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Human Neonatal Fc Receptor Mediates Transport of IgG into Luminal Secretions for Delivery of Antigens to Mucosal Dendritic Cells

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Summary

Mucosal secretions of the human gastrointestinal, respiratory, and genital tracts contain significant quantities of IgG. The mechanism by which IgG reaches luminal secretions and the function of IgG in these locations are unknown. Here, we find that the human neonatal Fc receptor (FcRn) is the vehicle that transports IgG across the intestinal epithelial barrier into the lumen where the IgG can bind cognate antigen. The FcRn can then recycle the IgG/antigen complex back across the intestinal barrier into the lamina propria for processing by dendritic cells and presentation to $CD4^+$ T cells in regional organized lymphoid structures. These results explain how IgG is secreted onto mucosal surfaces and scavenges luminal antigens for recognition by the immune system.

Introduction

Humoral immunity as mediated by secretory (s)IgA and IgG plays an important role in mucosal tissues as a first line of defense against microorganisms. Within the gastrointestinal and genitourinary tracts and lung, access of immunoglobulins to mucosal secretions must occur in the context of a barrier imposed by epithelial cells that separates the host from the external environment (Madara, 1998). sIgA is transported across epithelial cells from tissue spaces into the lumen through an active unidirectional process involving the polymeric Ig receptor pIgR (Johansen et al., 1999). IgG can also be detected within the intestinal lumen of the adult human that, in certain tissue locations, may reach levels approximating that observed for sIgA. In fact, the levels of IgG reached in fluids can be quite significant ranging from approximately 300 μg/ml in nasal secretions (Hanson and Brandzaeg, 1980) to 800 μg/ml in the human rectum (Kozlowski et al., 1997). Recent reports have also shown that passive administration of neutralizing IgGs can prevent mucosal human immunodeficiency virus transmission in rhesus macaques or macaque neonates (Robert-Guroff, 2000). Together, these observations support the concept that IgG along the mucosal surfaces can serve an important role in mucosal protection. However, the mechanisms(s) by which IgG gains access to the lumen and the biological functions of IgG once present in this location remain undefined.

Recent evidence has pointed toward a role for the neonatal Fc receptor (FcRn) in these processes. FcRn is structurally related to MHC class I molecules and consists of a heterodimer composed of a glycosylated heavy (α) chain in noncovalent association with β2-microgloblin (β2m) (Gastinel et al., 1992; Simister and Mostov, 1989). This molecule was originally identified in the intestinal epithelium of suckling rats as the receptor responsible for the well-known transport of maternal IgG across the intestinal epithelium into the bloodstream (Abrahamson and Rodewald, 1981; Brambell, 1966; Jones and Waldmann, 1971). This ability to move IgG in an apical to basolateral direction across the intestinal epithelium is a major distinguishing cell biologic feature of FcRn in comparison to the pIgR. Although FcRn was originally described as being developmentally regulated in rodent intestine in that its functional expression at birth was notably downregulated approximately 1000-fold at the time of weaning (Ghetie et al., 1996), it has recently become apparent that FcRn continues to be detectable at significant levels in many adult cell types in both rodents and humans (Blumberg et al., 1995; Dickinson et al., 1999; Israel et al., 1997; Leach et al., 1996; Spiekermann et al., 2002; Zhu et al., 2001). In in vitro cell models that express FcRn and that also exhibit cellular polarity, FcRn-mediated IgG transport exhibits bidirectionality predicting that adult expression of FcRn may endow upon polarized epithelia the ability to move FcRn-bound cargo from either the lumen or tissue spaces into the opposite pole of the cell with a steady-state distribution of transport in vivo that is directed abluminally into the tissue (Claypool et al., 2002; Dickinson et al., 1999; McCarthy et al., 2000; Praetor et al., 1999). In this context, it is of interest that FcRn has been not only functionally linked to the passive acquisition of immunity in neonatal rodents through the transport of maternal IgG but also to IgG-mediated immune surveillance based upon the indirect morphologic observation made more than two decades ago that FcRn is also capable of transporting antigen-antibody complexes across the intestinal epithelium from the lumen during neonatal rodent life (Abrahamson et al., 1979). This raises the hypothesis that a major function of FcRn in adult human life is to transport IgG into the apical region of the epithelium for retrieval of antigens, whereupon FcRn can scavenge these complexes for transport back into the lamina propria. Such a pathway could significantly contribute to the regulation of immune re-

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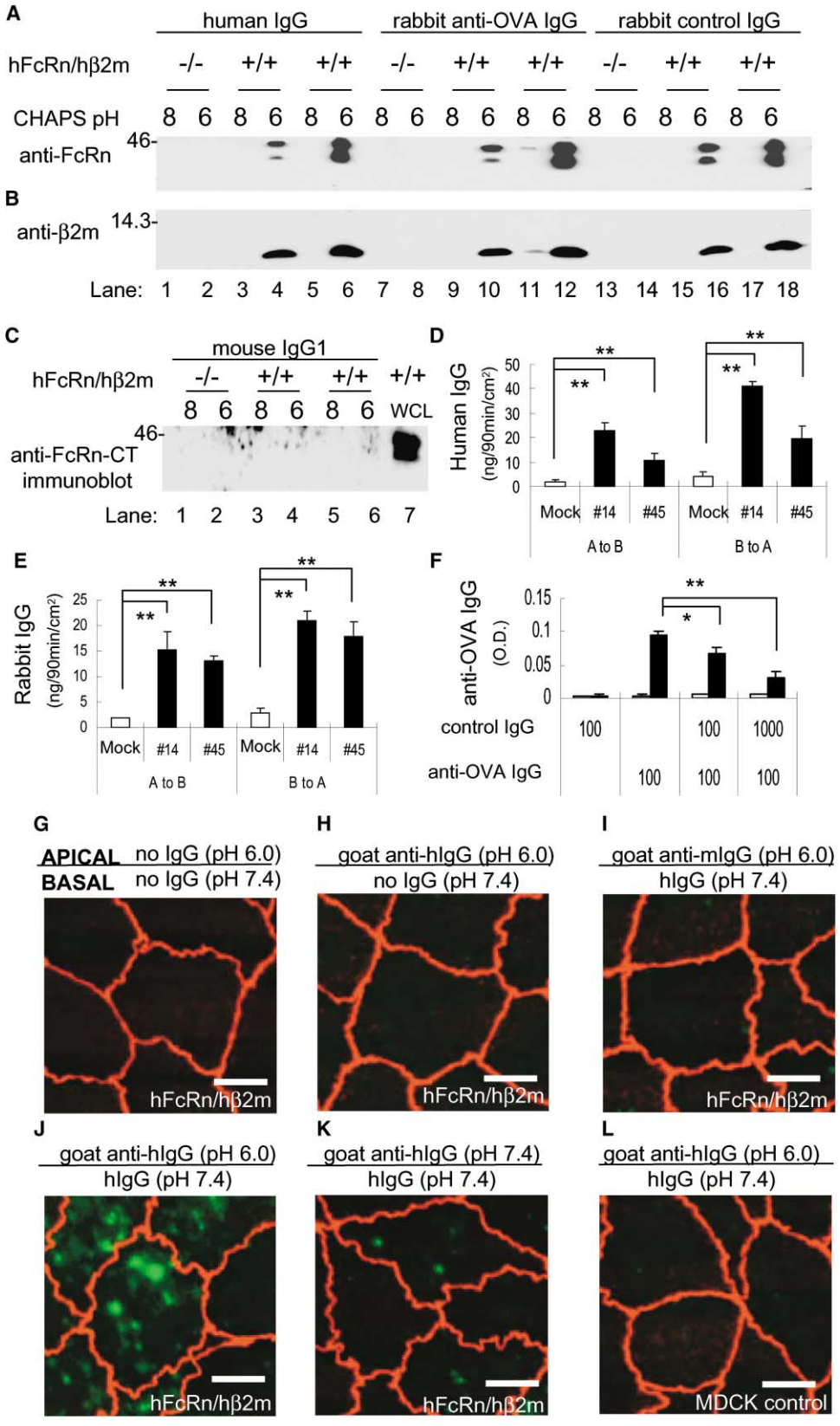

Figure 1. Specific Binding of Rabbit IgG to hFcRn

Mock-MDCK and hFcRn/hβ2m-MDCK were lysed in CHAPS buffer (pH 6.0 or 8.0), and each lysate preincubated with IgGs was incubated with protein G-Sepharose. Bound proteins were analyzed by SDS-PAGE under nonreducing conditions and immunoblotted for the HA tag

sponses by providing a mechanism for luminal antigen(s) to gain access to professional antigen-presenting cells, such as dendritic cells, which are known to be both present at this location and capable of interacting with $CD4^+$ T cells. Through an examination of in vitro and in vivo model systems, this report provides direct evidence in support of this hypothesis.

Results

Transport of Human and Rabbit IgG across Polarized MDCK Cells Expressing hFcRn and hβ2m

FcRn has been recently demonstrated to be capable of transporting IgG bidirectionally across epithelial barriers. Given the likelihood that luminal- or epithelial-associated IgG would be expected to interact with local antigens of the appropriate specificity, we predicted that FcRn is capable of retrieving luminal antigen/IgG complexes for transport back into the lamina propria. In an initial series of studies we determined whether hIgG and rabbit anti-OVA IgG could be recognized by hFcRn expressed in the transfected MDCK cell line. Both hIgG, rabbit control IgG, and rabbit anti-OVA IgG but not mouse IgG1 were capable of associating with and pulling down hFcRn as a complex with hβ2m at pH 6.0 but not at pH 8.0 (Figures 1A–1C), consistent with previous studies, which have demonstrated that hFcRn can bind to rabbit IgG but not mouse IgG1 (Ober et al., 2001). The existence of two FcRn bands was consistently observed and represents hFcRn containing mature (upper band in Figure 1A) and immature (lower band in Figure 1A) carbohydrate side chain modifications as previously described (Claypool et al., 2002).

We next examined whether hIgG and rabbit IgG are transported across a polarized monolayer in an FcRn-dependent manner. To do so, hIgG or rabbit anti-OVA IgG was applied to either the apical or basolateral reservoir. By this method, both hIgG and rabbit anti-OVA IgG could be shown to be bidirectionally transported by two different hFcRn/hβ2m-MDCK (#14 and #45) but not mock-MDCK cell lines (Figures 1D and 1E). Further demonstrating the receptor-mediated origin of this transport, rabbit control IgG (Figure 1F) or hIgG (data not shown) dose dependently suppressed the transport of the rabbit anti-OVA IgG. In contrast, chicken IgY (2.5 mg/ml), which is unable to bind hFcRn (Claypool et al., 2002; Dickinson et al., 1999), failed to abolish this transport (data not shown). These observations confirm that, like hIgG, rabbit IgG can bind and be transcytosed by hFcRn.

Antibody/Antigen Complexes Can Be Transported across Polarized MDCK Cells in an FcRn-Dependent Pathway

We next determined whether hFcRn contributes to the transport of immune complexes across an epithelial barrier. Goat anti-hIgG $F(ab')_2$ or goat anti-mIgG $F(ab')_2$ acting as model antigens due to their lack of Fc regions were applied to the apical reservoir, and hIgG was also applied to the basolateral reservoir. Strong uptake of goat anti-hIgG $F(ab')_2$ was detectable when goat anti-hIgG $F(ab')_2$ was applied to the apical surface and hIgG was applied to the basolateral surface of hFcRn/hβ2m-MDCK cells (Figure 1J) but not mock-MDCK cells (Figure 1L). In contrast, when goat anti-mIgG $F(ab')_2$ was applied to the apical chamber or when goat anti-hIgG $F(ab')_2$ was applied apically without application of hIgG basolaterally, no green signal was detectable (Figures 1H and 1I). Consistent with the work of others (Claypool et al., 2004; Praetor et al., 1999), buffering the input chamber of the apical reservoir to pH 6.0 significantly enhanced the ability to detect the goat anti-hIgG intracellularly within the hFcRn/hβ2m-MDCK cells in the presence of basolaterally applied hIgG (Figures 1J and 1K). Also consistent with previous observations (Claypool et al., 2004), buffering the basolateral chamber to pH 6.0 was not necessary to observe apically directed transcytosis from the basolateral chamber. These preliminary studies show that hFcRn can mediate the transport of IgG from the basolateral to apical compartment for retrieval of antigens as an immune complex.

To test whether such an immune complex can be transcytosed, we prepared immune complexes by incubating rabbit anti-OVA IgG or nonspecific rabbit IgG and biotin-conjugated OVA (B-OVA) for 30 min and applied these to the apical reservoir of a transwell system. As expected, rabbit IgG could be detected in the hFcRn/hβ2m MDCK cells exposed to control IgG and anti-OVA IgG (Figure 2A). Although small quantities of B-OVA as a baseline signal could be detected in either the absence of any rabbit IgG (data not shown) or the presence of control rabbit IgG in both MDCK cells (Figure 2B), only in the presence of both specific anti-OVA IgG and the hFcRn/hβ2m-MDCK cell line could high-level transport of B-OVA (Figure 2B) and OVA/anti-OVA IgG immune complexes (Figure 2C) be detected. Similar observations were made when immune complex transport was assessed in the basolateral to apical direction consistent with the bidirectionality of this transport process as previously shown for IgG antibodies (data not shown

(A), hβ2m (B), and hFcRn-CT (C). (C) Whole-cell lysate (WCL) of the hFcRn/hβ2m-MDCK cells. Bidirectional transcytosis of human and rabbit IgG by hFcRn in MDCK cell lines expressing hFcRn/hβ2m. (D–F) Human IgG (100 μg/ml) or rabbit IgG (anti-OVA IgG; 100 μg/ml) was added to either apical or basolateral reservoir on transwell filters of mock-MDCK (open bars) and hFcRn/hβ2m-MDCK (#14 and #45; closed bars) and cultured for 90 min. The output solution was collected from the opposite reservoir and subjected to ELISA for measuring the concentrations of hIgG (D) and rabbit IgG (E). (F) To further demonstrate receptor-mediated transport, rabbit control IgG at a final concentration of 100 μg/ml or 1000 μg/ml was added to the apical reservoir with 100 μg/ml anti-OVA IgG. After 90 min incubation, the output solution was collected and the concentration of rabbit anti-OVA IgG was assessed. The bars represent the mean ± SD. *$p < 0.05$ and **$p < 0.01$. Enhanced goat anti-hIgG was internalized from the apical membrane when hIgG was added in the basolateral reservoir in hFcRn/hβ2m-MDCK cells. Either goat anti-hIgG F(ab′)2 (40 μg/ml) or goat anti-mIgG F(ab′)2 (40 μg/ml) buffered at pH 6.0 (G–J and L) or pH 7.4 (K) was internalized from the apical chambers and hIgG (40 μg/ml) buffered at pH 7.4 was (I–L) or was not (G and H) added to the basolateral reservoir of filter-grown hFcRn/hβ2m-MDCK cells (G–K) and control MDCK cells (L) for 2 hr. The cells were fixed, permeabilized, and stained with anti-goat IgG (green) and ZO-1 (red) and the photos prepared at the level of tight junction (ZO-1). The schemas above each photo represent the condition of the apical and basolateral reservoirs. Scale bars, 5 microns.

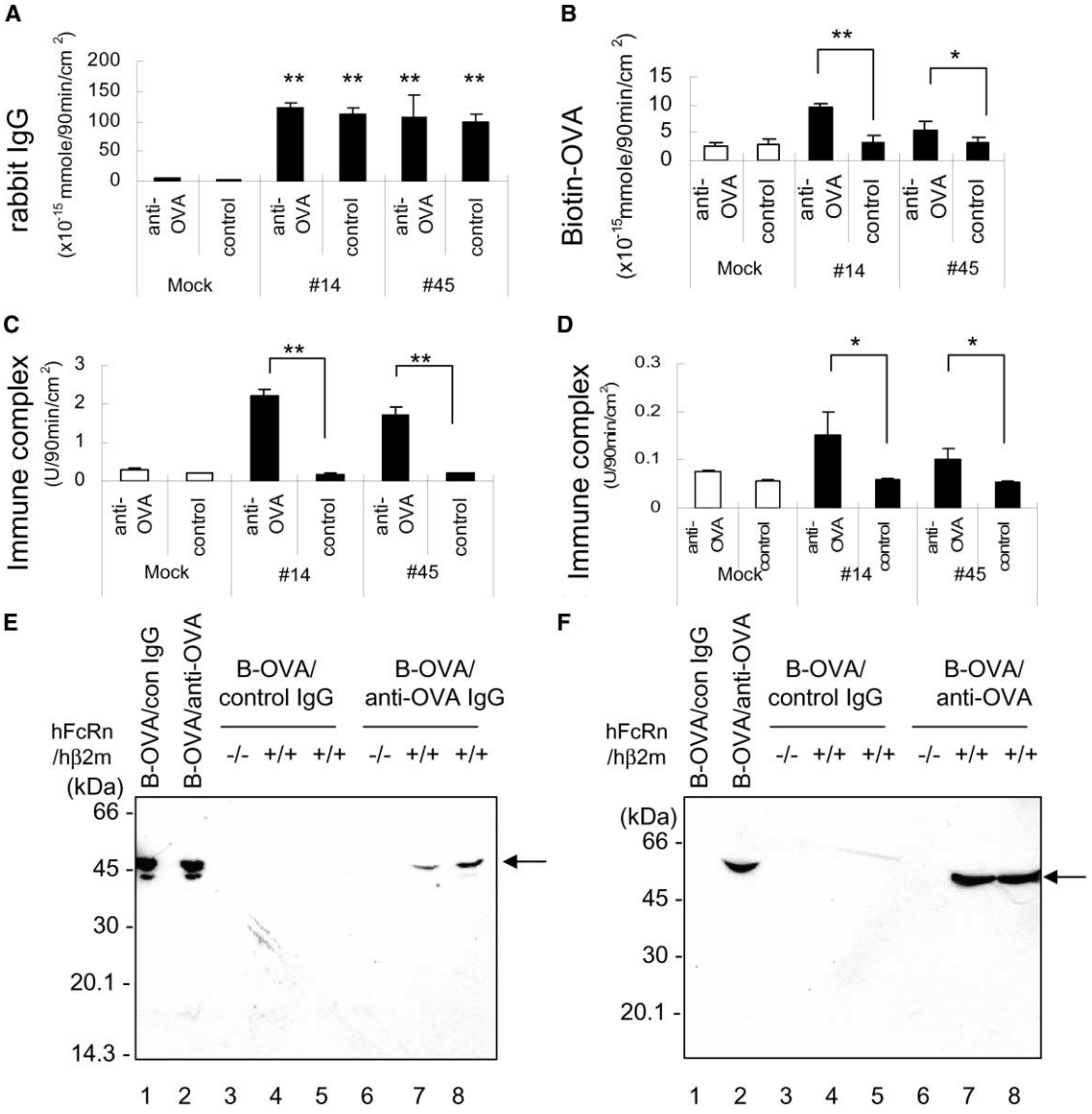

Figure 2. Transcytosis of Antigen/IgG Complexes by FcRn in MDCK Cell Lines Expressing hFcRn/hβ2m

Mock-MDCK (open bars) and hFcRn/hβ2m-MDCK (closed bars) cells were grown on transwell filters until confluent. (A–C) B-OVA (33 μg/ml)/control IgG (100 μg/ml) or B-OVA/anti-OVA IgG was added to the apical reservoir. After 90 min incubation, the supernatant obtained from the basolateral reservoir was subjected to ELISA. (D) B-OVA was added to the apical reservoir (100 ng/ml) and either control IgG or anti-OVA IgG was added to the basolateral reservoir (100 μg/ml). After 180 min incubation, the bottom reservoirs were washed with cold PBS and incubated with fresh HBSS containing 1% BSA (pH 7.4). After an additional 180 min incubation, the supernatant obtained from the bottom reservoir was subjected to ELISA. The bars represent the mean ± SD (*$p < 0.05$, **$p < 0.01$). (E and F) Detection of B-OVA/anti-OVA IgG complex by immunoblot. An equal quantitity (100 ng) as determined by B-OVA ELISA was obtained from the basolateral reservoir of a transwell system that had been incubated apically with B-OVA/anti-OVA IgG or B-OVA/control IgG. As controls for immunoblot, B-OVA with rabbit control IgG and B-OVA with rabbit anti-OVA IgG were also used. The supernatants were incubated with avidin-agarose beads and the bound proteins subjected to immunoblot analysis using mouse anti-OVA antibodies followed by HRP-conjugated anti-mouse IgG1 (E) or HRP-conjugated donkey anti-rabbit IgG antibodies (F). The arrow in (E) indicates the molecular weight of OVA and the arrow in (F) indicates that of the IgG heavy chain. The samples are as follows: lane 1, B-OVA (100 ng/well) with rabbit control IgG (30 ng/well); lane 2, B-OVA (100 ng/well) with rabbit anti-OVA IgG (30 ng/well); lane 3 and 6, mock-MDCK cells; lanes 4, 5, 7, and 8, two different hFcRn/hβ2m-MDCK clones.

and Figures 1D and 1E). These results indicate that hFcRn can transport an immune complex.

Next, to assess whether IgG transported in a basolateral to apical direction could retrieve antigens from the apical reservoir for transport as an immune complex back into the basolateral reservoir, B-OVA was applied to the apical reservoir and either anti-OVA IgG or control IgG was applied to the basolateral reservoir. Only in the presence of both specific anti-OVA IgG and the hFcRn/hβ2m-MDCK cell line could significant transport of B-OVA as a complex be detected (Figure 2D). These results indicate that the observed transport of immune complexes across an intact epithelial barrier described above is likely to be physiologic given the ability of

hFcRn to mediate the retrieval of free antigen from the apical luminal milieau when antigen-specific IgG is present basolaterally as modeled in the transfected MDCK cells.

We also directly tested whether B-OVA/IgG complexes can be detected by immunoblot to show the fact that intact OVA could be transported by FcRn-expressing epithelial cells. To do so, B-OVA and B-OVA/IgG complexes were retrieved from the basolateral reservoir by concentration with avidin-agarose. When OVA was transported apical to basal across the hFcRn/hβ2m-MDCK monolayer as an immune complex with specific IgG, it was detected as a single, 45 kDa band, consistent with the intact molecular weight of OVA (Figure 2E, arrow). In either the presence of control IgG or when applied as a specific immune complex to mock-MDCK cells, intact OVA could not be detected (Figure 2E). To exclude the possibility that the positive signal observed in Figure 2E represented the transported IgG heavy chain, the same membrane was reprobed with an HRP-conjugated donkey anti-rabbit IgG antibody. As seen in Figure 2F, the donkey anti-rabbit antibody detected a band consistent with the heavy chain of rabbit IgG (arrow) only when present as an immune complex with its specific cognate antigen either before (Figure 2F, lane 2) or after (Figure 2F, lanes 7 and 8) transport across the hFcRn/hβ2m MDCK cells. These results indicate that intact OVA/IgG immune complexes can be transported across polarized epithelial cells in an apical to basolateral direction via binding to hFcRn.

Antigen/IgG Complexes Transported by hFcRn Stimulate CD4$^+$ T Cells

We next determined whether the OVA transported as an immune complex with IgG by an FcRn-mediated pathway has preserved its antigenic activity as defined by the ability to drive MHC-restricted antigen presentation to antigen-specific CD4$^+$ T cells. To test the efficiency of an in vitro assay, naive CD4$^+$ T cells from DO11.10 mice with antigen-presenting cells (APCs) were cocultured with different concentrations of antigen or antigen/IgG complexes (Figure 3A). The expression of CD69 was examined following 60 hr of incubation, since preliminary studies that examined the time course of CD69 expression revealed a progressive increase of CD69 expression for up to 72 hr after incubation (data not shown). OVA as an immune complex with specific IgG upregulated CD69 expression on the OVA-specific CD4$^+$ T cells at significantly lower doses than either OVA alone or OVA/control IgG. These studies indicate this assay has a sensitivity to detect 10 ng/mL or more of OVA/anti-OVA IgG complexes.

To examine whether the OVA transported could be presented to T cells, MDCK cells were exposed apically to either B-OVA alone, B-OVA/anti-OVA IgG, or B-OVA/control IgG and the basolateral reservoir sampled after incubation. After standardizing the quantities of B-OVA transported as defined by ELISA, 1.0 μg/ml of B-OVA from each collected supernatant was cultured for 24 hr with naive CD4$^+$ T cells from DO11.10 mice and APCs and the levels of IL-2 assessed as a measure of T cell activation. When cells were pulsed with medium from the hFcRn/hβ2m-MDCK cells incubated with OVA/anti-OVA IgG but not control IgG IL-2, production was increased, (Figure 3B) but not with medium from mock-MDCK cells treated with OVA/anti-OVA IgG. To confirm the physiologic nature of the antigen presentation observed, the naive CD4$^+$ T cells from DO11.10 mice were also cultured for 60 hr. The OVA-specific CD4$^+$ T cells were only stimulated, as indicated by the production of IFN-γ (Figure 3C) and IL-10 (Figure 3D), when cells pulsed with the basolateral medium from hFcRn/hβ2m-MDCK cells incubated with OVA/anti-OVA IgG.

These results indicate that OVA transported across polarized epithelial cells in the apical to basolateral direction via hFcRn as an IgG-antigen complex is delivered as a macromolecule that retains its antigenicity for CD4$^+$ T cells.

Basolateral to Apical Transport of IgG into the Lumen In Vivo Is Dependent upon hFcRn

We next determined whether FcRn contributes to the transport of IgG across an epithelial barrier in vivo. To do so, we utilized a transgenic mouse expressing both hFcRn and hβ2m (hFcRnTg/hβ2mTg). To avoid any influence of the endogenous mouse FcRn, the hFcRnTg/hβ2mTg mice were backcrossed on to FcRn-deficient mice. An immunoblot analysis confirmed the expression of hFcRn and hβ2m in the small intestinal tissues of hFcRnTg/hβ2mTg/mFcRn$^{-/-}$ mice but not mFcRn$^{-/-}$ mice (Figure 4A). The expression levels of hFcRn within the intestinal epithelium of hFcRnTg/hβ2mTg/mFcRn$^{-/-}$ mice were similar to those of native human intestinal epithelial cells consistent with the fact that hFcRn in the transgenic mice is under the control of the endogenous human promoter (Figure 4B). Immunohistochemical analysis further confirmed that hFcRn expression was detectable in the epithelium of the small (data not shown) and large intestine (Figure 4C) in the hFcRnTg/hβ2mTg/mFcRn$^{-/-}$ mice but not mFcRn$^{-/-}$ mice. The detection of hFcRn expression in the epithelium of the adult transgenic mice is consistent with the fact that, in contrast to rodents, FcRn continues to be expressed at significant levels by the intestinal epithelium of adult humans (Dickinson et al., 1999; Israel et al., 1997; Zhu et al., 2001). Moreover, as modeled by in vitro cell lines, expression of hFcRn is associated with the bidirectional transport of IgG. This latter point predicts that expression of FcRn may account, at least in part, for the presence of IgG in mucosal luminal surfaces in vivo.

Therefore, to examine whether hIgG transport in the basolateral to apical direction is hFcRn dependent, EPO-hIgG-Fc and the EPO-hIgG-Fc/IHH mutant, which is unable to bind hFcRn (Spiekermann et al., 2002), were used. IgG binding assays showed that EPO-hIgG-Fc could bind to hFcRn in contrast to the EPO-hIgG-Fc/IHH mutant, which could not (Figure 4D). Therefore, hIgG, EPO-hIgG-Fc, or the EPO-hIgG-Fc/IHH mutant were injected intraperitoneally, and the quantities of IgG secreted into the feces compared. EPO-hIgG-Fc could be detected at significantly higher levels in the hFcRnTg/hβ2mTg/mFcRn$^{-/-}$ mice in comparison to the levels of the EPO-hIgG-Fc/IHH mutant injected into the same mice (Figure 4E). These observations confirm the fidelity of the hFcRn promoter in recapitulating the expression of hFcRn within the adult intestinal epithelium and the

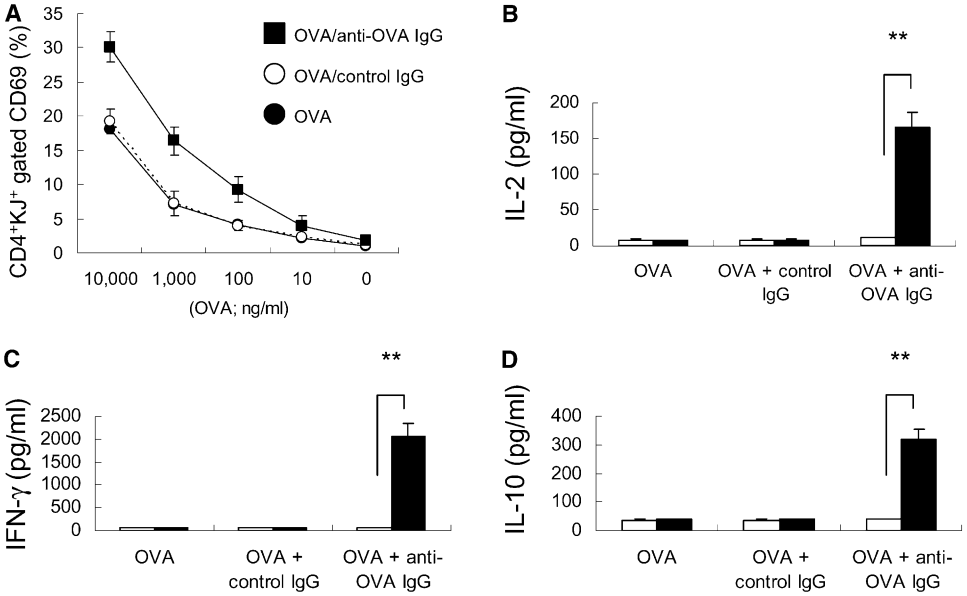

Figure 3. Transported Antigen/IgG Complexes Effectively Stimulate Antigen-Specific $CD4^+$ T Cells

(A) Naive $CD4^+$ T cells from DO11.10 mice and APCs were cultured with different doses of either OVA, OVA/control IgG, or OVA/anti-OVA IgG. After 60 hr incubation, cells were collected and the surface expression of CD69 determined after gating with a DO11.10Tg-TcR-specific antibody (KJ1-26) and anti-mouse CD4 antibody as determined by flow cytometry. Cytokine production by OVA-specific $CD4^+$ T cells (B–D). The medium from the basolateral reservoir of a transwell system in which mock-MDCK cells (open bars) and hFcRn/hβ2m-MDCK cells (closed bars) were incubated apically with B-OVA applied either alone or with anti-OVA IgG or control IgG were normalized to a B-OVA content of 1.0 μg/ml by ELISA and cultured with naive $CD4^+$ T cells from DO11.10 mice and APCs. The culture supernatant was subjected to ELISA to detect of IL-2 (B), IFN-γ (C), or IL-10 (D). The bars represent the mean ± SD (**$p < 0.01$) ($n = 3$).

utility of this mouse/human chimera in examining the in vivo function of hFcRn (Roopenian et al., 2003). Moreover, these studies show that hFcRn mediates the specific transport of IgG from tissue spaces into luminal secretions of the intestine.

Formation of Antigen/IgG Complexes in the Lumen as a Consequence of IgG Secretion

To examine whether secreted IgG could form complexes with antigen in the lumen, either anti-OVA IgG or control IgG were injected i.v. into hFcRnTg/hβ2mTg/$mFcRn^{-/-}$ mice; a quantity of IgG was injected, which achieved physiological levels of antibody in the serum (0.59 ± 0.06 versus 0.61 ± 0.05 mg/ml, respectively) based upon previous studies on the normal levels of IgG in the serum of wild-type mice (Telleman and Junghans, 2000). Six hours after the i.v. injection, B-OVA was administered orally. The concentrations of rabbit IgG and immune complexes retained in the small intestinal fluid were determined 30 min after the oral administration of antigen by ELISA. Although no significant differences could be detected in the concentrations of anti-OVA IgG and control IgG in the small intestinal fluid of the hFcRnTg/hβ2mTg/$mFcRn^{-/-}$ mice (Figure 4F), only in the presence of both specific antigen and anti-OVA IgG were immune complexes detected within the small intestinal fluid (Figure 4G). These results indicate that IgG, which gains access to the intestinal lumen via FcRn-mediated transport, is capable of forming immune complexes with the cognate antigen of the secreted IgG.

Intestinal Lumen-Derived Antigen/Antibody Complexes Can Be Transported into the Lamina Propria by hFcRn In Vivo and Can Be Taken up by $CD11c^+$ Cells

To examine whether hFcRn can transport OVA/IgG immune complexes from the intestinal lumen into the lamina propria across intestinal epithelial cells in vivo, FITC-conjugated OVA as an immune complex with anti-OVA IgG was administered intragastrically into mice. One hour after administration, positive signals indicative of the presence of FITC were detected in the epithelium of the small intestine in hFcRnTg/hβ2mTg/$mFcRn^{-/-}$ (Figure 5B, arrow) but not $mFcRn^{-/-}$ mice (Figure 5A). By 2 hr after administration of FITC-OVA with anti-OVA IgG to hFcRnTg/hβ2mTg/$mFcRn^{-/-}$ mice, positive signals could be detected in cells within the lamina propria (Figure 5D). As a control, FITC-OVA with control IgG was administered intragastrically into hFcRn/hβ2mTg/$mFcRn^{-/-}$ mice. Positive signals for FITC could not be detected in either the epithelium or lamina propria of the small intestine at 2 hr after administration in this circumstance (Figure 5C). These data indicate that hFcRn can direct the transport of antibody/antigen complexes across the epithelium of the small intestine and suggest that the transported complexes can be subsequently taken up by cells that are present within the lamina propria.

We next examined whether the cells capturing the FITC-OVA within the lamina propria of the small intestine were indeed DC. Therefore, 4 hr after inoculation of FITC-OVA with anti-OVA IgG or control IgG, cells were obtained from the MLN and liver and examined. OVA

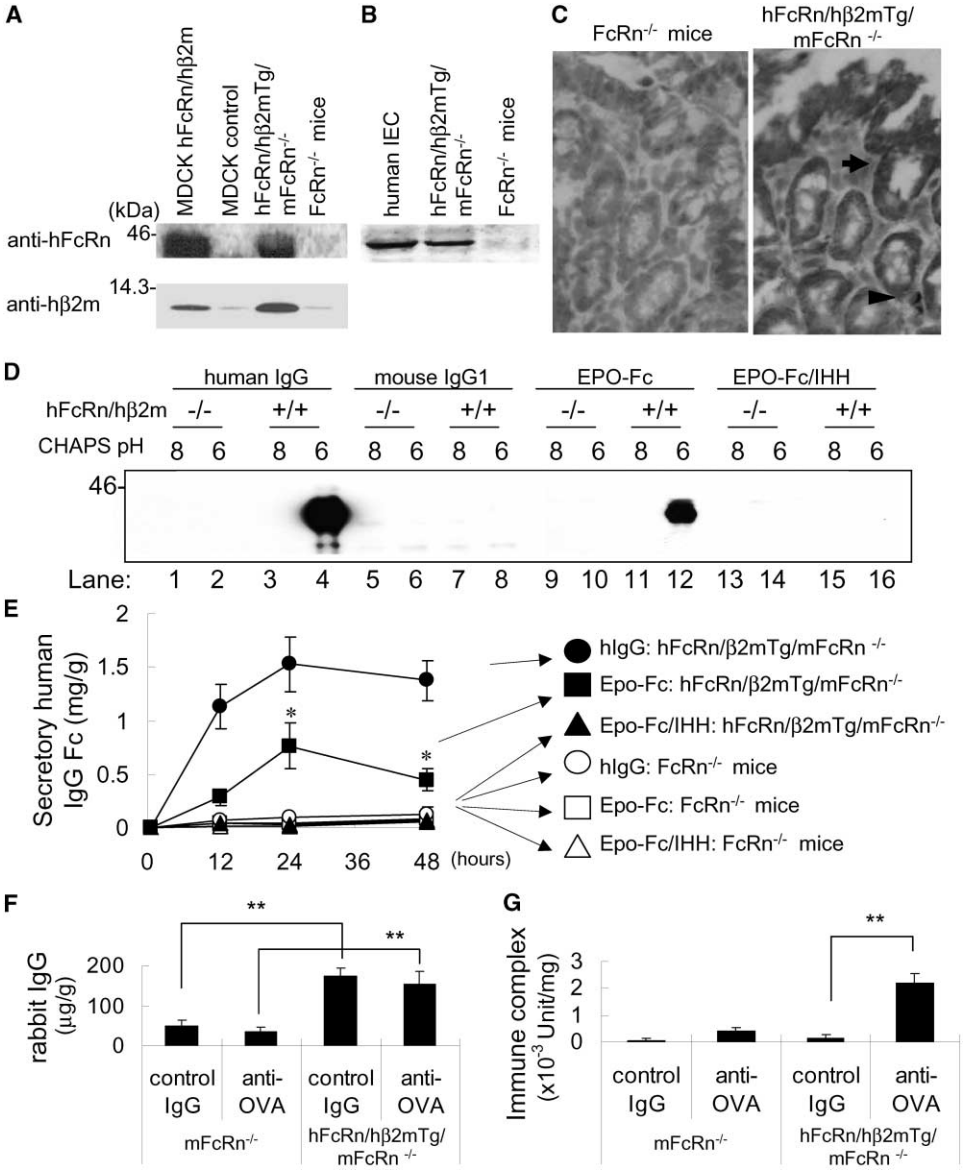

Figure 4. Analysis of hFcRn and hβ2m in hFcRn/hβ2m Transgenic Mice on an mFcRn$^{-/-}$ Background

(A) The lysates from hFcRn/hβ2m-MDCK and mock-MDCK cell lines and the small intestine of 8-week-old hFcRnTg/hβ2mTg/mFcRn$^{-/-}$ and mFcRn$^{-/-}$ mice were subjected to immunolot using anti-human FcRn-CT or anti-hβ2m antibodies.

(B) The lysates from human small intestinal epithelial cells and the small intestine of hFcRnTg/hβ2mTg/mFcRn$^{-/-}$ and mFcRn$^{-/-}$ mice were subjected to immunoblot using anti-human FcRn-CT antibody.

(C) Immunohistochemical analysis of hFcRn expression in the large intestine of 8-week-old hFcRnTg/hβ2mTg/mFcRn$^{-/-}$ mice and mFcRn$^{-/-}$ mice. hFcRn expression was detected within the entire epithelium of the large intestine (arrow) and lamina propria cells (arrowhead) in hFcRnTg/hβ2mTg/mFcRn$^{-/-}$ mice but not mFcRn$^{-/-}$ mice.

(D) Specific binding of EPO-hIgG-Fc to hFcRn but not EPO-hIgG-Fc/IHH mutant. Confluent monolayers of MDCK cells transfected as indicated were lysed in CHAPS lysis buffer (pH 6.0 or 8.0) and each lysate preincubated with 200 μg of hIgG, mouse IgG1, or 100 μg of EPO-hIgG-Fc or EPO-hIgG-Fc/IHH mutant for 4 hr prior to the addition of protein G-Sepharose. Bound proteins were analyzed by SDS-PAGE under nonreducing conditions and immunoblotting for the HA tag. Samples are as follows: lanes 1, 2, 5, 6, 9, 10, 13, and 14, mock-MDCK; lanes 3, 4, 7, 8, 11, 12, 15, and 16, hFcRn/hβ2m-MDCK. The images are the result of one representative experiment (n = 2). The molecular mass in kilodaltons is indicated on the left of each gel.

(E) Intestinal secretory human IgG-Fc concentration by ELISA. Human IgG (3 mg/mouse, circle), EPO-hIgG-Fc (1.5 mg/mouse, square), or EPO-hIgG-Fc/IHH mutant (1.5 mg/mouse, triangle) were injected i.p. into groups of five 8-week-old hFcRnTg/hβ2mTg/mFcRn$^{-/-}$ (closed marks) or mFcRn$^{-/-}$ mice (open marks), and the concentration of the retained human IgG-Fc tracer from serially sampled feces was determined by ELISA. Data are shown as the mean ± SD. The asterisk indicates significance for EPO-hIgG-Fc into hFcRnTg/hβ2mTg/mFcRn$^{-/-}$ versus EPO-hIgG-Fc/IHH mutant into hFcRnTg/hβ2mTg/mFcRn$^{-/-}$ (* $p < 0.05$).

(F and G) The concentrations of intestinal secretory rabbit IgG alone and as an immune complex with OVA by ELISA. Rabbit control IgG (3 mg/mouse) or anti-OVA IgG (3 mg/mouse) were injected i.v. into groups of four either hFcRnTg/hβ2mTg/mFcRn$^{-/-}$ or mFcRn$^{-/-}$ mice, and, 6 hr after the i.v. injection, B-OVA (1 mg/mouse) was administered orally. The concentrations of the rabbit IgG (F) and IgG-OVA immune complexes (G) in the fluid of the small intestine 30 min after the oral administration was determined by ELISA. Data represents a mean ± SD (**$p < 0.01$).

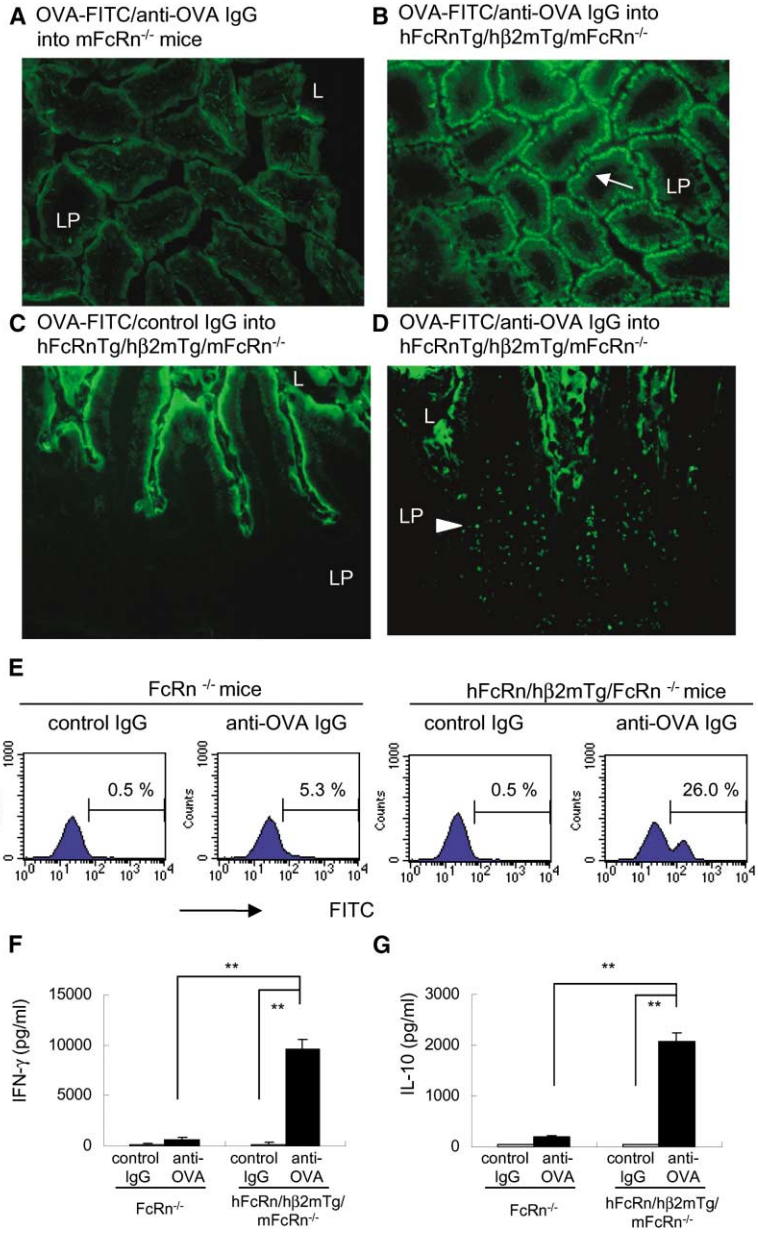

Figure 5. Antigen/IgG Complexes Are Transported across Intestinal Epithelial Cells via hFcRn In Vivo

FITC-OVA/anti-OVA IgG (A, B, and D) or FITC-OVA/control IgG (C) was administered intragastrically into mFcRn$^{-/-}$ mice (A) or hFcRnTg/hβ2mTg/mFcRn$^{-/-}$ mice (B–D). Tissue from the mid-small intestine was collected 1 hr (A and B) and 2 hr (C and D) after inoculation and examined by fluorescence microscopy. (A and B) Transverse section of small intestine. (C and D) Horizontal sections of small intestine. Arrow in (B), intestinal epithelial cells transporting FITC-OVA with rabbit anti-OVA IgG; arrowhead in (D), FITC-positive lamina propria cells. L, lumen; LP, lamina propria. (A–D) The magnification of images shown is ×200. Presence of FITC signals in CD11c$^+$ cells of liver 4 hr after the OVA/IgG complex administration (E). FITC-OVA with either control IgG or anti-OVA IgG were administered intragastrically into mFcRn$^{-/-}$ mice or hFcRnTg/hβ2mTg/mFcRn$^{-/-}$ mice. The cellular populations from liver were isolated 4 hr after inoculation, stained with PE conjugated anti-CD11c and 7-AAD to exclude the dead cells, and then examined by flow cytometry. Data are representative of four mice analyzed in each group. (F and G) Antigen-specific activation of OVA-specific CD4$^+$ T cells by antigen-bearing cells. The CD11c$^+$ cells purified from the MLN of hFcRnTg/hβ2mTg/mFcRn$^{-/-}$ (BALB/c) and mFcRn$^{-/-}$ (BALB/c) mice were prepared 4 hr after inoculation of FITC-OVA plus either anti-OVA IgG or control IgG OVA and cultured with naive CD4$^+$ T cells from DO11.10 mice for 60 hr. The culture supernatants were assessed for IFN-γ (F) and IL-10 (G) production by ELISA. The mean ± SD was shown for each group (n = 4) (**p < 0.01).

uptake into antigen-presenting cells in MLN was evident by the increased mean fluorescence intensity (MFI) in the CD11c$^+$ population within MLN (OVA/control IgG, 34.0 ± 0.9 versus OVA/anti-OVA, 43.2 ± 1.4, $p < 0.01$) obtained from hFcRnTg/hβ2mTg/mFcRn$^{-/-}$ mice. In comparison, the MFI in CD11c$^+$ cells from MLN of FcRn$^{-/-}$ mice that received anti-OVA IgG was not significantly different from that observed in the CD11c$^+$ cells from FcRn$^{-/-}$ mice that received control IgG (OVA/control IgG, 33.6 ± 1.8 versus OVA/anti-OVA, 36.1 ± 1.8). In the liver, in contrast to the MLN, a discrete population of CD11c$^+$ cells could be detected in the hFcRnTg/hβ2mTg/mFcRn$^{-/-}$ mice that contained high quantities of the FITC signal (Figure 5E). Such a population of cells was not evident in the CD11c$^+$ cells in liver from either FcRn$^{-/-}$ mice under any conditions examined or hFcRnTg/hβ2mTg/mFcRn$^{-/-}$ mice that received control IgG (Figure 5E).

Antigen Presentation by CD11c$^+$ Cells to CD4$^+$ T Cells after the Transport of OVA/IgG Complexes In Vivo

To determine whether the CD11c$^+$/FITC$^+$ cells present within the MLN can present OVA to CD4$^+$ T cells, CD11c$^+$ cells purified from MLN after inoculation were cultured with naive OVA-specific CD4$^+$ T cells. The CD11c$^+$ cells purified from the MLN of the hFcRnTg/hβ2mTg/mFcRn$^{-/-}$ mice that were inoculated with FITC-OVA/anti-OVA IgG but not control IgG-stimulated IFN-γ (Figure 5F) and IL-10 production (Figure 5G). However, CD11c$^+$ cells from MLN of mFcRn$^{-/-}$ mice failed to stimulate OVA-specific T cells under either condition (Figures 5F and 5G). These findings indicate that after transport of specific OVA-IgG complexes from the intestinal lumen into the lamina propria by FcRn-expressing epithelial cells, CD11c$^+$ cells, which have captured antigen and are capable of presenting the transported antigen to CD4$^+$ T cells, can be detected within the MLN.

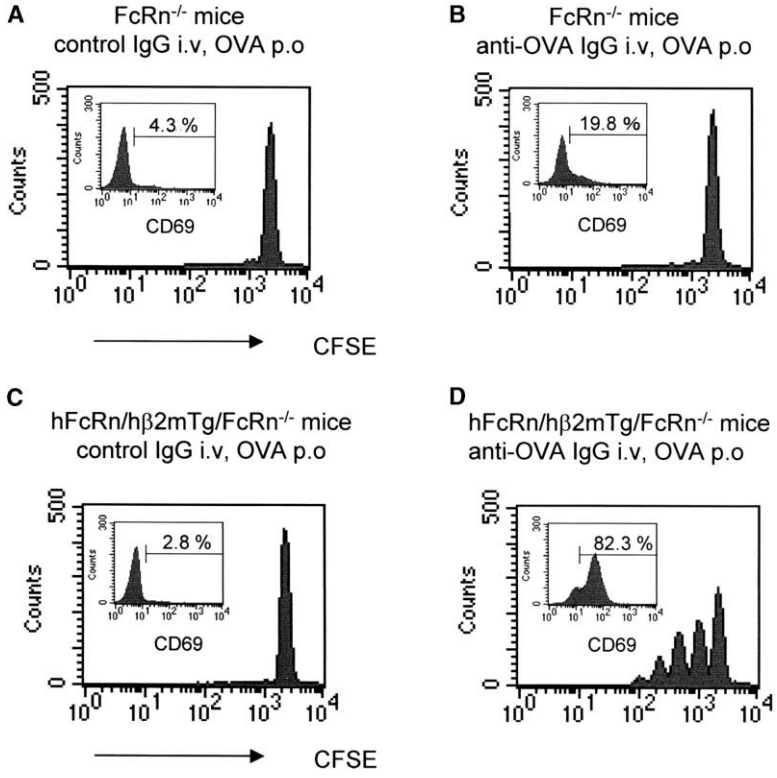

Figure 6. Oral OVA Administration Activates $CD4^+DO11.10^+$ T Cells in the MLN of $hFcRnTg/h\beta2mTg/mFcRn^{-/-}$ that Were Injected with Anti-OVA IgG

$hFcRnTg/h\beta2mTg/mFcRn^{-/-}$ (BALB/c) and $mFcRn^{-/-}$ (BALB/c) mice that were adoptively transferred with CFSE-labeled $CD4^+$ DO11.10 T cells for the detection of cell division or nonlabeled $CD4^+$ DO11.10 T cells for the examination of CD69 expression were injected i.v. with 3 mg of either anti-OVA IgG or control IgG and fed OVA (0.5 mg/mouse). Forty-eight hours later, mononuclear cells from MLN were analyzed for the CFSE intensity and CD69 expression of the $CD4^+$ DO11.10 T cells using flow cytometry. Data are representative of four mice analyzed in each group.

Detection of In Vivo Expansion of $CD4^+$ T Cells in Regional Lymphoid Tissues of Intestine after Antigenic Luminal Challenge by Adoptive Transfer System

We next established a model system that would reflect the native in vivo context. To confirm the relevance of these observations, we sought to determine whether antibody at physiological concentrations in the serum could retrieve antigens from the lumen for the activation of T cells in the presence of hFcRn. To do so, $hFcRnTg/h\beta2mTg/mFcRn^{-/-}$ BALB/c mice which received CFSE-labeled DO11.10 T cells by adoptive transfer were injected i.v. with anti-OVA IgG or control IgG followed by a single OVA feeding (0.5 mg/mouse). The administration of OVA caused an expansion of $CD4^+$ DO11.10 T cells as defined by the detection of multiple cell divisions in CFSE-loaded transgenic T cells and an upregulation of CD69 expression on $CD4^+$ DO11.10 nonlabeled T cells in the MLN (Figure 6D) of the $hFcRnTg/h\beta2mTg/mFcRn^{-/-}$ mice that received anti-OVA IgG intravenously. No significant expansion or upregulation of CD69 expression could be detected in either the MLN of $hFcRnTg/h\beta2mTg/mFcRn^{-/-}$ mice that received control IgG (Figure 6C) or $mFcRn^{-/-}$ mice that received anti-OVA IgG (Figure 6B).

Accumulation of $CD4^+$ T Cells in Regional Lymphoid Tissues of the Airway after Antigenic Luminal Challenge in the Context of Circulating Antigen-Specific IgG

To determine whether the results observed could be extended to other FcRn-bearing mucosal surfaces, we performed the following studies. We have previously demonstrated transport of the Fc domain of IgG across the mucosal surface of normal mouse lung from the lumen to the serum (Spiekermann et al., 2002). Based upon these studies, we examined and detected hFcRn expression in the nasal (Figure 7A) and respiratory (data not shown) epithelium of the $hFcRnTg/h\beta2mTg/mFcRn^{-/-}$ but not $mFcRn^{-/-}$ mice by RT-PCR. To assess whether IgG transported in a basolateral to apical direction could retrieve antigens from the lumen into the submucosa of the respiratory system as an immune complex in vivo, B-OVA was administered by the nasal route to mice that had been injected i.v. with either rabbit anti-OVA IgG or control IgG. Only in the presence of both specific circulating anti-OVA IgG and luminal B-OVA could transport of B-OVA be detected in $hFcRnTg/h\beta2mTg/mFcRn^{-/-}$ but not $mFcRn^{-/-}$ mice as a complex with IgG (Figures 7C and 7D). In addition, administration of OVA (0.5 μg/mouse) through the nasal route led to a significant expansion of CFSE-labeled $CD4^+$ $DO11.10^+$ T cells in the nasal-associated lymphoid tissue (NALT) in $hFcRnTg/h\beta2m/mFcRn^{-/-}$Tg mice injected i.v. with anti-OVA IgG (Figure 7F). No such expansion of CSFE-labeled $DO11.10^+$ T cells was observed in $hFcRnTg/h\beta2mTg/mFcRn^{-/-}$ mice that received control IgG i.v. (Figure 7E) and $mFcRn^{-/-}$ mice injected with anti-OVA IgG (data not shown). These results indicate that the observed transport of immune complexes across an intact epithelial barrier is likely to be physiologic given the ability of hFcRn to mediate the retrieval of free antigen from the lumen of multiple FcRn-bearing mucosal tissues when antigen-specific IgG is present in vivo.

Discussion

The recent recognition that FcRn expression continues within several mucosal epithelial surfaces into adult life

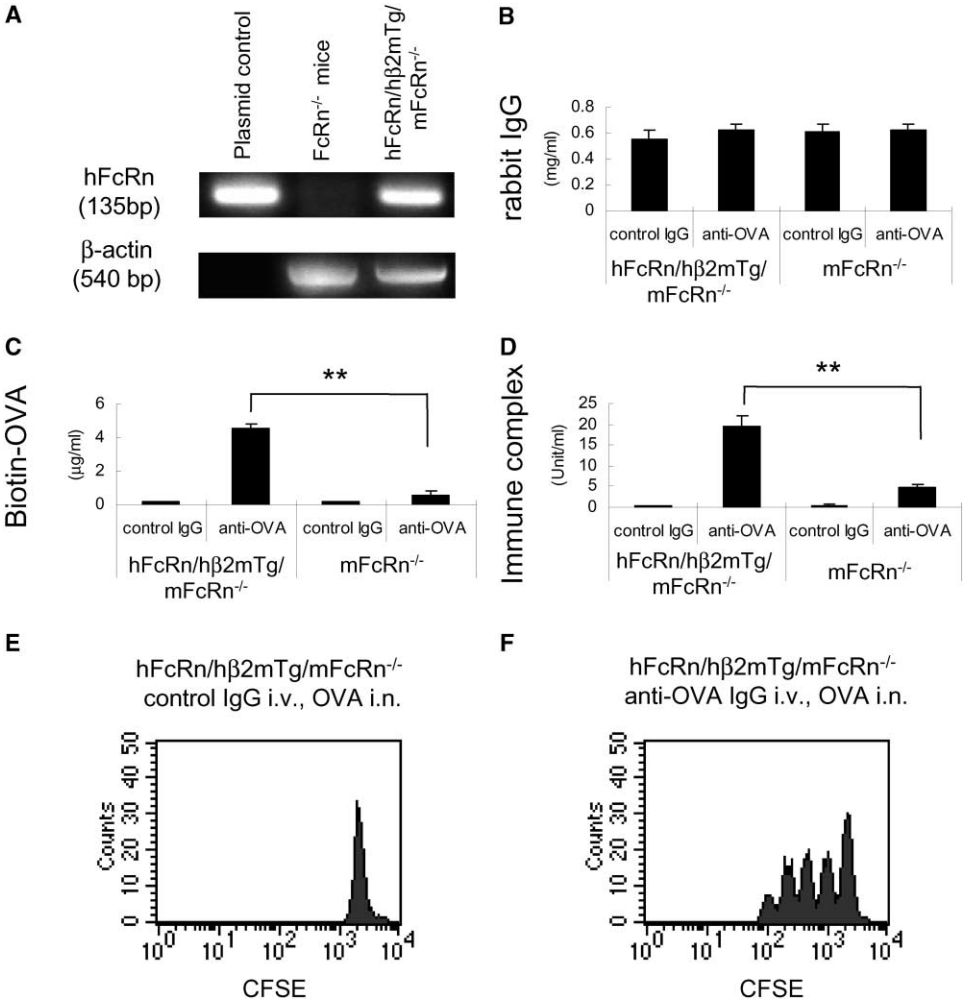

Figure 7. Detection of Physiologic Expansion of OVA-Specific $CD4^+$ T Cells in the Regional Lymphoid Tissues of the Upper Airway

(A) PCR amplification of hFcRn mRNA. Total RNA (20 ng) from nasal tissue of hFcRnTg/hβ2mTg/mFcRn$^{-/-}$ mice and mFcRn$^{-/-}$ mice was amplified by RT-PCR. The specific RT-PCR products representing hFcRn (135 bp) and β-actin (540 bp) are shown.

(B–D) Efficient transcytosis of antigen from nasal route into sera of hFcRnTg/hβ2mTg/mFcRn$^{-/-}$ mice that were injected i.v. with antigen-specific IgG. hFcRnTg/hβ2mTg/mFcRn$^{-/-}$ or mFcRn$^{-/-}$ mice were injected i.v. with anti-OVA IgG (3 mg/mouse) or control IgG and administered B-OVA (1 mg/mouse) through the nasal route 2 hr after the i.v. injection. Three hours later, the sera was collected and subjected to ELISA to measure the concentrations of rabbit IgG (B), B-OVA (C), or B-OVA/IgG immune complex (D). The bars represent the mean ± SD (**$p < 0.01$).

(E and F) Nasal OVA administration activates $CD4^+$ DO11.10 T cells in the NALT of hFcRnTg/hβ2mTg/mFcRn$^{-/-}$ that were injected with anti-OVA IgG. hFcRnTg/hβ2mTg/mFcRn$^{-/-}$ (BALB/c) mice that were adoptively transferred with CFSE-labeled $CD4^+$ DO11.10 T cells were injected i.v. with 3 mg of either anti-OVA IgG (F) or control IgG (F) and administered OVA (0.5 μg/mouse). Forty-eight hours later, mononuclear cells from NALT were analyzed for the CFSE intensity of the $CD4^+$ DO11.10 T cells using flow cytometry. Data are representative of four mice analyzed in each group.

at a time when passive acquisition of IgG-mediated immunity is nonessential and that, based upon in vitro cellular models, FcRn expression in epithelial cells is linked to a cell biologic pathway characterized by the bidirectional transport of IgG (Claypool et al., 2002; Dickinson et al., 1999; McCarthy et al., 2000; Praetor et al., 1999), the notion has been raised that the in vivo function of FcRn in this context is unique. In contrast to the mouse, which significantly downregulates FcRn expression in the intestinal epithelium at the time of weaning, adult humans continue to express significant levels of FcRn in the epithelium (Dickinson et al., 1999; Israel et al., 1997; Zhu et al., 2001). Therefore, in this report, we modeled adult human expression of FcRn in intestinal epithelium by utilizing genetically engineered mice to study the in vivo function of hFcRn in the absence of the mFcRn homolog. In this study, we have utilized mouse/human chimera mice in which hFcRn, driven by its endogenous promoter, and hβ2m are expressed in the absence of endogenous mouse FcRn expression. We demonstrate here that hFcRn can transport not only IgG but also intestinal luminal antigens as a complex with IgG across epithelial barriers into the lamina propria. This conclusion is further supported by our in vitro studies which demonstrate that OVA in a complex with a rabbit IgG specific for OVA but not control IgG is transported in the apical to basolateral direction across MDCK cells transfected with hFcRn and hβ2m but not

control vectors. The fact that IgG can be transported into the lumen from the lamina propria in an FcRn-dependent pathway and that IgG applied to the basolateral surface of a model polarized epithelial cell monolayer and to the serum of hFcRnTg/hβ2mTg/mFcRn$^{-/-}$ mice in vivo can retrieve antigen from the apical luminal milieu for transport back to the basolateral surface as an immune complex suggests that this transcytotic pathway associated with hFcRn is physiologic. The fact that we observed the transport of IgG in the basolateral to apical direction, as revealed by the presence of IgG in intestinal secretions in mice expressing the human transgene, further suggests that hFcRn is responsible, to a significant extent, for the presence of IgG within luminal secretions.

In the current report, we have also utilized a transfected model system utilizing MDCK cells. A detailed characterization of the cell biology of human FcRn as expressed in the hβ2m+ MDCK cells used here has been recently reported (Claypool et al., 2004). The fraction of hFcRn on the plasma membrane (most FcRn resides within the endosomal compartment; Berryman and Rodewald, 1995) is almost exclusively localized to the basolateral plasma membrane in these cells. However, we have observed that hFcRn traffics transiently to the apical plasma membrane (Claypool et al., 2004). Importantly, the steady-state localization of hFcRn in the MDCK model is the same as is detected in two different polarized human intestinal epithelial cell lines, T84 and Caco-2, which express hFcRn endogenously. The membrane distribution of the hFcRn expressed in the transgenic mice is currently unknown. By fluorescence microscopy, the bulk of the signal detected occurs in an endosomal compartment located in the subapical region of the epithelial cell. This is entirely consistent with the observed distribution of hFcRn in human intestinal epithelial cells in vivo and in vitro (Dickinson et al., 1999).

We have previously determined that while the presence of an experimentally established pH gradient is required for apical to basolateral transcytosis in the MDCK clones utilized in these studies, significant basolateral to apical transport of IgG relative to mock-transfected MDCK cells can be detected in the absence of a pH gradient, albeit to a lesser extent than when a pH gradient is present (Claypool et al., 2004). As such, we buffered the apical chamber to pH 6.0 to enhance FcRn-mediated transcytosis as previously described by us and others (Claypool et al., 2002, 2004; McCarthy et al., 2000; Wu and Simister, 2001). We believe that this is reasonable model for FcRn function for the following reasons. The pH in the duodenum to jejunum of neonatal rats is 6–6.5 (Rodewald, 1976). Therefore, maternal IgG in milk can bind FcRn on the luminal surface of epithelial cells in the neonatal rat model. However, IgG may also bind its receptor following uptake in the acidic endosomal system (Benlounes et al., 1995). This is the probable route of IgG entry in cells bathed in a neutral environment, including the fetal yolk sac of rodents (Roberts et al., 1990) and the human placental syncytiotrophoblast (Leach et al., 1996). In fact, monensin which breaks down Na^+ and H^+ gradients in endosomal and lysosomal compartments, raising the pH of endocytic vesicles from 5.5 to greater than 7, almost completely inhibited transcytosis of Fc across FcRn transfected rat inner medullary collecting duct cells (McCarthy et al., 2000). In addition, our group has reported that bafilomycin A, which similarly neutralizes the acididity of the endosomal compartment, abrogates transport of IgG across the polarized human intestinal cell line, T84 (Dickinson et al., 1999). Furthermore, it is likely that the pH of the total fluid within the lumen of the intestine is not the important driving force for transcytosis of IgG and IgG/antigen complexes. Although fluid phase endocytosis and binding in acidic endosomes may be expected to be the primary mode of ligand binding through the basolateral surface, the presence of an acidic microenvironment immediately adjacent to the apical plasma membrane exists at all mucosal surfaces that express the sodium-hydrogen exchanger NHE3 (Tse et al., 1993). In the intestine, the acidic microenvironment established by NHE3 at the brush border is used to drive both peptide and $Fe2^+$ absorption via proton-coupled transporters and is predicted to drive IgG binding to hFcRn for basolaterally directed transcytosis from the apical cell surface as has been shown for transport of peptides and $Fe2^+$ as noted. As such, we suggest that hFcRn is involved in the entire process of IgG transport into the lumen, antigen/IgG complex uptake from the lumen, and transcytosis from the lumen to basolateral surface.

To show that the function of FcRn is physiologic in vivo in our system, we injected rabbit IgG to achieve a total concentration of 0.5–0.6 mg/ml. Since the rabbit IgG utilized in the experiments described in the current report was polyclonal, we can further predict that approximately 10%–20% of the antibody is specific for OVA, indicating that the specific antibody levels in the experimental model used may be estimated to be in the range of 0.05–0.12 mg/ml. Since the serum levels of total endogenous IgG in normal 8- to 12-week-old mice is 3.0–8.0 mg/ml (Telleman and Junghans, 2000) and the total endogenous IgG levels in mFcRn$^{-/-}$ and hFcRnTg/hβ2mTg/mFcRn$^{-/-}$ mice are lower than wild-type mice, varying between 0.3 to 0.8 mg/ml due to the loss of FcRn-mediated protection of mouse IgG from catabolism (Roopenian et al., 2003), we can estimate that the specific rabbit anti-OVA IgG levels examined represent 1.25%–3.3% of the circulating antibody pool in normal mice and 12.5%–33.3% in mFcRn$^{-/-}$ and hFcTnTg/hβ2mTg/mFcRn$^{-/-}$ mice. This should be contrasted with the serum levels of total IgG in a hyperimmunized state, a situation in which the concentration of antibody are at least 50–100 times greater than the levels examined here (Christianson et al., 1997). As a corollary, the dose of antigen administrated in the GALT experiments (0.5 mg/mouse) and in the NALT experiments (0.5 μg/mouse) would be equivalent to 1.5 g and 1.5 mg of protein to a 60 kg human, respectively. Therefore, the quantities of circulating IgG and mucosally administered antigen that we investigated can be considered to be within the physiologic range, making it likely that FcRn-mediated uptake of antigens is a normally operative mechanism of mucosal antigen handling.

Perhaps most importantly, the FcRn-mediated transport of antigen/IgG complexes from the intestinal lumen is associated with antigen capture by antigen-presenting cells within the intestinal lamina propria. M cells, a specialized epithelial cell type which is present in the follicle-associated epithelium, have been previously considered as the major "professional" antigen sam-

pling cell type that is capable of delivering luminal antigens into the lamina propria (Iwasaki and Kelsall, 1999). In addition, intestinal DC have been demonstrated to directly capture antigens present in the intestinal lumen by opening the tight junctions between epithelial cells and sending dendrites into the lumen for antigen capture and retrieval (Hayday and Viney, 2000; Rescigno et al., 2001). Our present findings demonstrate an additional pathway for intestinal antigen sampling that is likely mediated by all FcRn-expressing epithelial cells in adult life given our ability to detect FcRn-mediated transport of immune complexes in both the GALT and the NALT. As a striking difference from other sampling pathways, FcRn delivers intestinal antigens as a complex with IgG. Moreover, as shown here, lamina propria cells efficiently capture these IgG/antigen complexes consistent with recent studies, which have demonstrated that immune complexes can be captured by DC (Mellman and Steinman, 2001).

After capturing antigens in the intestinal lamina propria, DC have been demonstrated to rapidly migrate into other tissues, such as MLN (Huang et al., 2000). Indeed, our present studies show that the lamina propria cells that captured OVA as a complex with specific IgG move into the MLN as well as the liver within 4 hr of antigen instillation in the intestinal lumen. In addition, our observation that a FITC signal, indicative of the incorporation of OVA/IgG complexes, can be detected in a significant proportion of $CD11c^+$ cells in these tissues suggests that the process described here is a major means by which antigen may be captured by a host that has previously raised an IgG immune response to a foreign antigen. Furthermore, the $CD11c^+$ cells in MLN that have captured OVA as a complex with IgG efficiently present the antigen to OVA-specific $CD4^+$ T cells. Recent studies have suggested that the manner in which antigen is taken up by a DC confers distinct functions on the DC (Mellman and Steinman, 2001). Interestingly, FcRγIIB-mediated capture of immune complexes are involved in the generation of dendritic cell-mediated immune tolerance (Kalergis and Ravetch, 2002). In addition, immunoglobulins have been proposed to suppress intestinal inflammation by facilitating phagocytosis of pathogenic antigens (Mizoguchi et al., 1997a) and IgG in the lumen of lung has been suggested to reduce allergen-induced asthma (Sehra et al., 2003). In our study, significant IL-10 production by $CD4^+$ T cells was observed when stimulated by antigen-presenting cells isolated from the MLN and liver that had received the OVA antigen after transcytosis via hFcRn. Therefore, it is possible that the sampling of intestinal luminal antigens by an FcRn-mediated process contributes to unique functions of mucosal effector and/or regulatory pathways involved in the maintenance of mucosal tolerance characteristic of this compartment or the development of intestinal inflammation as might occur if the transported antigen has adjuvant qualities. Further studies will be needed to clarify these important issues.

In this study, we provide an insight into the role of hFcRn in the transport of luminal antigens as a complex with antigen-specific IgG by absorptive intestinal epithelial cells and epithelial cells of the upper airway that express hFcRn. Antigen capture stimulates the migration of $CD11c^+$ dendritic cells to regional organized lymphoid tissues thus allowing for interactions with $CD4^+$ T cells. Such a mechanism defines a major pathway of antigen entry into mucosal tissues.

Experimental Procedures

Cells and In Vitro Transcytosis Assays

The MDCK cells transfected with full length HA-tagged hFcRn and hβ2m (hFcRn/β2m-MDCK) and mock-MDCK have been established, and transcytosis assays were carried out as previously described (Claypool et al., 2002).

Determination of IgGs and Antigen/IgG Complexes by ELISA and Immunoblot

Mouse anti-hIgG (Zymed) and mouse anti-hIgG-HRP (Zymed) for the hIgG, mouse anti-rabbit IgG (Sigma) and goat anti-rabbit IgG-HRP (Piece) for the rabbit IgG, OVA (Sigma) and donkey anti-rabbit IgG-HRP for rabbit anti-OVA IgG, avidin (Piece), rabbit anti-OVA IgG and donkey anti-rabbit IgG-HRP for the B-OVA, and avidin and donkey anti-rabbit IgG-HRP for B-OVA/anti-OVA IgG complex were used, respectively. For the detection of B-OVA/anti-OVA IgG complex by immunoblot, mouse anti-OVA IgG (clone OVA-14, Sigma) followed by goat anti-mouse IgG1-HRP was used.

IgG Binding Assay

IgG binding assays were performed as previously described (Claypool et al., 2002). Human IgG, rabbit control IgG, rabbit anti-OVA IgG, mouse IgG1, erythropoietin conjugated hIgG-Fc(EPO-hIgG-Fc), or EPO-hIgG-Fc/IHH mutant containing three point mutations that abrogate Fc binding to FcRn (Spiekermann et al., 2002) were utilized.

Immunocytochemistry and Confocal Microcsopy

Immunocytochemistry of MDCK cells grown on Transwell was performed as described (Claypool et al., 2004). Goat IgG was detected with rabbit anti-goat IgG-HRP, followed by Tyramide Signal Amplification $Alexa^{488}$ kit (Molecular Probe), and ZO-1 was revealed with mouse anti-ZO-1 (Zymed), followed by goat anti-mouse IgG-$Alexa^{568}$ (Molecular Probe). The images were gathered using a MRC1024 laser scanning confocal system (Bio-Rad Laboratories).

Preparation of Cell Suspensions from Spleen, MLN, and Liver

T cells from spleen, MLN, and NALT were prepared as previously described (Fukuyama et al., 2002). Dendritic cells were prepared from MLN and liver using a digestion buffer as described previously with a modification (Watanabe et al., 2002). In short, MLN and liver were digested with collagenase (1 mg/ml) and DNase I (15 μg/ml) for 30 min at 37°C and then passed through a cell strainer. Sample was centrifuged and the pellet resuspended in 4 ml 40% Percoll in PBS, underlaid with 2 ml of 60% Percoll in PBS, and centrifuged at 2200 rpm for 20 min. Cells were carefully collected from the interface, washed with PBS, and utilized.

Antigen-Specific Stimulation by Antigen/IgG Complex Transported across MDCK Cells

Naive splenic $CD4^+$ T cells (1×10^6/ml) purified from DO11.10 mice by a purification column (R & D Systems) and mitomycin C treated BALB/c splenocytes ($1x10^7$/ml) as antigen-presenting cells (APCs) were cultured with different doses of either OVA, OVA/control IgG, or OVA/anti-OVA IgG (the ratio of OVA:IgG is 1:3 weight/volume). For cytokine assays, the same quantity of B-OVA from the basolateral reservoir was used, and cells were cultured for 24 hr (IL-2) or for 60 hr (IFN-γ and IL-10). Detection levels of the cytokine ELISAs are 16.5 pg/ml (IFN-γ) and 7.8 pg/ml (IL-10).

Multicolor Flow Cytometric Analysis

PE-labeled anti-CD11c (clone HL3), FITC-labeled CD69 (clone H1.2F3), PE-labeled anti-mouse DO11.10 T cell receptor (clone KJ1-26; CALTAG Laboratories), and PerCP-Cy5.5-labeled CD4 (clone RM4-5) were used in this study. For the DC staining, Via-Probe (7-amino-actinomycin D; 7-AAD; PharMingen) was added to gate out the dead cells. The cells were analyzed using a flow cytometer (FACSort, Becton-Dickinson).

Mice

FcRn-deficient mice (mFcRn$^{-/-}$) and the hFcRn transgenic (Tg) mice under the control of the human endogenous promoter were established previously (Roopenian et al., 2003). The hFcRnTg mice were crossed to hβ2mTg mice (Krimpenfort et al., 1987) and further crossed with a FcRn$^{-/-}$ mouse of both C57BL/6 and BALB/c backgrounds (hFcRnTg/β2mTg/mFcRn$^{-/-}$). For the OVA studies, DO11.10 mice expressing a transgenic T cell receptor that recognized a peptide fragment of OVA in the context of IA^{d} (Murphy et al., 1990) were used. All mice were housed under specific pathogen-free conditions at the Animal Facilities of Harvard Medical School.

Detection of the hFcRn by Immunoblot and Immunohistochemistry

The expression of hFcRn and hβ2m was assessed by immunoblot analysis using a rabbit antibody specific for the hFcRn-CT (Claypool et al., 2002) and a goat antibody specific for hβ2m (Santa Cruz), respectively. Normal adult human small intestine was obtained from patients undergoing gastric bypass surgery, and epithelial cells were nonenzymatically isolated as described (Blumberg et al., 1991) under a protocol approved by the Human Studies Section of the Brigham and Women's Hospital. The expression of hFcRn was assessed by immunohistochemistry as previously described (Mizoguchi et al., 1997b). The frozen sections were stained using rabbit anti-FcRn antibodies, which were kindly provided by Dr. Neil E. Simister (Brandeis University).

Detection of Secretory Human IgG-Fc, Rabbit IgG, and Rabbit Anti-OVA IgG

Human IgG, EPO-hIgG-Fc, or EPO-hIgG-Fc/IHH mutant were injected intraperitoneally into mice, feces were collected at the indicated time points, and were suspended with PBS, homogenized, and centrifuged. Each supernatant was analyzed by ELISA using goat anti-human Fc (ICN) and goat anti-human Fc-HRP (Piece). The determined quantity of hIgG-Fc was further normalized to the protein concentration of each sample as determined by BCA protein assay (Piece). To determine whether antigen/IgG complex was formed in the intestinal lumen, either control IgG or anti-OVA IgG was injected i.v. into mice, and B-OVA was administered through the oral route 6 hr after the i.v. injection. The small intestinal fluid was collected, and the concentrations of rabbit IgG and an immune complex with OVA were determined by ELISA.

Preparation of FITC-Conjugated OVA and In Vivo Transcytosis of Antigen/IgG Complex

For the detection of the transported antigen and IgG in vivo, heat-denatured OVA was used to increase the signal of FITC. In short, OVA (50 mg/ml in PBS) was boiled for 5 min at 100°C, i.e., mechanically shattered. The heat-denatured OVA preserves antigenicity for DO11.10 CD4^{+} T cells when administered into skin and mucosal sites of BALB/c mice reconstituted with DO11.10 CD4^{+} T cells (Yamori et al., 2004). The heat-denatured OVA was incubated with 1 mg/ml of FITC solution in 0.05 M carbonate-bicarbonate buffer at pH 9.6 for 1 hr. After washing with PBS at pH 6.0, FITC-OVA (20 mg) was preincubated with 5 mg of either anti-OVA IgG or control IgG for 30 min. Samples including OVA (20 mg/mouse) and IgG (5 mg/mouse) were administered intragastrically into mice and the mid-small intestine collected 1 and 2 hr after the inoculation. After cutting into 6 μm sections, FITC signals were examined using a Nikon Eclipse E600 microscope (Nikon). For the detection of antigen-bearing cells and ex vivo stimulation of DO11.10 T cells, dendritic cells were prepared from MLN and liver 4 hr after oral administration and the cells analyzed using flow cytometry or purified CD11c^{+} cells by MACS beads were cultured with naive OVA-specific CD4^{+} T cells. The culture supernatants was used for the measurement of cytokines.

Reverse Transcriptase-PCR for hFcRn

Total RNA from nasal epithelial cells was prepared using Trizol™ reagent (Life Technologies). Amplification for hFcRn was carried out with OneStep RT-PCR kit (Qiagen) by using specific primer sets for hFcRn and β-actin as described previously (Zhu et al., 2001).

In Vivo Transport Experiment

To assess the physiological transport of antigen as an immune complex from the lumen into the serum by FcRn in vivo, mice were injected i.v. with either rabbit anti-OVA IgG or control IgG and administered B-OVA per the nasal route 2 hr after i.v. injection. After 3 hr, the sera was collected and subjected to ELISA.

Adoptive Transfer and CFSE Labeling of CD4^{+} DO11.10 T Cells and Antigenic Challenge

As described previously (Yoshida et al., 2002; D'Souza and Lefrancois, 2003), 8- to 12-week-old hFcRnTg/hβ2mTg/mFcRn$^{-/-}$ (BALB/c) or FcRn$^{-/-}$ (BALB/c) mice were reconstituted with 1×10^{7} CFSE-labeled T cells from DO.11.10/Rag1$^{-/-}$ mice. The recipient mice were injected i.v. with either anti-OVA IgG or control IgG 24 hr after transfer, followed by oral administration of 0.5 mg OVA 24 hr after IgG injection. In NALT experiments, 0.5 μg of OVA were administered intranasally into the recipient mice as described above. The mononuclear cells were prepared from MLN or NALT 2 days after the oral feeding.

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