25); they also modulate the antigen process pathway by regulating the endosomal/lysosomal microenviroment and promoting the translocation of MHC II-peptide complexes from endocytic vesicles to cell surface (26). Moreover, innate signals down-regulate CCR6 but up-regulate CCR7, enabling dendritic cells to migrate to peripheral lymph nodes and present

peptides to cognate naïve $CD4^+$ and $CD8^+$ T cells (27, 28). Upon arrival at the lymph nodes, DCs migrate to T cell zone and contact with T cells in the context of MHC molecules. In the T cell zone, DCs provide naïve T cells with two signals for their activation. The first signal is the peptide loaded on the MHC molecule on DCs to be recognized by antigen specific TCRs of T cells, while the second one is the costimulatory molecules like CD80 and CD86, which will activate CD28 signal pathway on T cells. Depending on the affinity and density of presented peptides, the strength of constimulation, the profiles of cytokines secreted by DCs, naïve T cells will differentiate into Th1, Th2, Th17 cells (29-31). Furthermore, innate signals have been found to promote T cell differentiation by counteracting the inhibitory effects of T regulatory cells (32, 33). When antigen specific naïve T cells become activated and differentiate into effector cells, chemokine gradients created by innate immune responses facilitate the recruitement of effector T cells from lymphoid tissues to the sites of infection (34, 35). It has also been reported that innate signals can directly control B cell activation and Ig isotype switch (36, 37). In addition, it was suggested that innate signals can influence the generation of T cell and B cell memory (34, 38).

Innate immune response is immediate at the exact site where a given pathogen is encountered, playing essential roles in the early control of the infections. However, it is relatively non-specific, recognizing only conserved molecules shared by a broad range of pathogens. Neither can it mount memory immunity to any particular pathogens. Innate immunity is highly effective, however, many microorganisms have evolved strategies to subvert, evade, or overwhelm innate immunity. In this regard,

another arm of immunity, adaptive immunity, is required to more effectively combat those pathogens.

Adaptive Immunity

Vertebrates evolved to be equipped with a second line of defense to fight infection, collectively termed “adaptive immunity”. This immune system utilizes special mechanisms to recombine a few of germline-encoded genes to generate an extraordinary diverse repertoire of receptors on lymphocytes to specifically recognize almost all the forms of antigens. It is termed as adaptive or acquired immunity because it adapts to the initial encounter of a given pathogen. When re-encountering the same pathogen, it will mount a faster, stronger and more effective immune response. The main functions of adaptive immunity are to recognize non-self antigens by means of antigen presentation and generate a complex range of immune responses that are tailored to maximally eliminate specific pathogen or pathogen infected cells. The initial encounter also leaves behind a long-lasting immunological memory, in which each encountered pathogen is “remembered” and will be eliminated more quickly and efficiently when re-infection occurs. The adaptive immunity mainly relies on two types of lymphocytes, T lymphocytes and B lymphocytes, to perform its functions.

T lymphocyte precursors are generated during hematopoiesis from hematopoietic stem cells in the bone marrow. Subsequently, they migrate to the thymus, where they will develop into mature naïve T cells. Microenvironments of thymus are assumed to direct T lymphocytes development. The site directed

recombinases, RAG-1 and RAG-2, act to rearrange germline-encoded genes to produce T cell receptors (TCRs) (39). Once TCR is delivered to the cell surface, each T cell precursor undergoes two selective steps before maturing into naïve T cells in the absence of pathogen: positive selection and negative selection. Positive selection takes place when precursor T cells bind cortical epithelial cells expressing MHC Class I or Class II plus self peptides with a high enough affinity to accquire the survival signal. Negative selection is driven when precursor T cells further move to cortico-medullary junction of the thymus and bind to bone-marrow derived APC (macrophages and dendritic cells) expressing MHC Class I or Class II plus self peptides with too strong binding to receive an apoptosis signal. Thus, after the two-step selection, only those T cells expressing a TCR that recognizes self peptide: MHC complexes with the appropriate affinity will survive while others will be deleted by undergoing apoptosis, leading to the formation of a TCR repertoire that is weakly self-reactive but is unable to be fully activated by self-ligands (40). T cells that survive from the two-step selection exit the thymus as mature naïve T cells, and re-circulate between the lymph and blood until they encounter with cognate antigens presented on APCs in the periphery lymphoid tissues (41).

Mature T cells include αβ T cells, γδ T cells and nature killer T cells (NKT). The majority of T cells are αβ T cells, in which their TCRs are composed of α chain and β chain. In γδ T cells, the TCR is made up of one γ and one δ chain. This group of T cells is much less (5% of total T cells) than their αβ counterparts, but they are abundant in the mucosa of gut, within a population of lymphocytes called

intraepithelial lymphocytes (IEL) (42, 43). NKT cells represent a special but very small population of T cells (about 0.2% of all peripheral blood T cells) that share properties of both T cells and NK cells. In αβ T cells, the TCR is highly variable MHC restricted and can only recognize peptide on MHC molecules of APCs. Conversly, in γδ T cells, the TCR is less variable, not MHC restricted and seems to be able to recognize whole antigen rather than peptide-MHC complexes on the cell surface of APCs (42, 43). NKT cells also have αβ TCR, but their TCRs are far more limited in diversity and can not recognize peptide-MHC complexes (44). Rather, they can recognize glycolipid presented by a molecule known as CD1d on APCs (45, 46).

The αβ expressing T cells can be subdivided into two different subsets based on the expression of either CD4 or CD8 co-receptors. Naïve $CD4^+$ T cells recognize exogenous peptides loaded on MHC class II on the cell surface of professional APCs (DC, macrophages and activated B cells) via both TCR and CD4 molecule. In order to be loaded up into MHC class II molecule, antigens must be internalized into endocytic vesicles, in which a array of proteases will cleave it into short peptides with different size, only those peptides containing between 8 and 25 amino acids can be accommodated on MHC class II molecules. Upon the assembly of peptide-MHC class II complex, the MHC class II molecules direct the translocation of the complexes from endocytic compartments into cell surface. $CD8^+$ T cells can recognize endogenously produced peptide in the context of MHC class I molecules. MHC class I molecules are expressed on all nucleated cells. Cells infected by viruses or other intracellular pathogens produce viral specific proteins in the cytoplasma, where they

will be marked with ubiquitin for further processing by proteasomes (47). The processed peptides will be retrograde transported to ER by antigen processing associated transporter, TAP1 and TAP2. Within ER, they will be further trimmed by enzymes into 8 to 10 amino acid peptides, in turn they will be loaded on MHC class I molecules. Once forming the complex with MHC class I, they will transverse the Golgi apparatus and reach the cell surface to be recognized by $CD8^+$ T cells (48). Some exogenous antigens can also enter the MHC class antigen presentation pathway, which is termed as cross-antigen presentation (49, 50).

Activation of T cells needs two signals. The first signal is generated through the specific recognition of antigen, while the second signal prevents inappropriate response to self antigen as self antigen seldom couples with co-stimulation simultaneously. In the absence of costimulatory signal, T cell receptor signaling alone leads to immunologic anergy. The TCR is a complex of several proteins, consisting of TCR alpha and beta (TCRα and TCRβ) chains and CD3 proteins. CD3 proteins are composed of CD3εγ and CD3εδ heterodimers and most important, a CD3ζ homodimer, which has a total of six immunostimulatory activated motifs (ITAM). Upon the interaction between TCR and peptide:MHC complexes and the formation of immunological synapases, the ITAM motifs on the CD3ζ are phosphorylated by Lck and in turn recruit ZAP-70. Lck and/or ZAP-70 can phosphorylate the tyrosines on many molecules, including CD28, LAT and SLP-76, which allows the formation of signalling complexes. Phosphorylated LAT recruits SLP-76 to the membrane, where it can then bring in PLCγ, VAV1, Itk and potentially PI_3K. Both PLCγ and PI_3K act

on PI(4,5)P_2 on the inner leaflet of the membrane to create the active intermediaries diacylglycerol (DAG), inositol-1,4,5-trisphosphate (IP3), and phosphatidylinositol-3,4,5-trisphosphate (PIP3). DAG binds and activates some protein kinase C, most important, PKCθ in T cells, leading to a process important for activating the transcription factors NF-κB and AP-1 (51). IP3 is released from the membrane by PLCγ and activates receptors on the ER, which triggers the release of calcium. The released calcium then activates calcineurin, and calcineurin activates NFAT, which then translocates to the nucleus (51). NFAT is a transcription factor, which activates the transcription of a pleiotropic set of genes, most notably, IL-2, a cytokine that promotes long-term proliferation of activated T cells (52). Although subtle difference may exist, CD4+ and CD8+ T cells share the majority of properties of the activation pathways (51).

Once activated, naïve CD4+ T cells proliferate and differentiate into various "help" effector T cell subsets (Th1, Th2, Th17 and Tfh) depending on the signals received and cytokine microenvironment (30, 31). Traditionally, Th1 cells are thought to orchestrate cell-mediated immunity, involved in activating cytotoxic CD8+ T cells, macrophages and NK cells against intracellular pathogen infection, Th2 cells regulate the development of humoral immunity and are important in the clearance of extracellular pathogens. T "Helper" effector cells perform their effector function mainly through secreting different groups of cytokines. The hallmark cytokines for Th1 cells are IFNγ and TNFα, while IL-4 and IL-13 are the major cytokines secreted by Th2 cells. Th1 derived IFNγ promotes the activation of macrophages by up-

regulating the reactive oxygen species (ROS) and inductible nitric oxide synthase(iNOS), leading to more efficiently killing intracellular pathogens (53), IFNγ can also prime macrophage mediated antigen presentation/cross-antigen presentation by up-regulating MHC class molecules and other molecules involved in antigen presentation, such as invariant chain (54), IFNγ can induce an anti-viral state by inducing a wide array of anti-viral genes, most notably, Protein Kinase R (PKR) (53), IFNγ orchestrates leukocyte recruiment to sites of infection and enhances NK cell activity and B cell functions, such as Ig production and class switch (53, 54). Recently, certain subsets of Th1 cells have found to act direct cytolytic functions, which are previously considered as unique effects of $CD8^+$ T cells and NK cells (55-57). Th17 cells are newly characterized effector $CD4^+$ T cells, which are associated with inflammation and also play roles in host defense against pathogens (58). The recently identified follicular T helper cells (Tfh) are specialized in facilitating B cell functions, such as affinity maturation, class switch, germinal center formation and the generation of long-lived memory B cells and plasma cells (30).

Upon activation, CD8+ T cells differentiate into cytotoxic T cells (also known as Tc or CTL). When exposed peptides in the context of MHC class I molecules, T_C cells can secret the cytotoxins, perforins and the caspase activating granzymes. Perforins are known to form pores in plasma membrane of target cells.Granzymes can get entry into the target cell through the existing pores made by perforin, which in turn activate the caspase cascade, eventually lead to apoptosis (programmed cell death) of target cells (59, 60). Upon activation, Tc up-regulates the expression of the

membrane protein *FAS ligand* (FasL), which can bind to its receptor, *Fas* protein expressed on the target cell (59), leading to the apoptosis of target cells. Activated $CD8^+$ T cells can also secrete a variety of cytokines, like IFNγ, TNFα and IL-2 etc, which has anti-viral and tumor functions (61, 62).

B cells represent another subset of lymphocytes in adaptive immunity, which play essential roles in humoral immunity. Unlike T cells, which mature and undergo positive and negative selection in the thymus, B cells development mainly occurs in bone marrow. B cell development needs several successive stages, from Pro-B cell, to Pre-B cell and immature B cells, and finally mature B cell, with each stage different in the antibody loci (63). Distinct from TCR, B cell receptor (BCR) is composed of membrane immunoglobulin (mIg) that contains two identical light (L) and heavy (H) chains with the 'V' (Variable) region and the 'C' (Constant) region. In the heavy-chain 'V' region, there are three segments: V, D and J. These segments recombine randomly, in a process called VDJ recombination, to produce a unique variable domain in the immunoglobulin of each individual B cell. Similar rearrangements occur in light-chain 'V' region except there are only two segments involved: V and J (39). When becoming mature, B cells exit bone marrow and migrate to peripheral lymphoid tissues.

B cells use BCR to detect antigens. BCR, representing the surface-expressed immunoglobulins, recognizes unprocessed soluble or membrane-bound proteins and other forms of antigens, while TCR recognizes processed peptide in the context of

MHC molecules. When encountering its cognate antigen, B cell can use its receptor to internalize antigen; within B cells, internalized antigen can be processed and presented to $CD4^+$ T helper cells. In most cases, the signal from antigen stimulation alone is not enough but needs to couple with the second signal from activated T helper cells for complete activation of B cells to start class switching and produce high affinity antibodies. Most antigens are T cell dependent, meaning that the second signal (co-stimulation) from helper T cells is required for maximal antibody production (64). Co-stimulation includes, but is not limited to, CD40-CD40L interaction between B cells and T cells, cytokines secreted from activated T helper cells, like IL-2 (64). Recently, a new subset of helper T cells, termed as follicular T help cells (Tfh) has been identified to specialize in helping B cells to be activated and differentiate into high affinity antibody producing cells in the germinal center. Most notably, those T helper cells can mediate the somatic hypermutation of the variable regions of immunoglobulin genes. This process will result in the increase of affinity of the BCR to its cognate antigen (65). Furthermore, only the population of B cells with BCRs of high affinity for a given antigen can be selected to survive and further differentiate into high affinity antibody producing plasma cells or memory B cells (66). Another type of antigens are called “T cell independent antigen”, representing that they can induce B cells to produce antibodies independent of T cell help. For example, many bacteria have repeating carbohydrate epitopes that directly stimulate B cells by cross-linking the IgM antigen receptors in the B cell, resulting in IgM synthesis in the absence of T cell help (67).

Upon activation and class switching, B cells differentiate into effector B cells, termed plasma cells. Plasma cells are terminally differentiated cells, specializing in producing antibodies. They are distinct from their precursors of many aspects: they cannot switch antibody classes, cannot act as antigen-presenting cells because they no longer display MHC-II, and they do not take up antigen because they no longer display significant quantities of immunoglobulin on the cell surface; they express relatively few surface antigens, and do not express common pan-B cell markers, such as CD19 and CD20 (68); they mainly reside in bone marrow with a long life span, some can even live all the life-long (69, 70). Four of structurally and functionally distinct antibodies are produced by plasma cells: IgG, IgE, IgM and IgA, but each individual plasma cell can only produce a single type. Those antibodies are essential players in mediating humoral immunity with broad diversity and magnitude.

Immune Memory

Upon the first encounter with a pathogen, lymphocytes generally undergo three distinct phases: (1) during the effector phase, lymphocyte precursors dramatically divide, expand and acquire effector functions, migrate to sites of infection and act on pathogen clearance; (2) during the contraction phase, companying with the resolution of infection, most effector cells die by apoptosis, leaving behind 5%-10% of the initial burst pool as memory lymphocytes; (3) during the phase of memory maintenance, memory lymphocytes are maintained as homeostasis for long years. Immune memory is the cardinal feature of adaptive immunity. The antigen-experienced memory cells exhibit with much higher frequency than naïve

counterparts and have a decreased threshold for re-activation. Thus, when re-exposure to the same pathogen, it will induce an accelerated, more robust immune responses, leading to either entirely preventing from infection, or more efficient clearance of infection or dramatically decrease the severity of clinical diseases; such unique character of memory immune responses lays the fundamental basis for vaccination.

Both humoral and cellular immune responses are equipped with the important capacity of generating immunological memory, and have evolved to perform fundamentally distinct effector functions. The humoral memory is composed of memory B cells and long-lived plasma cells, while cellular immune memory include long-lived central $CD4^+$ T cell memory and short-lived effector $CD4^+$ T cell memory, and $CD8^+$ T cell central and effector memory. Although T cells and B cells contribute to protective immunity from the re-infection through fundamentally distinct effector functions, their memory constitutes share many similar characteristics. These include but not limited to the generation of memory pools from activated precursors, heterogeneity in function, anatomical location and phenotype.

Memory B cells (MBCs) and long lived plasma cells (LLPCs) constitute of humoral immunological memory. MBCs are primarily located in secondary lymphoid organs, display higher frequencies in comparison with naïve B cells; they can not actively produce antibodies but express higher affinity BCRs (71). They respond to re-infection by rapidly dividing and differentiating into antibody-secreting cells, while simultaneously replenishing the MBC pool (72). In contrast, LLPCs reside

mainly in the bone marrow; LLPCs are terminally differentiated cells, constitutively producing antibodies in the absence of antigen stimulation (69). Following initial contact with antigens, naïve B cells proliferated at marginal zone of lymph node. Then, activated B cells either retain in the marginal zone and differentiate into short-lived plasma cells (mainly producing IgM), or enter the B-cell follicles, subsequently $CD4^+$ T-cell help (most likely, Tfh cells) coordinated follicular B cells to initiate a germinal center (GC) reaction (72). In the GC, activated B cells undergo somatic hypermutation, affinity maturation and selection, eventually resulting in the generation of high affinity MBCs and LLPCs. In the absence of $CD4^+$ helper T cells, or without CD40, CD28 or inducible costimulatory signals from Th cells, humoral immune responses are highly impaired and fail to generate GCs, MBCs and LLPCs (73, 74). CD40 signal and transcriptional factor PAX5 favor activated B cells differentiated into MBCs (75). Conversely, transcriptional factors Blimp-1, XBP-1 can drive activated B cells to differentiate into LLPCs (76, 77). However, the exact programs governing both distinct differentiations are still poorly understood. Both MBCs and LLPCs can be maintained for a long time even in the absence of antigen (78). But factors that influence the homeostasis of MBCs remain largely unknown. Bone marrow niches are thought to provide LLPCs with survival signals, such as the bone marrow stromal cell-derived IL-6 and VLA-4 (79). In human, MBCs can maintain for decades and PPLCs are believed to be sustained for life long (80, 81).

Memory T cells (both $CD4^+$ and $CD8^+$) include two different cell types: effector memory T cells (T_{EM}) and center memory T cells (T_{CM}) (82). T_{CM} mainly reside in

lymphoid tissues, expressing lymphoid tissue homing chemokine receptor CCR7 and CD62L; T_{CM} exhibit stem cell like qualities of self-renewal and respond to antigen by rapidly dividing and differentiating into effector cells; In contrast, T_{EM} ($CCR7^-$ $CD62L^-$) mainly reside at peripheral tissues and mount the immediate effector response when re-infection occurs (83). Thus, both T_{EM} cells and T_{CM} cells play essential roles in the protective immunity depending on the nature and route of infection. The conventional model of memory T cell differentiation is the linear differentiation model, which was proposed that memory cells are derived directly from effector cells (84-86) . However, the differentiation pathway of memory $CD4^+$ T cell is still controversial. Similar to MBCs, memory T cells can maintain their numbers in the absence of antigen stimulation by way of homeostatic proliferation. Furthermor, cytokines, like IL-7, IL-21 and IL-15, are demonstrated to be involved in this process (87-89).

Immunoglobulins and Their Receptors

Overview

Immunoglobulins are the key mediators of humoral immune responses. Immunoglobulin possesses a wide variety of effector functions in anti-infection immunity as followings: (1) stand as the first line of defense against infection by neutralize invading pathogens; (2) fix particulate antigen and activate complement cascades; (3) opsonize microorganisms to facilitate the phagocytosis by phagocytes; (4) flag-pathogen infected cells for NK cell mediated ADCC activity; (5) enhance the antigen presentation of invading pathogens on APCs. The broad and diverse effector functions of immunoglobulin depend on its specific

and high affinity binding to antigen and immunoglobulin Fc receptors expressed on the effector cells. In total, there are five isotypes of immunoglobulins in mammals: IgM, IgD, IgG, IgA and IgE. IgM and IgD, serving as B cell receptor (BCR), are expressed on the surface of naive B cells and play essential roles in the activation of B cells. Pentameic IgM is also the earliest antibody to be secreted upon infection or vaccination. The secreted form of IgD is very unusual in the serum in contrast to other isotypes. IgG is the predominant immunoglobulin in serum, lymphatic fluid, peritoneal fluid and cerebrospinal fluid. It accounts for 75% of total serum immunoglobulin (over 30 mg/kg/d). Most notably, IgG is the only immunoglobulin that can pass through the human placenta to provide protection to the fetus. The majority of synthesized IgA are secreted to bath the vast area of mucosal surfaces. IgE is present in serum at the lowest concentration among all isotypes of immunoglobulin. It plays a vital role in the clearance of parasites, while also resulting in the unfortunate consequence of allergy.

Immunoglobulin exerts the maximal effector functions primarily depending on Fc receptors expressed on leukocytes or epithelial cells. The Fc receptors expressed on phagocytes such as neutrophils and macrophages can facilitate the phagocytosis of antibody opsonized pathogens and activat the maturation of phagosomes to destroy phagoctyosed pathogens (90); Fc receptors on NK cells can mediate ADCC to induce apoptosis of infected cells (90, 91); Fc receptors on B cells can regulate antibody responses (92); Fc receptors expressed on polarized epithelial cells can mediate the transport of certain isotype of immunoglobulin to mucosal lumens (93).

Polymeric immunoglobulin receptor (pIgR) is the receptor for both IgM and IgA, responsible for the trans-cellular delivery of IgM and IgA to the mucosal surfaces. The secreted IgA (sIgA) has been known as the predominant immunoglobulin in the mucosal secretion. The sIgA is produced by plasma cells residing in the lamina propria as dimmers associated with a 15-kDa J chain. Dimeric IgA (dIgA) binds to pIgR expressed on the basolateral domain of poloarized epithelial cells, and the IgA-pIgR complex is shuttled from basolateral to apical surfaces, where proteolytic enzymes cleave the pIgR to release sIgA to the lumen (94). Once in the secretions of the lumen, IgA can bind to pathogen particles, inhibiting their attachment with susceptible epithelial cells, thus blocking subsequent infections (95, 96). In addition to neutralizing viruses in the lumen, it has been proposed that IgA can also neutralize viruses within epithelial cells during its transcytotic pathway (97, 98). Moreover, it has been reported that pIgR can move IgA-virus complexes from basolateral surface to apical lumen to attenuate the viral infection in the lamina propria (99). Besides pIgR, there is another receptor, known as Fcα receptor (FcαR, CD89) for IgA. FcαR is expressed on the cell surface of neutrophils, monocytes, and eosinophils, which can mediate the phagoctosis and release of inflammatory cytokines upon interacting with IgA-opsonized targets (93, 100).

There are two receptors for IgE, the high-affinity Fcε receptor I (FcεRI) and the low -affinity FcεRII (CD23). The high-affinity receptor FcεRI is a member of the immunoglobulin superfamily (it has two Ig-like domains), composed of one α chain associated with one β chain and two γ chains. FcεRI exhibit extremely high affinity to IgE (Kd~10^{-10} M), resulting in readily binding to monomeric IgE molecule even at very low concentration (101). FcεRI is

expressed on epidermal Langerhans cells, eosinophils, mast cells and basophils (102). When cross-linking of IgE that is pre-bound to FcεRI by cognate antigen, mast cells or basophils are activated that is triggered by ITAM on the C-terminal of β chain and γ chain of FcεRI, resulting the degranulation and ensuing immediate hypersensitivity reactions (101, 102). FcεRI can also be found on antigen-presenting cells (DCs and Macrophages), and is believed to mediate the production of inflammatory cytokines (101). The low-affinity receptor FcεRII (CD23) is indeed a C-type lectin. It exists in two isoforms: CD23a and CD23b (101). They are widely expressed by monocytes, macrophages, dendritic cells, mast cells, eosinophils, epithelial cells and B cells. FcεRII has multiple functions as a membrane-bound or soluble receptor; it controls B cell growth and differentiation and blocks IgE-binding of eosinophils, monocytes, and basophils (103).

IgG and Fcγ Receptors

Immunoglobulin G (IgG) is known as the Ig isotype with the highest concentration and longest half-life in the serum. IgG has four different subtypes: IgG1, IgG2, IgG3 and IgG4 in humans (IgG1, IgG2a, Ig2b and IgG3 in mice). Those distinct subtypes are not produced randomly, but depending on the milieu in which B cells are activated and differentiated. For example, in mice, Th1 immune responses are believed to induce B cell producing IgG2a or IgG3, while Th2 immune responses mainly induce the production of IgG1.The different subtypes differ from each other with different structures, including the length and flexibility of the hinge region and the complexity of Fc fragment. The different structure results in distinct binding

properties between Ig subtype and their receptors, IgG Fcγ receptors (FcγR), which determines the outcomes of IgG mediated immunity (**Table I.I**).

FcγRs belongs to the immunoglobulin superfamily. To date, four different FcγRs, FcγRI, FcγRIIB, FcγRIII and FcγRIV, have been found in mice. More complicated, several orthologous proteins in humans have also been identified, known as FcγRI, FcγRIIA, FcγRIIB, FcγRIIC, FcγRIIIA, FcγRIIIB (104). The FcγRs can be distinguished from each other by the affinity for the ligands, the membrane adaptor proteins they are associated and the signaling pathways they induce. FcγRI (CD64) is composed of a ligand-binding chain associated with the homodimer of a signal-transducing-γ chain. Within the extracellular region of the FcγRI α chain, three Ig-like domains exist while other FcγRs only have two such domains (90). This unique characteristic makes FcγRI as the only high affinity FcR with Kd~10^{-9}M, allowing it bind to monomeric IgG molecule (104). FcγRI is an active receptor, widely expressed on innate effector cells, such as monocytes, macrophages, dendritic cells and neutrophils. Upon cross-linking by antigen, the ITAMs on the γ chain of FcγRs are phosporylatad by Src kinase family, resulting in the recruitment of SYK-family, followed by the activation of various downstream molecules, finally leading to the activation of innate effector cells (105, 106).

Table I.I Characteristics of the IgG Isotypes.

CHARACTERISTICS OF IgG	
Subclasses	**IgG1, IgG2, IgG3, IgG4 (human)** **IgG1, IgG2a, IgG2b, IgG3 (mouse)**
Concentration in Sera	**IgG1 > IgG2 > IgG3 > IgG4 (human)**
FcγR Binding	**High affinity receptor -can bind monomeric IgG** **FcγRI: IgG3, IgG1 >IgG4>>IgG2 (human)** **IgG2a>IgG2b, IgG3>>IgG1 (mouse)** **Low affinity receptors –bind IgG-immune complexes** **FcγRII: IgG1, IgG2, IgG3 (human)** **FcγRIII:IgG1, IgG3 (human)** **FcγRIV:IgG2a, IgG2b >> IgG1, IgG3 (mouse)**
FcRn Binding	**IgG4>IgG1>IgG3>IgG2 (human)** **IgG2a>IgG1>IgG2c>IgG2b (rat)** **IgG2a>IgG1>IgG3>IgG2b (mouse)**
Th Response	**Th1: IgG1, IgG3 (human)** **IgG2a (mouse)** **Th2: IgG4 (human)** **IgG1 (mouse)**

FcγRIIb (CD32) is an inhibitory and low-affinity receptor, which contains an ITIM motif within its cytosolic domain. Unlike FcγRI, FcγRIIb only has the ligand-binding a chain. When stimulated with IgG immune-complex, the ITIM motifs of FcγRIIb are phosphorylated, followed by the recruitment of phosphotase SHIP-1 and SHP-1, leading to the inhibition of FcR signalings (107). In B cells (only expressesing the inhibitory FcγR), FcγRIIB has been reported to be involved in the inhibiting B cell activation, inducing B cell apoptosis and regulating plasma-cell survival (103). Like FcγRI, all other low-affinity FcγRs (Kd ~10^{-6}M) are activating receptors; they share very similar signaling pathways either through the ITAMs on α chain of its own (FcγRIIA and FcγRIIC in human) or through associated with signal-transducing γ chain (FcγRIII in mice and FcγRIIIA in human) after receptor cross-linking by immune complexes. Those receptors are differentially involved in a variety of cellular functions, including, but not limited to phagocytosis, endocytosis and antigen presentation of immune complexes on different types of cells. Notably, FcγRIIIA (FcγRIII in mice) are exclusive FcγRs expressed by NK cells, mediating the potent antibody-dependent cytotoxicity (ADCC) reactions, leading to the killing of antibody marked infected cells by apoptosis (104, 108).

The Neonatal Fc Receptor, FcRn

For decades, it has been known that IgG predominates in the serum and maternal IgG can be transported to immune-incompetent fetus or newborns. Over 45 years ago, Brambell et al. proposed a hypothesis that there existed cellular receptors that can

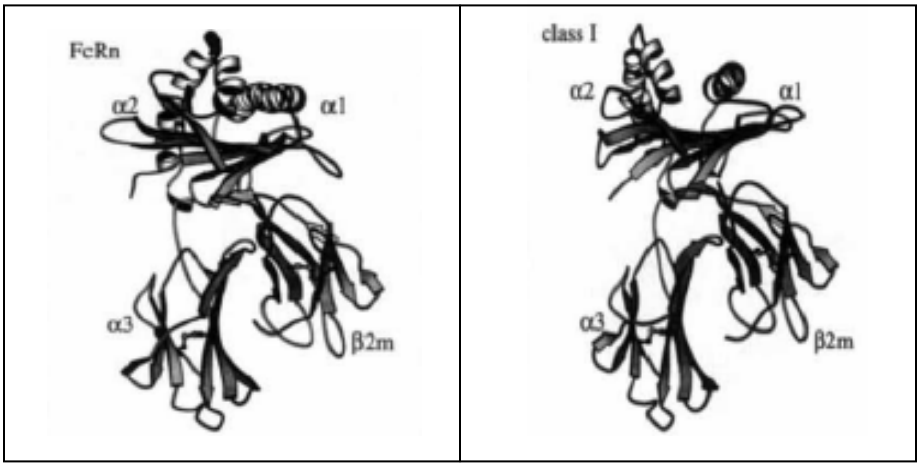
bind and recycle pinocytosed IgG back into the serum (109); In addition, they proposed that these receptors were involved in the transmission of maternal IgG to fetus or newborns. The putative receptors were believed to be saturable, so that any receptor un-bound IgG following pinocytosis were destined for degradation (109). This hypothesis was put forward in the absence of any knowledge of the receptor or cell type. However, it explained the homeostasis of IgG in the serum. Until 1989, Simister and Mostov first cloned the neonatal Fc receptor (FcRn) gene from neonate rats (110). To date, it is widely accepted that FcRn is the corresponding receptor predicted by Brambell, responsible for IgG homeostasis and transcellular transmission.

Simister and Mostov had found that rat FcRn was a heterodimer, comprised of β2-microglobulin and a protein with molecular weight 45-53 kDa (110). Association with b2-microglobulin suggested that FcRn heavy chain might be a major histocompatibility complex (MHC) class I related protein because it was common to all MHC class I molecules. They confirmed this possibility by sequencing FcRn gene and then comparing with MHC class I molecule. It turned out that all three extracellular domains and the transmembrane domain of FcRn shared sequence similarity with corresponding domains of MHC class I (110). This was surprising because it was the first time to assign an MHC class I related molecule as an IgG receptor. Despite the fact that FcRn is MHC class I-like protein, the α heavy chain of FcRn is encoded outside of the MHC gene complex (111).

The X-ray crystallographic structure of the FcRn heavy chain revealed by Bjorkman et al further confirmed that the overall three-dimensional structure of FcRn resembles the structures of molecules of MHC class I family (**Figure 1.1**)(112). However, the antigen binding groove in FcRn is rearranged, primarily due to the presence of proline at position 165 of the α2 domain, resulting in the occlusion of the peptide binding platform (112, 113). This correlates with the functional divergence between FcRn and MHC class I: FcRn functions as the IgG Fc receptor while MHC class I is involved in the antigen presentation.

To date, the genes encoding FcRn alpha chains have been isolated in many different mammalian species (114). It has been found that all of those FcRn sequences share homology (114, 115). This leads to the suggestion that mammalians may use the same strategy to maintain IgG homeostasis and IgG transport. Notably, the identification of FcRn functionally expressed in human syncytiotrophoblast indicated that FcRn plays a role in the placental transport of IgG from mother to fetus (116). More recently, Bjorkman and colleagues found that chickens employ a distinct receptor to transport IgY antibody across the yolk-sac membranes into the developing egg to confer passive immunity to the young (117); this receptor is known as FcRY, which is structurally unrelated to mammalian FcRn and MHC class I molecule. The fact of distinct receptors with similar functions in mammalians and chickens suggests that IgG transport by FcRn is a recent adaptation of the MHC class I family (117, 118).

A.



B.

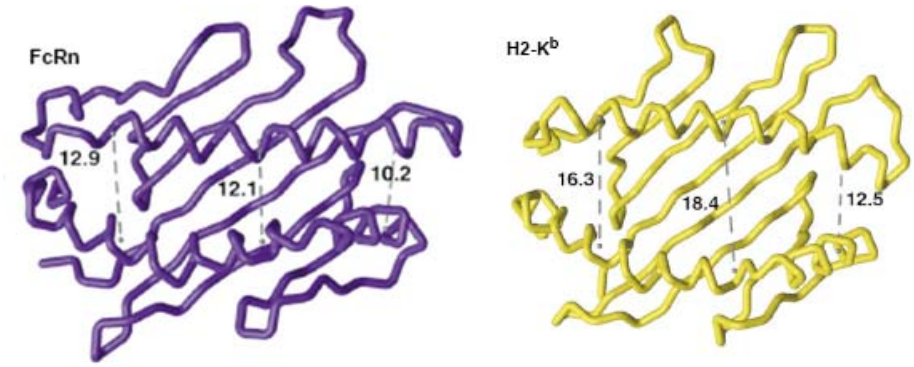


Figure 1.1. The structural comparison between FcRn and MHC class I. A. the overall structure of FcRn and MHC I, from Burmeister et al., ***Nature*** 372**,** 336 - 343 (1994); **B.** the α1-α2 domain of FcRn and MHC I, from Zeng et al., 1997, **Science**: 277:339-345.

The Analysis of IgG-FcRn Interaction

In contrast to other FcγRs, FcRn binds to the Fc region of IgG in a strictly pH-dependent manner (118, 119). At physiological pH7.4, FcRn can not interact with IgG at detectable levels, but at acidic pH condition (pH6.0-6.5), FcRn binds to IgG with high affinity with Kd~10^{-9}M. FcRn does not exhibit a dramatic conformational change upon binding to IgG (120). The pH dependent binding between FcRn and IgG is indispensible of histidine residues in the CH2-CH3 interface of IgG and their subsequent interaction with acidic residues on the surface of FcRn (120). From the side of IgG, mapped by using site-directed mutagenesis, several Ile253, His310 in CH2 domain of IgG have been found as the key amino acids involved in the interaction of IgG with FcRn, but the His435 in CH3 played a more significant role in the mouse FcRn-IgG interaction (121, 122). Plus, the sequence -H-N-H-Y (AA 433-436) of the CH3 domain is important for pH-dependent binding and these residues are found to be highly conserved across species (123, 124). Further analyses by employing mutated recombinant Fc fragments demonstrated that amino acids at position 257 and, to a lesser extent, positions 307 and 309 in proximity to the CH2-CH3 domain interface also play a role in the FcRn-Fc interaction. However, the role of residues 257, 307, and 309 is relatively less than that of Ile253, His310, and His435 (123, 125, 126). The amino acids of Fc region involved in the binding to FcRn were summarized in **Table I.II**. Furthermore, it has been found that fragment B of staphylococcal protein A shares the identical binding sites on Fc region with FcRn, leading to the competitive IgG binding between FcRn and IgG (127).

Crystallographic structure on the FcRn-Fc complex has revealed that the anionic residues Glu117, Glu132 and Asp137 on the α2-helix of FcRn form salt bridges with these protonated histidines of IgG at acidic pH (126). Besides, the hydrophobic Ile 253 of IgG engages with Trp133 of FcRn; Furthermore, Ile1 of β2-microglubolin was also found to be involved in bind to IgG. Thus, both α2 domain of FcRn and β2-microglubolin are crucial for the pH dependent binding of IgG (121, 126). Site-directed mutagenesis and crystallography structure studies have suggested that the FcRn and FcγRs bind to Fc region of IgG at distinct sites (128, 129). The residues in the lower hinge region of Fc are critical for IgG binding to FcγRI, FcγRIIa and FcγRIII (130, 131). Other residues close to lower hinge spatially, such as Pro331 and Glu318 on Fc are also involved to bind to FcγRs (130, 131).The N-linked glycosylation of Fc region of IgG plays vital roles for FcγR binding, which is not necessary for FcRn binding (104, 132). Thus, unlike FcRn which bind to Fc via CH2-CH3 region, FcγRs interact with IgG mainly through the interface between the lower hinge region and the upper CH1 domain (104).

Table I.II Key amino acids of IgG sequences in the Fc region involved in the binding of FcRn (124)**.**

	Position	252	253	254	255	256	257	307	308	309	310	311	433	434	435	436
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
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
	IgG2a	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

FcRn transports IgG Accross Mucosal Barriers

Of the five antibody class, IgG has long been known to be the only one that is actively transported from mother to offspring (133-135). Later on, the FcRn was found to perform such unique function (110). In human, the offspring acquires maternal IgG mainly through placenta. FcRn expressed on placental syncytiotrophoblasts can accumulate IgG in the endosomes via pinocytosis (136, 137). The IgG-containing endosome is then gradually acidified thereby allowing IgG to bind tightly to FcRn present in this compartment. The vesicle then fuses with the membrane on the fetal side of the syncytiotrophoblast, where the physiological pH promotes the dissociation of IgG from FcRn (116, 137, 138). Nevertheless, neonates in rodents accquire maternal IgG via the intestine (133, 134). Maternal IgG from ingested milk passes through the stomach of rodent neonates, in turn maternal IgG contained in the slightly acidic stomach contents reaches the duodenum, where IgG is bound to FcRn that is most efficiently expressed by the epithelial cell lining the intestine prior to the weaning (122). The FcRn-IgG complexes are then internalized by receptor-mediated endocytosis, subsequently transcytosed across the polarized barrier, eventually release IgG at the lamina propria, where it is at physiological pH (122, 139). In β2-microglobulin$^{-/-}$ or FcRn $^{-/-}$ mice, no maternal IgG was transported (140, 141). These studies confirm that FcRn is the receptor involved in the transport of IgG from mother's milk to newborns. More recently, it was reported that there is also a minor route for IgG transport in rodent. The successful cloning FcRn from rat yolk sac endoderm indicates that this Fc receptor is also involved in the maternal IgG transfer in rodents (142).

Notably, the FcRn expression pattern in the intestine is different between human and

rodent. In humans, FcRn is constitutively expressed by intestinal epithelial cells regardless of age and developmental stages (143, 144). The intestinal expression of FcRn in rodent is highest on the epithelial cells of the proximal small intestine during the neonatal period and declines rapidly to unappreciable level after weaning (139, 145). The intestinal expression of FcRn in adult human suggests additional roles in FcRn besides the maternal IgG transfer. One possibility is that FcRn can transport IgG from lamina propria to the intestinal lumen. This is the novel function for FcRn because previous evidences focused on the transport from the apical to basolateral direction. Subsequently, several in vitro experiments using intestinal and other cell lines have confirmed the possibility that FcRn can transport IgG across polarized epithelium bi-directionally (146-148).

To date, bi-directional transport of immunoglobulin is a unique function solely discovered for FcRn among all other Fc receptors, including pIgR. This suggests that FcRn may transport pathogen specific IgG induced by infection or vaccination to mucosal sites to protect host from mucosal infection. Furthermore, more recently, Blumberg and colleagues showed that human FcRn can transport IgG–bacteria complexes across the epithelium barrier by using a human transgenic mice model (149). Human FcRn in the mouse intestine was able to carry the immune complexes across the epithelial layer, releasing the immune complex at lamina-propria, which in turn was captured by DCs. DCs then efficiently processed and presented antigen to antigen-specific T‑cells in the draining lymph node (145, 149).

FcRn Protects IgG from Degradation

IgG possesses the longest half-life (6-8 days in mice and 21 days in human) as well as

highest concentration in the serum (1-2mg/ml in mice and 10-12mg/ml in human) relative to all other four classes of immunoglobulin. FcRn is believed to function to maintain IgG homeostasis. This was proved by showing that FcRn knockout mice or β2-microglobulin knockout mice have significantly lower IgG levels in serum (141, 150, 151). Furthermore, the IgG half-life in the serum of knockout mice decreased dramatically to 1 day compared to 6-8 days in wild type mice (141). It has been proposed that IgG of high concentration in the serum is pinocytosed into acidic endosomes, where FcRn will capture pinocytosed IgG from the serum; Subsequently, FcRn will recycle IgG back to the plasma due to the physiological pH condition. For those unbound IgGs, they will be destined to degradation in the lysosome (152). To some extent, this hypothesis has been confirmed by several findings: (1). FcRn resides mainly in early endosomes, rather than at cell surface (152, 153); (2). Recombinant IgG with FcRn binding site mutations was found mostly in lysosomes and eventually degraded revealed by confocal microscopy analysis (154, 155); (3). In the in vitro experiment, living images have been performed to track IgG salvage pathways successfully (156, 157).

Although FcRn is widely accepted as an IgG salvager, until recently it has been shown that FcRn extend the IgG half-life carrying out on both hematopietic monocytes, macrophages, dendritic cells as well as endothelial cells (158, 159). Despite monocytes, macrophages and dendritic cells have been long found to express FcRn, only recently they were reported to be involved in the IgG homeostasis (159). By taking advantage of irradiated bone-marrow chimeric mice, it was observed that bone-marrow-derived cells can protect IgG from degradation (158). Endothelial cells have the most possibility to become sites of IgG

protection. However, its definite role in IgG salvage could not be confirmed due to the lack of endothelial FcRn specific knockout mice. Most recently, this issue has been addressed by Ward and colleagues (159). They generated endothelial FcRn knockout mice, and as expected, the IgG level in mice with conditional deletion of FcRn in endothelial cells was significantly lowered (159). Thus, endothelial cells as well as myeloid monocytes, macrophages and dendritic cells play vital roles in the homeostasis of IgG in the serum.

Macrophages and dendritic cells are known as professional antigen presentation cells. It is interesting to probe whether FcRn on those cells are involved in the antigen presentation of immune complex besides their role in IgG protection. Indeed, a recent report demonstrated that FcRn can promote the antigen presentation of immune complex on DCs to $CD4^+$ T cells (160). In FcRn knockout mice, the presentation of immune complex was largely impaired (160). However, the underlying mechanism needs to be further investigated.

Recently, FcRn has also been reported to bind albumin and extend its half-life (161). Albumin is the most abundant plasma protein with long half-life in the serum, accounting for 70% of the total serum protein. It is not unusual if a receptor is involved in the maintenance of such high level of serum protein. Indeed, FcRn has been proved to play such a role. In FcRn-deficient mice, the serum albumin concentration is reduced to 40% of the normal level and the half-life of albumin is shortened to 1 day from 6-8 days in the wild type mice (161). It is intrigueing that FcRn uses the distinct sites to bind albumin as compared to binding to IgG. Furthermore, FcRn binding to albumin is also pH-dependent. Therefore, FcRn can simultaneously protect both proteins of high concentration without competition in the serum

(162-164).

FcRn Trafficking to Endosomes Is Critical For FcRn Function

FcRn are widely expressed on a variety of cell types, including epithelial, endothelial and antigen presenting cells, governing IgG homeostasis and trancellular transport (118, 153). FcRn binding to IgG exclusively occurs at acidic pH condition (119, 121). It correlates that FcRn mainly resides in acidic endosomes at steady state (152, 153, 155). It is logical to conclude that in order to perform IgG protection and transcytosis function, FcRn has to traffick into endosomes either directly from trans-golgi-networks (TGN) or endocytosed from cell membrane. Nevertheless, the mechanisms of the regulation of FcRn trafficking into endosomes are not fully understood.

Trafficking of transmembrane proteins to endosomes depends on sorting signals within the cytosolic domains of the protein (165). They are shown as short and linear sequences of amino acid residues. So far, several sorting signals responsible for endosomal traffic have been identified. One is referred to as tyrosine-based sorting signals, characterized as the NPXY or YXXϕ consensus motifs (166, 167). These motifs are phosporylated, then recognized by the adaptor protein complexes, like AP-1, AP-2 and AP-3 and AP-4 (168-170). A large array of endosomal proteins contain tyrosine based sorting signals. For example, LDL receptor and insulin receptor contain NPXY-type sorting signals, whereas LAMP1, CD63, CD1d and transferrin receptor possess YXXϕ–type signals (171, 172). The second type of endosomal sorting signals is known as dilucine-based signal, consisting of [DE]XXXL[LI] or DXXLL consensus motifs (172-174). [DE]XXXL[LI] motif is believed to

be recognized by adaptor protein complexes, whereas DXXLL is in engagement with another family of adaptors known as GGAs (174). A large group of endosomal proteins contains such sorting signals, such as LamP, LIMP II, invariant chain ([DE]XXXL[LI]) and CI-MPR, CD-MPR, GGA (DXXLL)(175). In addition to sorting signal peptide motifs, unbiquitination of the lysine residune within the cytoplasmic domain of the protein is another type of sorting signal (176, 177). This machinery may regulate the endocytosis of cell membrane protein to endosomes, like some cell surface receptors, EGF, MEF and CSF-1 (177). Some proteins, such as MHC II, do not contain any sorting signals within their cytoplasmic domain, but they are able to reside in endosomes by forming the complexes with accessory proteins which possess sorting signals, like invariant chain which contains a dilucine based sorting motif (178).

Presumably, two factors may influence the endosomal trafficking of FcRn. First, the cytoplasmic domain of FcRn contains the putative diluencine motif-based endosomal sorting singal: GDDTGVLLP, which is DE]XXXL[LI] type (179). Such signal sequence is conserved across different species, including human, mouse, rat bovine and porcine, suggesting its importance in FcRn trafficking (180). Second, FcRn may also form complexes with partner protein, which may be involved in the regulation of FcRn trafficking. The first hypothesis was partially resolved by Neil and colleagues (179). They reported that both dilucine and tryptophan based motifs were involved in the endocytosis of FcRn, but it was lack of direct evidence that such signals were also responsible for the sorting of FcRn from TGN to the endosomes (179). Thus, both hypotheises need to be further investigated.

Modulating the Interaction of IgG with FcRn

Because FcRn functions as IgG salvager and transporter, it is apparent that FcRn can be targeted to modulate the IgG level or improve the pharmacokinetic efficacy of therapeutic antibodies or deliver therapeutic agents in conjugation with Fc fragment to specific tissue or organs. A wide variety of autoimmune diseases are caused by excessive pathogenic self antibodies, such as myasthenia gravis, bullous pemphigoid, idiopathic thrombocytopenic purpura (ITP) and systemic lupus erythematosus (SLE). It is possible to modulate the IgG-FcRn interaction to decrease the pathogenic antibody level. Several attempts have been made to test this hypothesis. The first method is to administer excessive innocuous IgG. The high dosage of exogenous IgG will compete with endogenous pathogenic IgG, leading to the saturation of FcRn by exogenous IgG. As a result, endogenous pathogenic IgG will drop off from FcRn and destine to degradation in the lysosome. In humans, high dosage of intravenous immunogloblulin (IVIG) has been approved for clinical use in treating autoimmune diseases (181). In mouse models, IVIG treatment has been shown to decrease pathogenic antibody levels and can interfere with the interaction between FcRn and pathogenic antibody (89). Another method to attenuate the FcRn-pathogenic antibody interaction is to employ FcRn specific antibody. It has been recently reported that a monoclonal antibody specific for FcRn heavy chain has been described to alleviate the disease symptoms in rats with experimentally induced myasthenia gravis (182). The third approach is to engineer Fc mutants with increased affinity to FcRn. Administration of such Fc mutants will out-compete endogenous antibodies. Indeed, humanized IgG monoclonal antibodies with unusually high affinity to FcRn leads to the degradation of endogenous antibodies and attenuate the arthritic lesions caused by pathogenic IgG (183). Last, some

screened small molecules or peptide inhibitors of the FcRn-Fc interaction would be promising to be exploited to decrease the severity of antibody caused autoimmune diseases (184).

Mucosal Barriers and Potential Roles of FcRn In mucosal Immunity

The Organization of Mucosal Barrier

Mucosal epithelia, such as in the gastrointestinal, respiratory, and genitourinary tracts, form an interface between the external environment and the internal milieu of the body. The polarized epithelial cells, lining these mucosal cavities contains stratified and single cell layers (94, 185). They have two separate plasma membrane domains, apical and basolateral, which are separated by tight junctions. These domains further form barriers that allow for the selective exchange of macromolecules between the lumen and submucosal tissue under physiological conditions. Specifically, the transcellular pathway involves endoctyic uptake of macromolecules, generally by the receptor mediated and/or fluid-phase endoctytosis, at the apical or basolateral membrane. These molecules are then transported through the cell in endoctyic vesicles to the opposite membrane surface where they are released into the extracellular space. Therefore, the transcellular pathway is a major route in moving soluble macromolecules across the mucosal epithelial barrier. Besides transcellular pathway of macromolecular transport, in intestinal mucosa and possibly in respiratory mucosa, microfold or M cells, which are localized over subepithelial lymphoid follicles, are currently believed to deliver soluble and

particulate antigens across their cytosol to the underlying lymphoid follicles (186). However, M cell mediated macromolecular transport through polarized epithelial monolayer is technically very limited, because M cells are sparsely scattered. Furthermore, the population of M cells is very rare, accounting for 0.1% of epithelial cells (187).

The mucosal surfaces can be divided into two types according to their primary role and anatomical features. Type I mucosal surfaces represent those of the intestine, lung, and uterus, and they are covered by a simple monolayer epithelium that serves physiological functions, such as absorption and respiration. Type II mucosal surfaces include those of the oral, esophagus and vaginal cavities, and they are covered by stratified squamous epithelia. The main function of type II mucosa is to provide physical protective barriers for the host species. For both type I and II mucosa, in order to perform their vitally physiological functions, such as absorption, respiration and reproduction, the host is inevitably exposed to environmental pathogens, including a variety of viruses. To counteract with such detrimental environment, the body has evolved delicate mucosal immune system, containing most of the body's lymphocytes (188). Anatomically the lymphocyte populations are divided into those present in epithelium (intraepithelial lymphocytes, IELs) and those in the underlying lamina propria (lamina propria lymphocytes, LPLs) (42, 189). The LPLs can be subdivided into those from inductive sites (organized lymphoid nodules) and those from effector sites (diffuse lamina propria) (185). Only type I mucosal surfaces contain organized lymphoid nodules, also known as mucosa-associated lymphoid

tissue (MALT) (190). The small intestine contains peyer's patches (PPs) and isolated lymphoid follicles (ILFs). The large intestine contains ILF and the appendix, and the nasal mucosa harbors the nasopharynx-associated lymphoid tissue (NALT). These MALTs occur directly beneath the mucosal epithelial cells and hence do not possess afferent lymphatics. These MALTs consist largely of B cell forming follicular structures interspersed by interfollicular regions in which the majority of T cells reside. Distinct DCs are also found in the follicles, IFRs, intraepithelial and lamina propria region, playing central roles in the recognition and presentation of antigens to T cells (191, 192).In contrast, type II mucosal surfaces do not possess MALT directly underneath the epithelial layer under steady-state conditions. However, both type I and II mucosal lamina propria constituents can be drained by locally mucosa-draining lymph nodes(190). For example, cervical lymph nodes (CLN) drain the oral and nasopharyngeal mucosa, mesenteric lymph nodes (MLN) can accumulate DCs from the LP and PP, bronchial /mediastinal lymph nodes can drain the respiratory epithelial mucosa, and iliac/inguinal lymph node drains the reproduction tract (190) .

Viral Infection at Mucosal Sites

In general, the mucosal surfaces are the primary sites for microorganism entry and infection due to their vast area and underlying various types of susceptible cells. In terms of viral infection, some viruses, for example influenza virus and rotavirus, are limited to the mucosa; in many other viruses, such as polio, measles and HIV, crosses the epithelial barrier to spread to other organs distal of initial entry. Since polarized epithelial cells are characterized by distinct apical and basolateral plasma

membrane, the polarized distribution of host cellular receptors determines whether a virus initiates infection at apical or basolateral side. For example, the entry of vesicular stomatitis virus (VSV) and reovirus is restricted to the basolateral surfaces, whereas SV40 and rotavirus infections only occur following attachment to the apical surfaces. However, influenza virus can initiate infection at both apical and basolateral domains, although the apical domain is preferred. In addition, the budding of progeny virus particles also occurs in a polarized fashion, for example, VSV budding occurs primarily from the basolateral surface, and sendai viruses released exclusively from the apical surface. Besides epithelial cells, marcrophages, dendritic cells, T and B lymphocytes underlying or within epithelium are also permissive for some viruses. For example, most T lymphocytes underlying mucosa are CD4+ and CCR5+, HIV can establish local infection immediately after crossing the epithelium by using these two receptors, subsequently effectively spread for systemic infection (98).

Immunoglobulins in the Mucosal Secretion

In comparison with other immunoglobulin, secretory IgA (sIgA) has been long considered as playing predominant role against viral infection at mucosal surfaces. sIgA is produced by plasma cells residing in the lamina propria as dimmers associated with a 15-kDa J chain. Dimeric IgA (dIgA) binds to the polymeric immunoglobulin receptor (pIgR) on the basolateral domain, and the IgA-pIgR complex is shuttled from basolateral to apical surfaces of polarized epithelium, where proteolytic enzymes cleave the pIgR to release sIgA to the lumen (94). Once in the secretions of the lumen, IgA can bind to viral particles, preventing them from

attachment to or fusion with susceptible epithelial cells, thus blocking subsequent infections (95, 96). To further define the defensive roles of IgA against mucosal viral infection, J chain, IgA and pIgR deficient mice were generated to be challenged with common mucosal viruses, such as influenza, rotavirus and herpes simplex virus. Unexpectedly, results suggested that sIgA was not necessarily required for the protection of viral infection at mucosal surfaces (193, 194). For example, the IgA knock out mice exhibit comparable level of influenza infection as $IgA^{+/+}$ littermates (193, 195). Furthermore, passively transfer of viral specific neutralization IgA, specific for influenza HA, could not block corresponding viral infection at mucosal surfaces, but passively IgG could (196). Taken together, those investigations suggested that besides IgA, other Ig isotypes, particularly IgG, are also involved in mucosal immunity.

IgG has frequently been detected in secretions of human mucosal surfaces, such as the oral mucosa, small and large intestine, lung, and genitourinary tract (**Tabel I.III**). IgG predominates or exhibits comparable level as IgA in certain human secretions (197-200). Furthermore, IgG and IgA antibodies are equally effective in preventing mucosal infections (201, 202). Several studies have demonstrated the importance of IgG in the clearance of or prevention of infections at mucosal surfaces (196, 203).The passive administration of rhesus macaques with a mixture of anti-HIV neutralizing IgG prevented the transmission of the virus (204, 205). For influenza infection, it has been reported that passive transfer IgG, rather than IgA can cure influenza virus pneumonia in murine model (196, 206).

Table I.III. Levels (μg/ml) of Igs in human mucosal secretions

Fluids	IgA	IgG
Nasal	70-864	8-304
Bronchoalveolar	3	13
Milk	470-1632	40-168
Duodenal	313	104
Colonic	162	34
Female Genital tract	140-862	651-3516

FcRn Mediated IgG Transport at Mucosal Surfaces

In contrast to pIgR-mediated dIgA transport, little is known about how IgG presents at mucosal surfaces. For decades, the mucosal IgG was considered to transude from the serum, or intestinal IgG from bile. However, it has been found that under some conditions, the isotype pattern and concentrations of IgG in mucosal secretions are distinct from those of serum, ruling out the transudation of serum antibody (207). In addition, mucosal IgG derived from locally plasma cells in lamina propria has been extensively detected (207, 208). All those data suggest that an active transport system may be responsible for the appearance of IgG in mucosal secretions. As discussed previously, FcRn is constitutively expressed by mucosal epithelial cells, mediating bi-directional transepithelial transport of IgG. Therefore, it is rational to presume that IgG shown at the mucosal sites is dependent of FcRn. Indeed, our unpublished data have shown that intra-peritoneal administration of biotin-labeled IgG can be detected at both respiratory mucosal lavage and vagina fluid solely in wild type mice, but not in FcRn knockout mice. However, the further investigations need to be done in the context of infection or vaccination.

Mucosal Vaccine Development

Mucosal surfaces of respiratory, gastrointestinal and urogenital tracts are lined by polarized epithelial cells. The polarized epithelial layers are distributed into apical and basolateral domains with distinct membrane compositions by tight junction. They form the first line of defense for the evasion of danger pathogens. Underneath the epithelial barrier, a huge population of immune cells either form organized mucosal

associated lymph tissues, or scatter in lamina propria, which trigger both local and systemic immunity against microbial infections. Due to the vast area of mucosal surfaces and frequent exposure to external environment when performing vital physiological functions, such as breathing and digestion, the mucosal tissues are the primary sites for microbial infections. Indeed, the majority of viruses initiate their entry into body at the mucosal surfaces. Some viruses can bind to cellular receptors expressed either on apical or basolateral surfaces of epithelium for internalization and subsequent replication, like influenza viruses, Sendai viruse and vesicular stomatitis viruses. In addition, some viruses, like human immunodeficiency viruses (HIV), can take advantage of the process of transcytosis of epithelial layer, then infect underlying lymphoid cells (98).

Vaccination is the most effective and efficient way to control viral infections. Despite a wide array of vaccines have been successfully developed for the prevention and control of systemic infections, many viruses that infect mucosal epithelial cells or initiate systemic infection at mucosal sites are still big challenges and lack of effective vaccines, such as RSV, HSV-2 and HIV (209). Pre-existing antibodies and/or immune memory lymphocytes at mucosal sites are required for the maximal protection from many pathogens that cause mucosal infection like HIV(210). In this regard, mucosal vaccination is more effective than parenteral vaccination which is assumed to poorly induce mucosal immune responses (99, 209, 211). In addition, mucosal immunization is also capable of inducing systemic immune responses

comparable to systemic vaccination, which is of greater value for the control of some pathogens which require both systemic and mucosal immunity (210, 211).

Even though mucosal vaccination is assumed as the most effective way to control mucosal infection, the development of mucosal vaccines progresses slowly. Several challenges for mucosal vaccine development have been proposed: (1) vaccine components are largely diluted by mucosal secretions; (2) vaccine constitutes are captured in mucus gels, resulting the degradation by proteases; (3) vaccines are excluded by epithelial barriers, insulating from antigen presenting cells; (4) it is lack of effective and safe adjuvants, which can break into the mucosal immune tolerance; To overcome those difficulties, a lot of strategies have been employed to develop effective mucosal vaccines, including the administration of relatively large dosage, taking advantage of novel materials for effective delivery of mucosal immunogens, targeting M cells for efficient sampling of antigens and combining TLRs or toxin components in the formula to alert the mucosal immune system. In despite of those attempts, little progress is made in the past decades and effective and safe mucosal vaccines are still of great need, in particular, for the prevention and control of HIV infection (209). The ideal mucosal vaccine candidates should induce broad immune responses, including both mucosal and systemic responses, both humoral immune responses (serum IgG and secretion IgA) and cellular immune responses (CTL) and long lived memory immune responses (constitutively produced antibodies, long-lived plasma cells, memory T cells and B cells)(211). Apparently, much work remains to be done for the development of effective and safe mucosal vaccines.

Specific Aims

FcRn are constitutively expressed by most mucosal epithelial cells, governing the transport of IgGs from lamina propria to lumen and vice versa, possibly playing unique roles in antiviral immunity. However, in order to function in the transport of IgG across mucosal barrier, FcRn must be first delivered into early or sorting endosomes. How FcRn traffick into early endosomes is incompletely known. FcRn has a putative dilucine motif in the cytoplasmic tail domain, which is widely believed to be a sorting signal well characterized in a variety of endosomal/lysosomal residing molecules. Besides intrinsic sorting signal, interaction protein partners, like adaptor proteins and chaperons, are also involved in protein trafficking, for example, MHC class II associated invariant chain (CD74) directs MHC II molecules into acidic endosomes where antigen loading to MHC II occurs (178). Although the structural similarity to MHC class I molecule, FcRn intracellular trafficking is more like MHC class II, both of which are synthesized in the endoplasmic reticulum, further modified in the Golgi, and sorted from TGN into endosomal compartments. Because invariant Chain (CD74) plays an essential role in MHC II trafficking, we reasoned that CD74 may also interact with FcRn and regulates its intracellular trafficking. Therefore, **specific aim 1 is to define the role of putative dilucine motif and possible CD74 association in regulating FcRn intracellular traffic.**

In contrast to pIgR mediated single way of dIgA transport, FcRn can transport IgG bidiretionally across polarized epithelium. To take advantage of this unique bidirectional transport pathway, we hypothesized that FcRn may deliver viral antigen

fused with IgG Fc across epithelial barrier into lamina propria and the circulation, leading to elicit both systemic and mucosal immunity against corresponding viral infection (**Figure I.II.A**). **Therefore, specific aim 2 is to determine whether FcRn functions as vehicle of subunit vaccine to deliver Fc fused viral antigens for mucosal immunization**.

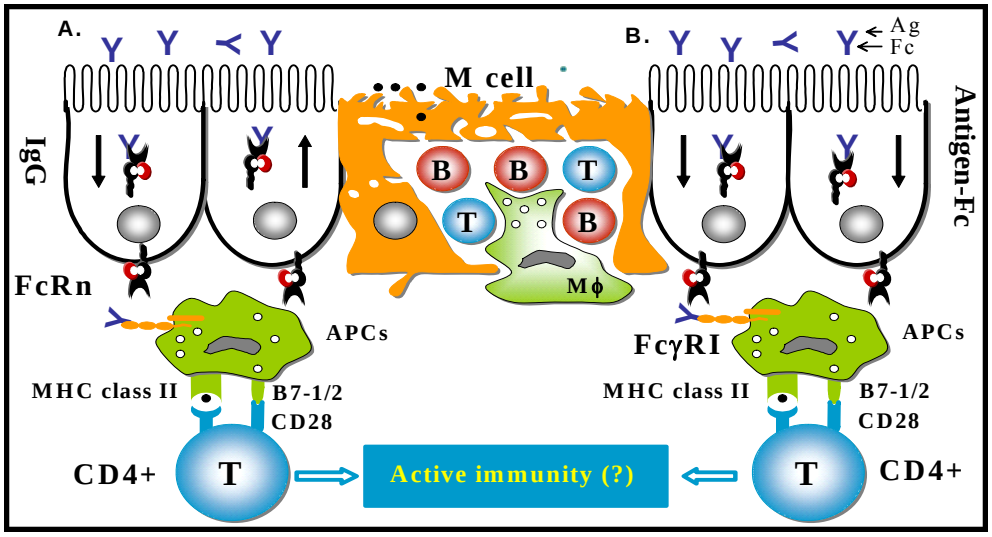


Figure I.II. **A**. FcRn can transport IgG bidirectionally across the polarized epithelial cells and IgG can be uptaken by FcγRs at APCs. **B**. Could FcRn transport Fc-fused viral antigens across the polarized epithelia and target it onto profession APCs to engender the protective immunity against viral infections?

Chapter 2: The MHC Class II Associate Invirant Chain Interacts with FcRn and Modulates Its Trafficking to Endosomal/Lysosomal Compartments

ABSTRACT

The neonatal Fc receptor for IgG (FcRn) transfers maternal IgG to the offspring and protects IgG from degradation. The FcRn resides in an acidic intracellular compartment, allowing it to bind IgG. In this study we found the association of FcRn and invariant chain (Ii) by immunoprecipitation. The interaction was initiated within the endoplasmic reticulum by Ii binding to either the FcRn HC alone or FcRn HC-β2m complex and appeared to be maintained throughout the endocytic pathway. The CLIP in Ii was not required for FcRn-Ii association. The interaction was also detected in IFN–$\tilde{\gamma}$ treated human macrophage-like THP-1, epithelial and endothelial cells, and mouse bone marrow-derived dendritic cells. A truncated FcRn, without the cytoplasmic tail, was unable to traffic to early endosomes; however, its location in early endosome was restored by Ii expression. FcRn was also detected in the late endosome/lysosome only in the presence of Ii or upon exposure to IFN-γ. In human monocyte- or mouse bone marrow-derived immature dendritic cells, FcRn failed to rescue in the late endosome/lysosome in the absence of Ii chain. Furthermore, the cytoplasmic tail of Ii conferred tail-less FcRn a route to both the early endosome and late endosome/lysosome in a hybrid molecule. Since the FcRn is expressed in

macrophage and dendritic cells or epithelial and endothelial cells where Ii is induced under inflammation and infection, these results reveal the complexity of FcRn trafficking in which Ii is capable of expanding the boundary of FcRn trafficking. Taken together, the intracellular trafficking of FcRn is regulated by its intrinsic sorting information and/or an interaction with Ii chain.

INTRODUCTION

The neonatal Fc receptor for IgG (FcRn) was first identified in the intestinal epithelial cells of a suckling rodent, where it is expressed at high level (212). However, its functional expression has recently been acknowledged in a diverse array of cell types and tissues, including epithelial cells, endothelial cells, macrophages, dendritic cells, and neutrophils of humans and rodents at all ages (118). FcRn is composed of a heavy chain (HC, 45 kDa in humans and 50 kDa in rodents) nonconvalently associated with a light chain β_2m (12 kDa)(153, 212). The HC comprises three extracellular domains that are anchored to the cell surface by a single transmembrane segment and a carboxyl-terminal cytoplasmic tail. The association of FcRn with β2m is critical for FcRn exit to the endoplasmic reticulum (ER) (213). As previously reviewed in this thesis, although FcRn shares extensive structural homology with MHC class I, it is unable to present antigenic peptides to cognant T cells due to its narrowed antigen binding groove. Instead, FcRn transfers maternal IgG across the polarized placental and/or intestinal epithelial cells, which allows newborns to obtain humoral immunity against antigens encountered by the mother before they develop their own immune system. In addition to its function as

a transporter, FcRn protects IgG and albumin by extending their life spans. Consequently, this Fc receptor establishes IgG (11-12 mg/ml for human) and albumin (30 to 50 mg/ml for human) as the most abundant proteins in the blood. This character ensures that IgG generated upon antigenic exposure or infection has a long-term protective immunity. On the other hand, FcRn could also prolong the life span of pathogenic or autoimmune IgG, which links FcRn to autoimmune diseases.

FcRn binds IgG isotypes in a pH-dependent manner, binging IgG at acidic pH (6.0-6.5) and releases IgG at neutral or higher pH. In the majority of cell types, FcRn resides primarily in the early acidic endosomal vesicles with limited cell surface expression. In early endoosmes, FcRn catches IgG that enters cell by pinocytosis or endocytosis (152). Subsequently, FcRn recycles IgG back to the cell surface in nonpolarized cells or transcytoses IgG to the opposite surface in polarized epithelial cells. The near neutral pH of extracellular environment causes IgG release from FcRn. The IgG that does not bind to the FcRn inside cells would move to lysosomes where it undergoes degradation. The FcRn-IgG transport pathway is further elaborated by recent studies that identify early endosomes as the major sorting location for FcRn-IgG complex in endothelial cells (152, 156). In most cases, FcRn does not appear in the late endosomal/lysosomal compartment of either endothelial or epithelial cells. Two targeting signals, a tryptophan-and a dileucine-based motif in the cytoplasmic tail of FcRn has been postulated to mediate internalization of FcRn from the plasma membrane or transport of FcRn from from the *trans*-Golgi network (TGN) to endocytic pathway (179). Recently, a

Ca2+-dependent calmodulin-binding motif in the cytoplasmic tail of FcRn in modulating the intracellular trafficking of FcRn was also reported (148). Despite these studies, it still remains uncertain about its detailed trafficking and regulation of its entry into the endocytic pathway under physiological and pathophysiological conditions.

Invariant chain (CD74, Ii) is a nonpolymorphic, type II integral membrane glycoprotein. Ii chains form a trimer in the ER, where, each Ii non-covalently binds to a MHC class II αβ heterodimer, thereby forming a nonameric complex $(\alpha\beta Ii)_3$ (214). The binding of Ii chain with MHC class II in the ER stabilizes the MHC class II and protect it from binding to endogenously generated peptides. When the nonamer reaches the trans-Golgi network (TGN), the complex is sorted away from the secretory pathway and routed to the endocytic pathway, ultimately to lysosome-like compartments, called MHC class II compartments (MIIC). The *N*-terminal cytoplasmic tail of Ii chain contains two acidic dileucine-based endosomal targeting motifs (D/EXXXLL), which direct MHC class II to the endocytic pathway (178). Upon entry of the nonameric complex to endosomes, Ii chain is gradually degraded by pH-dependent cathepsin proteases (215). Consequently, a small fragment, called class II-associated invariant chain peptide (CLIP), is left in the peptide-binding groove of MHC class II. The removal of CLIP from MHC class II, which is catalyzed by HLA-DM in humans (216), facilitates the binding of antigenic peptides derived from internalized antigens for antigen presentation. Thus, Ii chain plays a critical role in the antigen presentation by MHC class II to $CD4^+$ T cells by

stabilizing MHC class II in the ER and directing MHC class II away from the default secretory pathway to endocytic pathway.

Although FcRn is structurally similar to MHC class I, its intracellular trafficking pathway is much more analogous to that of MHC class II. Since Ii chain plays such an important role in MHC class II trafficking to the endocytic pathway, we hypothesized that Ii molecules could play an additional role in directing FcRn trafficking within the endocytic compartments by physical association with FcRn. We were surprised to find that the Ii was indeed capable of associating with FcRn and regulating FcRn trafficking. Although dispensable under a certain circumstance, the interaction of Ii chain with FcRn offers an additional targeting signal that directs FcRn into the endosomal/lysosomal compartments, especially under immunological, inflammatory, and infectious conditions. Our results provide discover a novel role of Ii chain in modulating FcRn intracellular trafficking pathway that is essentially involved for IgG transport and homeostasis.

MATERIALS AND METHODS

Cell lines, antibodies and mice

Human epithelial T84, HT-29, Caco-2, HeLa, and Chinese hamster ovary (CHO) cell lines were grown in complete DMEM. Macrophage-like cell line THP-1 and melanoma FO-1 (β2m-deficient) cell lines were grown in RPMI 1640 (Invitrogen Life Technologies) complete medium. All complete media were supplemented with 10 mM HEPES, 10% fetal calf serum (Sigma), 1% L-glutamine,

nonessential amino acids, and 1% penicillin/streptomycin. Cells were grown in a 5% CO_2 at 37°C.

Rabbit-anti-FLAG epitope (DYKDDDDK, a single letter for amino acid) or mAb LN-2 for human CD74 was purchased from Sigma. HRP-conjugated rabbit anti-mouse or donkey anti-rabbit antibody was from Pierce (Rockford). The hybridoma 12CA5, which reacts with the influenza hemagglutinin (HA) epitope, was purchased from ATCC. Biotin-labeled mAb for Ii was from Southern Biotechnology. Anti-lysosome-associated membrane glycoprotein-1 (anti-LAMP-1; mouse IgG1, clone H4A3, rat IgG2a, clone 1D4B, developed by Drs. T. August and J. Hildreth) was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the National Institute of Child Health and Human Development and maintained by the University of Iowa (Iowa City, IA). Mab anti-EEA1, FITC-conjugated mAb anti-LAMP-1, rat anti-mouse Ii (In-1), and rat anti-mouse transferrin receptor were obtained from BD Biosciences. Alexa 488, Alexa 555, and Alexa 633 fluor-conjugated secondary antibodies were from Molecular Probes. Recombinant human IFN-γ, IL-4 and GM-CSF were from R&D Systems.

Homozygous Balb/c $Ii^{-/-}$ mice (217), originally obtained from Dr. Elizabeth Bikoff (University of Oxford, England, UK), were bred in a specific pathogen free facility at the National Institutes of Health animal facilities. BALB/c/J mice were purchased from Jackson Laboratory. All mice were maintained under AAALAC approved conditions.

Production of Affinity-purified FcRn antibodies

The human FcRn codons corresponding to the 299-343 or mouse FcRn codons 427-472 were amplified by PCR and subcloned into the pGEX4T-1 (Amersham Pharmacia Biotech) expression vector. Production of affinity-purified glutathione *S*-transferase fusion proteins was as previously described (153). For production of anti-FcRn peptide antibody, peptide *CLEWKEPPSMRLKARP* was synthesized by Invitrogen. The immunization of rabbits with purified fusion protein or FcRn peptides coupled with keyhole limpet hemocyanin was carried out by Rockland Immunochemicals. Anti-FcRn antibodies were affinity-purified from rabbit sera with affinity columns, respectively.

Reverse Transriptase (RT)-PCR analysis

For total RNA extraction, cells were pelleted and resuspended at a 10^6 cells/ml in Trizol Reagent (Invitrogen Life Technologies). The human Ii gene was amplified by primers (5'-TCCCAAGCCTGTGAGCAAGATG-3', 5'-CCAGTTCCAGTGACTCTTTCG-3') with one step RT-PCR kit (Qiagen). The mRNA was also amplified by glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-specific primers (5'-GAGAAGGCTGGGGCTCAT-3', 5'-TGCTGATGATCTTGAGGCTG-3') as an internal control to monitor the quality of the RNA purification and cDNA synthesis. The PCR products were analyzed by 1.5 % agarose gel electrophoresis and stained with ethidium bromide.

Construction of FcRn wild-type, mutant, and invariant chain expression vector

The constructions of human β_2m and FcRn expression plasmids, pCDNAβ_2m and pcDNA-FLAGFcRn, have been described previously (153). The pcDNA-FLAGFcRn construct fused with a preprotrypsin signal sequence, FLAG epitope, into the amino terminus of the human FcRn gene (1-342 aa). PCR primer pair (5'ATAAGAATGCGGCCGCGGCAGAAAGCCACCT CTCCCT-3', 5'-TGCTCTAGATTACCTCATCCTTCTCCACAACA-3') was used to construct a plasmid encoding FLAG-tagged FcRn mutant (1-302 aa) that lacks the cytoplasmic tail. The DNA fragment was digested with *Not* I and *Xba* I and ligated into the plasmid pCDNAFLAG to generate the plasmid pcDNAFLAGFcRn-TD. To generate pcDNAFLAGFcRn-LL/AA, FcRn cDNA in a pCDNAFLAG was used as the template for site-directed mutagenesis by an *in vitro* Transformer Site-directed Mutagenesis Kit (Clontech). The oligonucleotide (5'-CACCGGGGTCGCCGCGCC CACCCCA-3') was used for mutation of leucine residues 320-321 to alanine residues (base substitutions are *underlined*). To construct pcDNAFLAGmFcRn, PCR primer pair (5'-TCACAAGCTTTCAGAGACCCGCCC CCCACTGATGTAT-3', 5'-TGATCTAGACTAGGAAGT GGCTGGAAAGGCA) was used. The DNA fragment was digested with *HIND* III and *Xba* I (*underlined* in primers) for cloning**.** PCR primer pair (5'-CCGCTCGAGTCAGAGGCTAGCATAA TCAGGAACATCATACGGATACATGGGGACT GGGCCCAGA, 5'-ATAAGAATGCGGCCGCT TCCGAGATGCACAGGAGGAGA-3') was used to construct pBUDCE4Ii plasmid encoding HA-tagged Ii. The plasmids phFcRn-

DMcyt and phFcRn-Iicyt were constructed by two-step PCR reactions cloning PCR fragments into pCDNAFLAG. The cytoplasmic tail codons (222-245 aa) of HLA-DM were amplified by primer pair (5'-GTAGGAGGAGCTCTGTTGTGGAGAGCTGGCCA CTCTAGTTA-3', 5'-TCTCTCTAGACTAG GAAATGTGCCATCCTT-3'). The Ii cytoplasmic tail (1-46 aa) was synthesized by Integrated DNA Technologies. The amplified HLA-DM or synthesized Ii chain cDNA fragments contain an overhanging sequence complementary to the FcRn cDNA. In the second step, fragments of Ii or HLA-DM were annealed to FcRn plasmid and a fusion product of both cDNA fragments was synthesized by PCR primer pairs specific for FcRn and Ii or HLA-DM. In all above cloning, the primer introduced a *Not* I or *Xba* I site (*underlined*) to facilitate cloning. The plasmid encoding the *N*-glycosylation mutant (FcRn N/S) of human FcRn was generated by replacing the consensus glycosylation sequence, Asn-X-(Ser/Thr) for change of asparagine 102 to a serine residue (213). All constructs were sequenced to verify the fidelity of the amplification and cloning. The human Ii.Δ91-99 or Ii.M91A in pCDNA3 vectors was a kind gift from Dr. Norbert Koch (218). The plasmid pTFR-GFP was obtained from Dr. Gary Banker (Oregon Health & Science University, Portland, Oregon). The plasmid pcEXV3-mIi 31, coding for wild-type mouse (m)Ii 31, was a kind gift from Dr. Ronald N. Germain (National Institutes of Health, Bethesda, Maryland).

Transfection and protein expression

The stable cell lines, HeLa^{FcRn}, FO-1^{FcRn} and FO-1$^{FcRn+\beta2m}$, have been described previously (153, 213). HeLa or HeLa^{FcRn} cells were transfected with pBUDCE4-Ii vector with Effectene transfection reagent (Qiagen). Stable transfectants were selected by G418 or G418 plus zeocin for single or double transfectants, respectively. Positive clones were tested for protein expression through Western blot using anti-FLAG or -HA Ab. Transfectants were maintained in medium containing G418 (500 μg/ml) and/or Zeocine (150 μg/ml). For transient transfections, cells were transfected with the 2 μg plasmid pBUDCE4-Ii. Protein expressions were examined 48 h after transfection.

Western blotting, immunoprecipitation, and gel electrophoresis

Cell lines and transfectants were lysed in 0.5% CHAPS in PBS with a cocktail of protease inhibitor (Roche). Protein concentrations were determined by Bradford assay (Bio-Rad Laboratories). The lysates were resolved on a 12% SDS-PAGE gel under a reducing condition. Proteins were electrotransferred onto a nitrocellulose membrane (Schleicher & Schuell). The membranes were blocked with 5% non-fat milk, probed separately with anti-FcRn or Ii Ab for 1 h, and followed by incubation with HRP-conjugated rabbit anti-mouse or donkey anti-rabbit Ab. All blocking, incubation, and washing were performed in PBST solution (PBS and 0.05% Tween 20). Proteins were visualized by the ECL (Pierce). Immunoprecipitation was done as described previously (219). Protein was precipitated with anti-FLAG or -HA mAb. The immunoreactive products were eluted from the protein G complex with gel loading buffer at 95°C.

Deglycosylation

Endo-*N* acetylglucosaminidase H (endo-H) (New England Biolabs) digestions were performed as described previously (219). For FcRn digestion in immunoprecipitates, proteins were resuspended in 0.5 ml of endo-H digestion buffer (100 mM sodium acetate, pH 5, 150 mM NaCl, 1% Triton X-100, 0.2% SDS, and 0.5 mM PMSF). Beads containing antibody-antigen complexes were pelleted and then eluted with reducing sample buffer. A mock digestion without enzymes was performed for each digestion. All digestions were performed for 18 h at 37°C. Proteins were analyzed on a 12% SDS-PAGE gel under reducing conditions and immunoblotted with anti-FLAG Ab.

IgG binding assay

IgG binding assay was performed as previously described (2, 6). Cells were lysed in PBS (pH 6.0 or 7.5) with 0.5% CHAPS (Sigma) and protease inhibitor cocktail on ice for 1 hr. Post-nuclear supernatants containing 0.5–1 mg of soluble proteins were incubated with human IgG-Sepharose (Amersham Pharmacia Biotech) at 4°C overnight. The unbound proteins were removed with PBS (pH 6.0 or 7.5) containing 0.1% CHAPS. The adsorbed proteins were boiled with reducing electrophoresis sample buffer at 95°C for 5 min. The eluted fractions were subjected to Western blot analysis as above described.

Isolation of human monocyte-derived dendritic cells (DC), knockdown of CD74 in DC by small interfering RNA (siRNA), and generation of bone marrow-derived DC (BMDC) from mice.

Human immature DCs were obtained by culturing the adherent fraction of normal human PBMCs (Institute of Human Virology, Baltimore, MD) in the presence of GM-CSF and IL-4 as previously described (6). Briefly, monocytes were isolated from PBMCs by adherence to plastic Petri dish for 2 h and then cultured for 6 days in RPMI 1640 complete medium containing human GM-CSF (50 ng/ml) and 100 ng/ml IL-4 (R&D). The medium was replaced every 3 days. The 1 X 10^6 immature DC cells were transfected with 2.5 ug mission^{TM} shRNA plasmid for human CD74 (Sigma) in 100 μl nucleofactor solution using a DC nucleofection kit (Amaxa, Cologne, Germany) according to the instructions of the manufacturer. Cells were then resuspended with RPMI 1640 medium and cultured on coverslip. After 24 h, cells were fixed, permebilized, and stained for confocal microscope analysis.

Bone marrow-derived DCs from wild type BALB/c and $Ii^{-/-}$ mice were generated as previously described (220). Immature DCs were purified from CD11c+ magnetic microbeads contained columns (MACS, German) and characterized by flow cytometry. The purified immature DCs were then used for experiments.

Confocal Immunofluorescence

Immunofluorescence was performed based on methods previously described (2, 10). Briefly, cells were cultivated on coverslips for 24 h. The cells were rinsed in PBS and cold-fixed in 3.7% paraformaldehyde (Sigma) in PBS for 30 min at 4°C and quenched with glycine for 10 min. After two washings with PBS, the coverslips were permeabilized in solution (PBS containing 0.2% Triton X-100) for 20 min, then blocked with blocking buffer containing 3% normal goat serum. Cells were incubated with affinity-purified primary antibody in PBST with 3% BSA for 1 h. Cells were then incubated with Alexa 555 Fluro-conjagated goat anti-mouse IgG and Alexa 488 Fluro-conjugated goat anti-rabbit IgG in blocking buffer. After each step, cells were washed at least three times with 0.1% Tween-20 in PBS. Coverslips were mounted on slides with ProLongTM antifade kit (Molecular Probes) and examined using a Zeiss LSM 510 confocal fluorescence microscopy. The images were processed using the LSM Image Examiner software (Zeiss, Germany). Quantitative colocalization measurement was performed using the Zeiss LSM 510 Examiner Software. Pearson's correlation coefficient was calculated for describing the colocalization correlation of the intensity distributions between two channels. In each quantitative experiment with transfected HeLa cells, 15 cells in total were analyzed. For immature DCs, 10 cells in total were analyzed. $P<0.05$ was considered as significant.

RESULTS

The association of FcRn with the invariant chain

HeLa is an Ii and FcRn double negative cell line. To test whether Ii chain interacts with FcRn molecule, $HeLa^{FcRn}$ or $HeLa^{mock}$ cells were transfected with plasmids encoding HA-tagged Ii cDNA. Cells were lysed with a low-stringency CHAPS buffer and subjected to immunoprecipitation with either an anti-FLAG (for FcRn) or anti-HA (for Ii chain) Ab. The immunoprecipitate was analyzed by blotting with an anti-HA (Fig. 2.1A) or anti-FLAG Ab (Fig. 2.1B). As shown in Fig. 2.1, anti-FLAG Ab co-immunoprecipitated Ii (Fig. 2.1A, lane 3). The anti-HA mAb co-immunoprecipitated FcRn HC (Fig. 2.1B, lane 1). Interaction between FcRn and Ii was further analyzed by confocal microscopy (Fig. 2.1C). As expected, both FcRn and Ii appeared as punctuate and vesicular staining. They were colocalized with each other (Fig. 2.1C, *right panel*). In addition, the association of FcRn and Ii could be maintained in the presence of other detergents such as 0.5% Triton X-100 (Fig. 2.1D, lane 1) and NP-40 (Fig. 2.1D, lane 2); however, the association was disrupted by 0.5% SDS (Fig. 2.1E, lane 3). Therefore, these data show that Ii specifically associates with FcRn.

Ii chain interacts with both FcRn HC alone and FcRn-β2m complex

The functional FcRn molecule consists of an FcRn HC and β2m. We have previously expressed FcRn alone, or, with β_2m together in FO-1 cells (213). FO-1 is a melanoma cell line that lacks β_2m expression. To test whether Ii chain interacts with FcRn HC alone or in an FcRn-β_2m complex, the cell lysates were generated from $FO\text{-}1^{mock}$, $FO\text{-}1^{FcRn}$, and $FO\text{-}1^{FcRn+\beta2m}$ cells that were transiently transfected with HA-Ii, respectively. The cell lysates were immunoprecipitated with an anti-

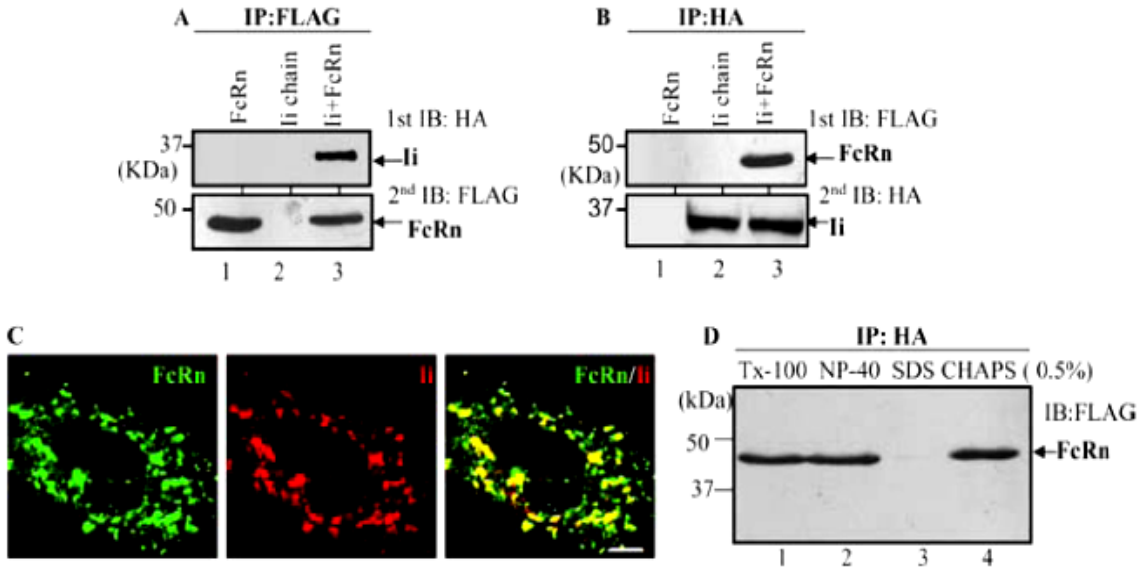


FIGURE 2.1. FcRn interacts with the Ii chain. Cell lysates were immunoprecipitated (IP) with Abs and the immunoprecipitates were subjected to 12% SDS-PAGE electrophoresis under reducing conditions, then transferred to a nitrocellulose membrane for Western blotting. Immunoblots (IB) were developed with ECL. Each experiment was performed at least twice.

A+B. The cell lysates from HeLa^{FcRn} (lane 1), HeLa^{Ii} (lane 2), and HeLa$^{FcRn+Ii}$ (lane 3) were immunoprecipitated by anti-FLAG M2 mAb or anti-HA mAb. The immunoprecipitates were subjected to Western blotting with anti-HA or FLAG mAb as indicated.

C. Colocalization of FcRn and Ii in HeLa$^{FcRn+Ii}$. Cells grown on glass coverslips were fixed with 3.7% paraformaldehyde and permeabilized in 0.2% Triton X-100. Subsequently, the cells were incubated with affinity-purified rabbit anti-FLAG (FcRn, *left panel*) or anti-HA (Ii, *middle panel*) specific Ab, followed by Alexa Fluro 488- or 533-conjugated IgG. Puncta that appear yellow in the merged images (*right panel*) indicate colocalization of FcRn with the Ii chain. Scale bar represents 5 μm.

D. HeLa$^{FcRn+Ii}$ cell lysates were generated by 0.5% Triton X-100 (lane 1), NP-40 (lane 2), SDS (lane 3), and CHAPS (lane 4) buffers and immunoprecipitated by HA-specific Ab. The immunoprecipitates were subjected to Western blot analysis. The location of the FcRn HC is indicated by an arrow.

FLAG Ab for FcRn or anti-β_2m mAb, BBM1. The immunoprecipitates were sequentially blotted with anti-HA for Ii chain (Fig.2. 2A, lanes 2 and 3) and FLAG Ab for FcRn (Fig. 2.2B, lane 3). The results showed that Ii was detected in either FLAG immunoprecipiates from FO-1^{FcRn} or anti-β2m immunoprecipiates from FO-1$^{FcRn+\beta2m}$. We were unable to co-immunoprecipitate the FcRn HC and Ii chain from the lysates of untransfected FO-1 cells by anti-β2m Ab, suggesting the specificity. Therefore, we conclude that Ii interacts with both FcRn heavy chain alone and FcRn-β2m complex. To further confirm the presence of FcRn–Ii complex in the ER and Golgi apparatus, HeLa$^{FcRn+Ii}$ cells were lysed in CHAPS buffer; anti-Ii (LN-2) immunoprecipitates were subjected to Endo H digestion. Human FcRn possesses one *N*-linked glycosylation site. As shown in Fig. 2C, FcRn HC in an Ii immunoprecipitate from HeLa$^{FcRn+Ii}$ cells exhibited a mixture of sensitivities to Endo H digestion (Fig. 2.2C, lane 5) as compared to mock digestion (Fig 2.2C, lane 4). Endo H sensitive FcRn HC had a similar mobility as FcRn N/S (Fig 2.2C, lane 1), an *N*-glycan mutant of FcRn (213). These data show that Ii-associated with both both endo H-sensitive and -resistant forms of FcRn, corresponding to post-ER modification of the glycan structure, and therefore, supporting the initiation of the FcRn–Ii interaction in the ER and maintenance during transport through the Golgi stack.

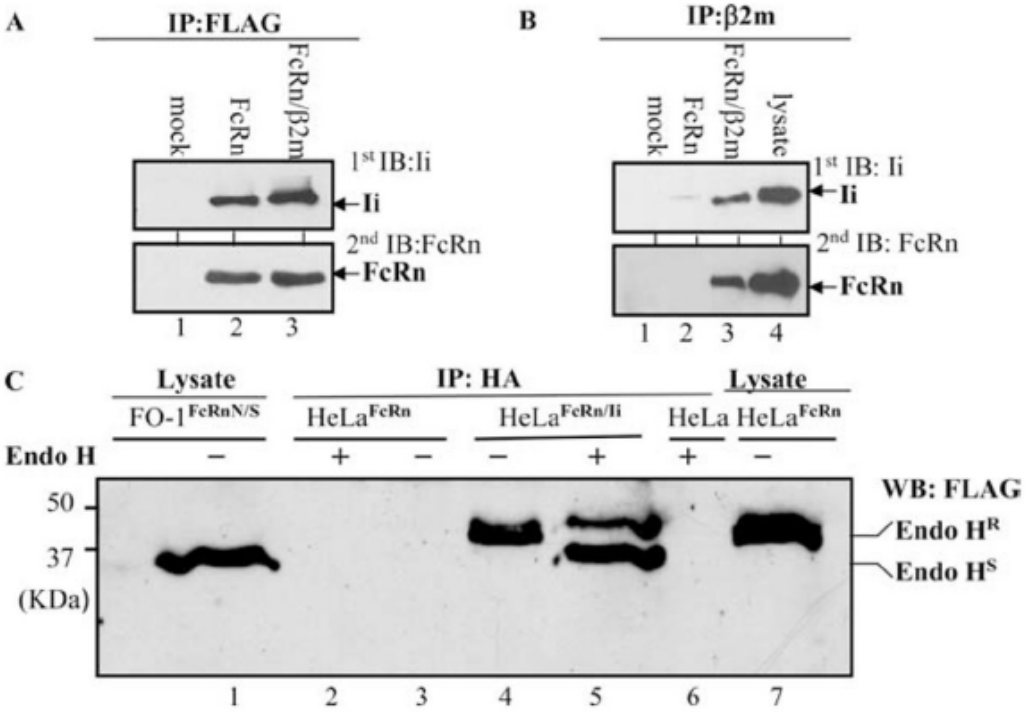


FIGURE 2.2 Ii chain interacts with both FcRn HC alone and FcRn H-β2m complex. All immunoprecipitates were subjected to Western blotting analysis. Blots were developed with ECL. Each experiment was performed at least three times. Only the region of interest in the gel is shown.

A+B. The FO-1^{mock} (lane 1), FO-1^{FcRn} (lane 2) and FO-1$^{FcRn+\beta2m}$ (lane 3) cells were transiently transfected with pBUDCE4Ii-HA. After 48 h, cell lysates were immunoprecipitated by anti-FLAG M2 mAb (***A***) or anti-β2m mAb BBM1 (***B***). The immunoprecipitates were blotted with anti-HA (Ii, 1st blot) or rabbit anti-FLAG (FcRn, 2nd blot) Ab. The cell lysates from Hela$^{FcRn+Ii}$ were used as a positive control for immunoblotting (B, lane 4).

C. Sensitivity of Ii-associated FcRn HC to Endo H digestion. The lysates (500 μg) from HeLa^{FcRn} (lanes 2 and 3), HeLa$^{FcRn+Ii}$ (lanes 4 and 5) and HeLa (lane 6) were immunoprecipitated by HA mAb. The immunoprecipitates were digested by mock (lanes 3 and 4) and Endo H (lanes 2, 5, and 6) for 18 h, respectively. Lane 5 represented a mixture of Endo H-sensitive (H^S) and -resistant (H^R) FcRn proteins. The cell lysates from glycan mutant FO-1$^{FcRnN/S}$ (lane 1) and HeLa^{FcRn} (lane 7) were used as controls.

FcRn associating with Ii chain can bind to IgG in acidic pH

FcRn binds IgG at acidic pH 6.0 and releases IgG at neutral pH. We tested whether the the association of FcRn with Ii affects its ability to bind to its natural ligand IgG. We incubated cell lysates from HeLa cells expressing FcRn and/or Ii with human IgG-Sepharose at either pH 6.0 or pH 7.5. Cell lysates from $HeLa^{FcRn}$ cells were used as positive control. The binding elutes and cell lysates were subjected to the Western blot analysis. As expected, FcRn from $HeLa^{FcRn}$ cells bound to IgG at pH 6.0 (Fig. 2.3, lane 2), but not at pH 7.5 (Fig.2.3, lane 1). Similarly, FcRn from $HeLa^{FcRn+Ii}$ cells bound to IgG at pH 6.0 (Fig. 2.3, lane 4), but not at pH 7.5 (lane 3). Both Ii and FcRn were detected in the binding elutes of human IgG beads, suggesting the presence of Ii in FcRn-IgG complexes. Hence, it is very likely that Ii-association does not interfere with FcRn function in the IgG binding assay.

CLIP is not required for the interaction of Ii with FcRn

Human Ii has a short, 30 amino acids (aa), *N*-terminal cytoplasmic tail, followed by a single 24 aa transmembrane region and an ~150 aa long lumenal domain (Fig. 2.4A). A segment of residues 81-104 is called class II associated invariant chain peptide (CLIP). The CLIP region contains a central 9 amino acid sequence, MRMATPLLM (91-99 aa), which is conserved among human, mouse and rat (Fig. 2.4A). Analysis of MHC class II supermotifs (221) and the crystal structures of CLIP complexed with MHC class II molecules (222) indicate the importance of methionine residues at positions 91 and 99 in its binding to MHC

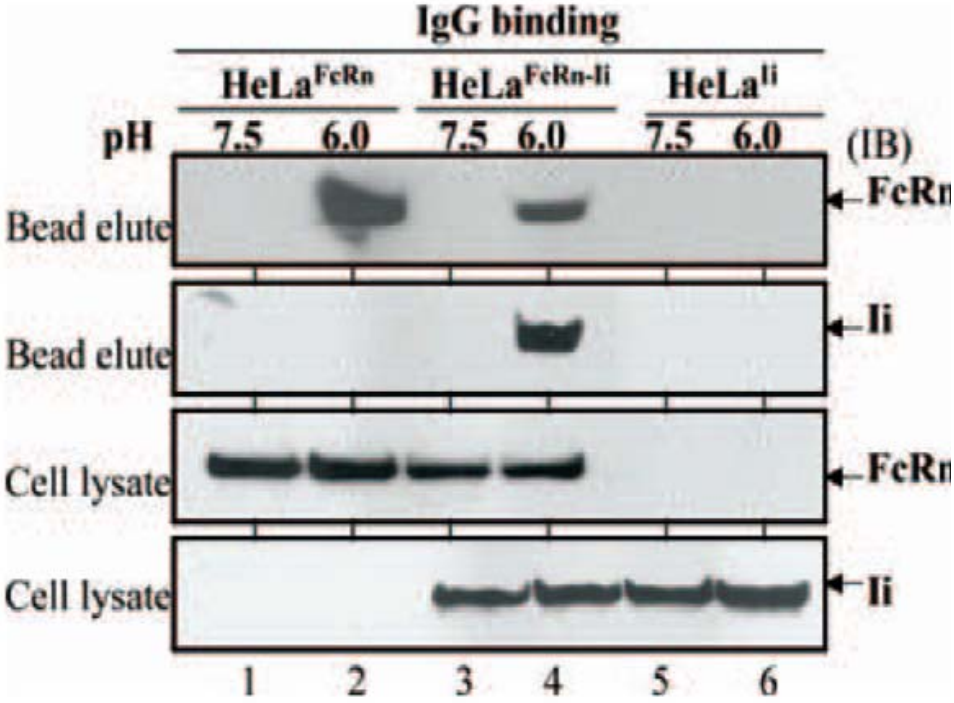


FIGURE 3.3. FcRn/li chain complex in IgG binding. HeLa transfectants as indicated were lysed in sodium phosphate buffer (pH 6.0 or 7.5) with 0.5% CHAPS and proteinase inhibitors. Approximately 0.5 mg of the soluble proteins was incubated with human IgG-Sepharose at 4°C. The eluted proteins or cell lysates were subjected to Western blotting analysis. Proteins were probed with rabbit anti-FLAG Ab or anti-HA mAb and developed with HRP-conjugated Abs and ECL. The locations of the human FcRn HC and Ii chain are indicated by arrows.

class II. In addition, different MHC class I alleles display different dependencies upon the sequence of CLIP (223). Therefore, it is pertinent to assess whether the CLIP or its methionine 91 is important for the interactions of Ii with FcRn. To do so, the CLIP region of Ii chain was deleted, or methionine 91 was replaced with alanine (construct Ii.M91A). The ability of these Ii mutants to associate with FcRn molecule is tested using $HeLa^{FcRn}$ cells transiently transfected with these Ii constructs. Deletion of CLIP (Fig. 2.4B, lane 3) or substitution of methionine at position 91 (Fig. 2.4B, lane 2) did not appreciably alter the ability of Ii association with FcRn, as shown by the immunoprecipitation-Western-blot analysis. $Hela^{FcRn}$ (Fig. 2.4B, lane 1) or isotype-matched IgG (lane 5) was used as a positive or negative control in this assay. Taken together, these data suggest that the CLIP (91–99) is not required for binding to the FcRn.

Both Ii and the cytoplasmic tail of FcRn targets FcRn into the early endosomes

Immunofluorescence microscopy studies showed that in $HeLa^{FcRn}$ transfectant, a high proportion of the FcRn colocalized with the early endosomal marker, EEA1 (Fig. 2.5A, *upper panel*), indicating a preferential distribution of FcRn to the early endosomes. The expression of Ii in $HeLa^{FcRn}$ cells did not significantly change the steady-state distribution of FcRn and EEA1 (Fig.2.5A, *bottom panel*). An FcRn mutant that lacks its cytoplasmic tail (FcRn-TD) was immunoprecipitated with Ii (Fig. 2.5B, lane 2), indicating that the cytoplasmic tail of FcRn is not necessary for its association with Ii chain.

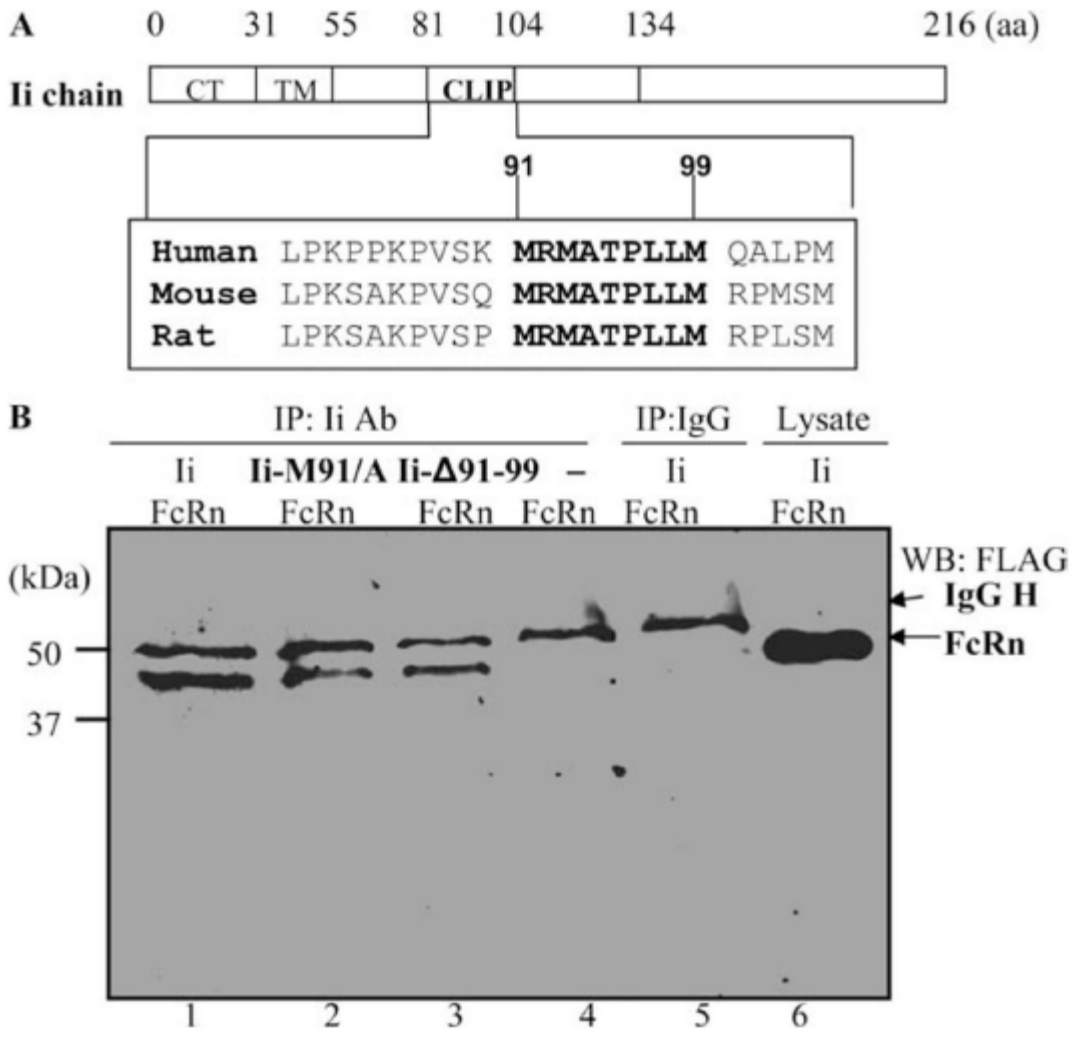


FIGURE 2.4. Function of CLIP in the Ii association with FcRn.
A. Schematic representation of the full-length Ii chains. TMD: the transmembrane domain, CLIP: the class II-associated Ii chain peptide. Ii chain sequence alignment of human (Accession No. 10835070), mouse (13097485) and rat (37589621) indicates conservation of the CLIP (91–99) regions.
B. CLIP (91–99) is not important for interaction with FcRn. $HeLa^{FcRn}$ cells were transiently transfected by plasmid pCDNA3 encoding the full-length Ii chain (Ii), Ii.Δ91-99, and Ii.M91A, respectively. After 48 hr, the cell lysates were immunoprecipitated by anti-Ii mAb (lanes 1-3) and an isotype-matched IgG control (lane 5). The cell lysates from HeLa (lane 4) or $HeLa^{FcRn+Ii}$ (lane 6) were used as a negative or positive control. The immunoprecipitates were subjected to Western blotting with FLAG-specific Ab and HRP-conjugated goat anti-rabbit Ab, and finally visualized with ECL.
Immunofluorescence studies showed that the tail deletion mutation profoundly

altered the cellular distribution pattern of FcRn appearing as honeycomb with rare colocalization with EEA1 (Fig. 2.5C, *upper panel*). However, HeLa$^{FcRn-TD}$ cells transfected with Ii chain resulted in a significant redistribution of FcRn-TD into an EEA1 positive intracellular compartment (Fig. 2.5C, *lower panel*; Fig. 2.5D). This result suggests that the association of Ii and FcRn is functional and has the ability to drive associated FcRn into the early endsomes independently of the FcRn cytoplasmic tail.

Ii directs FcRn to the late endosomes/lysosomes

In HeLa^{FcRn} cells, the majority of FcRn appeared in the early endosomal compartment (Fig. 2.5A, *upper panel*) and did not colocalize well with LAMP-1 marker (Fig. 2.6A, *upper panel*), a marker for late endosomal/lysosomal compartments. To determine the effect of Ii expression on the cellular distribution of FcRn, we examined the intracellular FcRn distribution by immunofluorescence staining in HeLa$^{FcRn+Ii}$ cells. In comparison with HeLa^{FcRn}, co-expression of Ii greatly increased the colocalization of FcRn with LAMP-1 (Fig. 2.6A, *lower panel; Fig. 2.6E*). As expected, FcRn-TD, when expressed alone, exhibited a honeycomb appearance and had limited colocalization with LAMP-1 (Fig. 2.6B, *upper panel*). Co-transfection of HeLa cells with both FcRn and Ii chain resulted in a redistribution of FcRn-TD into an intracellular compartment that largely overlapped with the LAMP-1 (Fig.2.6B, *lower panel*; Fig. 2.6E).

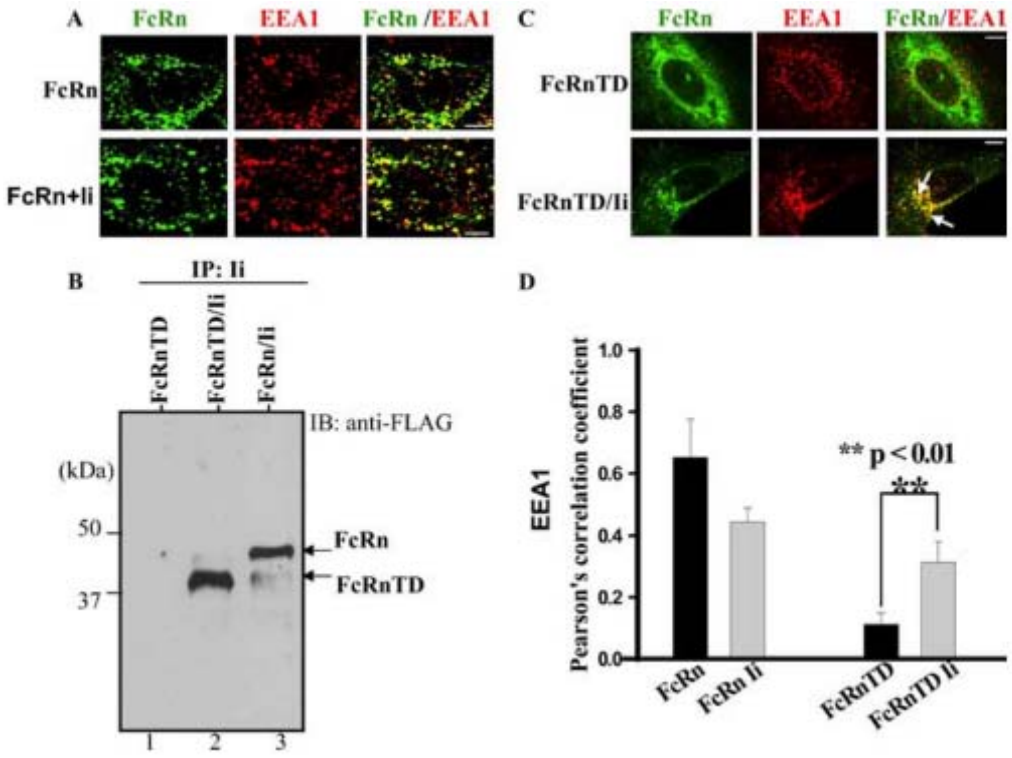


FIGURE 2.5. Ii chain redirects tailless FcRn-TD to the early endosome. Scale bars represent 5 μm.
A. The endocytic trafficking of FcRn in HeLa^{FcRn} and HeLa$^{FcRn+Ii}$ transfectants. HeLa^{FcRn} cells were transiently transfected with pBUDCE4 or pBUDCE4-Ii vector. Cells were fixed, permeabilized and co-stained for FcRn (*in green*) or early endosomal marker EEA1 (*in red*). Puncta that appear yellow in the merged images (*right panel*) indicate colocalization of FcRn with the EEA1.
B. The association of tailless FcRnTD with the Ii chain. The cell lysates from HeLa^{FcRnTD} (lane 1), HeLa$^{FcRnTD/Ii}$ (lane 2), and HeLa$^{FcRn/Ii}$ (lane 3) were immunoprecipitated by anti-HA mAb. The immunoprecipitates were subjected to Western blot. Immunoblots (IB) were blotted with rabbit anti-FLAG Ab and HRP-conjugated goat anti-rabbit Ab. The blot was developed with ECL.
C. Immunofluorescence analyses of HeLa cells expressing either tailless FcRn-TD alone (*upper panel*) or FcRn-TD and Ii (*lower panel*). Transfected cells were fixed, permeabilized, and stained with an Ab to FLAG (*in green*) or EEA1 (*in red*). The arrows indicate the colocalization (*in yellow*) of the proteins.
D. Averages of the colocalization coefficients in panel ***A*** and ***C***. Pearson's correlation coefficient were calculated. For each experiment, fifteen cells were analyzed in three different optical regions.

It is possible that over-expression of Ii non-specifically alters the endocytic system (224), consequently resulting in a redistribution of tailless or full-length FcRn into the LAMP-1^+ compartment. Hence, we followed the effect of over-expression of Ii on the cellular distribution of GFP-fusion of transferrin receptor (TfR-GFP), an endosome marker. TfR-GFP was found to be predominantly accumulated in early endosomes in either the presence (Fig. 2.6C, *upper panel*) or absence (Fig. 2.6C, *lower panel*) of Ii expression, and did not significantly route to the LAMP-1^+ compartment in $HeLa^{FcRn}$ (Fig. 2.6D, *upper panel*) or $HeLa^{FcRn+Ii}$ cells (Fig. 2.6D, *lower panel*; Fig. 2.6E). This suggests that Ii over-expression did not result in the redistribution of TfR from early endosome to the LAMP-1+ compartment. These results indicate that the Ii specifically targets FcRn from the early endosome to the late endosomes/lysosomes without significantly modifying the endocytic pathway.

To further study the effect of Ii on FcRn trafficking in cells naturally expressing both FcRn and Ii molecules, we examined the intracellular distribution of FcRn in PBMC-derived DCs using immunofluorescence with affinity-purified FcRn-specific Ab. In immature DCs, FcRn colocalized with both EEA1 (Fig. 2.7*A*, *upper panel*) and LAMP-1 markers (Fig. 2.7A, *lower panel*), verifying the cellular location of FcRn in both early and late endosomes/lysosomes. The colocalization of FcRn (green) and Ii (blue) with LAMP-1 (red) was observed in immature DCs, which appeared as white (Fig. 2.7*B*, *lower panel*). To test the role of Ii in targeting of FcRn to the late endosomal/lysosomal compartment in human immature DCs, we knocked down Ii expression using siRNA. In Ii-knockdown immature DCs, the

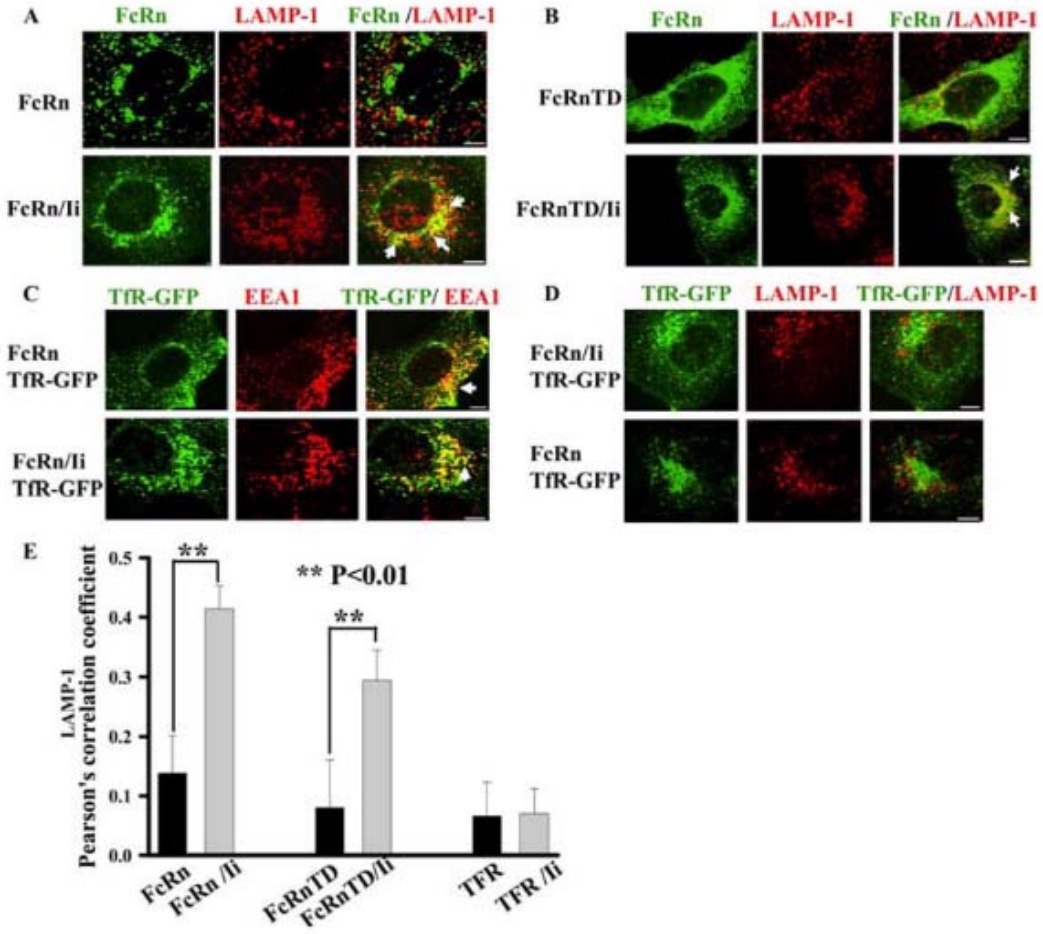


FIGURE 2.6. Ii chain directs FcRn to the late endosome/lysosome compartment. Merged images are shown in the right panels. The yellow in the merged images indicate colocalization of FcRn with EEA1 or LAMP-1 marker. Similar experiments were observed from three independent experiments. The arrows indicate the colocalization of the proteins. Scale bars represent 5 μm.

A+B. Lysosomal sorting of full-length FcRn (***A***) or tailless FcRn-TD (***B***) in the presence of Ii chain expression. HeLa^{FcRn} or HeLa$^{FcRn+Ii}$ cells were fixed, permeabilized, and immunostained for LAMP-1 (*in red*) or FcRn (*in green*), followed by Alexa 555 or 488 Fluro-conjagated IgG.

C+D. Localization of transferrin receptor (TfR) in HeLa^{FcRn} or HeLa$^{FcRn+Ii}$ cells. HeLa^{FcRn} or HeLa$^{FcRn+Ii}$ cells were transiently transfected with pcDNA-TfR-GFP, fixed, permeabilized, and immunostained for EEA1 (***C***) or LAMP-1 (***D***) marker. Transferrin (TfR) was directly visualized by GFP fluorescence (*in green*), EEA1 or LAMP-1 is shown in red. Merged images are represented in the third panel of each row.

E. Averages of the LAMP-1 colocalization coefficients in panel ***A***, ***B***, and ***D***. Pearson's correlation coefficient was measured. The fifteen cells were analyzed.

colocalization of FcRn with LAMP-1 staining was significantly decreased (Fig. 2.7B, *upper panel*; Fig. 2.7C), suggesting FcRn failing to colocalize with LAMP-1 marker when Ii was knocked down.

To examine the role of mouse Ii chain in mouse FcRn (mFcRn) trafficking. We first determined the interaction of mFcRn and ii using co-immunoprecipitation. The anti-FLAG Ab for mFcRn immunoprecipitate was analyzed by immunoblotting for Ii chain (Fig.2.8A). Anti-FLAG Ab co-immunoprecipitated mouse Ii chain (Fig. 2.8A, lane 1) in CHO cells expressing both mFcRn and murine Ii chain, but not in CHO cells expressing Ii (lane 2) or mFcRn (lane 3) alone, further confirming the association of mFcRn with Ii. To test the IgG-binding ability of mouse FcRn-ii complexes, we incubated cell lysates from mouse bone marrow-derived DC (BMDC) at either pH 6.0 or pH 7.4 with IgG-Sepharose. Cell lysates from CHO^{mFcRn} cells were used as a positive control. The binding elutes or cell lysates were analyzed using Western blot for either Ii or mFcRn. Both Ii and mFcRn were detected in the elute from IgG beads (Fig.2. 8, lane 1). As expected, mFcRn-Ii from BMDC cells bound to IgG at pH 6.0 (Fig. 2.8, lane 1), but not at pH 7.4 (Fig. 2.8, lane 2). These data confirm that like human Ii chain, the murine Ii chain also interacts with the mFcRn-IgG complexes. To examine the role of mouse Ii in the routing of mFcRn to the late endosomes/lysosomes, we compared the cellular distribution of mFcRn in BMDCs from wild type and $Ii^{-/-}$ mice. In immature BMDC from $Ii^{-/-}$ mice, the colocalization between mFcRn and LAMP-1 staining was significantly decreased (Fig. 2.8C, *upper panel* and Fig. 2.8D) in comparison

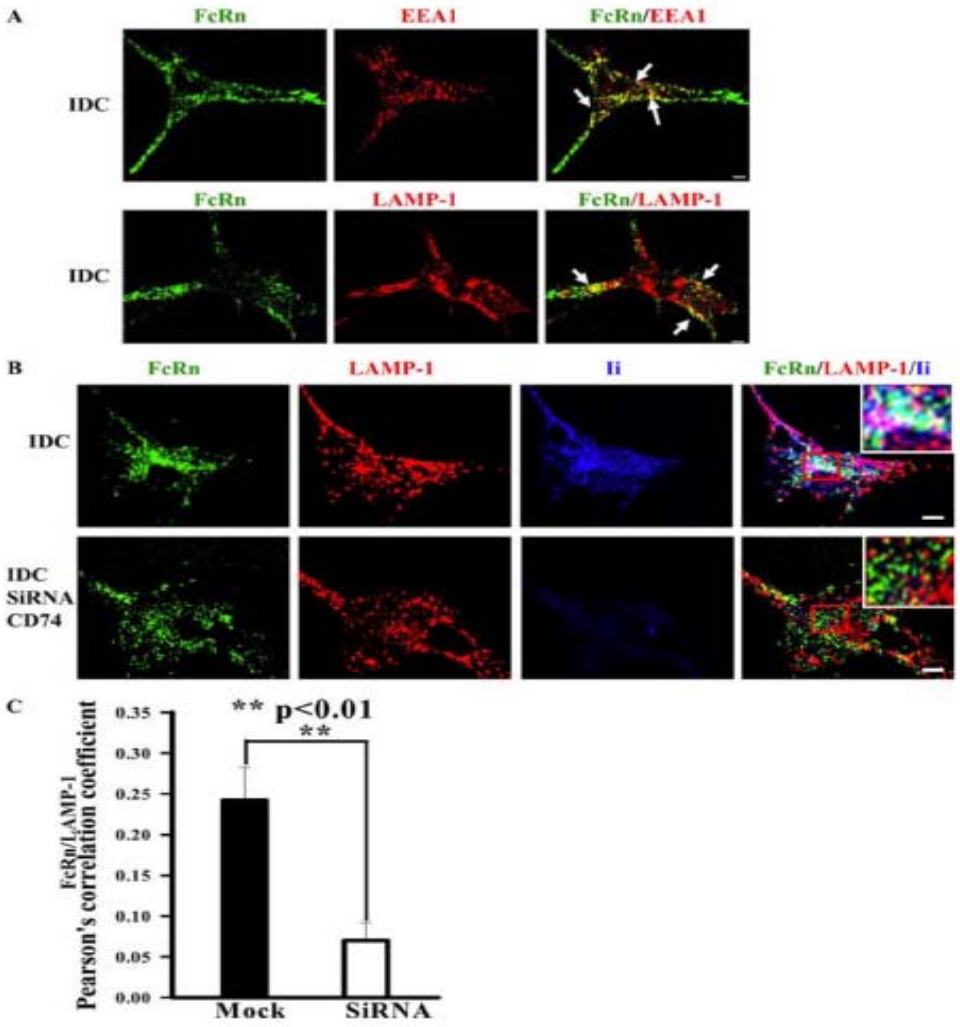


FIGURE 2.7. FcRn appearance in the late endosome/lysosome of human immature dendritic cells (IDCs) is dependent on the Ii chain expression. Human IDCs were obtained by culturing human PBMCs in the presence of GM-CSF and IL-4 for 6 days. Cells grown on glass coverslips were fixed and permeabilized before staining. The bars represent 5 μm. ***A***. Colocalization of FcRn (*in green*) and EEA1 or LAMP-1 (*in red*) in DC cells. The IDCs were incubated with affinity-purified rabbit anti-FcRn, mAb anti-EEA1 or LAMP1 antibody, followed by Alexa 555 or 488 Fluro-conjagated IgG of the corresponding species. Punctuate staining that appears in yellow in the merged images indicates (*right panels*) the colocalization of FcRn with the endosomal or lysosomal marker. The arrows indicate the colocalization of the proteins.***B.*** FcRn trafficking to the late endosome/lysosome becomes less in Ii-depleted IDC. Human IDCs were transfected with Ii siRNA plasmid (*top pane*) or vehicle (*bottom panel*)**.** Cells were immunostained for FcRn (*in green*), LAMP-1 (*in red*), and Ii (*in blue*). Colocalization of all three molecules appears in white (lower panel, inset). Similar results were observed from at least three independent experiments.
C. Averages of the LAMP-1 and FcRn colocalization coefficients in IDC transfected with vehicle (mock) or Ii siRNA plasmid in panel ***B***. Pearson's correlation coefficient was measured. The ten cells were analyzed in three different optical regions in each experiment.

with the colocalization level in BMDC from wild-type mice (Fig. 2.8C, *lower panel* and Fig.2. 8D), suggesting the majority of mFcRn fails to colocalize with LAMP-1 marker when Ii is knocked out. These data confirm that the targeting of endogenous FcRn in human immature DCs (Fig. 2.7C) or mouse BMDC (Fig. 2.8D) to the late endosomes lysosomes is significantly dependent on Ii.

Ii chain interacts with FcRn and directs FcRn into the late endosomes/lysosomes under inflammatory conditions

In addition to the expression in macrophages and DCs, FcRn is also expressed in epithelial and endothelial cells. Under physiological conditions, most epithelial and endothelial cells are Ii-negative. However, the Ii chain can be induced for expression in these types of cells by proinflammatory cytokines, such as IFN-γ or bacterial infections (225). Hence, we verified the induction of Ii in the intestinal epithelial HT-29 and T84 (Fig. 2.9A), primary endothelial (Fig. 2.9B), and HeLa cells upon exposure to IFN-γ. Caco-2 cells showed Ii chain expression by PCR even in absence of induction by gamma-IFN (Fig. 2.9B). Co-immunoprecipitation of FcRn with anti-Ii Ab from the lysates of $HeLa^{FcRn}$ or THP-1 cells treated with IFN-γ showed the Ii–FcRn association in IFN-γ-stimulated $Hela^{FcRn}$ (Fig. 2.9C) or THP-1 cells (Fig. 2.9D). However, we were unable to co-immunoprecipitate the FcRn HC from the lysates of $HeLa^{FcRn}$ cells that were not treated with IFN-γ by anti-Ii mAb, which served as a negative control (Fig. 2.9C).

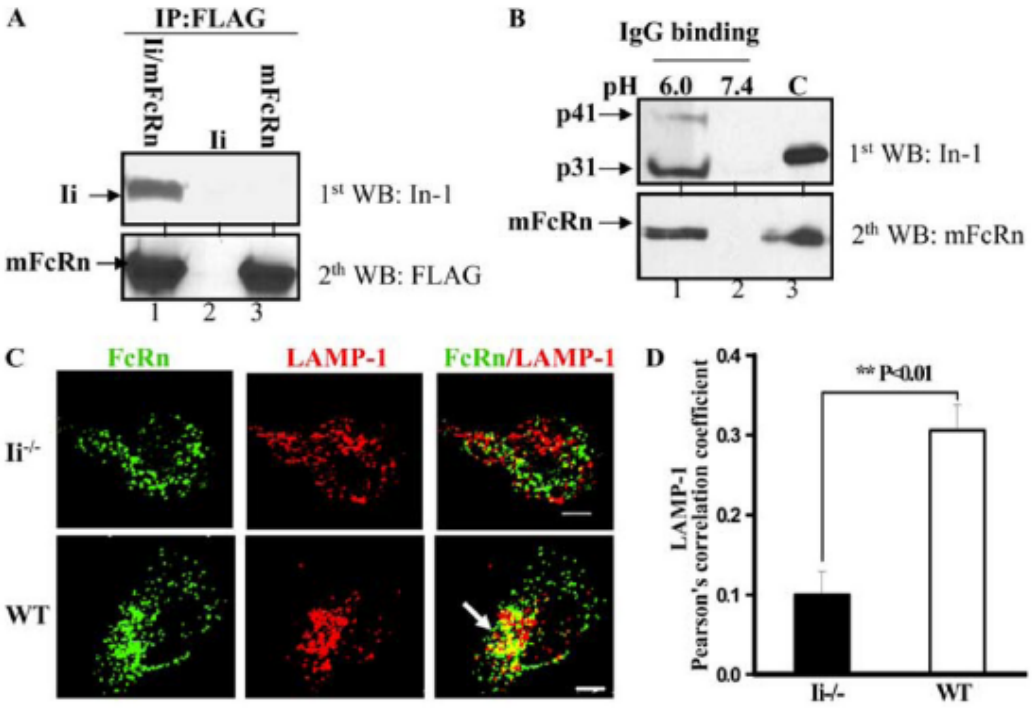


FIGURE 2. 8. Mouse FcRn appearance in the late endosome/lysosome of bone marrow-derived dendritic cells (BMDC) is significantly dependent on the Ii expression.
A. Mouse FcRn associates with mouse Ii chain. The cell lysates from CHO cells transiently with plasmids encoding murine Ii and mFcRn (lane 1), Ii (lane 2), and mFcRn cDNA (lane 3) were immunoprecipitated by anti-FLAG M2 mAb. The immunoprecipitates were subjected to Western blotting with anti-Ii (In-1) or FLAG mAb as indicated. Immunoblots were developed with ECL.
B. The mFcRn/li complex in IgG binding. BMDCs were lysed in PBS (pH 6.0 or 7.5) with 0.5% CHAPS and proteinase inhibitors. Approximately 0.5 mg of the soluble proteins was incubated with IgG-Sepharose at 4°C. The eluted proteins (Lanes 1 and 2) or cell lysates (lane 3) were subjected to Western blotting. Proteins were probed with anti-Ii (In-1) or rabbit anti-FLAG Ab and developed with HRP-conjugated secondary Abs and ECL. The locations of mFcRn HC and Ii (p31 and p41) are indicated by arrows.
C. Colocalization of FcRn (in green) and LAMP-1 (in red) in BMDC cells. The BMDC from Ii -/- (*top panel*) or wild type (*bottom panel*) mice was incubated with affinity-purified rabbit anti-FcRn or mAb anti-LAMP-1 Ab, followed by Alexa 555 or 488 Fluro-conjagated IgG of the corresponding species. The yellow and arrows indicate the colocalization of FcRn with LAMP-1. Scale bars represent 5 μm.
D. Averages of the LAMP-1 and FcRn colocalization coefficients in BMDC. Pearson's correlation coefficient was measured for the colocalization correlation of the intensity distributions between two channels. The ten cells were analyzed in three different optical regions in each experiment.

Furthermore, immunofluorescence staining showed that the colocalization of FcRn with Ii and LAMP-1were observed in Hela^{FcRn} cells upon exposure to IFN-γ stimulation (Fig.2. 9E and 2.9F, *lower panels*), but not in mock-stimulated cells (Fig. 2.9E and 2.9F, *upper panels*). This was further confirmed by Pearson's colocalization coefficients of FcRn, Ii, and LAMP-1 staining (Fig.2.9G). Statistical data showed the significance of FcRn and LAMP-1 colocalization (Fig.2. 9G). Therefore, we conclude that the intracellular trafficking of FcRn can be regulated by inflammatory cytokine through induction of Ii expression.

The cytoplasmic tail of Ii is responsible for regulating FcRn trafficking to the endosomal and lysosomal compartments

We further examined how Ii chain regulates FcRn trafficking inside cells. The key signals determining the subcellular location of most membrane proteins are in the cytoplasmic tail. The cytoplasmic tail of both human and murine FcRn contains a dileucine-based motif (Fig. 2.10A), which is existed in a number of endosomal/lysosomal resident proteins (Fig. 2.10A). To examine whether the dileucine-based motif of human FcRn is the early endosome targeting signal of FcRn, the two leucine residues were mutated into alanine (Fig. 2.10B). The FcRn-LL/AA mutant showed a honeycomb distribution with little colocalization of the EEA1 (Fig. 2.10B, *upper panel*) or LAMP-1 (Fig. 2.10B, *lower panel*) marker in HeLa$^{FcRnLL/AA}$ cells. This distribution pattern was similar to that of tailless FcRn (Fig. 2.10B, *upper panel*). Therefore, the dileucine motif in the FcRn is responsbiling for targeting of FcRn to the early endosome.

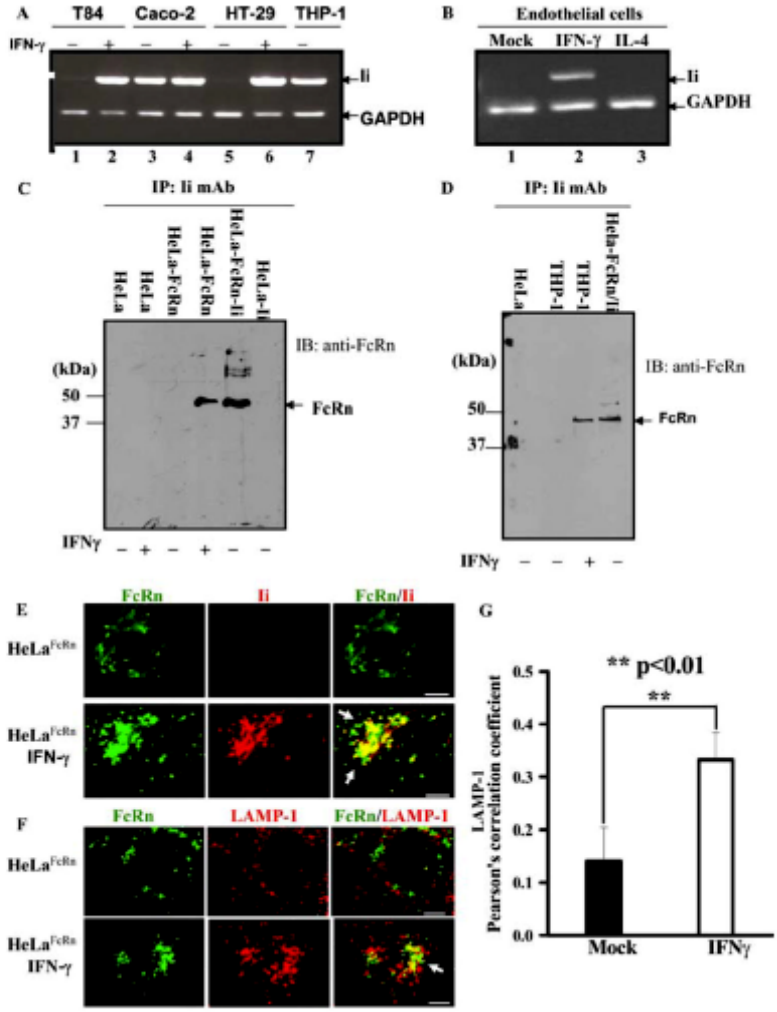


FIGURE 2.9. Ii chain can interact with FcRn under IFN-γ stimulation. ***A+B***. Ii chain was expressed in IFN-γ stimulated epithelial and endothelial cells. Intestinal epithelial (***A***) and endothelial (***B***) cells were incubated with IFN-γ (50 ng/ml) for 48 hr. THP-1 cells were used as a positive control for the expression of Ii. Total RNA was extracted. RT-PCR was performed for amplification of FcRn. ***C+D***. Association of Ii chain and FcRn in IFN-γ-treated HeLa^{FcRn} and THP-1 cells. HeLa, HeLa^{FcRn} (***C***) and THP-1 (***D***) cells were stimulated with or without IFN-γ for 24 h. The cell lysates were immunoprecipitated by anti-Ii mAb. Immunoprecipitates were subjected to Western blotting with affinity-purified rabbit anti-FcRn Ab. Immunoblots (IB) were developed with ECL. ***E+F***. Colocalization of FcRn and Ii (***E***) or LAMP-1 (***F***) in HeLa^{FcRn} cells without (*upper panel*) or with (*lower panel*) IFN-γ treatments. Cells were stimulated with or without IFN-γ for 24 h. HeLa^{FcRn} cells were incubated with anti-FLAG (*in green*), mAb anti-Ii or LAMP-1 (*in red*) antibody, The yellow and arrows (*right panels*) indicates the colocalization of FcRn with the Ii or LAMP-1 marker. Scale bar represents 5 μm.
G. Averages of FcRn and LAMP-1 colocalization coefficients in panel ***F***. Pearson's correlation coefficient were calculated. For each experiment, fifteen cells were analyzed.

The two dileucine motifs in the cytoplasmic tail of the Ii molecule are required for targeting MHC class II molecules to the endocytic pathway directly from the TGN. This suggests the possibility that the cytoplasmic tail of the Ii molecule is also responsible for targeting FcRn to the endocytic pathway. To test this hypothesis, we expressed a chimeric protein fusing the cytoplasmic tail of Ii to the extracellular domain of FcRn (FcRn-Iicyt) (Fig. 2.10C). Since the Ii chain is a type II glycoprotein, we also generated a similar chimeric protein to replace the cytoplasmic tail of FcRn with that of HLA-DM as a control (Fig. 2.10C). A tyrosine-based motif in the cytoplasmic tail of HLA-DM chain has been shown to target HLA-DM to the endocytic compartments (226). Similar to full-length FcRn in $HeLa^{FcRn}$ cells, the chimeric protein FcRn-Iicyt bound IgG at pH 6.0 (Fig. 2.10C, lane 2) but not at pH 7.4 (lane 1) in an IgG binding assay, suggesting that the fusion of the Ii cytoplasmic tail did not affect the integrity of FcRn structure. As shown in Fig. 2.10D, the cytoplasmic tail of Ii chain targeted FcRn into an intracellular compartment that largely overlapped with either $EEA1^+$ (left panel) or the LAMP-1^+ (right panel) marker. As a control, the cytoplasmic tail of HLA-DM chain directed FcRn into the LAMP-1^+ late endosomal/lysosomal compartment (Fig. 2.10D), although a relatively small portion of FcRn appeared in the $EEA1^+$ compartment in HeLa cells transfected with FcRn-DMcyt. Person's correlation coefficient analysis shows the significance of colocalization of hybrid FcRn-Iicyt molecule with endosomal or lysosomal marker (Fig. 2.10E). Taken together, these data suggest that cytoplasmic tail of Ii chain, likely two dileucine-based motifs, is responsible for directing FcRn trafficking into the endocytic pathway.

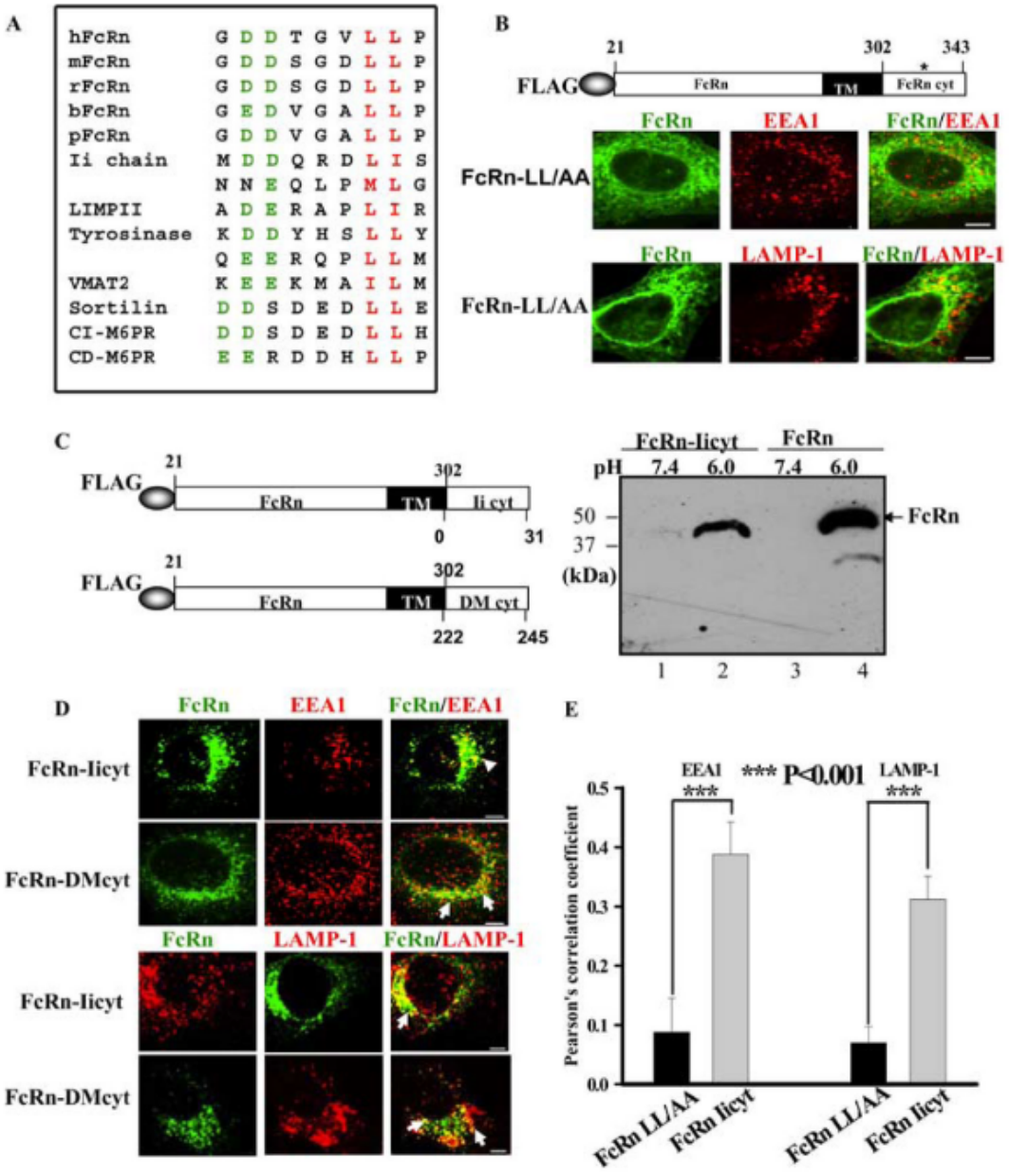

A
hFcRn G D D T G V L L P
mFcRn G D D S G D L L P
rFcRn G D D S G D L L P
bFcRn G E D V G A L L P
pFcRn G D D V G A L L P
Ii chain M D D Q R D L I S
N N E Q L P M L G
LIMPII A D E R A P L I R
Tyrosinase K D D Y H S L L Y
Q E E R Q P L L M
VMAT2 K E E K M A I L M
Sortilin D D S D E D L L E
CI-M6PR D D S D E D L L H
CD-M6PR E E R D D H L L P
B
21
302
343
FLAG
FcRn
TM
FcRn cyt
FcRn
EEA1
FcRn/EEA1
FcRn-LL/AA
FcRn
LAMP-1
FcRn/LAMP-1
FcRn-LL/AA
C
FLAG
21
FcRn
302
TM
Ii cyt
0
31
FLAG
21
FcRn
302
TM
DM cyt
222
245
FcRn-Iicyt
FcRn
pH 7.4 6.0 7.4 6.0
50
37
(kDa)
FcRn
1 2 3 4
D
FcRn
EEA1
FcRn/EEA1
FcRn-Iicyt
FcRn-DMcyt
FcRn
LAMP-1
FcRn/LAMP-1
FcRn-Iicyt
FcRn-DMcyt
E
EEA1
***
*** P<0.001
LAMP-1
***
Pearson's correlation coefficient
0.5
0.4
0.3
0.2
0.1
0.0
FcRn LL/AA
FcRn Iicyt
FcRn LL/AA
FcRn Iicyt

FIGURE 2.10. The cytoplasmic tail of Ii chain can direct FcRn trafficking to both the early endosomal and late endosomal/lysosomal compartments. The arrows indicate the colocalization of the proteins. Scale bars represent 5 μm.
A. Dileucine-based motifs involved in intracellular sorting and/or cell-surface endocytosis that present upstream acidic residues are shown. The critical Lulea (lie, Met, Val) pairs are shown in red face and acidic residues at positions upstream of the dileucine are in green face. Sequences were taken from the indicated proteins: human, mouse, rat, bovine, and swine FcRn, Ii chain, LIMPII (lysosomal integral membrane protein II), tyrosines, sportily VMAT2 **(**vesicular monoamine transporter 2**)**, CI-M6PR (action independent mannose 6-P receptor), and CD-M6PR (action dependent mannose 6-P receptor).
B. Immunofluorescence analyses of HeLa cells expressing FcRn-LL/AA. The dileucine motif was replaced by alanine residues (*) in FcRn cytoplasmic tail (*top panel*). Transfected cells were immunostained with a mAb to FLAG, EEA1 or LAMP-1, followed by Alexa 555 or 488 Fluro-conjagated IgG.
C. Schematic representation of the chemeric FcRn-Iicyt or FcRn-HLA-DMcyt. *Left panel*: the extracellular domain of FcRn (21-302 aa) was in frame fused to the cytoplasmic tail of Ii (0-31 aa) or HLA-DMβ (222-245 aa) in pCDNAFLAG. *Right panel:* The chmeric FcRn-Iicyt bound IgG. HeLa cells expressing FLAG-tagged FcRn-Iicyt (lanes 1 and 2) or $HeLa^{FcRn}$ cells (lanes 3 and 4) were lysed at both pH 6.0 and pH 7.4**.** The IgG binding assay was performed. The eluted proteins were subjected to Western blotting with anti-FLAG Ab. The blot was visualized by ECL method.
D. Immunofluorescence analyses of HeLa cells expressing FcRn-Iicyt and HLA-DMcyt. Transfected cells were fixed, permeabilized and immunostained with a mAb to FLAG, EEA1 or LAMP-1, followed by Alexa 555 or 488 Fluro-conjugated IgG.
E. Averages of endosomal or lysosomal colocalization coefficients between FcRn LL/AA and FcRn FcRn-Iicyt in panels ***B*** and ***D***. Pearson's correlation coefficient were calculated. For each experiment, fifteen cells were analyzed in three different optical regions.

DISCUSSION

The Ii chain was thought to mainly function as an MHC class II chaperone, which prevents the binding of endogenous peptides to MHC class II in the ER and directs MHC class II to endocytic compartments, where they are loaded with antigenic peptides generated from endocytosed proteins (227, 228). However, Ii has recently been shown to have additional functions by interacting with other molecules, such as with CD44 (229), MHC class I (230), and CD1d molecules (231) during T or NKT cell-mediated responses, the macrophage migration-inhibitory factor to induce the phosphorylation of the extracellular signal-regulated kinase-1/2 (232), *Helicobacter pylori* urease B subunit to stimulate IL-8 production (225), and HIV-2 Vpx (233). In this study, we showed a novel role of Ii chain in FcRn-mediated IgG transport and catabolism by regulating intracellular trafficking of FcRn.

This study provides several lines of evidence to demonstrate the interaction of Ii chain with FcRn. First, Ii was co-immunoprecipitated with FcRn in HeLa transfectant (Fig.2.1). Furthermore, FcRn colocalization with Ii in HeLa transfectant suggests their association in vivo. Second, although the Ii is not constitutively expressed in certain types of epithelial and endothelial cells, FcRn/Ii complexes were coimmunoprecipitated from these cells when Ii chain expression was induced by IFN-γ Fig. 9, suggesting the interaction of FcRn and Ii in epithelial or endothelial cells can be caused under inflammatory conditions. Third, the FcRn/Ii complexes were also present in both Endo-H-sensitive or -resistant form. This

biochemical evidence suggests that the FcRn/Ii complexes were formed as early as in the ER and remained as a complex when passing through the Golgi stack. Fourth, Ii chain interacted with the nascent FcRn HC alone or with FcRn-β2m complex (Fig. 2.2), suggesting that the Ii chain may play a role in retaining FcRn HC in the ER until assembly of the complex of HC with β2m. This is in agreement with the studies showing that Ii chain retains incompletely-folded CD1d HC in the ER (231), but in disagreement with studies showing that the Ii–MHC class I association in the ER appears to require essentially fully-folded MHC class I because free HC does not associate efficiently with Ii chain (223, 234).

The CLIP fragement of Ii bind to the antigen binding groove of MHC class II or I (223). In contrast to the interaction of Ii with MHC class II, the CLIP of Ii is not required for interaction of Ii with FcRn due to a narrowed antigen binding groove of FcRn. This raises an interesting question whether CLIP is important for the association of Ii with FcRn. Our finding that mutation or deletion of the CLIP did not disrupt FcRn-Ii interaction suggests that the CLIP fragment is not directly involved in the FcRn-Ii association (Fig. 2.4). It is still possible that the CLIP positions itself above the binding groove of FcRn by the flanking sequences. This would result in the engagement of Ii to FcRn at multiple sites other than the peptide binding groove. The multiple binding sites between Ii and FcRn may enhance the interaction. Indeed, the FcRn-Ii complex resisted multiple detergents (Fig. 2.1E). Further study is required for mapping FcRn-Ii interaction as described for MHC class II and Ii (235).

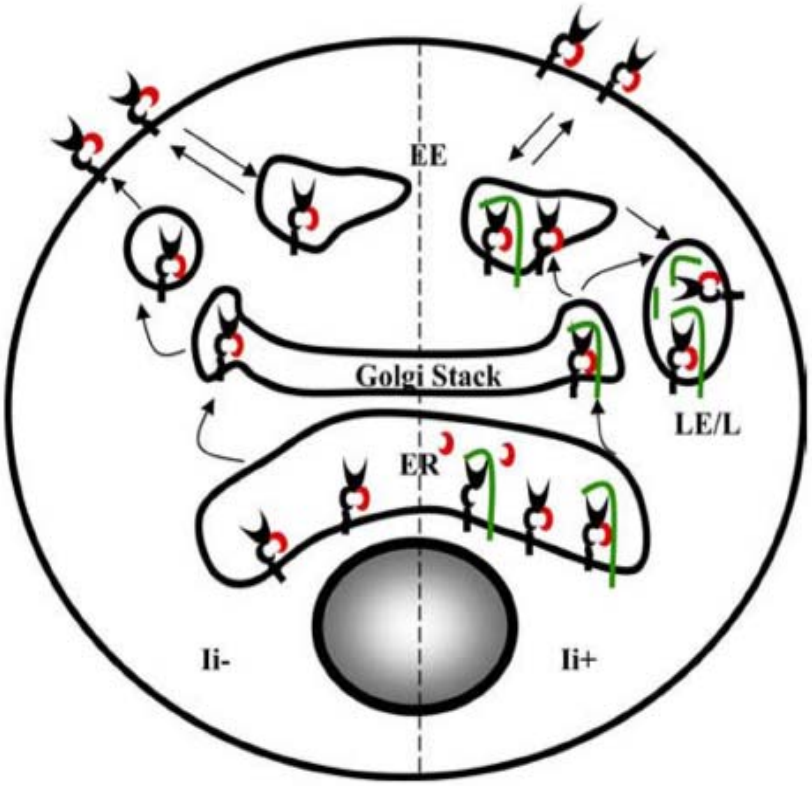


FIGURE 2.11. Two pathways for intracellular trafficking of FcRn. In cells without Ii expression, FcRn may reach the cell surface through the secretory pathway and recycle between the plasma membrane and endosomes via endocytosis. In the presence of Ii expression, a portion of FcRn molecules is associated with the Ii in the ER and is targeted to the endosomes and lysosomes via Golgi stack. In the endosome/lysosome, Ii was released from FcRn, presumably, by proteolytic cleavage. Ii represents in green, FcRn heavy chain in black, and β2m in red. ER, endoplasmic reticulum; EE, early endosome; LE, late endosome; L, lysosome.

The association of Ii chain with FcRn clearly expands the distribution of FcRn within the endocytic pathway. FcRn has been predominantly detected in the early endosomes. The deletion of FcRn cytoplasmic tail alters the distribution of FcRn to the cell surface and cytomplasm. The cellular location of tailless FcRn was partially restored by the expression of Ii chain (Fig. 2.5). Furthermore, Ii directs both tailless and intact FcRn to LAMP-1^{+} late endosomes/lysosomes (Fig. 2.5). This observation was further verified by the fact that FcRn appeared in the late endosomes/lysosomes in wild-type immature DCs (Fig. 2.7 and 2.8) or IFN-γ-treated $HeLa^{FcRn}$ cells (Fig.2.9F), but not in Ii-deficient DC (Figs. 2.7 and 2.8). In this aspect, the role of Ii in FcRn intracellular trafficking mirrors its role in directing the MHC class II intracellular trafficking. Furthermore, our data showed the transplantation of Ii cytoplasmic tail conferred tailless FcRn to appear in both early endosome and late endosome/lysosome (Fig.2.10D), suggesting the dileucine-based motif in the Ii molecule would compensate for the sorting function absent in the tailless FcRn. Mutations of the dileucine-based motif in the cytoplasmic tail of FcRn associated with a profound loss of its early endosomal location (Fig. 2.10B), suggesting this motif is sufficient to direct FcRn to the endosomes at the steady state. Dileucine signals have been reported to bind adaptor protein (AP) complexes that are essential for intracellular protein sorting (171). The additional two dileucine-based motifs in Ii would further enhance the interaction of FcRn-Ii complex with AP complexes, leading FcRn trafficking from the early endosome to the late endosome/lysosome. We propose that the intracellular locations of FcRn may be controlled by two different mechanisms, which lead to either early endosomal or late

endosomal/lysosomal targeting of FcRn. This provides flexibility for FcRn traffic in Ii-positive or negative cell types. In Ii negative cell types, FcRn probably traffics directly to the cell surface from the TGN and is subsequently internalized into the early endosomes. In Ii expressing cell types, Ii-associated FcRn is likely to be segregated from the secretory pathway at the TGN and directly targeted to the endocytic pathway without access to the plasma membrane (Fig. 2.11), although further studies are needed to test this hypothesis.

What would be the biological consequences of Ii-FcRn association and the expanded intracellular trafficking of FcRn as conferred by Ii chain particlularly in heightened inflammatory circumstances? Several speculations can be made. FcRn has two known biological functions: transcytosis of IgG across the polarized epithelial cells and maintainence of IgG or albumin homeostasis. Under physiological conditions, the cytoplasmic tail of FcRn clearly predominates its trafficking in the majority of Ii-negative epithelial or endothelial cells to early endosomes. However, this pattern of trafficking may be significantly altered in inflammatory conditions since Ii expression is induced in epithelial and endothelial cells by inflammatory cytokines or during bacterial and viral infections (225). FcRn transports normal or pathogen-specific neutralizing IgG across polarized epithelial cells, potentially 'seeding' mucosal immunity. It remains to be deteremined precisely how the altered trafficking to late endosomal/lysosomal compartments in Ii expressing epitheleial and endothelial cells would influence the IgG transport and homeostasis and the local immune response. Neveretheless, from the aspect of IgG

protection, one can envision that due to the association of FcRn with Ii, FcRn might bind IgG not only in the early endosome, but also in the late endosomal/lysosomal compartment, thus extending its boundary for sampling IgG. The evidence that FcRn was capable of binding IgG under lysosomal conditions (pH 5.0) (Zhu et al., unpublished) makes this event highly likely. Hence, the appearance of FcRn in the late endosome/lysosome may function as a second line that salvages the IgG from degradation. Second, since MHC class II are most efficiently targeted into proximity with antigenic peptides by Ii chain in antigen presenting cells (APCs); it is interesting to bring into perspective the known functions of Ii in conventional APCs in order to understand the role of FcRn/Ii association in influencing antigen presentation. Our previous findings show that the FcRn is expressed in Mϕ and DCs (153). Since FcRn appears to associate with Ii chain, FcRn and MHC class II might occupy the same acidic compartments. In Mϕ and DCs, FcγRs can promote the internalization of immune complexes into the endosomes and lysosomes to increase the efficiency of MHC class II presentation to $CD4^+$ T cells (236). FcRn, on the other hand, may mediate the antigen presentation by binding the immune complexes in these antigen processing compartments. The evidence that FcRn is able to bind immune complexes and IgG in the pH range of endosomes and lysosomes (Zhu et al., unpublished data) supports this probability. Therefore, the association of Ii chain and FcRn could further influence the antigen presentations. Third, FcRn was involved in IgG-mediated phagocytosis with its expression in the phagolysosomes in human neutrophils (237). It would be interesting to know whether FcRn-Ii association in the neutrophils mediates the translocation of FcRn

into the phagolysosomes. Fourth, an *N*-terminal product, liberated from Ii chain via regulated intramembrane proteolysis, has been recently shown to function as a transcription factor in activating the NF-κB-dependent transcription program, at least in B lymphocytes (238, 239). Our recent finding revealed that the activation of NF-□B signaling upregulates the level of FcRn expression (240). As a result, co-expression of Ii chain and FcRn might regulate the FcRn expression.

In conclusion, this study delineated a novel intracellular destination for FcRn trafficking and demonstrated a novel, unexpected function of Ii chain. Our study suggests that the intracellular trafficking pathway and functions of FcRn may be altered by the association of Ii chain during physiological and inflammatory conditions, and points to a potential effect of Ii in modifying IgG functions of FcRn. Thus, the association of FcRn with Ii chain is physiologically relevant, and appreciation of this process is important to understanding how IgG is transported and how IgG levels are maintained throughout the body. Given that Ii chain may function at different conditions, it is of interest to examine how these various effects will ultimately manifest in terms of IgG transcytosis and protection.

Chapter 3: Mucosal Delivery of Subunit Vaccine Using an IgG Transfer Pathway

ABSTRACT

Vaccine strategies to prevent invasive mucosal pathogens are being sought due to the fact that 80-90% of infectious diseases are initiated at mucosal surfaces. However, our ability to deliver an intact vaccine antigen across the mucosal barrier for induction of the effective immunity is limited. The neonatal Fc receptor (FcRn) mediates the transport of IgG across polarized epithelial cells lining mucosal surfaces. By mimicking IgG transfer at mucosal surfaces, intranasal immunization with a model antigen herpes simplex virus type-2 (HSV-2) glycoprotein gD fused with an IgG Fc fragment in the combination of adjuvant CpG resulted in a complete protection of wild type, but not FcRn knockout mice that were intravaginally challenged with virulent HSV-2 186. The immunization induced efficient mucosal and systemic antibody, B and T cell immune responses, including memory immune responses which remained stable at least 6 months post-vaccination. These results at first demonstrate that the FcRn-IgG transcellular pathway may represent a novel mucosal vaccine delivery path for a subunit vaccine against mucosal pathogens in humans.

INTRODCUTION

Most infectious pathogens initiate their infections through mucosal surfaces of the respiratory, gastrointestinal and genital tracts; an ideal vaccine should not only

elicit systemic humoral and cellular immunity, but also engender mucosal immune responses. However, most current vaccines are administered through intramuscular or subcutaneous route (209). While these parental immunizations are efficient at inducing strong systemic IgG responses, they usually engender low mucosal immunity. The continued emergence of clinic patients suffering mucosal infections emphasizes the urgent need to develop a vaccine targeted at mucosal-associated lymphoid tissues. To achieve this goal, the passage of vaccine antigens into or across the epithelial barrier is the first step in the complex sequences of events that lead to mucosal, and potentially, systemic immunity. Mucosal and system immune responses can be more efficiently achieved by directly applying antigens of vaccines onto mucosal surfaces (209). The close associations among epithelial, immune effector and antigen presenting cells create an efficient mechanism for sampling lumenal vaccine antigens. This ideal sampling should trigger the induction of mucosal immunity and result in protection of mucosal surfaces. However, epithelial monolayers lining the mucosal surfaces are impervious to macromolecule diffusion, due to the presence of intercellular tight junctions at the apical poles (185). In this way, mucosal barriers insulate cross-talk between lumenal vaccine antigens and the immune effectors cells within the laminar propria, resulting in the exclusion of vaccine antigens from transport across the epithelial barrier or from taking up for processing and presentation to subjacent lymphoid tissue within the epithelium, lamina propria and draining lymph nodes. Different approaches have been taken in an exploratory way, such as targeting mucosal vaccines onto the specially differentiated microfold (M) cells at mucosal surfaces (241). However, the population of M cells is

relatively rare (only accounting for 0.1% of epithelial cells) and sparsely scattered, leading to the less efficient delivery of antigens. Therefore, it is necessary to explore novel pathways to efficiently deliver vaccine antigens across mucosal barriers.

The neonatal Fc receptor for IgG (FcRn) was first identified in the intestinal epithelial cells of a suckling rodent, where it is expressed at high level. FcRn transfers maternal IgG across the polarized placental and/or intestinal epithelial cells (124), which allows newborns to obtain maternal IgG against antigens encountered by the mother before they develop their own immune system. However, its functional expression in a variety of cells and tissues, including epithelial cells lining mucosal surfaces, has recently been identified in adult animal and humans (118). Further work has showed that FcRn is also responsible for shuttling IgG antibodies across mucosal surfaces. In addition to its function as a transporter, FcRn protects IgG by extending their life span (124, 141). FcRn binds IgG isotypes in a pH-dependent manner, binding IgG at acidic pH (6.0-6.5) and releases IgG at neutral or higher pH (124). Amino acid residues, I253, H310, and H433 located at the interface between CH2 and CH3 domains of IgG, are of particularly functional significance to this pH-dependent binding (124). In the majority of cell types, FcRn resides primarily in the early acidic endosomal vesicles with limited cell surface expression. In early endosome, FcRn binds IgG that enters cell by pinocytosis or endocytosis. Subsequently, FcRn recycles IgG back to the cell surface in nonpolarized cells or transcytoses IgG to the opposite surface in polarized epithelial cells. The near neutral pH of extracellular environment causes IgG release from FcRn. The IgG that does not bind to the FcRn inside cells

moves to lysosomes where it undergoes degradation.

Observations on IgG transport within mucosal epithelia by FcRn imply that FcRn may transport antigen, if fused with the IgG Fc, across the mucosal barrier. Several lines of evidence suggest that this pathway for direct shuttling of Fc-antigen may be feasible. For example, in the rodent, IgG-mediated immune complexes can be transported from the gut lumen (149). Therefore, FcRn-mediated mucosal vaccine delivery, if possible, might allow the host to specifically sample an Fc-fused subunit vaccine in the mucosal lumen, followed by transport across the mucosal epithelial barrier. Such an idea has never been tested for delivering a subunit vaccine across mucosal barrier against a particular pathogen. Herpes simplex virus type-2 (HSV-2) causes sexually-transmitted disease and the primary site of HSV-2 infection is the mucosa of the genital tract. The development of HSV-2 subunit vaccines is focusing on their major envelope glycoproteins, because of their key roles in the early steps of viral infection and their being major targets for both humoral and cellular immunity. Of these glycoproteins, gD is proposed as a principal vaccine candidate, since gD induces a more consistent and stronger cellular immune response and gD-specific antibody has the highest complement independent neutralization titer (242). In this study, we were elected to determine the ability of FcRn to deliver the model antigen, HSV-2 gD-Fc fusion protein, across the respiratory mucosal barrier to probe immune responses to this mucosal immunization and to define protective immune responses and mechanisms against mucosally-administered virulent HSV-2 challenge. We found that FcRn-targeted mucosal immunization were efficient at inducing gD-

specific antibody responses in both serum and mucosal secretions and maintaining high levels of protective immune responses for at least six months in mice. The memory response was domonstrated by antibody secreting plasma cells, memory B cells, and IFN-γ and IL-2 producing T cells with antigen-specific proliferative potential and superior cytokine production profiles. These results are the first to demonstrate that the FcRn-IgG transcellular pathway may represent a novel mucosal subunit vaccine delivery against mucosal pathogens in humans.

MATERIALS AND METHODS

Cells, antibodies, and virus

Chinese Hamster Ovary cells were grown in DMEM with 10% FCS (Gibcol), when necessary complemented with 400 μg/ml of G418. Inner Medullary Collecting Duct (IMCD) cell line expressing rat FcRn was gift from Dr. Neil Simister in the Brandies University, grown and maintained in DMEM with 10% FCS. Splenocyte suspensions or bone marrow cells ($5x10^5$/ml) were incubated overnight at 37°C and 5% CO_2 in a humidified incubator in complete medium (RPMI 1640 medium with 10% (vol/vol) FCS, penicillin (100 units/ml), streptomycin (100 μg/ml) and 2-mercaptoethanol (50 μM; Sigma). Herpes Simplex Virus-2 186 (HSV-2) strain was from Dr. Lawrence Stanberry (Columbia University, New York, NY) and virus stocks were prepared by infection of Vero cell monolayers at a multiplicity of infection of 0.01. All epithelial and CHO cells were maintained in DMEM complete medium (Invitrogen Life Technologies) supplemented with 10 mM HEPES, 10% fetal calf

serum, 2 mM L-glutamine, nonessential amino acids, and penicillin (0.1 μg/ml)/ streptomycin (0.292 μg/ml) in a humidified atmosphere of 5% CO_2 at 37°C.

Antibody specific for mouse FcRn was prepared as previously descirbed (247). HRP-conjugated donkey anti-rabbit or rabbit anti-mouse Ab was purchased from Pierce, purified mouse IgG and chicken IgY was from Rockland Laboratories, and HRP-conjugated goat anti-mouse IgG1, IgG2a and IgG3 were from Southern Biotech. All DNA modifying enzymes were purchased from New England Biolab. Purified HSV-2 glycoprotein D was purchased from Meridian Life Science.

Expression of gD-Fc Fusion Proteins

cDNA encoding the extracellular domain of HSV-2 gD (26aa-340aa) was amplified by PCR from a plasmid provided by Dr. Patricia G. Spear (Northwestern University) using the forward primer, 5'-CCCAAGCTTAAAATGGGGCGTTTGACCTCCGGC-3', and backward primer ,5'-AGATCCCGAGCCACCTCCTCCGGACCCACCCCCGCCTGATCCGCCCGGGTTGGCTGG-3' to introduce a COOH-terminal extension with fourteen codons for glycine and serine residues (GSSGGGSSGGSSS). The Fc-fragment of mouse IgG2a containing hinge, CH2 and CH3 domains was amplified from the OKT3 hybridoma. The mutant Fc (HQ310 and HN433), unable to bind mouse FcRn, was made by oligonucleotide site-directed mutagenesis (Clontech). To construct a nonlytic Fc fragment, oligonucleotide site-directed mutagenesis was used to replace the C1q

binding motif Glu^{318}, Lys^{320}, Lys^{322} with Ala residues. Fusions were then performed in PCR-based gene assembly approach by mixing the cDNA for gD and the Fc fragment. All these DNA fragments were ligated into the pCDNA3 vector. Each construct was verified by DNA sequencing.

The plasmid containing the chimeric gD-Fc fragment was transfected into Chinese hamster ovary (CHO) cells. G418-resistant clones were selected for secretion of gD-Fc. SDS-PAGE and Western blot were performed to assess the recombinant fusion proteins in serum-free medium (SFM, Invitrogen). The highest secreting clones were screened. Recombinant proteins were purified from CHO cell supernatants by affinity chromatography using Protein A Sepharose 4 Fast Flow (Amersham Pharmacia Biotech) or goat anti-mouse IgG affinity column (Rockland). Protein concentration was measured with Brafold Kit (Pierec) using mouse IgG2a as standard.

In vitro and in vivo transcytosis

The in vitro IgG transport was performed as a modification from previously-described methods (147). IMCD cells expressing rat FcRn were grown onto transwell filter inserts (Corning Costar) to form a monolayer exhibiting transepithelial electrical resistances (TER, 400 $\Omega.cm^2$). TER was measured using a tissue-resistance measurement equipped with planar electrodes (World Precision Instruments, Sarasota, FL, USA). Monolayers were equilibrated in Hanks' balanced salt solution. The 50 μg/ml of fusion proteins were applied to the apical compartment, and

incubated with DMEM medium supplied with or without 1mg/ml of mouse IgG or chicken IgY as competitors for 2 hr at 37°C degree. Transported proteins were sampled from the basolateral chamber and analyzed by reducing SDS-PAGE and Western blot-ECL. For in vivo transport, the biotinylated 20 μg of fusion proteins or gD alone in 20 μl of PBS were intranasally (i.n.) administered into the mice that were anethesitized with isoflourane. 8 hr later or at indicated time points, transported proteins in sera were determined by ELISA. NIH Image software (National Institutes of Health, Bethesda, MD) was used to determine relative band intensities of a blot.

Mice immunization and virus challenge

Female inbred C57BL/6 mice aged 6 to 8 weeks were purchased from the Charles River. The B6.129x1-Fcrgttm1Dcr ($FcRn^{-/-}$) were from the Jackson Laboratory. All mice were housed in the animal resources facility at the University of Maryland. All animal studies were reviewed and approved by the Institutional Animal Care and Use Committee. To overcome the possible mucosal immune tolerance (243), all proteins and PBS were loaded with immunostimulatory DNA rich in CG motifs (CpG). Groups of 5 mice were intranasally immunized with 20 μl of 20 μg gD-wtFc, gD-mutFc, or recombinant gD alone in combination with 20 μg CpG ODN1826 (5′-TCCATGACGTTCCTGACGTT-3′) (Invivogen) per immunization at weeks 0 and 2 under an anesthesia condition with isofluorane. One group of 5 mice was mock-immunized with PBS following the same schedule. Mice were kept on their backs under the anesthesia to allow the inoculum to be taken up.

Mice were inoculated with viruses intravaginally as described previously (244). Briefly, 7 days prior to inoculation, mice were treated with 3 mg of medroxyprogesterone acetate (Depo-Provera, Pfizer) subcutaneously in a 10-day period. Hormonal pretreatment was necessary to induce susceptibility of mice to genital HSV-2 inoculation, which may reflect thinning of the genital epithelium or induction of the HSV entry receptor, nectin-1, on vaginal epithelial cells. Avertin (Sigma) anesthetized mice were infected intravaginally with 1×10^4 pfu of wild-type HSV-2 strain 186 in a total volume of 20 µl. Mice were kept on their backs under the influence of anesthesia for 45 min to allow infection. Mice were monitored for 14 days for the pathology and death. For virus titration, virus were inoculated into Vero cells, incubate for 45 minutes at 37°C. After washing, 1% methcellulose in DMEM containing 2% FCS were added to overlay the cells. The cells were cultured for additional 3 days, and the overlay was removed and fixed with 3.7% formaldehyde for 1 hr, then staining with 1% crystal violet.

Preparation of single-cell suspensions from lymph nodes, **spleen, lung, and vaginal tissues**

Spleens and lymph nodes were made into single-cell suspensions by passage through a sterile mesh screen. Cells were resuspended in Hanks' balanced salt solution (HBSS) and counted by trypan blue dye exclusion. For each experiment, LNC and spleens were generally pooled from 3 mice. For the preparation of single-cell suspension from lung, mice were administered with 400 ul of Avertin through i.p. injection. Lungs were perfused with 10 ml PBS through the right ventricle, minced

with blades, and incubated with HBSS (Hyclone) containing 2.5 mM Hepes and 1.3 mM EDTA at 37°C for 30 minutes, followed by treatment at 37°C for 1 hr with 2.5 mg/ml collengase D (Roche) in RPMI 1640 medium containing 5% FBS. A single-cell suspension was prepared after RBC lysis. The resulting cells were filtered through a 70-um cell strainer (BD) and used for FACS analysis.

For isolation of vaginal lymphocytes, the vagina was excised, the cervix was removed, and the remaining vaginal tissue was cut longitudinally and minced with a sterile scalpel in complete RPMI 1640 culture medium. Minced tissues (epithelium and lamina propria) were digested in complete medium with sterile 0.25% collagenase type IV (Sigma Chemical Co., St. Louis, Mo.). Digestion was accomplished with shaking incubation at 37°C for 30 min. After digestion, tissues and cells were filtered through a sterile gauze mesh and washed with RPMI 1640 medium, and additional tissue debris was excluded by slow-speed centrifugation for 1 min. Cells were collected from the supernatant by centrifugation, resuspended in HBSS, and counted by trypan blue dye exclusion.

Flow cytometry

Single cell suspensions from the spleen, lung or vaginal tissues were collected and cells were spun down. Erythrocytes were then lysed in 0.14 M NH4Cl, 0.017 M Tris-HCl at pH 7.2 on ice for 10 min. Cells were preincubated with an Fc block (mAb to CD16–CD32, 2.4G2, PharMingen, San Diego, CA) and washed in FACS buffer (PBS, 0.5% bovine serum albumin, 0.01% sodium azide). Cells were incubated with

specific antibody (0.25 μg/10^6 cells/100 μl) directly conjugated to fluorsecein isothiocyante (FITC), phycoerythrin (PE), washed, and analyzed using a FACScan (Becton Dickinson, Mountain View, CA). The mAbs (PharMingen) we used were anti-CD3ε, 500A2; anti-CD4, RM4- 5; anti-CD8, 53-6.7; anti-IFN-γ, XMG1.2, anti-B220, RA3-6B2, FAS, Jo2, PNA (Sigma). Purified HSV-2 gD proteins were labeled with Alexa Fluro647 protein labeling kit (Invitrogen) according to the manufacture's instruction. Cells incubated with rat IgG2a, rat IgG2b, or hamster IgG isotype control antibodies were used to determine the background fluorescence. The isotype control antibodies included in each experiment were considered the true baseline fluorescence used to evaluate and illustrate the results for the cell-specific antigen markers. Cells were washed in FACS buffer and analyzed using a FACSAire and FlowJo software (Becton Dickinson, Mountain View, CA).

T cell proliferation

Single cell suspensions from mouse spleen were suspended in RPM-1640 with 1% FCS, 2.5mM Hepes at 10^7/ml. Carboxyfluorescein diacetate succinimidyl ester (CFSE, 5mM in stock, Invitrogen) was 10-fold diluted with PBS, 4 μl of diluted CFSE was then added into 10^7/ml cells for a 2 μM final concentration. The reaction was incubated for 10 min at 37°C. The 1 ml cold FCS was added incubated on ice for 5 min to stop the reaction. The cells were washed twice with RPMI-1640 with 10% FCS. The $5x10^5$ labeled cells were plated into 96 well plates in 200 μl of medium and cultured for 4 days. The cells were then harvested and subjected to flow cytometry assay.

Intracellular cytokine staining

Intracellular IFN-γ production by primed $CD4^+$ and $CD8^+$ T cells was evaluated using bulk splenocytes or isolated lung or vagina infiltrating lymphocytes incubated for 8 hr with 25 μg//ml of the purified gD protein or medium alone. Cells were then cultured for another 10 hr in the presence of brefeldin A (Sigma, St. Louis, MO). Then cells were washed and incubated with anti-CD16/CD32 antibody to block Fcγ receptors, and stained with anti–mouse CD4, CD8, and CD3 antibodies for 15 min at 4°C. After fixation and membrane penetration with Cytofix/Cytoperm Plus (BD Biosciences), cells were stained for intracellular IFN-γ for 30 min on ice. All mAbs were purchased from BD Biosciences. Cells were washed three times, resuspended in FACS buffer, and analyzed by flow cytometry. Data was collected using FACSAire and analyzed using FlowJo software (Tree Star).

Enzyme-linked immunosorbent assay (ELISA), plasma cell ELISPOT, and neutralization test

For the detection of gD-specific antibodies in serum, bronchial lavage and vagina fluid of mice, high-binding ELISA plates (Maxisorp, Nunc) were coated with 5 μg/ml of recombinant gD protein in PBS and incubated overnight at 4 °C. Plates were then washed three times with PBS-Tween 20 0.02% and blocked with PBS-BSA 1% for 1 h at room temperature. Serial dilutions of the samples or controls in PBS-BSA 0.25% were incubated for 2 h at room temperature and visualized with rabbit anti–mouse IgG antibody conjugated to horseradish peroxidase (1:2,000; Pierce) or

anti–mouse subclass-specific antibodies (1:5000; SouthernBiotech), followed by colorimetric assay using tetramethyl benzidine. OD_{450} was measured using a Victor III microplate reader (Perkin Elmer). Titers represent the highest dilution of serum showing an OD_{450} having 2 fold of control. Neutralizing antibodies were measured by a standard virus neutralization assay. Sera were heat-inactivated, diluted 10-fold, then in twofold steps in MEM with 2% FBS. Fifty PFU of HSV-2 per well were added and incubated at 37°C for 1 hr. Methylcellulose (0.8%) in DMEM containg 2% FCS were added and incubated for 72 hr at 37°C. The titers were expressed as the reciprocal of twofold serial dilution decreasing the half of plaque number of control group. Each assay was done in triplicate.

For measuring anti-gD antibody-producing plasma cells, the 96-well ELISPOT plates (Multiscreen^{TM} HTS HA; Millipore) were coated with gD (5 μg/ml) and blocked with RPMI 5% FCS (Invitrogen) for 90 min at 37°C and 5% CO_2. Serial dilutions of bone marrow single-cell suspensions were prepared in RPMI 5% and incubated in the coated wells for 24 hours at 37°C in 5% CO_2. Cells were removed, and plates were washed with PBS-T for 5 times, then incubated with biotin labeled goat anti–mouse IgG-specific antibody (1:1500; Sigma) for 2 hours. After washing with PBS, the avidin conjugated to horseradish peroxidase (1:2,000; Vector Laboratories) was added and incubated for 1 hour, followed by substrate from the AEC kit (BD Biosciences). Spots were counted with Elispot Reader and analyzed with software (Zesis). Mouse cytokines IFN-γ, IL-2, and IL-4 from the cell culture

supernatant were analyzed by ELISA according to the manufacturer's instructions (BD Biosciences).

Immunofluorescence

Immunofluorescence was performed based on methods previously described (245). Briefly, frozen serial sections of the lung were cold-fixed in 3.7% paraformaldehyde (Sigma) in PBS for 30 min at 4°C and quenched with glycine for 10 min and stained with anti-PNA (germinal centers, red) and anti-B220 (B cells, green), followed by Alexa 555 or 488 Fluro-conjagated IgG of the corresponding species. After each step, cells were washed at least three times with 0.1% Tween-20 in PBS. Coverslips were mounted on slides with ProLongTM antifade kit (Molecular Probes) and examined using a Zeiss LSM 510 confocal fluorescence microscopy. Images were manipulated in Adobe Photoshop 7.0.

Western blot and SDS-PAGE gel electrophoresis

The proteins in the medium or extracted from lung tissue were resolved on a 12% SDS-PAGE gel under a reducing or non-reducing condition. Proteins were electro transferred onto a nitrocellulose membrane (Schleicher & Schuell). The membranes were blocked with 5% non-fat milk, probed separately with anti-gD, anti-IgG Fc Ab or anti-mouse FcRn for 1 hr, and followed by incubation with HRP-conjugated rabbit anti-mouse or donkey anti-rabbit Ab. All blocking, incubation, and washing were performed in PBST solution (PBS and 0.05% Tween 20). Proteins were visualized by the ECL (Pierce).

Passive transfer of immune sera

Sera were collected weekly from the mice 4 weeks after immunization, then pooled , heat inactivated at 56°C for 30 minutes and stored frozen at -80°C until use. Mice received a single intraperitoneal (i.p.) injection of 0.3 ml immune sera 24 hours prior to challenge to allow distribution and equilibration of antibody to all tissues prior to virus inoculation. Mice were challenged intravaginally with 1 X 10^4 PFU HSV-2 186 strain.

Statistics

To compare survival curve, Kaplan-Meier log-rank analyses were used. Ab titers, concentration of gD in serum, cytokine concentration and virus titers were assessed by using the unpaired two-tailed t test. GraphPad Prism 5 provided the software for the statistical analysis.

RESULTS

Production of HSV-2 gD-Fc fusion proteins

To determine whether antigens targeted to FcRn in vivo produce antibody and cellular immune responses, we generated a fusion protein HSV-2 gD-wtFc by cloning the extracellular domain of gD in frame with the carboxyl terminus of the heavy chain of the mouse IgG2a antibody (Figure 3.1A). We elected to use mouse IgG2a Fc fragment since mouse IgG2a, but not IgG1, Fc is capable of binding mouse FcγRI. We also engineered the heavy chain of the mouse IgG2a that does not bind FcRn,

generating a gD-mutFc. In this mutant, point mutations (HQ310 and HN431) were introduced into the Fc-domain known to inhibit binding to FcRn (122). In all these constructs, the constant regions of the mouse IgG2a were also modified to remove complement C1q-binding motif (246) to produce nonlytic fusion proteins.

The fusion proteins were produced by CHO transfection with the gD-Fc constructs. The secreted gD-Fc fusion proteins formed monomers under reducing conditions, but exhibited as disulfide-linked homodimers under non-reducing conditions in Western blot, using both the affinity-purified anti-gD and anti-Fc antibodies (Figure 3.1B). A functional test of the Fc-domain was confirmed in vitro by immunoprecipitation with Staphylococcal protein A beads for gD-wtFc. It has been shown that protein A makes contact with overlapping amino acids of IgG Fc for binding to the FcRn (118, 124). As expected, protein A effectively to fusion protein. Therefore, this suggests the Fc portion of IgG on the gD-wtFc maintains all structures necessary for binding FcRn.

Transcytosis of gD-wtFc fusion proteins

In nature, FcRn transports maternal IgG across the placenta and intestine with a high affinity (118). Two criteria were applied to demonstrate FcRn-mediated transport of gD-wtFc. First, an IMCD-FcRn cell line was used to transport gD-wtFc fusion protein in transwell.

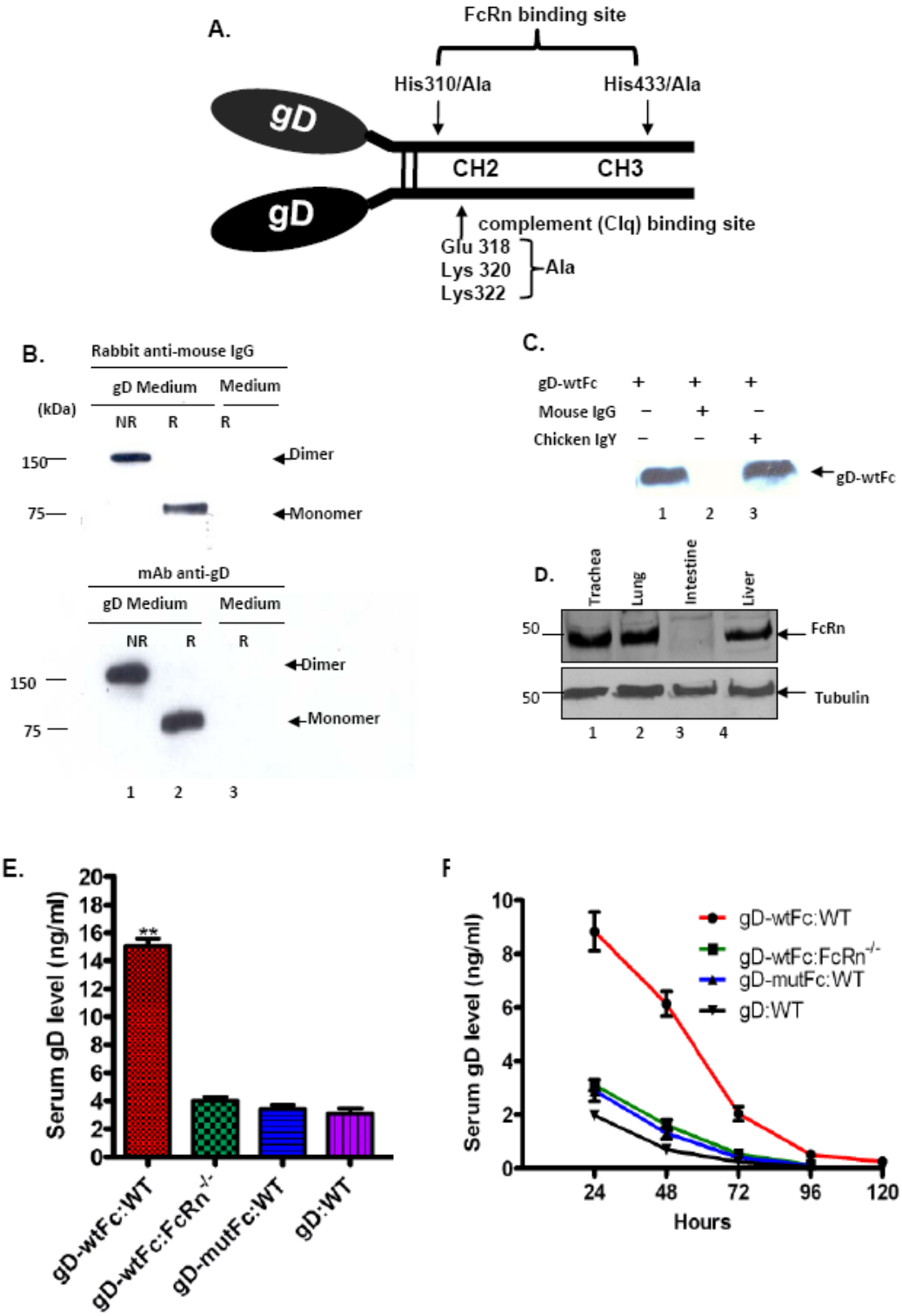

A.
FcRn binding site
His310/Ala
His433/Ala
gD
gD
CH2
CH3
complement (Clq) binding site
Glu 318
Lys 320
Lys322
Ala
B.
Rabbit anti-mouse IgG
gD Medium
Medium
(kDa)
NR
R
R
150
75
Dimer
Monomer
mAb anti-gD
gD Medium
Medium
NR
R
R
150
75
Dimer
Monomer
1
2
3
C.
gD-wtFc + + +
Mouse IgG − + −
Chicken IgY − − +
gD-wtFc
1
2
3
D.
Trachea
Lung
Intestine
Liver
50
FcRn
50
Tubulin
1
2
3
4
E.
Serum gD level (ng/ml)
20
18
16
14
12
10
8
6
4
2
0
**
gD-wtFc:WT
gD-wtFc:FcRn-/-
gD-mutFc:WT
gD:WT
F
Serum gD level (ng/ml)
10
8
6
4
2
0
gD-wtFc:WT
gD-wtFc:FcRn-/-
gD-mutFc:WT
gD:WT
24
48
72
96
120
Hours

Figure 3.1. Design and characterization of HSV-2 gD fused to IgG Fc fragment.
(**A**). Schematic illustration for the genetic fusion of HSV-2 gD and murine Fcγ2a cDNA to create a gD-Fc fusion gene. Mutations were made in the CH2 domain of Fcγ2a fragment by using site-directed mutagenesis to replace Glu318, Lys320, and Lys322 with Ala residues to remove complement C1 q binding site, and His 310 and His 433 with Ala residues to eliminate FcRn binding sites.
(**B**). The gD-Fc fusion proteins were secreted by CHO cells. The gD-Fc was recognized by either rabbit anti-mouse IgG (top panel) or a mAb anti-gD. The fusion protein was exhibited as a dimer under non-reducing (NR) or a monomer under reducing (R) condition.
(**C**). Transport of gD-wtFc fusion proteins in rat IMCD-FcRn cell lines. IMCD-FcRn cells were plated onto 24-mm transwells and grown for 3-6 days to allow a polarized monolayer with resistance greater than 300 Ω cm^2 to form. The purified gD-wtFc (50μg/ml) was applied to the apical reservoir and allowed for transcytosis for 2 hr. The proteins were collected from the basolateral reservoir and blotted with anti-gD antibody under reducing condition. The gD-wtFc fusion protein (lane 3) was transported from the apical to basolateral surface. The transport was inhibited by an excessive amount of mouse IgG (lane 2), but not by chicken IgY (lane 3).Wt: wild-type.
(**D**). Expression of mouse FcRn in the epithelial cells of mouse trachea and lung. The epithelial cell lysates from trachea, lung, intestine, and liver of 6 week C57B6/L mice was blotted with anti-mouse FcRn and anti-tubulin antibody. Molecular weight markers are indicated in kDa.
(**E**). Purified, gD, gD-wtFc and gD-mutFc proteins (20 μg) were intranasally inoculated into wild-type and FcRn KO mice as indicated. 8 hr later, the mouse sera were collected, the gD or gD-Fc protein concentration was measured by ELISA. Inoculation conditions are displayed at the bottom of the panel. Star (**) denotes $p<0.01$.
(**F**). Persistence of the transported gD-Fc fusion protein in the mouse serum. Proteins (20 μg) were intranasally inoculated into wild-type and FcRn KO mice as indicated. The mouse sera were collected and the gD or gD-Fc protein concentrations were measured by ELISA as indicated time.

The IMCD cells expressing rat FcRn have been shown to specifically and saturably transport murine IgG when grown in transwell (147). FcRn-dependent transcytosis of gD-wtFc in either purified or crude medium to the apical reservoir was detected by Western blotting of material collected from the basolateral reservoir under reducing conditions. The gD-wtFc fusion protein (Figure 3.1C, lane 3) was transported from the apical to basolateral surface. The transport was inhibited by an excessive amount of rodent IgG (lane 2), but not by chicken IgY (lane 1). Chicken IgY does not bind FcRn. Lane 4, representing gD-wtFc, was used as a positive control. For in vivo transcytosis, we first determine the expression of FcRn in the epithelial cells of trachea, lung, and liver by Western blot (Figure 3.1D). It was revealed that the whole respiratory tract expressed FcRn. Given that FcRn higly expressed by respiratory epithelial cells (247), we wanted to see whether FcRn can deliver the gD-Fc across mucosal barrier and reach the sera after i.n. inoculation. To this end, 20 μg of the gD-wtFc, gD-mutFc, or gD proteins were administered i.n. and measured 8 hr later using ELISA. As shown in Fig. 1E, gD-wtFc could readily be detected in sera of wt mice but not FcRn KO animals (Figure 3.1E). In addition, smaller amounts of gD-mutFc or gD alone was detected in the sera of wt mice in comparison with that of the gD-wtFc, indicating that i.n. administered gD-wtFc efficiently crosses the respiratory barrier and reaches the sera. Furthermore, FcRn functions in protecting IgG from degradation (124). For this reason, we measured the persistence of gD-Fc fusion proteins in the sera of inoculated mice. The results showed that the transported gD-wtFc in the sera of wild type mice persisted about 5 days, much longer than other proteins (Figure 3.1F). Taken together, we conclude

rodent FcRn can transport the gD-wtFc fusion proteins in the polarized epithelial cell lines and in the respiratory tract of mice, further suggesting gD-wtFc maintains structural integrity and biological function for interacting with FcRn. In addition, inactivating complement C1q binding motif has no effect on the FcRn binding potency of the fusion proteins.

Protection against HSV-2 challenge

To test the theory that FcRn functions as a receptor to transport subunit vaccines across mucosal surfaces in adult mice, we i.n. immunized mice with gD-Fc or gD proteins and boosted them similarly after 2 weeks. To determine if immunity to FcRn-mediated mucosal vaccine delivery leads to protection, we challenged the mice with a lethal dose of 1×10^4 pfu of HSV-2 186 strain 30 days following the boost. Mice immunized with the gD-wtFc were protected from a lethal challenge and showed 100% survival (Figure 3.2.A). The gD-wtFc immunized wt mice showed significantly higher survival compared to the gD-mutFc group, gD-wtFc-immunized FcRn KO group, and gD immunize group (Figure 3.2A). As expected, PBS control mice succumbed to lethal infection. Additionally, viral titers measured in the vaginal washs taken after infection showed that the gD-wtFc immunized mice had completely controlled viral titers by day 4 after challenge compared to other groups (Figure 3.2B). Overall these results demonstrate that i.n. administration of gD-Fc antigens efficiently induce a protective immunity.

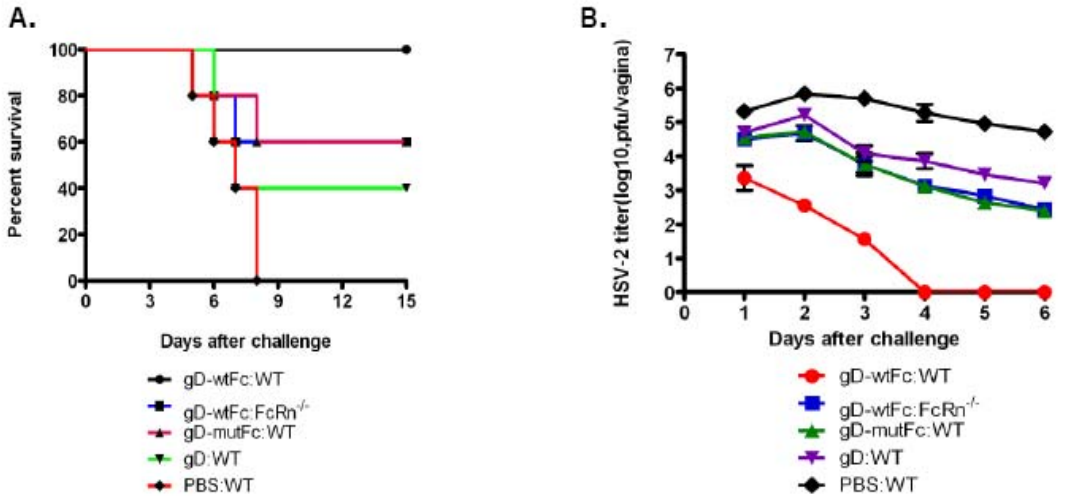


Figure 3.2. FcRn-targeted mucosal vaccination provides protective immunity to intravaginal challenge with virulent HSV-2 186. Groups of five mice were immunized i.n. with 20 μg gD-wtFc, gD-mutFc, or gD alone. Initial immunization conditions were as follows: gD-wtFc in wt mice (♦), gD-wtFc in FcRn KO mice (■), gD-mutFc in wt mice (▲), gD alone (*), and PBS (x). Mice received boost, and after 1 month, they were challenged intravaginally with 1×10^4 pfu of HSV-2 strain 186.
(**A**) Mean survival following genital HSV-2 challenge. Percentage of mice from protection on the indicated days calculated as the number of mice survival divided by the number of mice in each group. Three weeks following immunization, mice were challenged intravaginally with 1×10^4 pfu of HSV-2.
(**B**) Mean of viral titers following HSV-2 challenge. Virus titer was measured in vaginal washes by taking vaginal swabs on the indicated days after HSV-2 inoculation. Viral titers were measured by plaque assay on Vero cell monolayers.

Strong anti-gD antibody responses after immunization with FcRn-targeted gD-wtFc

To further elucidate the protective immune mechanism, we first analyzed the humoral immunity elicited by gD-Fc fusion protein adjuvanted with CpG. HSV-2 gD has major and minor immunodominant serological epitopes (248, 249). To determine whether antigen targeting to FcRn by gD-wtFc elicits antibodies to potential vaccine target antigen gD, we measured antibody immune responses to the gD antigen. Immune responses in vaccinated animals, including adjuvant controls and naive mice, were assessed at various time points up to 56 days after the primary immunization by measuring the gD-specific IgG. Mice were bled at indicated days, and the IgG antibody titers in sera were measured by ELISA against the gD. On each post-vaccination, gD antibody displayed similar kinetics among primed groups. However, significantly higher titers of IgG were seen in the gD-wtFc immunized mice when compared with other groups (Figure 3.3A). To further characterize the antibody responses, we determined the subclass specificity of the anti-gD IgG response. Immunization with the gD-wtFc elicited antibodies with mainly restricted to the IgG2a subclass (Figure 3.3B). We conclude that immunization by targeting antigens to FcRn together with a CpG adjuvant produces strong humoral immune responses to HSV-2 gD.

To examine whether the sera from the immunized mice have the neutralizing activity, we performed in vitro neutralization test. The sera from the gD-Fc immunized mice exhibited the stronger neutralizing activity than that of other groups

(Figure 3.3C). To further examine the protection provided by the serum antibody in vivo, groups of five mice received 0.3 ml the immunized sera 24 hours prior to ivag HSV-2 challenge. B6 mice receiving the sera from the gD-wtFc immunized mice were significantly protected compared to PBS-immunized animals and other groups respectively, Figure3.3D). Administration of the sera from the gD-wtFc immunized mice to B6 mice resulted in significantly lower HSV-2 titers in the vaginal epithelium on day 2 after inoculation compared to PBS-treated controls.

Strong T cell responses to FcRn targeted mucosal immunization

Naive C57BL/6 mice were immunized with 20 μg gD-wtFc or gD-mutFc in the presence of a DC maturation stimulus CpG because the latter is considered to be effective in producing T cell help for antibody responses. 4 days after the boosting, spleen cells isolated from immunized mice were pulsed with purified gD, and the frequency of IFN-γ^+ T cells was measured by flow cytometry. We detected significant number of IFN-γ secreting $CD4^+$ (Figure 3.4A, upper panel) and $CD8^+$ (Figure 3.4A, bottom panel) T cells in response to gD in the group immunized with gD-wtFc, but rarely in any of the other groups, including mice immunized with gD-mutFc. In the gD-wtFc immunized mice, about 0.88 % of the $CD4^+$ and 1.6% of the CD8 T cells became responsive to stimulation. Targeting the gD-wtFc via FcRn was more effective at initiating IFN-γ–producing $CD4^+$ and $CD8^+$ T cell immunity (Figure 3.4A) than control proteins. The cytokine responses were dominated by T cells with cytokine IFN-γ and IL-2 and a lack of the IL-4 production when splenocytes were incubated with different MOI of inactivated virus (Figure 3.4B).

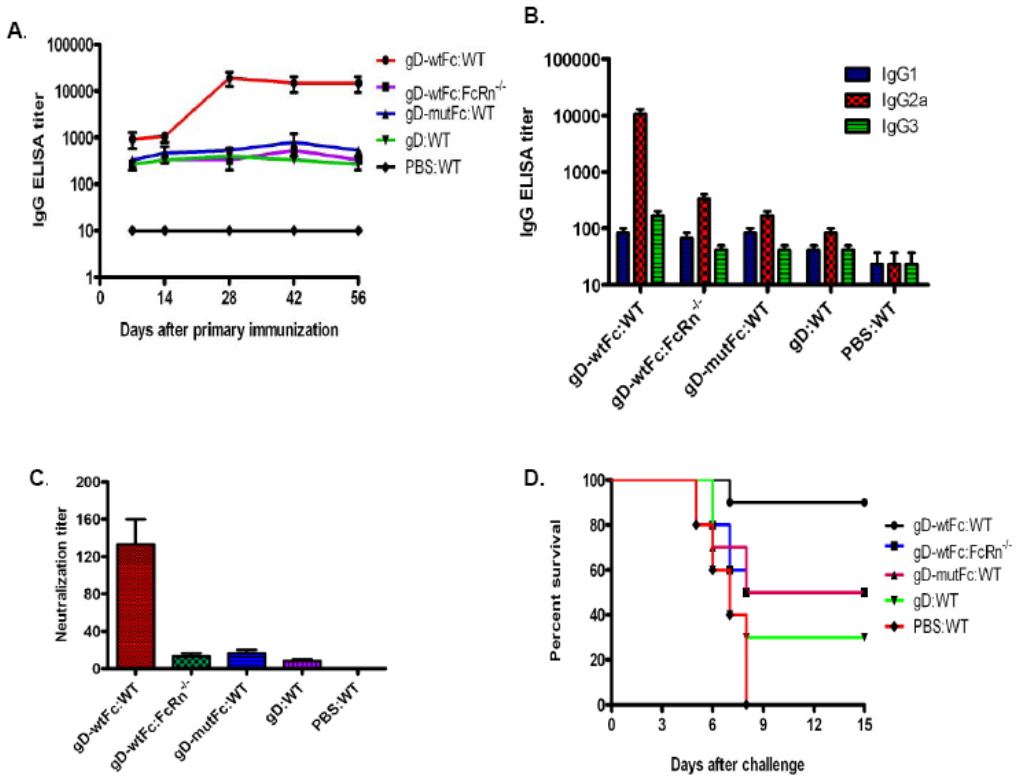


Figure 3.3. FcRn-targeted mucosal vaccination induces enhanced antibody responses. Antigen-specific antibody titers were determined for each mouse in a group (n = 3) from serum harvested at indicated days post vaccination.
(**A+B**). Measurement of anti-HSV-2 gD-specific IgG antibody titers in serum before and after the boost immunization (mice was boost immunized 14 days after primary immunization). Blood samples were taken from mice by tail bleeding. HSV-2 gD-specific IgG antibody as indicated days (A) or IgG isotype 21 days post boost was measured in serum by ELISA. Immunization conditions are displayed at the right. The curves represent mean values for each group (±S.E.M.). (**C**). Test of neutralizing activity in the immunized sera. Sera were heat-inactivated, diluted 10-fold, then in twofold steps in MEM with 2% FBS. Fifty PFU of HSV-2 were added and incubated at 37°C heat inactivated serum for 1 hr. Finally, Vero cells were added and incubated for 48 hr at 37°C. The titers were expressed as the reciprocal of twofold serial dilution reducing the half of the CPE compared to control serum. Each assay was done in triplicate. Star (*) denotes $p<0.01$.
(**D**). Survival of mice following passive transfer of immunized mouse sera. Groups of five mice received 0.3 ml of immunized sera prior to ivag inoculation with 1 X 10^4 PFU HSV-2. 24 hours following transfer, mice were challenged intravaginally with 1×10^4 pfu of HSV-2. Percentage of mice from protection on the indicated days is presented.

We conclude that immunization with gD-wtFc fusion protein induces strong $CD4^+$ and $CD8^+$ T cell responses, whereas the immunization with gD-mutFc or the gD-wtFc immunized wt mice do not.

Induction of local mucosal immune responses

The mediastinal lymph nodes (MLN) drain the lung and are usually the site where mucosal immune responses are initiated against antigens reaching the lung. At first, to ascertain the ability of the FcRn-targeted mucosal delivery of the gD-wtFc generates the germinal center (GC) in the MLN, it was removed 10 days later after the boost. The GC is antigen-specific as characterized by the presence of peanut agglutinin (PNA)-positive areas and Fas apoptotic death receptor is highly expressed in activated and GC B cells (250, 251). Cells from the MLN were gated on $B220^+$ cells and stained for the presence of PNA- and Fas-positive B cells. As shown in Figure 3.5A, the gD-wtFc immunization efficiently induced GC, displaying much higher frequency of $FAS^+PNA^+B220^+$ B cells than all other groups. Therefore, we conclude that antigenic stimulation induces the activation of B cells to form GC in draining MLN by FcRn-targeted mucosal vaccine delivery.

It has been previously shown that vaccine or antigens can induce bronchus-associated lymphoid tissue (iBALT), which is very similar to germinal centers in perpherial lymphoid tissues, and contribute to the mucosal surveillance and protection (250).

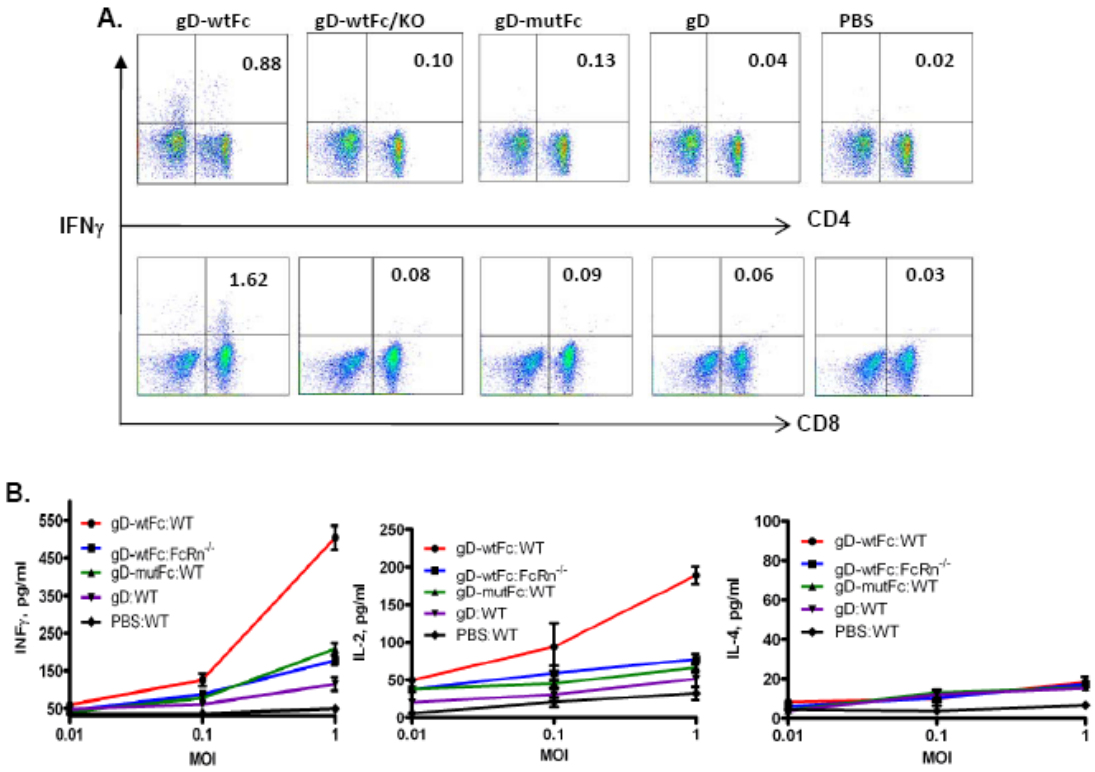


Figure 3.4. FcRn-targeted mucosal vaccination enhances HSV-2 gD–specific T cell responses. 20 μg of fusion protein or gD with 20 μg CpG were administered i.n. into wild-type or FcRn KO mice.14 days later, mice were booster immunized. Spleenocytes were harvested 4 days post boost. The percentage of IFN-γ^+ T cells in gated $CD3^+$ splenic T cells were assessed after co-culture with 10μg/ml of gD

(**A**) IFN-γ production by $CD4^+$ and $CD8^+$ T cells is shown as dot plots of gated $CD3^+$ T cells stained for CD4 and CD8 and intracellular IFN-γ in the spleen of the immunized mice. Spleen cells from immunized mice were stimulated for 24 hr with purified gD or medium control. Lymphocytes were gated by forward and side scatter and T cells labeled with anti-CD3 and identified by their respective surface markers CD4 and CD8. Immunization conditions are displayed on the top. Number in each quadrant represents the percentage of IFN-γ^+ $CD3^+$ $CD4^+$ (top panels) or IFN-γ^+ $CD3^+$ $CD8^+$ (bottom panels) T cells

(**B**) Cytokine secretions from re-stimulated spleen T cells. Spleenocytes from immunized mice were restimulated in vitro specifically with different multiplicity of infection (MOI) of inactivated HSV-2 virus as indicated. Cytokines IFN-γ, IL-2, and IL-4 secreted into the culture supernatant were detected by ELISA and are presented as picograms/ml of culture supernatant. Data are representative of three experiments with three mice pooled in each experiment.

To address the possibility of iBALT being induced by FcRn targeted immunization, lungs of i.n. immunized animals were analyzed immunohistologically by confocal microscope. As shown in Figure 3.5B, such germinal center-like structure could be steadily detected in the lung of the gD-wtFc immunized mice, but it was absent from the gD-mutFc, gD alone, or PBS immunized mice. The gD-wtFc immunized FcRn KO mice exhibited a low intensity staining in the lung, but it did not reach the significant difference. This indicates that FcRn targeted gD-wtFc delivery can induce strong mucosal immunity.

Antibodies, in particular sIgA and IgG, represent a first line of defense on mucosal surfaces. In order to assess the ability of the FcRn-targeted immunization to induce gD-specific antibody in mucosal secretions, the bronchial alveolar lavage (BAL) were collected two weeks following the boost and analyzed for gD-specific IgG and IgA by ELISA. In order to determine if the antibody responses induced by i.n. immunization were disseminated to distant mucosal sites, vaginal washes were also taken and subjected to antibody analysis. Significantly increased levels of gD-specific IgG in the lung lavages (Fig. 3.5C, middle panel) and vaginal washes (Fig. 3.5C, right panel) were observed in the gD-wtFc immunized group. Low level of gD-specific IgG was detected in the lung lavages and vaginal washings of mice immunized with the gD/Fc/mut or gD alone. Only wt but not FcRn KO mice that received the gD-Fc i.n. had high levels of gD-specific IgG antibodies in BAL and vaginal washings ($p < 0.01$, Figure 3.5C), suggesting the appearances of mucosal IgG is FcRn mediated. In marked contrast, we only detected a small amount of IgA in

both BAL and vaginal washings, although we were able to detect significant amount of IgA in nasal washings (Figure 3.5A, left panel).

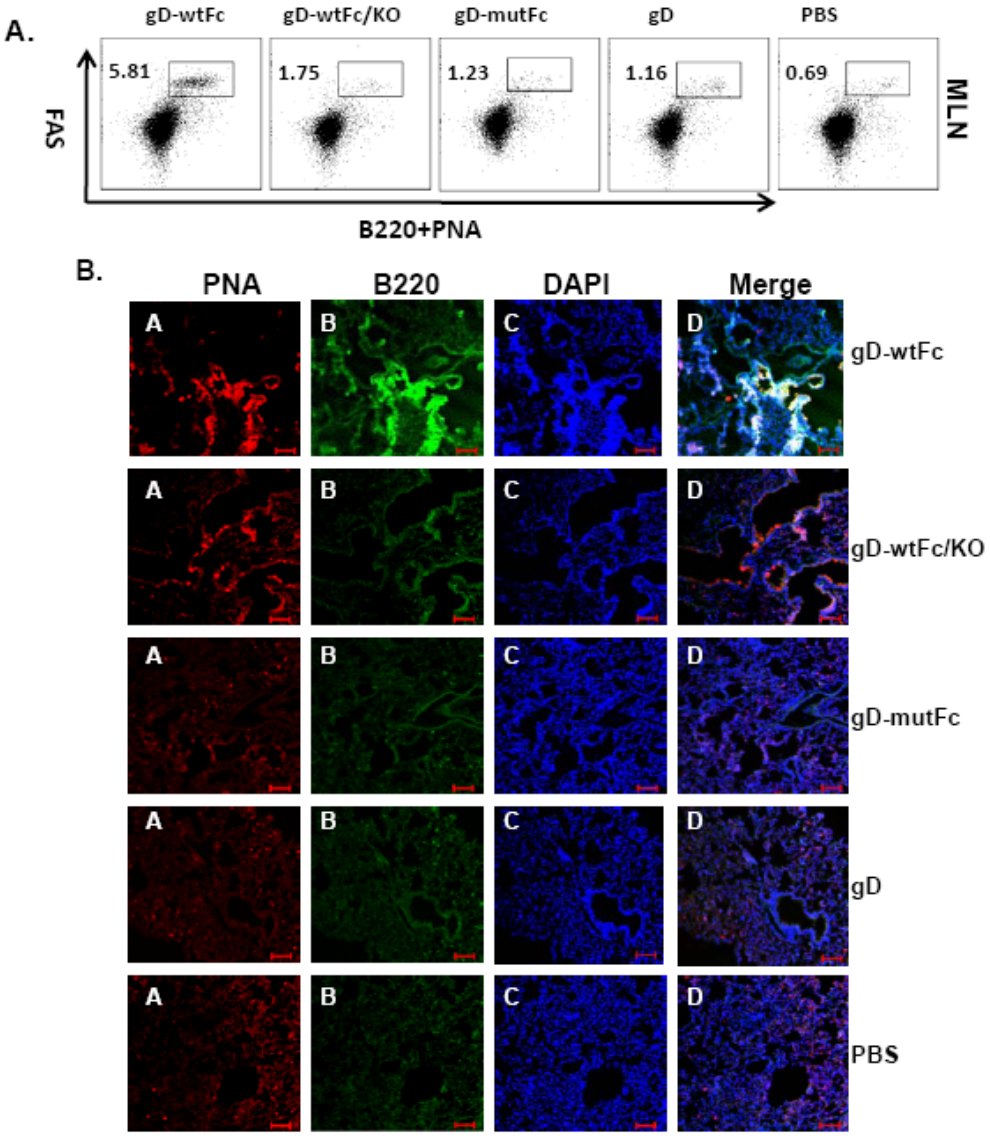
To further show whether FcRn targeted mucosal delivery vaccine can induce T cell immune responses in the lung and vaginal tissues, 4 days after the boosting, lung lymphocytes isolated from the immunized mice were pulsed with purified gD, and IFN-γ secreting cells were measured by flow cytometry. We detected significant number of IFN-γ secreting $CD4^+$ (Figure 3.5D, upper panel) and $CD8^+$ (Figure 3.5D, bottom panel) T cells in response to gD in the group immunized with gD-wtFc in comparison with other groups, including mice immunized with gD-mutFc or FcRn KO mice immunized with the gD-wtFc. Similarly, vaginal lymphocytes isolated from the challenged mice displayed significant number of IFN-γ secreting $CD4^+$ (Figure 3.5E, left panel) and $CD8^+$ (Figure 3.5E, right panel) T cells in the mice immunized with gD-wtFc in comparison with other groups. We conclude that immunization with gD-wtFc fusion protein induces strong IFN-γ producing $CD4^+$ and $CD8^+$ T cell responses at the mucosal sites, whereas the immunization with gD-mutFc does not.

FcRn targeted mucosal immunization elicits long-lived humoral immune responses

A hallmark of humoral immune responses is the activation of GC B cells leading to the further differentiation into memory B cells and long-lived plasma cells (252). At first, to ensure the ability of the FcRn-targeted mucosal vaccine generates GC B cells in the spleen of the immunized mice, spleens were removed 10 days later

after the boost. Cells from the spleen were gated on $B220^+$ cells and stained for the presence of PNA- and Fas-positive B cells. The mice immunized by the gD-wtFc, but not by the gD-mutFc or gD alone proteins, efficiently developed the comparable number (5% of total isotype-switched B cells) of $FAS^+PNA^+B220^+$ B cells, suggesting the further differentiation into memory MBCs and LLPCs (Figure 3.6A). In addition, the gD-wtFc immunized FcRn KO mice failed to show the comparable number of $FAS^+PNA^+B220^+$ B cells. Therefore, we conclude that FcRn-targeted mucosal delivery of the gD-wtFc induces the activation of GC B cells.

Of the clonally expanded B cells, some differentiate to plasma cells that secrete antibodies at high rate and persist in niches in the bone marrow, whereas others become memory B cells. The latter can rapidly respond to antigenic restimulation and it has been suggested that they may contribute to maintaining the plasma cell pool and therefore serum antibody levels over prolonged periods of time. To determine whether antigen targeting to FcRn leads to long-lasting memory B cell immune responses, spleen cells isolated from 6 months post immunization were analyzed with Alexa647 labled gD protein. Memory B cells were barely observed after vaccination with control gD or PBS (Figure 3.6B). However, gD-wtFc immunized mice were shown to have a distinct population of the memory B cells in contrast to the gD-wtFc immunized FcRn KO or the gD-mutFc immunized WT mice. To determine whether antigen targeting to FcRn-mediated IgG transfer pathway also leads to long lived plasma cells that secret gD-specific antibodies, the number of IgG-producing cells from the bone marrow was measured by ELISPOT.


A.
gD-wtFc
gD-wtFc/KO
gD-mutFc
gD
PBS
5.81
1.75
1.23
1.16
0.69
FAS
MLN
B220+PNA
B.
PNA
B220
DAPI
Merge
A
B
C
D
gD-wtFc
gD-wtFc/KO
gD-mutFc
gD
PBS

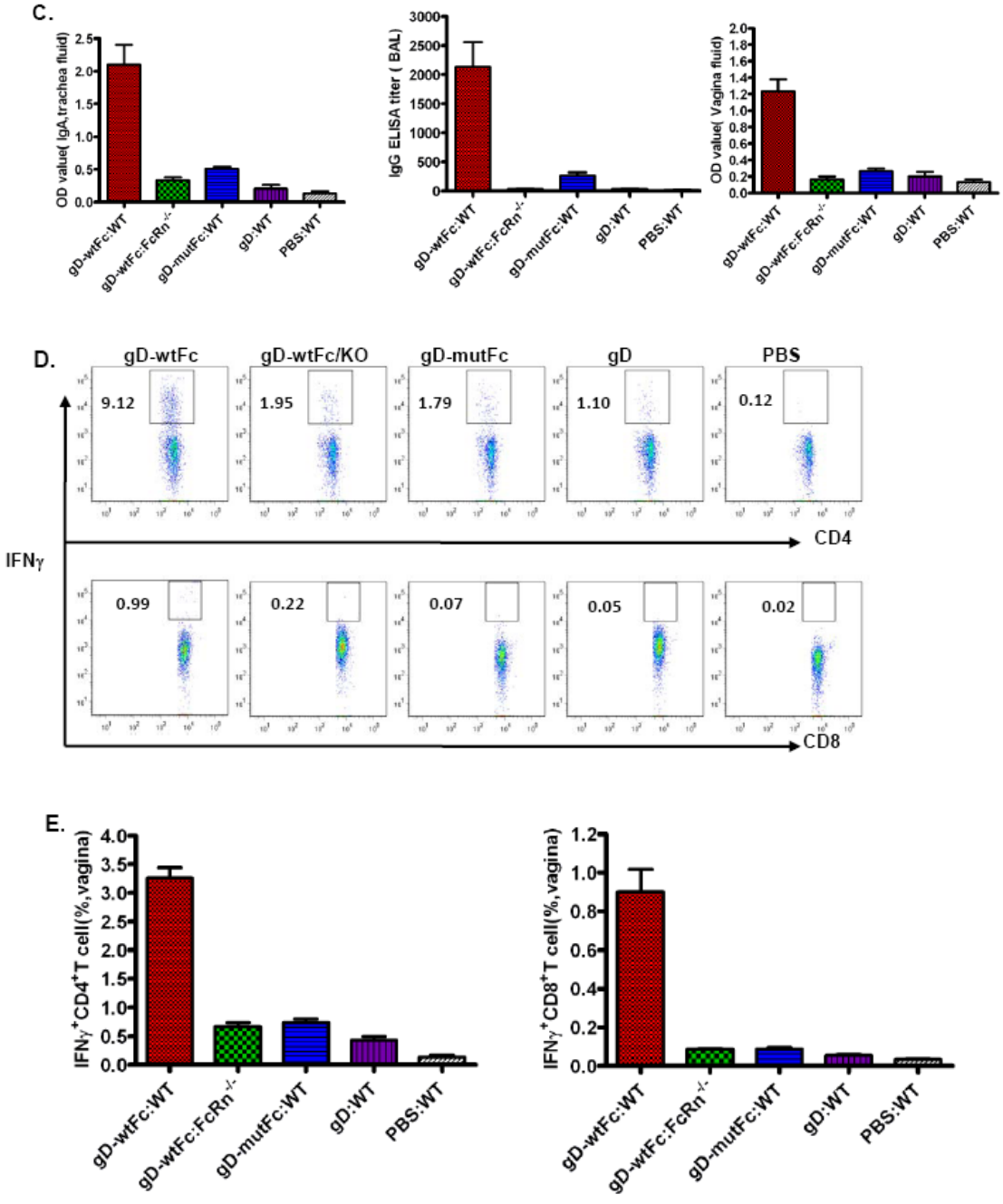

C.
OD value(IgA,trachea fluid)
IgG ELISA titer (BAL)
OD value(Vagina fluid)
gD-wtFc:WT
gD-wtFc:FcRn-/-
gD-mutFc:WT
gD:WT
PBS:WT
D.
gD-wtFc
gD-wtFc/KO
gD-mutFc
gD
PBS
9.12
1.95
1.79
1.10
0.12
0.99
0.22
0.07
0.05
0.02
IFNγ
CD4
CD8
E.
IFNγ+CD4+T cell(%,vagina)
IFNγ+CD8+T cell(%,vagina)

Figure 3.5. Local immune responses induced by FcRn-targeted mucosal immunization. Immunization conditions are displayed at the top or bottom of each panel.
(**A**). Detection of activated B cells in the germinal center (GC) in the gD-wtFc immunized mice by flow cytometry. Representative flow cytometric analyses of GC B cells among B220+ B cells in the MLN 7 days after immunization. Numbers are the percentage of activated GC B cells (PNA+FAS+) among gated B220+ cells.
(**B**). Inducible bronchus-associated lymphoid tissue (iBALT) is formed in the gD-wtFc immunized mice. Frozen serial sections of the lung were stained with biotin-PNA (germinal centers, red) and anti-B220 (B cells, green), followed by Alexa 555-Avidin or 488 Fluro-conjugated goat anti-rat IgG The nucleus is stained with DAPI (blue). Colocalization of all three red, green, and blue appears in white. The germinal center-like structure is shown in the mergered panel that appears in white color. The data are representative of sections from at least three independent mice. Images were originally obtained at 10× magnification. Scale bars represent 100 μm. Immunization conditions are displayed at the right.
(**C**). HSV-2 gD-specific antibody response in bronchial lavage and vaginal secretions following the immunization. The nasal washings (left panel), bronchial lavage (middle panel) and vaginal washes (right panel) were obtained from mice on the 10 days after the boost and gD-specific IgG and IgA titers were determined by ELISA. Antibody titers for five mice from a representative experiment of three performed were quantified by endpoint titer. Titers of HSV2 gD-specific IgG antibody from serum and vaginal washes of naive mice always fell below the limits of detection and are omitted from the figure for clarity. The data shown are representative of three independent experiments. Asterisk (*) indicates significant difference among groups ($P \leq 0.05$).
(**D**). Presence of HSV-2 gD-specific T lymphocytes in the lung. Lung cells isolated from mice 4 days after i.n. second immunization were incubated with 10μg/ml of gD. Lymphocytes were gated based on their forward scatter (FSC) vs. side scatter SSC profile. Intracellular staining for IFN-γ was performed after surface staining of CD4 and CD8 molecules. The profiles shown are representative of three pooled mice from one of two separate experiments. Numbers in the quadrants indicate percentages of IFN-γ-secreting T lymphocytes from gated CD4+ and CD8+ T cells. (**E**). Increased presence of HSV-specific T lymphocytes in the vaginal epithelium after immunization. Lymphocytes were harvested from collagenase-digested vaginal tissues pooled from 3 normal or immunized mice 6 days after intravaginal inoculation of 10^4 pfu of virus. Intracellular staining for IFN-γ expression on CD4+ and CD8+ T cells was analyzed after gating on viable CD3+ lymphocytes. The numbers in each column show the percentage of IFN-γ-positive T lymphocytes from gated $CD4^+$ or $CD8^+$ T cells. Data shown are of a representative experiment of three repeat using three mice per experiment.

We found the significant number of gD-specific IgG antibody-producing cells in the bone marrow of mice immunized with the gD-wtFc in comparison with that of other groups (Figure 3.6C).

To further examine whether the increase in germinal centre B cells, antibody-secreting plasma cells, and memory B cells correlates with an increase in gD specific IgG production, mice were bled six months, the longest point we tested, after the boost, and IgG antibody responses in sera were measured by ELISA. High titers of gD-specific IgG antibodies were elicited in mice immunized with the gD-wtFc, but not with the gD-mutFc or gD alone (Figure 3.6D), indicating the long persistence of gD-specific antibody after a mucosal immunization.

FcRn targeted mucosal immunization induces long-term memory T cell responses

An important feature of memory T cells is their proliferative potential upon reencounter with an antigen. To test if long-term memory T cells could be detected six months following the immunization, we measured $CD4^+$ and $CD8^+$ T cell proliferation by CFSE dilution in response to gD antigen stimulation. Spleen cells isolated six month post-vaccination were CFSE-labeled and stimulated in vitro with the recall antigen (Figure 3.7). After 4 day incubation, the CFSE profiles were read on CD4- or CD8-gated T cells and subsequently analyzed by flow cytometry.

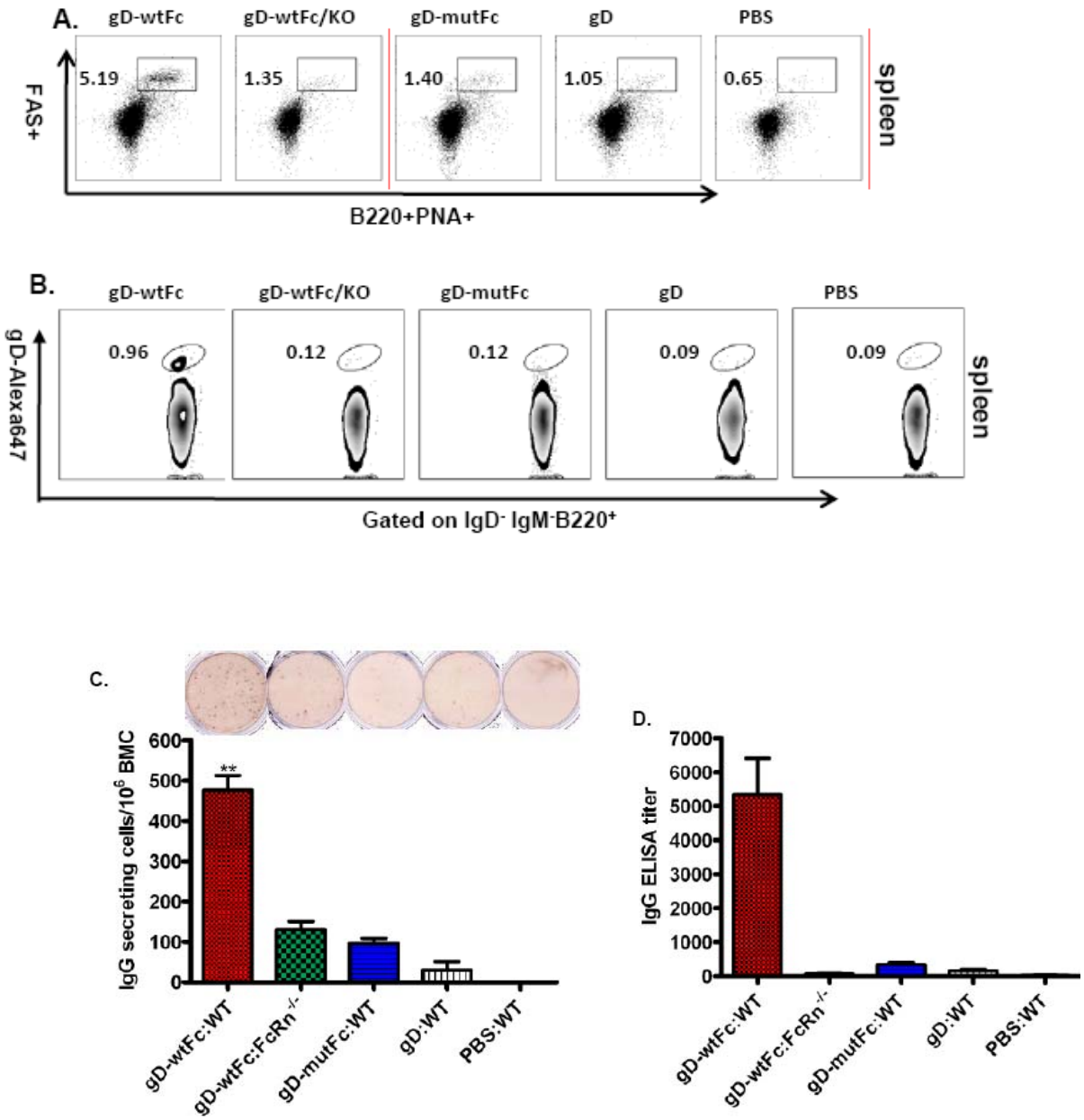

A.
gD-wtFc
gD-wtFc/KO
gD-mutFc
gD
PBS
5.19
1.35
1.40
1.05
0.65
spleen
FAS+
B220+PNA+
B.
gD-wtFc
gD-wtFc/KO
gD-mutFc
gD
PBS
0.96
0.12
0.12
0.09
0.09
spleen
gD-Alexa647
Gated on IgD- IgM-B220+
C.
IgG secreting cells/10^6 BMC
600
500
400
300
200
100
0
**
gD-wtFc:WT
gD-wtFc:FcRn-/-
gD-mutFc:WT
gD:WT
PBS:WT
D.
IgG ELISA titer
7000
6000
5000
4000
3000
2000
1000
0
gD-wtFc:WT
gD-wtFc:FcRn-/-
gD-mutFc:WT
gD:WT
PBS:WT

Figure 3.6. Increased memory humoral immune responses in FcRn-targeted mucosal immunization. The groups of five mice were i.n. inoculation of proteins or PBS as indicated. (**A**). Accumulation of activated B cells in germinal center (GC) in the spleen of the gD-wtFc immunized mice. $B220^{+}PNA^{high}FAS^{high}$ cells are B cells that exhibit the phenotypic attributes of GC B cells. Representative flow cytometric analyses of GC B cells among B220+ B cells in the spleen 10 days after second immunization. Numbers are the percentage of activated GC B cells (PNA+FAS+) among gated B cells, and are representative of three independent experiments and are representative of three independent experiments.
(B). Induction of gD specific memory B cells in the spleen. The frequency of gD-specific memory B cells was assessed 6 months post primary immunization. Memory B cells, defined as $B220^{+}$ IgG (gD-specific)-$surface^{+}$, present 6 months after the boost were analyzed by FACS. Purified gD proteins were labeled with Alexa Fluro647. Spleen cells (2×10^{5}) were isolated from 6 months post primary immunization and incubated with the 1 μg of Alexa Fluro647-labeled gD proteins. Numbers in the quadrants are the percentage of HSV-2 gD-specific memory B lymphocytes from gated class switched IgD^{-} $IgM^{-}B220^{+}$ B cells. Data are representative of three experiments on the same batch of immunized mice. (**C**). Long-term HSV gD-specific antibody-secreting cells in the bone marrow. Bone marrow cells removed 6 months after the primary immunization were placed on HSV-2 gD-coated plates and quantified by ELISPOT analysis of gD-specific and IgG-secreting plasma cells. Data were pooled from two to three separate experiments with three mice in each experiment. The graphs are plotted based on the average ELISPOTs for replicate wells. Values marked with asterisk are significantly greater ($P < 0.01$) from the gD-wtFc fusion protein-immunized mice than those for other groups as indicated. No HSV-2 gD-specific and IgG-secreting plasma cells were detected in bone marrows from naive animals.
(**D**) Durability of HSV-2 gD-specific serum IgG response. In two separate experiments, HSV-2 gD-specific IgG was quantified by ELISA in serum by endpoint titer from 3 mice at 6 months after the boost. HSV-specific IgG antibody was not detected in naive mice.

We detected significant number of $CD4^+$ (Figure 3.7A, upper panel) and $CD8^+$ (Figure 3.7A, bottom panel) memory T cell proliferation in response to gD recall stimulation in the group of mice immunized with gD-wtFc, but not in any of the other groups, including the FcRn KO mice immunized by gD-wtFc proteins, indicating that the gD-specific T cells had maintained a significant proliferative potential at this late time point post-vaccination. A recall IFN-γ and IL-2 secretions within 48 hr of gD antigen restimulation were detected in spleen cells from mice immunized with the gD-wtFc, but not with other immunized groups (Figure 3.7B). Collectively, these results lead us to conclude that mucosal immunization by antigen targeting to FcRn is effective in eliciting long term memory T cell immune responses.

DISCUSSION

Mucosal subunit vaccines are assumed as most effective and safe agents to prevent or control the mucosal infections. However, to date, we are unable to develop such vaccines. One of the challenges of mucosal subunit vaccine design is lack of efficient delivery tools (Reviewed in chapter 1). In this study, we propose to target FcRn as a vehicle to deliver Fc fused antigen as mucosal subunit vaccine, given the fact that FcRn is capable of transporting IgG from apical to basolateral compartment of different mucosal barriers, like placenta and intestine. In this regard, we fused the Fc fragment of IgG2a with HSV-2 gD protein and intranasally applied such fusion protein to wild-type and FcRn KO mice respectively, and it turned out that FcRn can efficiently transport gD-wtFc fusion proteins across mucosal barrier, evidenced by the presence of fusion protein in the circulation of wild type mice (Figure 3.1).

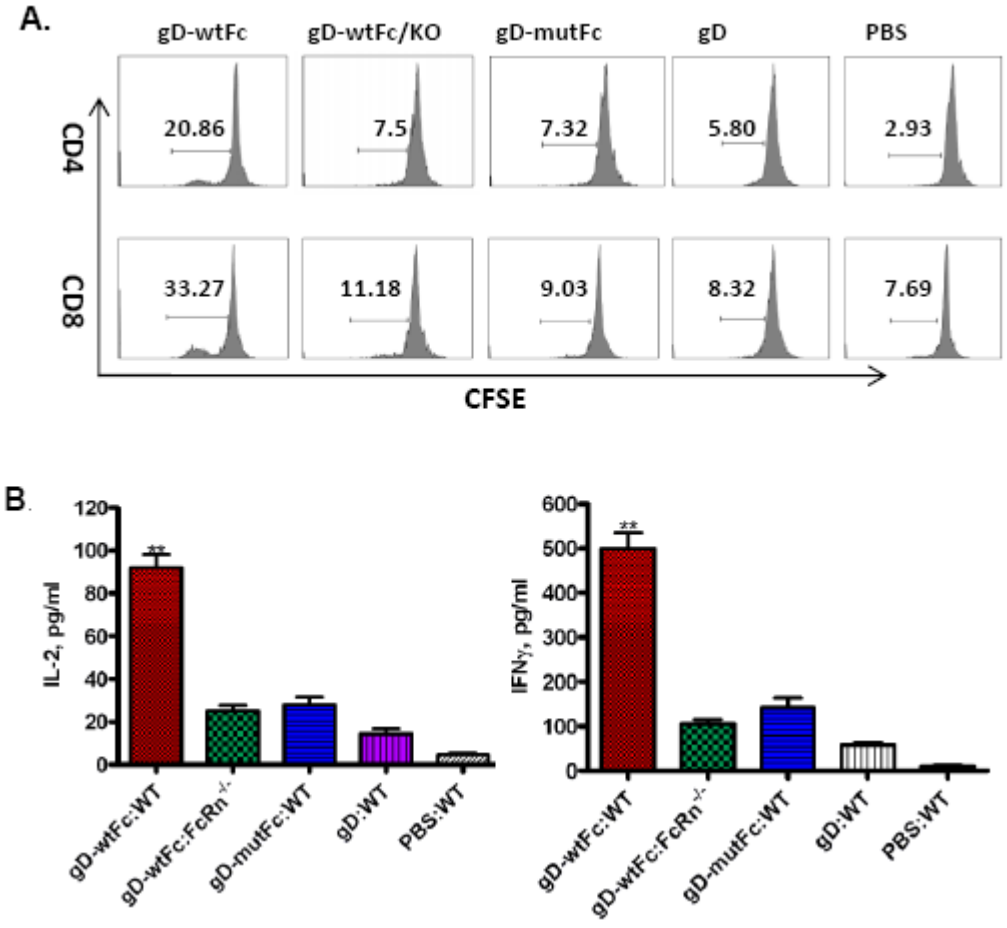


Figure 3.7. Long-lived gD-memory T cells to FcRn-targeted mucosal vaccination. C57BL/6 mice or FcRn KO mice were i.n. immunized with two doses of 20 µg fusion protein or gD. Representative flow cytometry profiles of three similar experiments with three mice per group were shown. Immunization conditions are displayed on the top or bottom. **(A).** Proliferative capacity of gD-specific T cells six months post-vaccination. Splenocytes were pooled from three control or vaccinated mice six month after the primary immunization, stained with CFSE, and stimulated in vitro with purified gD for 4 days. Cells were analyzed by flow cytometry, where CFSE dilution on CD4- and CD8-gated cells was used as a readout for gD-specific proliferation. Numbers are the percentage of $CD4^+$ and $CD8^+$ proliferating T cells. Data are expressed in CFSE histograms of fluorescence intensity versus the number of fluorescing cells, indicating the percentage of the cell population positive for CD4 and CD8 antigen. Data are representative of two experiments on the same batch of immunized mice. **(B).** Cytokine secretions from re-stimulated spleen T cells. Spleen T cells were collected and pooled from three mice per group 6 months post-vaccination. Cells were restimulated in vitro specifically with purified gD protein. Cytokines secreted into the culture supernatant were detected by ELISA. Data are representative of 3 experiments with 3 mice pooled in each experiment.

The delivery function of FcRn was further verified by the fact that the gD-wtFc protein was failed to be transported across mucosal barrier in FcRn KO mice. In addition, the transported gD-wtFc in wild type C57/B6 mice was persisted much longer in the sera. However, it should be noticed that the detected fusion proteins in the mouse sera may not reflect the actual amount of fusion proteins transported in mice since we were unable to quantify the fusion proteins deposited in the different tissues and pinocytosed or endocytosed proteins inside of cells.

Preferably, an effective mucosal subunit vaccine should give both humoral and cell-mediated immunity, not only at the mucosal delivery site, but also all through the body, including systemic compartments as well as distal mucosal tissues from the mucosal immunization sites. Therefore, following the successful mucosal transport, we are interested in determining whether gD-Fc proteins directed to the FcRn-mediated transport pathway can be perceived by the immune system and induce strong mucosal and systemic antigen-specific antibody and cellular immune responses. Our data clearly showed that FcRn-targeted delivery of mucosal subunit vaccine was able to mount strong systemic and mucosal humoral immune responses. Likewise, the gD-wtFc fusion proteins that we tested also induced broad and strong cellular immune responses, shown as the induction of IFN-γ–producing $CD8^+$ and $CD4^+$ T cell responses in both lymphoid tissue and mucosal sites. We also determined that FcRn was essential for such efficacy by employing both mutant fusion protein and FcRn knockout mice. Thus, the use of FcRn targeting greatly increases the efficiency with which gD antigens engenders both humoral and cellular immunity

when given together with CpG stimuli for DC maturation and mucosal tolerance disruption. In terms of humoral responses, we noted that the FcRn-targeted mucosal vaccine was superior in inducing the high titer of IgG antibody production in sera relative to the gD-mutFc or gD alone (Figure 3.3A). IgG2a, but not IgG1, is a major isotype detected (Figure 3.3B). This is not surprising because the helper type 1 cytokine IFN-γ is associated with the production of IgG2a, whereas the helper type 2 cytokine IL-4 supports switching to IgG1. In this study, IL-4 was less produced by T cells (Figure 3.4B).

It is crucial to notice that an important consequence of FcRn-targeted mucosal delivery of subunit vaccine elicited strong mucosal immune responses. This conclusion is supported by several evidences, such as the existing of significant amount of IgG antibody in the lung lavages and vaginal washings and IgA in the trachea wash, and IFN-γ producing $CD4^+$ and $CD8^+$ T cells in the lung and vaginal tissues in the gD-wtFc immunized mice. This observation was paralleled by the higher numbers of activated B cells in the germinal centers found in MLN (Figure 5A) and the presence of induced bronchial lymphoid tissues (iBALT) in the lung tissue (Figure 3.5B) of the gD-Fc immunized mice. Appearance of the germinal centers in MLN as well as iBALT in the lung is best explained by local induction due to the presence of the transported gD-wtFc antigen in the MLN and lung. The IgG detected in BAL may be transported from serum to the luminal secretions actively by FcRn. Of note is the observation that BAL- or vaginal IgG levels were much higher than that of IgA detected, despite high IgA levels in the nasal washings (Figure 3.5C,

middle panel). Indeed, IgG appears a major isotype of immunoglobulins in the lower respiratory tract and reproductive tracts (253). Thus, the IgG antibodies detected in BAL and vaginal washings may be both locally produced as well as serum-derived. Overall, both locally and serum-derived IgG are important for controlling viral infection in the lower respiratory and genital tract. We conclude that antigen targeting to FcRn combined with a mucosal adjuvant produces strong mucosal T cell as well as humoral immune responses. Considering that an efficient protective vaccine should induce mucosal in the mucosa in order to hinder pathogen penetration and spreading, the data presented here suggest that FcRn-targeted mucosal vaccine delivery may provide a very promising technology for the development of protective mucosal vaccines in humans.

Notably, FcRn-targeted mucosal immunization can confer protection against mucosal challenge by virulent HSV-2 virus. Mice inoculated intranasally with an attenuated strain of HSV-2 develop vigorous antibody and cell-mediated immune responses that have been shown to protect the animals from death following intravaginal inoculation with lethal doses of fully virulent HSV-2. We take advantage of this system as a paradigm to examine whether the immune responses elicited by FcRn-mediated subunit vaccine delivery via intranasal inoculation for the protection of the distal vaginal mucosa. The gD-wtFc subunit vaccine described in the present study induced complete protection and significantly reduced virus shedding in the vaginal lumen, but not the gD-mutFc or gD alone subunit vaccine, throughout the experimentation period (Figure 3.2B). In addition, the gD-wtFc-immunized FcRn KO

mice were failed to provide the complete protection (Figure 3.2A). These data indicate that protection is FcRn dependent. Several mechanisms may account for the role of the immune responses elicited by this novel pathway in protection of the distal vaginal mucosa. First, mucosal and systemic humoral immune responses elicited by FcRn transported gD-Fc may play an important protective role. This conclusion was supported by the evidences that the significant amount of gD-specific antibody appeared in the vaginal secretions (Figure 3.5C) and the sera passively transferred from the gD-Fc immunized mice conferred high level of protection. It has been reported (254) that IgG was the main protective antibody in mouse vaginal secretions after vaginal immunization with attenuated herpes simplex virus type 2 . Second, T lymphocytes were present in the vaginal epithelium of HSV-2 infected mice at a time coincident with virus clearance. In the current study, the gD-wtFc subunit vaccine induced a high frequency of IFN-γ secreting $CD4^+$ and $CD8^+$ T cells in either the lung (Figure 3.5D) or vaginal tissues (Figure 3.5E). The mechanisms by which such IFN-γ-positive cells exert their function may be diverse. IFN-γ is clearly indispensable for resistance to HSV-2 infections (255). It is also possible that the strong $CD4^+$ T cell responses induced by FcRn targeting provides direct resistance, e.g., through the direct lysis of MHC class II– bearing infected epithelial cells. Another possiblility is that CTL may contribute to the protection of HSV-2 infectin in the vaigina.Another interesting consequence of the FcRn-targeted vaccine was that protective $CD4^+$ T cell immunity was elicited at a mucosal surface of the genital tract, which was distal to the intranasal vaccination site. Several studies found that mucosal immunization using the intranasal route is effective for generating antibody and T cell immune responses in

the female genital tract. Perhaps intranasal immunization stimulates cells in the nasal lymphoid tissue (NALT) and its draining cervical lymph nodes, MLN, leading to the migration of antibody secreting cells and T cells generated in the airways into the female genital tract (256).

FcRn targeted mucosal immunization elicits long-lived memory immune responses. Immunological memory to a specific vaccine antigen or pathogen is a hallmark of the vertebrate immune system. It is characterized by increased levels of effector T and B cells and, functionally, the ability to respond faster and more vigorously to a second encounter with the pathogen or vaccine antigens than during the primary response (79). Hence, an important success criterion for any vaccine is the formation and maintenance of a reservoir of memory lymphocytes of both adequate size and quality to maintain efficient immune surveillance for prolonged periods. A popular paradigm theory is that continuous antigen exposure provided by a live viral or bacterial vaccine would be necessary for the maintenance of efficient immunological memory. An obstacle for the successful implementation of any subunit vaccine is the production and maintenance of a pool of memory lymphocytes with adequate size and duration. This feature has been a special cause of concern in a subunit vaccine development. Hence, it has been extremely difficult to implement subunit strategy to many global infections because its preparation could elicit high levels of immunity immediately after vaccination but that immunity waned rapidly over time. However, the most striking feature is that the FcRn mediated delivery of mucosal subunit vaccine in this study promoted and sustained high levels of exact

gD-specific plasma cells and memory B and T cells at least 6 months post-vaccination (Figures 3.6 and 3.7). Long-lived plasma cells in the bone marrow (Figure 3.6C) and memory B cells in the spleen (Figure 3.6B) should explain for the appearance of high level of serum antibodies (Figure 3.6D). Given the relatively short half-life of immunoglobulin in vivo, antibodies have to be continuously secreted by plasma cells. In the cases of polio and diphtheria vaccines, serum antibody levels are maintained at high levels for prolonged periods of time after vaccination. Although the reason for the high memory T cell activity is not completely clear, cytokine IL-2-producing memory T cells formed in the responding T cell population (Figure 3.7B) may be important since IL-2 plays an important role in the successful long-term survival of primed $CD4^+$ T cells in vivo, and the expression of IL-2 has been associated with predominantly central memory T cells (257). In addition, the FcRn *per se*, in addition to be a highly efficient epithelial transporter for the gD-wtFc, acts as a protector for prolonging the half-life of Fc-fused vaccine antigens (258). It is generally assumed that a slow, low-level release of vaccine antigen over prolonged time can promote good long-term memory responses. Future studies are planned to address the potential role of the long-term memory response after the gD-wtFc subunit vaccination in modulation of reactivation from latency. These important features may represent a major advantage for the practical implementation of this FcRn-targeted mucosal vaccine in humans.

Overall, given the fact that the FcRn targeted mucosal vaccination differs notably between wt and FcRn KO mice or the gD-wtFc and the gD-mutFc immunized

mice in terms of protection results, mucosal and systemic immune responses, cytokine expression profiles, and the long-term memory and protective efficacy, this study shows that mucosal administration of the vaccine antigens targeted to FcRn is sufficient to elicit strong and long-lasting mucosal and systemic antibody and T cell responses as well as immune memory specific for vaccine antigens. The knowledge gained from this study will be useful in development of effective mucosal vaccine strategies for broad mucosal pathogens, such as human immunodeficiency virus-1, Chlamydia, influenza etc., that infect at or invade across mucosal surfaces. Taken together, these promising results may lay the groundwork for introducing FcRn-targeted vaccines to mucosal infections, such as HIV and HSV-2, also may emerge as a realistic and safer alternative implementation to live virus or bacterium vaccines in the near future.

Chapter 4: Summary and Perspectives

Since FcRn was cloned in 1989 by Neil Simister, much progress has been made to elucide the biological characteristics of FcRn. To date, FcRn is defined as a unique IgG Fc receptor and member of MHC class I family, due to following features: (1) FcRn plays essential roles in both IgG homestasis and IgG transport; (2) FcRn binds to IgG exclusively at acidic pH condition; (3) FcRn mainly resides in the endocytic vesicles of cells; and (4) FcRn transports IgG across polarized mucosal barrier bi-directionally. However, many questions remain unknown. Which signals determine FcRn to transport IgG across mucosal barrier bi-directionally? What kind of epigenetic modifications regulate FcRn expression in newborn and adult intestine? What are exact roles of FcRn in maintaining the autoantibody in autoimmune diseases ? What kind of roles does FcRn play in the process of viral or bacterial infection at mucosal sites?

Particularly, I am interested in how FcRn traffick into endosomes, because the residency of FcRn in endosomes is the prerequisite for its IgG protection and transport function. In Chapter 2, by utilizing the combination of a variety of biochemical and cell biology tools, we identified that the putative dilucine motif within the cytoplasmic domain of FcRn was involved in the FcRn trafficking into early endosomes. Furthermore, we also found that MHC class II associated invariant chain can interact with FcRn and modulate FcRn trafficking into both endosomes and lysosomes. This is demonstrated by multiple approches: FcRn can be specifically pulled down by invariant chain antibody from FcRn/invariant chain (human or

mouse) double transfected HeLa cells or CHO cells. This also holds true for primary cultured human PBMCs or mouse bone marrow derived dendritic cells. More significantly, such interaction can regulate FcRn trafficking into both early endosomes and lysosomes. FcRn has been known as early endosomal receptor, but our data challenged this dogma, arguing that in the absence of invariant chain like in epithelial cells at steady state, FcRn mainly resides early endosomes, however in the presence of invariant chain, such as in the APCs which constitutively express invariant chain or epithelial/endothelial cells at inflammatory condition in which the expression of invariant chain is dramatically upregulated, a significant fraction of FcRn was diverted from endosomes to lysosomes. This notion was further confirmed by the observation that FcRn seldom trafficks into lysosomes in the bone marrow derived dendritic cells (mouse) or PBMC derived dendritic cells (human) when invariant chain expression was abolished by gene knockout or silence. Our data discover that invariant chain expands the boundary of FcRn residence. This was surprising, because invariant chain is known as MHC class II but not MHC class I chaperon.

Nevertheless, the biological significance of FcRn trafficking into lysosomes is unkown. However, by virtue of FcRn's IgG protection and transport function, we can propose the following hypothesises. First, FcRn may extend its IgG protection function into lysosomes, building up the second line of IgG protection, which is potentially important for the protection of pathogen specific IgG during infection. Microbial infection caused inflammation upregulates invarianct expression, leading to

the lysosomal traffic of FcRn. Because normal IgG likely occupy most FcRn in early endosomes due to a high concentration, anti-micorbial IgGs would most likely be delivered to lysosomes where lysosomal FcRn may capture the antigen specific IgG and transport FcRn-IgG complex out of cells via exocytosis. However, such a hypothesis needs to be carefully tested in the future. Nontheless, FcRn may also protect normal IgG in lysosomes. This hypothesis may be addressed by comparing the serum IgG level between wild type and Ii knockout mice in the future under the physiological or inflammatory conditions . Second, since MHC class II are most efficiently targeted into proximity with antigenic peptides by Ii chain in antigen presenting cells (APCs); it is interesting to bring into perspective of the known functions of Ii in conventional APCs in order to understand the role of FcRn/Ii association in influencing antigen presentation. Our previous findings show that the FcRn is expressed in Mϕ and DCs (153). Since FcRn appears to associate with Ii chain, FcRn and MHC class II might occupy the same acidic compartments. In Macrophages and DCs, FcγRs can promote the internalization of immune complexes into the endosomes and lysosomes to increase the efficiency of MHC class II presentation to $CD4^+$ T cells (236). FcRn, on the other hand, may mediate the antigen presentation by binding the immune complexes in these antigen processing compartments. The evidences that FcRn is able to bind immune complexes (65) and IgG in the pH range of endosomes and lysosomes (Zhu et al., unpublished data) support this probability. Therefore, the association of Ii chain and FcRn could further influence the antigen presentations. Third, FcRn was involved in IgG-mediated phagocytosis with its expression in the phagolysosomes in human neutrophils (237).

It would be interesting to know whether FcRn-Ii association in the neutrophils mediates the translocation of FcRn into the phagolysosomes.

FcRn is known as the only IgG transporter among Fc receptors, which can shuttle IgG across mucosal barrier bi-directionally. In Chapter 3, we investigated whether we can use FcRn as vehicle to transport viral-immunogen-fused Fc from mucosal surface to lamina propria to induce protective immunity to combat mucosal viral infections. This is of great significance because so far we are in great need but lack of safe and effective mucosal vaccines (209). In this project, we used herpes simplex virus type II as mucosal infection model, and we fused HSV-2 envelope protein gD with Fc fragment of mouse IgG2a. It was observed that such fusion protein can be efficiently transported by FcRn from mucosal lumen to the circulaton. More significantly, such transport can induce strong systemic and mucosal immunity, including both cellular and humoral immunity, evidenced by the induction of antigen specific $CD4^+$, $CD8^+$ T cell and antibody. As expected, the much stronger immunity was induced in wild type fusion protein intranasally administrated wild type B6 mice but not in FcRn knockout mice, mutant protein and gD immunized mice. In addition, we also observed that strong immune memory including long lived plasma cells, memory B cells and memory $CD4^+$ and $CD8^+$ T cells was left behind and lasted a long period. Most important, wild type mice can be completely protected from IVAG lethal viral challenge after intranasal immunization. Although we analyzed many aspects of immune responses induced by Fc fused protein in details, some information need to be further clarified in the future. First, it would be of great

interest to know what types of dendritic cells or macrophages uptake fusion proteins in the lamina propria. Different subsets of dendritic cells have been reported to induce the different consequences of immune responses (190). Second, we have shown that the fusion protein can induce strong CTL responses, it is reasonable to determine whether $CD8\alpha^+$ dendritic cells can acquire our fusion protein because $CD8\alpha^+$ DCs are known as lymphoid residential subsets, specialized in the crosspresentation of antigens (259). Third, it would be interesting to investigate the protection through respiratory challenge with respiratory virus models, like influenze virus infection. In this regard, more detailed information of mucosal CTLs and antigen secreting cells can be documented. Collectively, our data revealed that FcRn-IgG transfer pathway is superior for mucosal subunit vaccine delivery. This finding would be of important value because we are in great need for safe and effective mucosal vaccines, in particular for STDs, like HSV-2 and HIV infections. Subunti vaccine is the safetest one, but so far we still do not have good candidates for mucosal subunit vaccine due to practical obstacles (209). Our study may shed light on the development of effective and safe mucosal vaccines in human in the future.

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