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Human dendritic cells accumulate in lung disease and contribute to serum IgE clearance via FcERI

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Human dendritic cells accumulate in lung disease and contribute to serum IgE clearance via FcERI

by

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DISSERTATION

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CONTRIBUTIONS OF CO-AUTHORS TO THE PRESENTED WORK

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Human dendritic cells accumulate in lung disease and contribute to serum IgE turnover via FcεRI

Alexandra Greer

ABSTRACT

Dendritic cells are crucial for the development of the adaptive immune response and have also been implicated in a number of inflammatory diseases. Much of what we know about dendritic cell function is limited to mouse models of disease, as human DCs remain difficult to identify consistently and samples can be difficult to acquire. We undertook a study to identify the prevalence of BDCA1$^+$ DCs in a panel of healthy and diseased lung donors by carefully and comprehensively identifying DCs in parenchymal lung tissue. We found a dramatic increase in the prevalence of BDCA1$^+$ DCs in the fibrotic lung diseases idiopathic pulmonary fibrosis (IPF) and chronic hypersensitivity pneumonitis (HP) by flow cytometry. We also saw a significant increase in BDCA1$^+$ DCs in Th2-high asthma by using the high-affinity IgE receptor, FcεRI and MHCII as identification markers of DCs in biopsy sections. These increased numbers of BDCA1$^+$ DCs could potentially contribute to disease and may represent a novel therapeutic target. Because of the potential role of FcεRI in promoting Th2-mediated allergy via DCs, we also investigated regulation of this receptor on human DCs. Surprisingly, we found that human DCs and monocytes constitutively endocytose FcεRI and IgE in the absence of crosslinking stimulus. Transgenic mice expressing human FcεRIα showed similar FcεRI regulation, which resulted in rapid human IgE endocytosis in blood DCs and monocytes in vivo. Serum IgE catabolism was accelerated in FcεRI-transgenic mice and was significantly influenced by DC and monocyte numbers. DCs and monocytes may therefore contribute to serum IgE control

through constitutive IgE endocytosis and could represent a novel therapeutic strategy for allergic conditions. Further work on the trafficking mechanism of FcεRI regulation may provide a significant novel strategy for control of IgE-mediated allergies.

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CHAPTER I: INTRODUCTION

Part A: Dendritic cells as orchestrators of the immune response

Dendritic cell development and homeostasis

Dendritic cells are part of the myeloid compartment of the immune system and are generated both in the bone marrow and in tissues. Because of their ease of study and the availability of various tissues for testing, most of what is known about DC differentiation has been generated by studying their development in the mouse. DCs, monocytes and macrophages are generated from the macrophage and DC precursor (MDP), which gives rise to the DC-specific common DC precursor (CDP) (Liu et al., 2009) and development into DCs is dependent on cytokines such as GM-CSF and Flt3L. In vitro cultures of whole bone marrow isolates from mouse with either GM-CSF or Flt3L are enriched for DCs, though with slightly different phenotypes (Xu et al., 2007): GM-CSF cultures generate a fairly pure population of CD11b+ DCs, while Ftl3L cultures generate a mixed population of conventional and plasmacytoid DCs (Waskow et al., 2008). In mouse, conventional DCs are divided into two major phenotypes: the typically inflammatory $CD11b^{+}CD8^{-}CD103^{-}$ DCs, and $CD11b^{-}CD8^{+}CD103^{+}$ DCs which are specialized to cross-presentation of antigens to $CD8^{+}$ T-cells (del Rio et al., 2010). After development in the bone marrow, DCs enter the blood, where they circulate before entering peripheral tissues. Upon entering the tissues, MHCII levels may increase slightly and the DCs are now considered fully differentiated, immature conventional DCs.

DCs can also be generated through intermediate cell types, such as monocytes. There is considerable evidence that in mouse, monocytes can differentiate into mucosal $CD11b^{+}$ DCs in

the steady state (Landsman et al., 2007; León et al., 2004; Varol et al., 2007; 2009) in addition to inflammatory conditions (Osterholzer et al., 2009; Strauss-Ayali et al., 2007; van Rijt et al., 2002). This process can occur both in the bone marrow and in peripheral tissues, where large numbers of monocytes traffic to during times of inflammation (Landsman et al., 2007; León et al., 2007).

Some DC subtypes, including Langerhans cells, are derived from local progenitors (Chorro et al., 2009). These cells are radio-resistant and are not replaced by donor cells in steady-state bone marrow transfer (Merad et al., 2002). Interestingly, however, monocytes can also contribute to some Langerhans cell populations in the context of inflammation or local depletion of skin Langerhans cells by UV-irradiation (Bennett et al., 2005; Merad et al., 2002). Overall, DC development is a complex process involving multiple possible pathways to DC generation that play complementary roles during health and disease.

Once in the tissues, DCs locally migrate within the tissue until moving to a lymph node whether or not they have encountered an activating stimulus (Wilson et al., 2008), where they can activate T-cells.

Dendritic cell function: T-cell activation

As professional antigen presenting cells, the main function of DCs is to activate T-cells against foreign protein antigens as a way to protect the host against a wide array of pathogens. As such, DCs are specialized in a number of ways to facilitate this process.

First, DCs are highly mobile and are localized in essentially every peripheral tissue in order to maximize their potential to encounter foreign antigens in the body. Localization in a variety of peripheral tissues is achieved through expression of different adhesion molecules and

chemokine receptors, which allow the cells to home and adhere to different tissues in the body. In fact, different subsets of DCs express unique repertoires of adhesion molecules that allow them to diversify their localization (Banchereau et al., 2000); furthermore, inflammation can induce DCs to express additional adhesion molecules and chemokine receptors that allow them to home to the specific site of inflammation. The process of DC migration can be very rapid – as quickly as one hr following antigen deposition (McWilliam et al., 1996), which allows these cells to quickly and efficiently respond to potential danger to the organism.

DCs are also highly adept at internalizing antigens through a variety of mechanisms. First, DCs are by nature macropinocytic and are capable of taking up small soluble and particulate antigens in the absence of specific recognition or inflammation (Thornton et al., 2012). In addition, DCs express a panoply of endocytic receptors meant to facilitate antigen uptake. C-type lectins are one common type of endocytic receptor present on DCs: DEC-205 is a C-type lectin that facilitates antigen uptake up to 100-fold over macropinocytosis (Mahnke et al., 2000). In fact, antigens targeted to DEC205 via anti-DEC205 antibody-fusion proteins can efficiently induce either T-cell tolerance or activity depending on the context in which the antigen is delivered (Ring et al., 2013). DCs also express a number of scavenger receptors that help take up cell debris, and Fc receptors allow for focused uptake of antigens bound to immunoglobulin molecules, which facilitates endocytosis of a wide range of immunogenic antigens.

Many of the endocytic receptors expressed by DCs are capable of transmitting an activating or maturing stimulus upon receptor ligation or crosslinking. Furthermore, DCs also express a number of pathogen-associated molecular pattern (PAMP) and danger-associated molecular pattern (DAMP) receptors, including TLRs, cytokine receptors, and other molecules

that help the DC detect the presence of danger and cause maturation of the cell (Guermonprez et al., 2002). Broadly, DC maturation results in three major changes in the DC phenotype: an increase in DC cytokine production and release, the expression of costimulatory molecules, and heightened expression of MHCII (Shin et al., 2006a). Concomitantly, DC maturation results in downregulation of macropinocytosis and many endocytic receptors (Sallusto and Lanzavecchia, 1994; Sallusto et al., 1995). Therefore, DC maturation results in facilitated presentation of antigens seen at the time of maturation and prevents the presentation of irrelevant antigens encountered after the activating stimulus. In addition to changes in the DC's phenotype, DCs also migrate to lymph nodes upon maturation due to increased expression of the chemokine receptor CCR7, which allows them to travel to lymph nodes via CCL19/CCL21 gradients present in the lymphatics (Randolph et al., 2005). Once in the lymph node, mature DCs provide three signals to the naïve T-cells inside: antigen recognition via an specific MHC-peptide: TCR interaction, binding of costimulatory molecules present on mature DCs, and signaling via cytokines (Figure 1). Without these three signals, T-cells are not adequately activated and may become anergic or deleted.

Dendritic cell function: T-cell skew

$CD4^+$ T-cells show a variety of specializations, called 'T-helper skew', that help generate uniquely directed immune responses against general types of pathogens. DCs are capable of influencing these types of T-cell responses both in the lymph node and in the periphery through direct interactions with naïve T-cells and the release of cytokines (Amsen et al., 2004; Guermonprez et al., 2002). In some cases, DCs require the assistance of additional cells such as basophils (Tang et al., 2010) in order to adequately skew T-cells, but there are many examples of

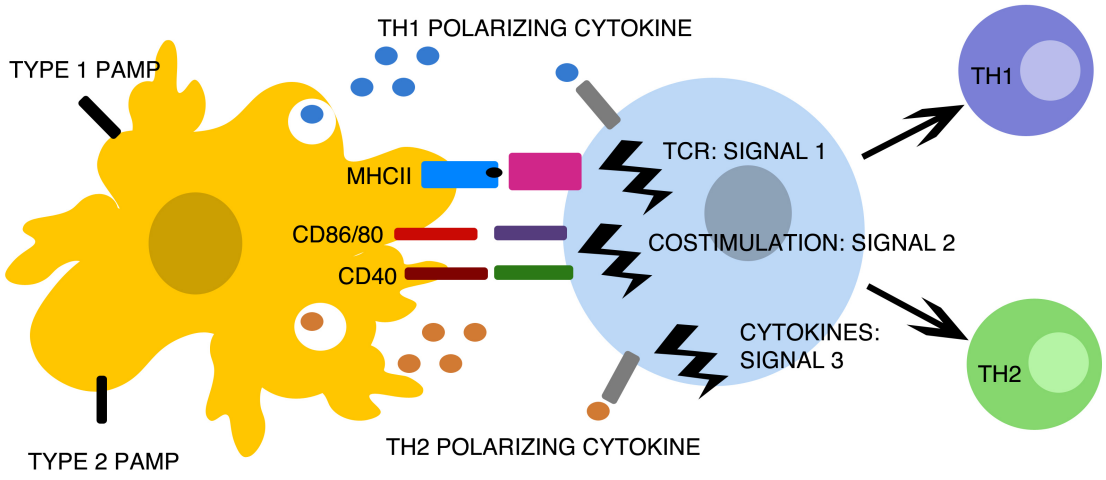

Figure 1: DCs activating and skewing T-cells (Figure adapted from Kapsenberg, 2003). DCs (left, in yellow) activated by PAMPs and DAMPs mature by upregulating MHCII, costimulatory molecules, and cytokine production, as well as traveling to local lymph nodes in order to activate cognate T-cells. Type I PAMPS (top) result in the release of cytokines and expression of costimulatory molecules that promote Th1 development, whereas Type 2 PAMPS (bottom) promote the development of Th2. Naïve T-cells (right, in blue) specific for presented antigens become activated through the transmission of three necessary signals: MHCII-TCR stimulation, costimulatory molecule binding, and cytokine binding. Depending on the specific types of costimulation and cytokines present, T-cells may become different T-helper phenotypes, of which Th1 (purple) and Th2 (green) are two examples.

DCs being sufficient to skew T-cell responses. Typically, the combination of specific expression of certain costimulatory molecules by the DC along with the secretion of specific cytokines induced upon DC activation via PAMPs and/or DAMPs contributes to differential T-cell skew (Figure 1).

For example, dsRNA, LPS and imiquimod activate DCs through TLR3, TLR4 and TLR7. This mode of activation causes significant IL-12 secretion by mature DCs and thus leads to a robust Th1-mediated response to gram-negative bacterial and viral pathogens (Kapsenberg, 2003). Exposure to TSLP released by damaged epithelial cells causes DCs to upregulate expression of the costimulatory molecule OX40L, which is sufficient to promote Th2 skew of T-cells in coculture studies (Ito et al., 2005). In addition, some reports indicate that DCs produce IL-13 in response to protein allergen exposure, thereby contributing to Th2 skew (Bellinghausen et al., 2003). In multiple mouse models of Th2-mediated allergic asthma, DCs were necessary for the induction of Th2 immunity (Hammad et al., 2010; Lambrecht et al., 2000; Plantinga et al., 2013). However, some groups have also found a necessary role for basophil secretion of IL-4 in Th2 induction following allergen sensitization (Tang et al., 2010), so it remains unclear whether DCs are both necessary and sufficient for both T-cell activation and subsequent Th2 skew.

Th17 describes a subset of T-helper cells that are highly inflammatory (Wilson et al., 2007) and are often associated with inflammatory autoimmune disorders (Hu et al., 2011). DCs incubated with schistosome eggs or activated via Dectin-1 promote Th17 through the release of IL-23, IL-6, and IL-1 (Shainheit et al., 2008) (LeibundGut-Landmann et al., 2007). Th17 can also be induced through interactions between ICOS-L on human DCs and ICOS on T-cells (Paulos et al., 2010).

Finally, Tregs can be induced by DCs through release of IL-27 and IL-10 (Ilarregui et al., 2009). It is unclear under what conditions DCs release these cytokines and some groups have hypothesized that a lack of DC maturations induces Treg development upon DC:T-cell interaction (Guermonprez et al., 2002). In addition to the induction of Tregs, DCs can also promote tolerance to antigens through deletion of self-reactive T-cells. By presenting antigen without the required secondary signals (such as costimulation and cytokine release), T-cells are inadequately activated and can become anergic or deleted.

Because of their potent ability to both activate and skew T-helper responses, DCs have been examined as potential therapeutics for a number of diseases. By influencing DC maturation, it may be possible to thereby influence downstream T-cell activation and the direction of the immune response. Many efforts have focused on targeting antigens to DCs in ways that generate regulatory T-cell responses as a way to tolerize the immune system against self- or harmless exogenous antigens. DEC-205, for example, has been examined extensively as a way to generate tolerogenic T-cell responses to antigen; by generating fusion proteins of anti-DEC205 antibodies with the peptide or protein of interest, antigens are efficiently targeted to $DEC205^{+}$ DCs and presented to T-cells in the absence of activatory stimulus (Mahnke et al., 2000). Delivery of antigen by this method has been shown to prevent and even treat experimental autoimmune encephalitis (EAE) in mice (Ring et al., 2013). On the other hand, by giving potent TLR stimuli to DCs with specific antigens, one can generate inflammatory T-cell responses that can overcome deficits in immune function. For example, there are a number of experimental cancer therapies that isolate monocytes from a patient, provide GM-CSF and IL-4 to cause them to differentiate into immature DCs, and provide a tumor antigen with inflammatory cytokines to induce maturation. Then, the now-mature DCs are injected back into the patient, where they

home to a local lymph node and activate inflammatory T-cells against the tumor antigen (reviewed in Gilboa, 2007).

Dendritic cell function: influencing the tissue environment

In addition to their crucial role in activating and skewing T-cells to initiate adaptive immune responses, DCs can also influence the immune response by releasing chemokines and cytokines in peripheral tissues and influencing the immune response of the periphery. DCs can release chemokines that attract immune cells to the periphery in both health and inflammation (Beaty et al., 2007; Lieberam and Förster, 1999; McIlroy et al., 2006). Furthermore, DCs are instrumental in initiating and perpetuating secondary lymphoid organs (GeurtsvanKessel et al., 2009; Halle et al., 2009), which significantly contribute to long-term inflammation in tissues. DCs also release cytokines that can locally activate other immune cells (reviewed in (Banchereau et al., 2000)) and that can act directly on the local tissue environment to promote or prevent inflammation (Gao et al., 2011).

Human dendritic cell development, subsets, and functions

The study of human DCs is a more nascent field and there is considerably less known about their development and function in vivo due to limitations on sample availability and experimental procedures on human subjects. Like in mouse, human DCs are indispensable for the generation of adaptive immunity against harmful pathogens; however, they have also been implicated in the development of potentially harmful autoimmunity. Unfortunately, though, inherent limitations have restricted our knowledge to observational studies of human DC prevalence and phenotype in disease conditions, in vitro tests of DC function, and mouse

modeling of DC involvement in human diseases. Even as such, the limited consensus on human DC identification strategies has left much yet unknown about the role of human DCs in disease.

Overall, DC development in humans is similar to their development in mouse: myeloid DCs arise from a $CD34^+$ stem cell progenitor along with macrophages, monocytes, and plasmacytoid DCs, then move into the circulation (Banchereau et al., 2000). After circulating in the blood, they move into peripheral tissues as immature DCs until they mature and migrate to a lymph node (LN) to activate cognate T-cells. During times of inflammation, human monocytes can also give rise to conventional DCs. TipDCs (TNF/iNOS-producing DCs) and infDCs (inflammatory DCs) are mouse and human subtypes of DCs that are thought to arise only in times of inflammation and come from a monocytic precursor (Chong et al., 2011; Lowes et al., 2005; Serbina et al., 2003).

There are multiple DC subtypes present in human tissues: $BDCA1^+$ ($CD1c^+$) conventional DCs, $BDCA3^+$ ($CD141^+$) conventional DCs, and $BDCA2^+$ or $CD123^+$ plasmacytoid DCs have been identified from blood (Dzionek et al., 2000), lung (Demedts et al., 2005; Masten et al., 2006), and LN (Segura et al., 2012), and correlate well in both function and gene expression patterns with the $CD11b^+$, $CD103^+$, and plasmacytoid DC populations also found in mouse (Robbins et al., 2008). $BDCA3^+$ DCs have been identified in human bronchiolar lavage fluid (BALF) (Kayserova et al., 2012) and are much rarer than $BDCA1^+$ pulmonary DCs; they are thought to specialize in cross-presentation (Jongbloed et al., 2010). $BDCA1^+$ conventional DCs also contain a subset of $Langerin^+$ ($CD207^+$), $CD1a^+$ DCs (Shortman and Liu, 2002), though not all $Langerin^+$ DCs are considered "Langerhans cells", depending on the presence of the defining Birbec granules (Van Pottelberge et al., 2010). Some $BDCA1^+$ DCs are also $CD14^+$ and all are $CD11c^+$, though these alone are not sufficient markers of classical DCs

because of their expression on monocytes (Masten et al., 2006) and macrophages (Demedts et al., 2005). Due to the plethora of partially specific markers of these cells, much of the literature is confusing at best in its discussion of conventional DC function (Figure 2) and there remains much room for improvement (Van Pottelberge et al., 2010). In fact, recent acknowledgement of the difficulty in conclusively identifying human DCs has led to new attempts to identify better markers of these cell types. For example, Segura et al. (2013) recently identified FcεRI as a potential marker for BDCA1$^+$ human DCs. Chapter II describes work in which we expanded on this idea by carefully and comprehensively identifying human lung BDCA1$^+$ DCs with a variety of markers, including FcεRI, to quantify their presence in a variety of lung diseases.

Human DCs are implicated in the development of T-cell and some B-cell mediated autoimmune diseases such as Type I diabetes, systemic lupus erythematosus (SLE), multiple sclerosis (MS), and arthritis. In each, increased human DC numbers in the affected organs are thought to contribute to disease progression (Blanco et al., 2008; Lebre et al., 2008). For example, increased numbers of conventional human DCs have been observed in the pancreas, but not peripheral blood, of Type I diabetics compared to healthy controls (Summers et al., 2003), implicating them in the development of specific T-cell responses against pancreatic tissue. Increased DC numbers have also been observed in the CSF of patients with neuroinflammatory disorders (Pashenkov et al., 2001), though in both cases, a lack of consensus regarding proper human DC markers has led to some confusion regarding the true prevalence of DCs in these diseases.

Human DCs can contribute to disease by aberrantly skewing recently activated T-cells towards a pro-inflammatory T-helper phenotype. For example, DCs can contribute to the exacerbation of leprosy through improper skew of activated T-cells towards a Th2 phenotype

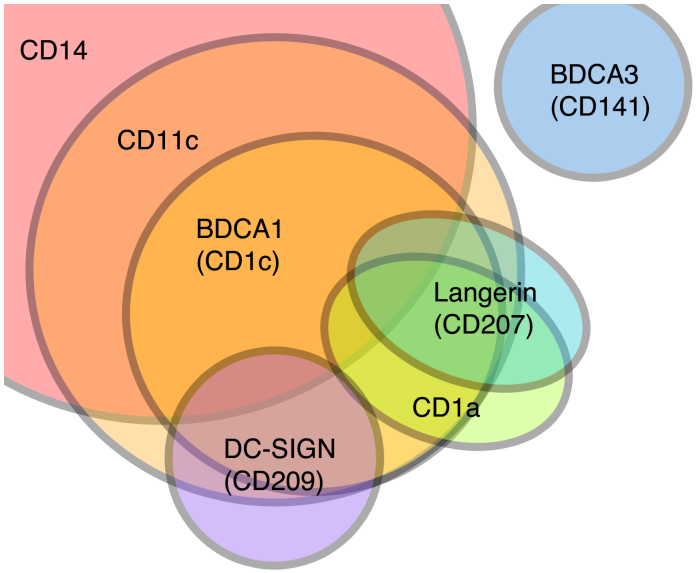

Figure 2: Current paradigms in human DC identification (adapted from Van Pottelberge et al., 2010). Human conventional DCs encompass a wide range of potential subsets, many of which overlap phenotypically. Recent publications have used a variety of different and often sub-optimal methods to identify conventional DCs, including CD11c (which also marks monocytes and macrophages, which are also $CD14^{+}$) and DC-SIGN, which marks only a subset of conventional DCs which may or may not overlap with other DC markers, such as CD1a and/or Langerin. Increasingly, researchers are using BDCA1 to identify a large and non-contaminated population of conventional human DCs. Note that circles are not necessarily drawn to the scale of representative cell populations and are likely to change depending on further studies.

instead of a bacteria-killing Th1 phenotype. In fact, human DC expression of CD1d strongly correlates with the development of effective immunity against *M. leprae*, implicating the importance of DC function in orchestrating the immune response (Sieling et al., 1999). DCs have also been implicated in the development of psoriasis. $CD11c^{+}BDCA1^{-}$ cells are increased in skin biopsies of psoriasis patients, which researchers concluded represents an influx of dermal DCs (Zaba et al., 2009). However, the $BDCA1^{-}$ cells did not express significant MHCII, CD11c also marks macrophages and monocytes, and the cells also expressed some macrophage markers that normal DCs do not express (Zaba et al., 2009). Regardless, $BDCA1^{+}$ DCs are potent at activating Th17 cells (Segura et al., 2013), which are significant contributors to psoriatic inflammation (Zaba et al., 2009). In fact, mouse models of psoriasis have shown that depletion of DCs, macrophages, and monocytes with clodronate is sufficient to resolve psoriatic lesions and arrest Th17 infiltration into the skin, implicating their importance in the development of psoriasis (Ward et al., 2011).

Human DCs have been implicated in numerous atopic diseases involving significant Th2 involvement, including atopic dermatitis (eczema) and allergic asthma. In atopic dermatitis, it is thought that epidermal barrier dysfunction leads to dermal exposure to antigens that typically do not have access beyond the epidermis. Accompanying PAMPs and DAMPs then activate local epithelial cells to secrete inflammatory mediators, which stimulate local tissue DCs to mature despite the lack of obvious infection (Soumelis et al., 2002). Epidermal $Langerin^{+}$ DCs, which are increased in eczematous skin and decrease upon treatment (reviewed in Novak, 2012) are more potent at activating T-cells, are more likely to skew them towards a Th2 phenotype (Klechevsky et al., 2008), and are less potent at stimulating Tregs (Novak, 2012). In asthma, DCs have also been implicated to initiate and exacerbate Th2 inflammation of the lungs through

careful studies with mouse models. In humans, BDCA3$^+$ DCs are increased in the bronchiolar lavage fluid (BALF) of allergic asthmatics (Kayserova et al., 2012), implicating a potential role in perpetuating asthma in humans as well as mouse.

Human DC release of cytokines has been implicated as contributing to multiple inflammatory diseases independently of their activity as T-cell activators. For example, human DCs release inflammatory cytokines into the blood in atherosclerosis and significantly contribute to the risk of myocardial infarction (MI). Inflammatory cytokines produced by atherosclerotic plaques can induce IL-12, matrix metalloproteinase (MMPs), and interferon release by circulating blood DCs, which can contribute to plaque cell apoptosis and the risk of cardiac vessel blockage and MI (Niessner and Weyand, 2010).

In many of these diseases, poor characterization of human DCs has precluded a full understanding of their prevalence and phenotype in health as compared to inflammatory diseases. Chapter II describes work in which we carefully quantified the prevalence of BDCA1$^+$ DCs in lung parenchyma and epithelium of a variety of lung diseases compared to healthy specimens, implicating their role in the inflammatory process of these diseases.

Part B: FcεRI structure and function

FcεRI structure

The high affinity IgE receptor, FcεRI, is crucial for the IgE-mediated allergic response. It binds IgE with high affinity and initiates a signaling cascade upon receptor crosslinking with IgE and allergen, resulting in cellular activation and the typical symptoms of allergies. FcεRI is made up of four, or three protein chains (Garman et al., 1998): an IgE-binding α-chain, two signal

transducing γ-chains, and a signal amplifying β-chain which in humans is not required for surface FcεRI expression or basal signaling (Ra et al., 1989).

The α-chain is a transmembrane glycoprotein that is necessary and sufficient for IgE binding (Hakimi et al., 1990). It adopts the shape of an inverted 'v' with two domains, the second of which is dominant in IgE binding (Garman et al., 1999). Interestingly, α-chain glycosylation is not directly required for IgE binding (Garman et al., 1999); even unglycosylated, immature FcεRIα (Albrecht et al., 2000; Kraft et al., 1998), and recombinant FcεRIα made in *E. coli* (Geha et al., 1985; Robertson, 1993) are capable of binding IgE with no significant loss in affinity; however, proper glycosylation is required for the receptor to traffic to the plasma membrane (Albrecht et al., 2000). IgE binds to FcεRIα in a 1:1 stoichiometric ratio with a remarkable affinity of $K_a = 10^{10}\ M^{-1}$ (Gould and Sutton, 2008) and also adopts a bent formation upon binding to FcεRI (Garman et al., 2000; Hunt et al., 2012; Wan et al., 2002)Figure 3A). This complicated conformation contributes to three phenomena unique to IgE, the first being the remarkably low dissociation rate of IgE from FcεRI as compared to other immunoglobulin receptor interactions (Wan et al., 2002). This low dissociation rate contributes to the long half-life of IgE on FcεRI-expressing cells (Kubo et al., 2003). Second, these complicated interactions require 37° C for IgE to effectively bind FcεRI (Chen et al., 2003), which causes significant difficulty with in vitro testing, as IgE binding and plasma membrane dynamics, including endocytosis, cannot be unlinked at that temperature. Third, this unique conformation places potential steric constraints on IgE epitopes (Gould and Sutton, 2008), which may explain some restriction in the IgE-recognizing antigen repertoire (Hunt et al., 2012).

The γ-chains of FcεRI are transmembrane proteins that are shared among Fc receptors and contain ITAMS that homodimerize via N-terminal cysteines (Ravetch and Kinet, 1991) and

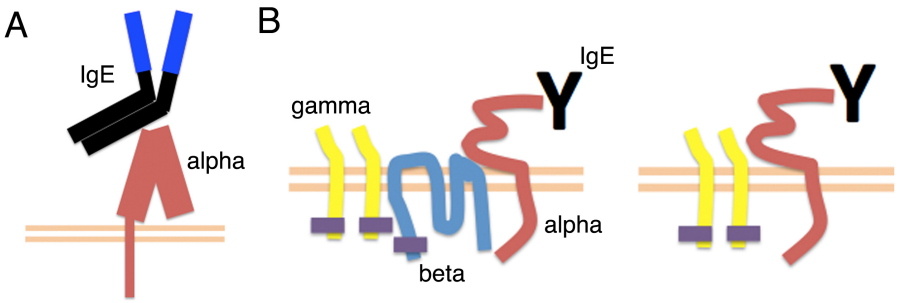

Figure 3: FcεRI-IgE binding (adapted from Gould and Sutton, 2008) and different forms of the receptor complexes (adapted from Kinet, 1999). (A) Simplified model showing the bent conformation of IgE (constant region in black and variable regions in blue) upon binding to FcεRIα (red). The uniquely bent conformation of the immunoglobulin on FcεRI is thought to contribute to the temperature sensitivity (Chen et al., 2003), long half-life (Gould and Sutton, 2008), and steric constraints of IgE binding to FcεRI (Hunt et al., 2012). **(B)** Different forms of FcεRI. Tetrameric FcεRI including the β-chain is shown on the left, and the trimeric form of the receptor is shown on the right. Tetrameric FcεRI is expressed on human and murine basophils and mast cells, and trimeric FcεRI is restricted to human myeloid cells including DCs and monocytes.

transduce activating signals via interactions with Syk. The γ-chains pair with the α- and β-chains in the ER before export to the cell surface. It is not thought that the γ-chains are required for IgE binding, but they are required for the α-chain to get to the cell surface and for signaling via FcεRIα due to the masking of an ER retention motif on FcεRIα.

The β-chain of FcεRI is a 4-transmembrane protein involved in receptor stabilization (Singleton et al., 2009) and signal amplification upon receptor crosslinking through interactions between the C-terminal β-chain ITAM and the tyrosine kinase Lyn (Dombrowicz et al., 1998; Lin et al., 1996). Paradoxically, the β-chain ITAMS can also associate with the tyrosine phosphatases SHP-1, SHP-2, and the inositol phosphatase SHIP, thereby dampening signaling upon FcεRI crosslinking (Kraft and Kinet, 2007).

FcεRI expression and regulation in human and mouse

In humans, FcεRI is expressed as either the trimeric or tetrameric form (Figure 3B) in a variety of cell types. Tetrameric FcεRI expression is restricted to mast cells and basophils due to the restricted nature of FcεRIβ chain expression; these cells express high levels of the receptor with a long half-life on the surface of the cell. While estimates of the density of FcεRI/cell for basophils differs depending on the type of model used, it is thought that healthy human basophils express a range of FcεRI densities around 25,000-200,000/cell (MacGlashan et al., 2001; Maeyama et al., 1988; Malveaux et al., 1978), whereas atopic individuals may have densities as high as 700,000/cell (MacGlashan et al., 2001; Malveaux et al., 1978).

As opposed to the restricted nature of tetrameric FcεRI expression, trimeric FcεRI is expressed on a wide variety of human cells. In the steady state, APCs such as blood and tissue plasmacytoid and conventional DCs, monocytes, and Langerhans cells are $FcεRI^{+}$ (Bieber et al.,

1992; Maurer et al., 1995; 1996; Vasudev et al., 2012; Wang et al., 1992). In inflammatory conditions, neutrophils (Gounni et al., 2001) and eosinophils also become FcεRI^{+} (Kayaba et al., 2001; Rajakulasingam et al., 1998), although there is still some question as to the extent of surface-FcεRI as opposed to intracellular FcεRI expressed (Seminario et al., 1999). FcεRI expression has even been reported on megakaryocytes and platelets (Joseph et al., 1997), although the function of FcεRI on these cells is poorly understood.

In mouse, the β-chain of FcεRI is required for surface expression, which typically limits FcεRI expression to mast cells and basophils and prevents expression of a trimeric FcεRI on APCs as measured by the anti-mouse FcεRI antibody MAR1, an Armenian hamster monoclonal IgG antibody generated against the mouse receptor α-subunit. Some groups have reported the presence of MAR1^{+} DCs in inflammatory conditions such as viral infection (Grayson et al., 2007) and exposure to house dust mite Derp1 allergen (Plantinga et al., 2013). However, there remains some controversy whether wild-type mice are capable of expressing trimeric FcεRI under any conditions due to potential issues with MAR1 antibody specificity (laboratory observations). Because of these limitations, many mouse studies have used human FcεRIα transgenic lines under either the endogenous human alpha proximal promoter (Dombrowicz et al., 1996) or under the mouse CD11c promoter, allowing for DC-specific, human IgE-binding FcεRI expression (Sallmann et al., 2011).

FcεRI complex formation is limited by the expression of its parts. Expression of the α-chain is regulated by a proximal and distal promoter. The transcription factors GATA-1 and PU.1 or YY1 promote FcεRIα expression in myeloid cells (Donohoe et al., 1999; Mak et al., 2011) via binding to the proximal promoter region in RBL cells, whereas Elf-1 inhibits FcεRIα expression via the proximal promoter when coexpressed with PU.1 and YY1 (Nishiyama et al.,

1999). Polymorphisms in the proximal promoter region of FcεRIα have been positively correlated with serum IgE levels in multiple recent studies (Bae et al., 2007; Kanada et al., 2008; Weidinger et al., 2008; Zhou et al., 2012); while it remains unclear exactly how these polymorphisms result in higher serum IgE, at least one SNP alters transcription factor binding to the promoter of the gene (Kanada et al., 2008), leading to speculation that FcεRI regulation can influence serum IgE levels. The distal promoter is negatively regulated through PU.1, YY1, and Elf-1; however, IL-4 positively regulates the distal promoter (Hasegawa et al., 2003) and enhances FcεRIα mRNA levels along with IL-13 in human monocytic cell lines (Reischl et al., 2000) as well as primary human monocytes (Gosset et al., 2001).

FcεRI β-chain expression is regulated by multiple transcription factors that act on both the β-chain promoter and (surprisingly) the 5' untranslated region (UTR). Like for the FcεRIα chain, myeloid expression of the transcription factors YY1 and Oct-1 are thought to promote transcription of FcεRIβ through interaction with either the β promoter (Nishiyama et al., 2004) or the 5' UTR (Akizawa et al., 2003). MZF negatively regulates FcεRIβ expression via a region in FcεRIβ's fourth intron along with the cofactor FHL3 and is thought to confer specificity of tetrameric FcεRIβ expression due to the ubiquity of MZF expression in a large number of hematopoietic cells (Ra et al., 2012; Takahashi et al., 2003). GM-CSF also causes an accumulation of FHL3 in the nucleus, which contributes to FcεRIβ suppression in non-mast cells and basophils (Ra et al., 2012). FcεRIβ can be expressed as an alternative form that paradoxically inhibits FcεRI expression: β_{var}, also referred to as βt, is a variant of FcεRIβ that is transcribed with its 5^{th} intron, which replaces the final C-terminal transmembrane domain and cytoplasmic ITAM motif with 16 intronic residues along with an early stop codon (Donnadieu et al., 2003; Fiebiger et al., 2005). β_{var} is expressed to varying degrees along with full-length β in

healthy humans and downregulates surface FcεRI levels by binding exclusively to immature FcεRIα and preventing its maturation and migration to the cell surface (Donnadieu et al., 2003). Instead, β_{var} is rapidly targeted for degradation via the proteasome (Donnadieu et al., 2003).

FcεRI complex expression is also regulated at the post-translation level. The α-chain protein is translated with a signal peptide that prevents its export from the ER until it pairs with two FcεRI γ-chains (Platzer and Fiebiger, 2010), which mask a retention motif present on the α-chain (Letourneur et al., 1995a) and prevent expression of a non-signaling competent FcεRI complex. FcεRI α and γ are also co-translated in the ER, which helps to coordinate complex formation (Fiebiger et al., 2005). FcεRI complex formation is furthermore dependent on glycosylation of FcεRIα (Albrecht et al., 2000; Letourneur et al., 1995b) (Reviewed in Figure 4).

Multiple in vitro and in vivo studies in both human (Conroy et al., 1977) and mouse have demonstrated that FcεRI surface levels correlate with available IgE. Mouse mast cells in vivo and in vitro increase surface FcεRI with increasing available IgE (Yamaguchi et al., 1997). Similarly, human mast cells (Yamaguchi et al., 1999) or FcεRI-transfected cell lines (Borkowski et al., 2001) cultured with IgE increase surface FcεRI expression in vitro. Conversely, treatment of human basophils and mast cells in vitro or in vivo with a blocking anti-IgE antibody (omalizumab) results in downregulation of surface FcεRI levels due to sequestration of available IgE (Beck et al., 2004; MacGlashan et al., 1997; Prussin et al., 2003; Saini et al., 1999). This correlation is due to a stabilization of the receptor on the cell surface that prevents receptor internalization and recycling through recycling endosomes whereas new receptor complexes continue to be brought to the cell surface (MacGlashan et al., 2001). In accordance with this observation, individuals with increased IgE levels tend to have higher FcεRI expression levels (Foster et al., 2003; Saini et al., 2000).

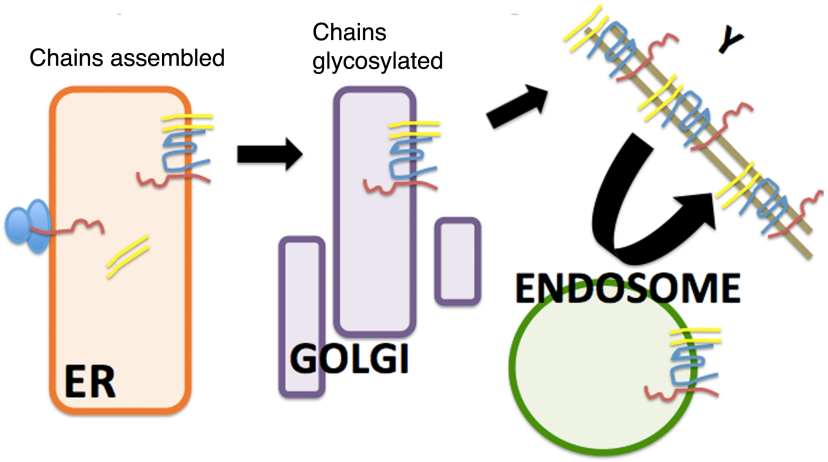

Figure 4: Intracellular FcεRI trafficking and post-translational regulation (adapted from Kraft and Kinet, 2007). FcεRI receptor chains are assembled in the ER (left) and move to the golgi complex for terminal glycosylation. Upon glycosylation, the receptor moves to the surface of the cell, where it recycles through recycling endosomes when unbound to IgE. Upon IgE binding, the receptor is stabilized on the surface of the cell, resulting in an accumulation of the receptor on the plasma membrane in high IgE conditions.

Canonical FcεRI functions in the allergic response

In many ways, IgE is a unique and poorly understood immunological molecule. Canonically, it is thought that IgE and FcεRI are designed to defend an organism against parasite infection. In fact, serum IgE levels dramatically increase upon parasite infection, though much of this IgE is not pathogen-specific (Turner et al., 1979). Murine eosinophil activation via FcεRI in vitro is important for parasite killing (Soussi Gounni et al., 1994), although in vivo mouse models have shown that FcεRI is dispensable for host resistance to *S. mansoni* infection (Jankovic et al., 1997). Ideally, IgE-deficient humans could help to isolate the function of this immunoglobulin in the immune response; IgE deficiency, however, often occurs concomitantly with other hypoglobulinemias (Schoettler et al., 1989; Smith et al., 1997), which makes the isolation of IgE's function difficult. Regardless, case studies of individual patients with isolated IgE deficiency have not found any gross defects in immune function (Levin et al., 2006; Levy and Chen, 1970), although their immune response to parasites has not been challenged. In the absence of evidence for the utility of this immune response, IgE remains the potent activator of allergic reactions against harmless antigens.

In humans, it is thought that FcεRI is required for IgE-mediated allergic responses. No other Fc receptors bind with similarly high affinity and the low-affinity receptor is expressed predominantly on B-cells. In mouse, the role of IgE in particular is not as clear: both FcεRI and FcγRIV are capable of binding IgE (Mancardi et al., 2008), and some groups have reported anaphylactic responses in the absence of FcεRI. Despite this, mice lacking FcεRIα and/or FcεRIβ have severely attenuated allergic responses (Dombrowicz et al., 1993).

In the steady state, IgE circulates in the body and binds to FcεRI expressed on various cell types. This primes these cells in case of exposure to cognate allergen but also can initiate a

number of cellular responses even when allergen is absent. For example, monomeric binding of IgE to FcεRI prolongs the life of mast cells (Kalesnikoff et al., 2001; Kawakami and Kitaura, 2005) through autocrine IL-3 secretion (Kohno et al., 2005). Only some clones of IgE are apparently capable of this poorly understood phenomenon and are referred to as 'cytokinergic' IgE (Kawakami and Kitaura, 2005; Kohno et al., 2005). In addition, as mentioned previously, monovalent IgE binding to FcεRI causes a change in the trafficking patterns of the protein that results in an accumulation of the receptor on the surface of the cell. This receptor stabilization, which is thought to be due to an arrest in receptor recycling (MacGlashan et al., 2001), is the reason why serum IgE positively correlates with surface FcεRI levels on basophils and mast cells (Conroy et al., 1977; Foster et al., 2003; Saini et al., 2000; Yamaguchi et al., 1997).

When exposed to a multivalent antigen that the IgE is specific for, IgE:FcεRI complexes are brought into close proximity to each other within the plasma membrane in a phenomenon known as receptor crosslinking which initiates a signaling cascade within the cell. Signal strength correlates with the duration of FcεRI crosslinking, which is influenced by the IgE-antigen affinity and on/off rate of the IgE and antigen. FcεRI signal strength also correlates with the valency of the antigen: a divalent antigen, such as an anti-FcεRI or anti-IgE antibody generates only a fraction of the maximum potential signal that a more multivalent antigen can generate; this is because with increasing valency of the antigen, more FcεRI:IgE complexes are involved in the resulting cluster of bound receptors. These clusters aggregate in lipid rafts of the plasma membrane (Field et al., 1997), which allows for easier association with signaling molecules such as Lyn and Syk (Sheets et al., 1999; Young et al., 2003). Once crosslinked, the tyrosine kinase Lyn, which regularly associates with the FcεRI β ITAM, phosphorylates the ITAM, which recruits Syk to the FcεRI γ-chains (Kraft and Kinet, 2007), although

autophosphorylation of Syk via γ-chain ITAMS alone is also possible. Autophosphorylation still generates a signal, but of less potency than possible with the β-chain (Lin et al., 1996). In either case, Syk activates the adaptors LAT and SLP76, which result in Calcium mobilization and many downstream effects (Kraft and Kinet, 2007).

A number of mechanisms are also in place to limit FcεRI signaling upon crosslinking with IgE and antigen. Surprisingly, in addition to Lyn, FcεRIβ can also interact with inhibitory signaling molecules such as SHP-1/2 and SHIP, which dampen signaling cascades initiated by FcεRI crosslinking. Furthermore, endocytosis of crosslinked tetrameric or trimeric FcεRI complexes can also help limit activation by sequestering the receptors from signaling molecules.

Endocytosis upon receptor crosslinking is rapid; as early as 30 mins post-crosslinking of FcεRI complexes on rat basophilic leukemia (RBL) cells with anti-DNP IgE and DNP-HAS antigen, most internalized FcεRI colocalizes with markers of early endosomes (Fattakhova et al., 2009). By 60 mins, most FcεRI complexes are found in late endosomes and in lysosomes by 2 hrs post-crosslinking (Fattakhova et al., 2009). Endocytosis occurs in a dynamin-dependent fashion (Fattakhova et al., 2006) and is dependent on ubiquitination via c-Cbl, an E3 ligase which ubiquitinates cytoplasmic lysine residues on FcεRIβ and γ (Molfetta et al., 2009) and initiates endocytosis through a variety of ubiquitin-interacting proteins (Molfetta et al., 2009; Raiborg et al., 2006). Endocytosed complexes are trafficked to terminal lysosomes where the receptor, along with IgE and the crosslinking antigen, are degraded (Fattakhova et al., 2009).

Downstream effects of FcεRI crosslinking are cell-dependent. In mast cells and basophils, crosslinking of FcεRI results in cell activation and release of preformed granules. These granules contain inflammatory mediators such as cytokines and prostaglandins, which mediate the early symptoms of the allergic response (Gould et al., 2003). Human neutrophils

respond to FcεRI crosslinking with IL-8 release (Gounni et al., 2001). In eosinophils of both human and transgenic mouse, crosslinking of FcεRI has been shown to cause the release of IL-10 (Kayaba et al., 2001); however, these results remain somewhat tentative because of the still controversial presence of FcεRI on eosinophils.

FcεRI expression and function on dendritic cells

Wild-type mice require expression of FcεRIβ in order to express a functional FcεRI complex on the surface of the cell in the absence of inflammation: therefore, FcεRI expression is restricted to basophils and mast cells. However, human FcεRIα-transgenic mice express the receptor on a repertoire of cells very similar to humans, including both conventional and plasmacytoid DCs in addition to monocytes, in both blood and multiple tissue environments. These mice have exacerbated allergic responses as measured by TNBS-induced colitis (Dombrowicz et al., 2001) compared to wild-type mice, indicating the role of FcεRI on non mast cells and basophils in allergic inflammation.

In humans, the trimeric form of FcεRI is expressed on a number of DC subtypes in the steady state, including both conventional and plasmacytoid DCs. Both $BDCA1^+$ and $BDCA3^+$ conventional blood DC subsets express FcεRI (Dehlink et al., 2010; Foster et al., 2003; Vasudev et al., 2012). In tissues, conventional and plasmacytoid DCs are also $Fc\varepsilon RI^+$. Studies have identified $Fc\varepsilon RI^+CD1a^+$ DCs in airway epithelium (Tunon-De-Lara et al., 1996), GI tract (Bannert et al., 2012), and epidermis/dermis (Bieber et al., 1992; Jürgens et al., 1995; Kraft et al., 1998; Wang et al., 1992). FcεRI is also expressed on human blood monocytes (Bubnoff et al., 2004; Maurer et al., 1994; Reischl et al., 1996; Sihra et al., 1997), albeit to varying amounts depending on atopic status and serum IgE levels.

Unlike basophils and mast cells, it is not clear whether increasing serum IgE influences surface FcεRI levels of DCs and monocytes. While some groups have found a positive correlation between the two (Foster et al., 2003; Sihra et al., 1997; Vasudev et al., 2012), others see no clear relationship between serum IgE and surface FcεRI expression (Dehlink et al., 2010; Saini et al., 2000). As such, DC and monocyte function upon FcεRI crosslinking is largely dependent on FcεRI surface levels, with low FcεRI-expressing donor cells having little or no stimulation upon crosslinking (Jürgens et al., 1995; Novak et al., 2001). Therefore, many studies of FcεRI function in DCs and monocytes have focused only on atopic individuals (Maurer et al., 1994; 1995; 1996), who tend to have higher FcεRI surface levels.

Numerous studies have examined the effects of FcεRI crosslinking on human DCs and monocytes. For monocytes, crosslinking of FcεRI prolongs survival (Katoh et al., 2000) but prevents differentiation into DCs (Novak et al., 2001). Crosslinking of the receptor on human DCs (at least from atopic donors) causes the cell to mature in an NFκB-dependent manner (Kraft et al., 2002). With that, DCs change their phenotype in a number of ways.

Upon crosslinking of FcεRI, the complex along with IgE and the cognate allergen are endocytosed and degraded in terminal lysosomes (Molfetta et al., 2010). As a result, both monocytes (Maurer et al., 1995) and DCs (Maurer et al., 1996) in vitro have shown an enhancement of T-cell activation and Th2 skew upon crosslinking of FcεRI. In vivo mouse studies using a CD11c-FcεRIα transgenic mouse model demonstrated that activation of DCs via FcεRI resulted in a slight exacerbation of the symptoms of allergic asthma, including mainly airway eosinophilia (Sallmann et al., 2011). Conversely, neutralization of IgE abrogates DC-dependent Th2 inflammation in mouse models (Schroeder et al., 2010).

FcεRI crosslinking also causes the production and release of a number of cytokines and chemokines that can act on surrounding tissues. Monocytes of atopic human donors have been reported to release IL-10 (Novak et al., 2001) and prostaglandins (Takenaka et al., 1995) upon crosslinking of FcεRI. Activation of human DCs via FcεRI crosslinking also causes an upregulation of TNF-α and the chemokine MCP-1 (Kraft et al., 2002).

One caveat of these early studies is that imperfect cell isolation methods from human tissues could result in contamination by FcεRI-expressing cells such as basophils or mast cells that can skew functional studies and give an inaccurate picture of FcεRI function on DCs and monocytes. For example, early studies that used a common method of DC purification involving gradient centrifugation claimed to have identified FcεRIβ expression in human Langerhans cells (Bieber et al., 1992); however, additional studies have found this not to be the case (Jürgens et al., 1995; Wang et al., 1992), which lends credence to the possibility that early studies of FcεRI on DCs were sometimes contaminated with tetrameric FcεRI-expressing cells. Indeed, (Jürgens et al., 1995) cite that 'over 50%' of cells isolated with lymphoprep centrifugation were Langerhans cells, indicating significant potential for contamination. Therefore, studies using older methods of DC isolation and enrichment should perhaps be viewed with some skepticism, as readouts of DC function in these tests could have been influenced by contaminating FcεRI-expressing cells. In conclusion, there are many remaining questions as to the function and purpose of FcεRI on human DCs and monocytes. Chapter III of this work describes a new role of FcεRI in serum IgE clearance through constitutive endocytosis of the receptor with IgE in human DCs. Through careful isolation and characterization of human DCs and basophils, we identified a unique pattern of intracellular lysosomal trafficking of FcεRI in human DCs and monocytes. This lysosomal trafficking resulted in FcεRI-dependent endocytosis of IgE in the absence of

crosslinking stimulus into lysosomes, where the IgE was degraded. In the human FcεRIα-transgenic mouse model, this trafficking resulted in a dramatic loss in serum IgE over time and implicates a new role of FcεRI trafficking in the control of IgE-mediated allergies.

CHAPTER II. HUMAN BDCA1$^+$ DENDRITIC CELLS ACCUMULATE IN INTERSTITIAL FIBROTIC LUNG DISEASE AND TH2-HIGH ASTHMA

The text of this chapter is a reprint of the material as it was submitted to *PLoS One*: "Accumulation of BDCA1$^+$ dendritic cells in interstitial fibrotic lung diseases and Th2-high asthma".

Abstract

Dendritic cells significantly contribute to the pathology of several mouse lung disease models. However, little is known of the contribution of DCs to human lung diseases. In this study, we examined DC infiltration of human lungs in patients with interstitial lung diseases or asthma. Using flow cytometry, we found that BDCA1$^+$ DCs, the major human DC subset, increased by 5 ~ 6 fold in the lungs of patients with idiopathic pulmonary fibrosis or hypersensitivity pneumonitis, which are both characterized by extensive fibrosis in parenchyma. The same DC subset also significantly increased in the lung parenchyma of patients with chronic obstructive pulmonary disease, although the degree of increase was relatively modest. By employing immunofluorescence microscopy using FcεRI and MHCII as the specific markers for BDCA1$^+$ DCs, we found that the numbers of BDCA1$^+$ DCs also significantly increased in the airway epithelium of Th2 inflammation-associated asthma. These findings suggest a potential contribution of BDCA1$^+$ DCs in human lung diseases associated with interstitial fibrosis or Th2 airway inflammation.

Introduction

Dendritic cells (DCs) play an important role in immune surveillance in the lungs. DCs located within or beneath the airway epithelia send projections toward the lumen to capture antigens in the airway (Jahnsen et al., 2006). DCs also reside in alveoli and capture antigens that have reached deep into the alveolar space (Thornton et al., 2012). Mice deficient in lung DCs exhibit significant defects in developing protective immunity against respiratory antigens (McGill et al., 2008), indicating that antigen capture and subsequent presentation by lung DCs plays an important role in inducing and propagating respiratory immunity. Interestingly, a number of studies suggest that DCs also play pathologic roles in several lung diseases, at least in mouse models. In a mouse model of viral lung infection, DCs produce chemoattractants that recruit Th2 cells that mediate persistent airway inflammation and mucus production (Grayson et al., 2007). DCs also both initiate and perpetuate Th2 inflammation driven by allergens in mice (van Rijt et al., 2005). Furthermore, DCs contribute to persistent inflammation in murine lungs by mediating development and maintenance of tertiary lymphoid organs, which promote local activation of T-cells (GeurtsvanKessel et al., 2009). However, relatively little is known about the contribution of DCs to lung diseases in humans.

Bronchial biopsies serve as an important source of accessible human lung tissue and immunohistochemistry is an important method to examine DCs in these small tissue samples. Previously, immunohistochemical analyses using antibodies to CD11c, DC-SIGN, CD1a, or Langerin have been performed to identify DCs in human bronchial biopsies (Marchal-Sommé et al., 2007). These analyses have suggested that asthma, chronic obstructive pulmonary disease, and fibrosis are associated with a significant increase in DCs (Jahnsen et al., 2001; Marchal-Sommé et al., 2007; Möller et al., 1996; Van Pottelberge et al., 2010). However, recent advances

in characterization of human DC subsets reveal that many of these antibodies are neither specific nor sufficiently label broad human DC subsets. For example, CD11c is not only expressed in DCs but also highly expressed in monocytes and macrophages in humans (Masten et al., 2006). DC-SIGN was found to be only expressed in monocyte-derived DCs, at least in mice (Cheong et al., 2010). CD1a and Langerin were found to be restricted to DC subsets specifically localized in the epithelium (Demedts et al., 2005). More recently, BDCA1 was found to be specifically expressed in the major DC subset in human blood (Dzionek et al., 2000). Since this finding, anti-BDCA1 antibodies have been used to label DCs in various tissues, including lungs (Demedts et al., 2005; Segura et al., 2012; 2013; Tsoumakidou et al., 2008). Although this antibody labels a significant proportion of lung DC subsets, including CD1a or Langerin-expressing epithelial DCs (van Haarst et al., 1994; 1996; Van Pottelberge et al., 2010), it additionally labels B cells (Dzionek et al., 2000). Thus, immunohistochemistry-based methods of DC identification require an improved staining strategy by which DCs of a broadly representative subset are labeled in a specific manner.

Another method useful for DC analysis in human lungs is flow cytometry. This method allows accurate identification of DCs by using multiple cell-specific markers and also provides a powerful means of generating quantitative analysis of the frequency of DCs. A major limitation, however, is that this method requires relatively large sized tissues due to the scarcity of DCs in the lungs. Although not common, lung transplantation is sometimes performed for patients with severe interstitial lung diseases (ILDs) (Orens et al., 2006). Resected lungs from these patients would be very useful sources for flow cytometric analysis of DCs, which may reveal potential association of DCs with these specific diseases.

In this study, we examined by flow cytometry the prevalence of BDCA1$^+$ DCs in the lungs isolated from patients with ILDs: idiopathic pulmonary fibrosis (IPF), hypersensitivity pneumonitis (HP), and chronic obstructive pulmonary disease (COPD). We also identified a new marker for BDCA1$^+$ lung DCs, developed a dual-fluorescence staining strategy, and quantified the DCs in bronchial biopsies from patients with asthma (Woodruff et al., 2009).

Materials and Methods

Ethics

All studies were approved by the UCSF Committee on Human Research, and written informed consent was obtained from all subjects. The interstitial lung disease study was approved by the UCSF IRB #10-00198. For the asthma study, subjects also provided consent for their biospecimens to be placed in the UCSF Airway Tissue Bank IRB #11-05176 for studies. Blood samples were collected as part of a larger study approved by the UCSF IRB #10-02596.

Interstitial lung disease (ILD) study population

The study population consists of 9 patients with idiopathic pulmonary fibrosis (IPF), 7 with chronic hypersensitivity pneumonitis (HP), 7 with COPD/emphysema, and 13 without lung disease who had died from a non-pulmonary cause. All ILD diagnoses were established thorough multidisciplinary review of clinical data, radiology, and pathology. Diseased lung tissues were obtained from donors undergoing lung transplantation. Normal lung tissues were from lungs originally designated for transplantation by the Northern California Transplant Donor Network, but ultimately not used for various reasons.

Asthma study population

Asthmatic subjects had been characterized by a prior physician diagnosis of asthma, airway hyper-responsiveness (methacholine PC20 < 8.0 mg/mL), and at least one of the following: asthma symptoms on 2 or more days per week; β-agonist use on 2 or more days per week; FEV1 less than 85 % predicted. Subjects had not taken inhaled or oral corticosteroids for 4 weeks before enrollment. Healthy control subjects had reported no lifetime history of pulmonary disease and lacked airway hyper-responsiveness. Criteria for exclusion for both healthy and asthmatic subjects included a demonstration of any previous history of lung disease other than asthma, a history of an upper or lower respiratory tract infection in the 4 weeks preceding the study enrollment visit, females who were pregnant or breast feeding, taking beta-blocker medication, current cigarette smoking or a total smoking history > 15 pack-years. Characterization of Th2-low and -high asthma was performed by microarray hierarchical clustering analysis on epithelial brushings as described previously (Woodruff et al., 2009).

Bronchial biopsy preparation

Endobronchial biopsy samples from healthy and steroid naïve asthmatic subjects were obtained from the Airway Tissue Bank at the University of California, San Francisco. These samples had been collected during multiple research studies performed at UCSF between 2007 and 2013 in which all characterization studies and biospecimen collection had followed standardized protocols. For immunofluorescence microscopy study, biopsies were collected at the lateral edges of 2^{nd} to 5^{th} -order carinae, washed in PBS, fixed in ice-cold 10% formalin, and suspended in paraffin wax. Tissue blocks were arranged with 2-6 biopsies per paraffin block and were cut at a thickness of 3 mm. For flow cytometric analysis, biopsies were not fixed but instead digested

with 1mg/mL collagenase I (Roche) in RPMI 1640 without phenol red for 2 hrs at 37° C, then dispersed with a syringe and passed through a cell filter. The resulting single-cell suspension was spun and resuspended in FACS buffer for staining.

Peripheral blood mononuclear cell (PBMC) isolation

Blood was collected from healthy anonymous individuals with written and informed consent. PBMCs were isolated by layering blood diluted 1:1 with HBSS over a Ficoll (GE Sciences) density gradient and subsequently centrifuging it.

Pulmonary mononuclear cell (PMC) isolation

Lungs were removed from the patient, and large vessels were perfused with PBS. The parenchyma was cut into 2 cm^3 to 5 cm^3 sections and placed in cold media for storage until use. Lung specimens were used within 72 hrs of isolation. To prepare single cell suspensions for flow cytometry, first the lung specimen was minced with scissors in warm digestion media (20 mL HBSS with 10 mg Collagenase D [Roche] and 2 mg DNAse I [Roche]). Minced tissue was rocked at 37 degrees for 45 mins. Then the tissue was pushed through a 75 mm-mesh cell strainer over a 50 mL falcon tube with a plunger. The resulting single-cell suspension was spun at 1300 rpm for 5 mins and washed with HBSS, resuspended in 20 mL HBSS, and layered over 15 mL of Ficoll and spun at 2400 rpm for 20 mins at room temperature with no brake. Red blood cells and platelets were discarded and the mononuclear cell layer was collected for FACS staining. The resulting cell population was washed once with FACS buffer before staining.

Flow cytometry

Samples were resuspended in FACS buffer (2 % FBS, 0.1 % sodium azide) and stained with the following mixtures of Biolegend antibodies: BDCA1-Percp/Cy5.5, CD14-APC, MHCII-Pacific Blue, CD3/19/56-FITC, CD123-PE/Cy7, and either FcεRIa-PE or mouse IgG_{2b}-PE, all at manufacturer's recommended concentrations. Some samples were stained with CD45-A700 to identify hematopoietic cells and CD203c-biotin (Biolegend, at manufacturer's recommended concentrations) followed by streptavidin-A647 (Invitrogen, at manufacturer's recommended concentration) to identify mast cells. Cells were stained with antibodies and propidium iodide (PI) (Biolegend) at 1:400 for 15 mins at 4 degrees, washed and spun at 1300 rpm, and resuspended in FACS buffer. At least $1x10^6$ cells were run on slow or medium speed on a BD LSRII machine and analyzed with Flowjo software.

Confocal microscopy

Tissue sections mounted on glass slides were rehydrated in 2 xylene baths for 5 mins each, 2 100% Ethanol baths for 3 mins each, and 1 bath of 95 % ethanol and 1 bath of 80 % ethanol for 1 min each. Afterwards, slides were rinsed with distilled water and dried. Samples were washed once with 10 % goat serum (MP Biomedicals) with 0.05 % saponin in PBS ('wash buffer') and then stained with 1:10 human IgG block for 1 hr. Slides were then washed 3 times with wash buffer for 5 mins each and then stained for 1 hr with 1:50 anti-FcεRI (CRA-1, Biolegend) and 1:200 anti-MHCII ("DRAB" polyclonal rabbit antisera). After 1 hr, slides were washed again and stained with 1:300 goat anti-mouse Alexa 568 and goat anti-rabbit Alexa 488 (Life Technologies) for 1 hr. After rinsing with wash buffer, slides were washed 3 times with PBS and 2 times with distilled water. Slides were dried thoroughly and mounted with Prolong Gold anti-fade mounting reagent with DAPI (Life Technologies). They were then left in the dark at room

temperature overnight to cure and were stored at - 20 degree for imaging. Slides were imaged using a Nikon C1si confocal microscope. First, overlapping images were taken at 20x and epithelial regions were identified using FIJI software. We calculated the area in microns of the epithelium for each biopsy for each patient. Once regions of epithelium were identified, slides were imaged at 60x to identify FcεRI$^+$, MHCII$^+$ DCs within the noted regions of epithelium. The numbers of double-positive cells and single-positive FcεRI$^+$ cells were counted and recorded for each area of epithelium, and the number of FcεRI$^+$ DCs per mm^2 was quantified for each donor's biopsies. Significance was determined via unpaired, two-tailed Student's T-test. The operator performing the microscopy experiments was blind to clinical status of donor who had provided the tissue section.

Results

BDCA1$^+$ DCs markedly increase in the lungs of patients with IPF or HP.

To examine whether DCs infiltrate the lungs in association with human interstitial lung diseases, we isolated pulmonary mononuclear cells (PMCs) from lung parenchyma of healthy deceased donors and patients with IPF, HP, and COPD (Table I) and identified DCs by flow cytometry. First, singlet, live cells were gated based on forward and side scatter and a lack of PI staining (Fig. 1A). A great majority of these cells, even in fibrotic lungs, were found to be of hematopoietic origin as they were CD45$^+$ (Fig. 1B). Next, BDCA1$^+$ DCs were identified by gating BDCA1$^+$lymphocyte marker$^-$ cells and further gating MHCII$^+$ cells (Fig. 1C). In contrast to blood BDCA1$^+$ DCs (Fig. 1D), we found that lung BDCA1$^+$ DCs were mostly CD14$^+$ for all donors tested (Fig. 1E). A quantitative analysis of DC frequencies revealed that BDCA1$^+$ DCs represented around 0.1-0.9 % of PMCs in healthy lungs (Fig. 1F) and that this frequency

	IPF	HP	COPD/Emphysema
Number	9	7	7
Age ± SD	60 ± 7	52 ± 14	62 ± 7
Gender M:F (% F)	5:4 (44%)	3:4 (57%)	4:3 (43%)
Ethnicity			
White	7	5	5
African American	0	0	2
Asian	0	0	0
Pacific Islander	0	0	0
Hispanic	2	2	0
FEV % predicted ± SD	59 ± 17	40 ± 10	18 ± 6

Table 1: Subject data for ILD cohort. Healthy discarded donor lungs or diseased lungs from transplantation were examined for DC content of pulmonary mononuclear cells (PMCs). All ILD diagnoses were made using accepted standard diagnostic criteria. Lungs were lavaged and stored at 4 degrees in media until use (<72 hrs from harvest). Age and FEV % predicted are shown as mean ± SD.

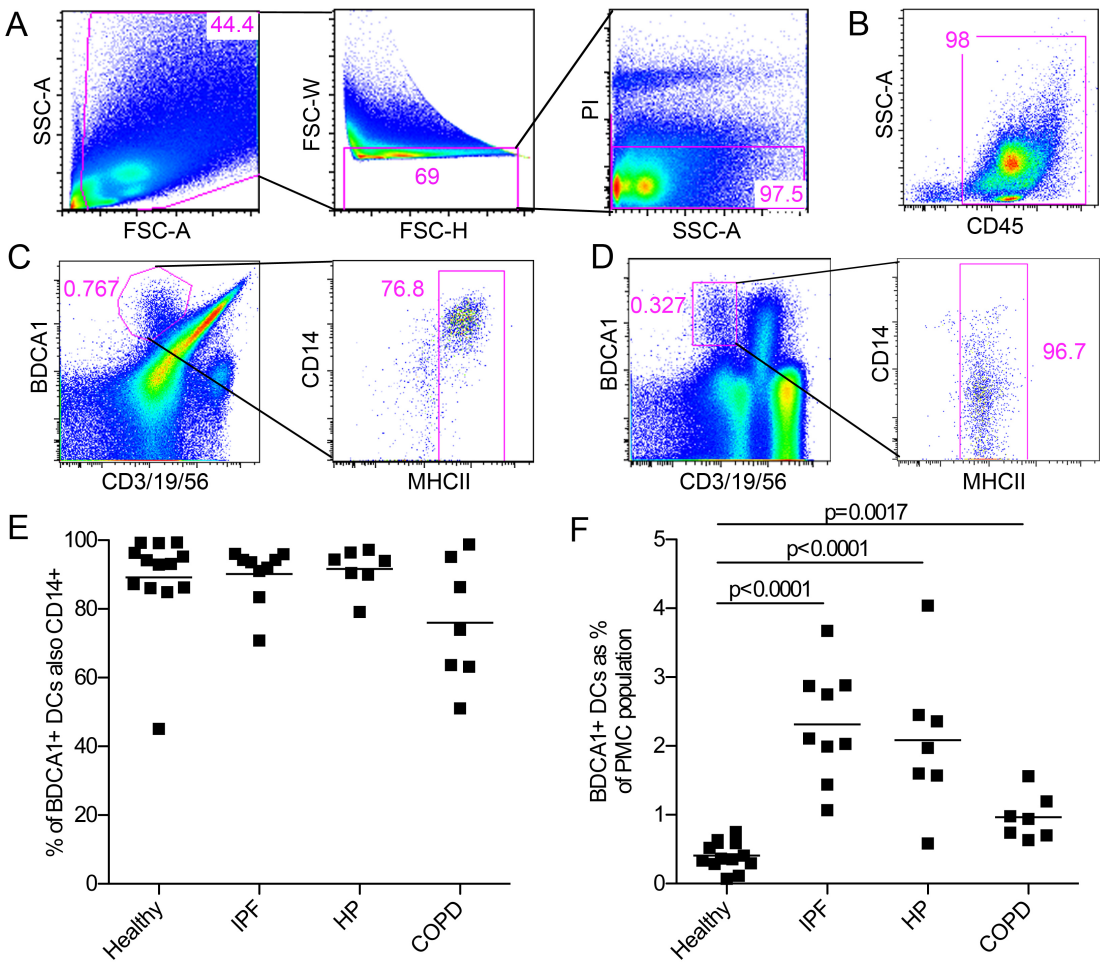

Figure 1: BDCA1$^+$ DCs markedly increase in the lungs of IPF and HP. (A) Pulmonary mononuclear cells were isolated from surgically resected parenchymal lung tissue. Live singlet cells were identified based on forward and side scatter and a lack of PI-staining. Data from one representative donor of over 20 are shown. Numbers in pink represent the gate population as a % of parent. **(B)** Live, singlet cells were stained with an anti-CD45 antibody to determine the prevalence of hematopoietic cells. Data from one representative donor of 3 healthy and 6 ILD patients are shown. **(C)** BDCA1$^+$ DCs were identified in PMC isolates by their expression of BDCA1, MHCII, and their lack of expression of the lymphocyte markers CD3, CD19, and CD56. Data from one representative donor of over 20 are shown. **(D)** DCs were identified from peripheral blood mononuclear cells in a similar fashion based on their expression of BDCA1, and their lack of expression of the lymphocyte markers CD3, CD19, and CD56. They were further examined for their expression of MHCII. Data from one representative donor of over 20 are shown. **(E)** Percentage of BDCA1$^+$ lung DCs that are also CD14$^+$ from a cohort of donors described in Table 1. Each dot represents a unique donor and bar represents mean. There are no significant differences between subject groups as measured by unpaired, two-tailed Student's t-test. **(F)** BDCA1$^+$ DCs were determined as a percentage of total PMCs from the same cohort of donors. Each dot represents a unique donor and bar represents mean. Statistical significance was determined by unpaired, two-tailed Student's t-tests.

increased in both of IPF and HP patients by five to six fold (Fig. 1F). COPD patients also had a significant increase in BDCA1$^+$ DCs but to a much lesser degree than patients with fibrotic diseases (Fig. 1F). Thus, IPF and HP are associated with a robust increase in BDCA1$^+$ DCs while COPD is associated with relatively a modest increase.

BDCA1$^+$ DCs express FcεRI in lung parenchyma and airways.

To aid in identification of BDCA1$^+$ DCs in microscopic studies using small lung biopsies, we searched for specific markers that could be used to better identify these cells. Previous studies have shown that the high affinity IgE receptor, FcεRI, is expressed not only in mast cells and basophils but also in BDCA1$^+$ DCs of human tissues. Blood and tonsil BDCA1$^+$ DCs and Langerhans cells, the skin epidermal DC subset, express FcεRI (Bieber et al., 1992; Foster et al., 2003; Wang et al., 1992). BDCA1$^+$ DCs from arthritic synovial fluid and malignant tumor ascites also express FcεRI (Segura et al., 2013). Whether lung BDCA1$^+$ DCs express FcεRI has not been examined, although CD1a$^+$ DCs in the airways have been shown to express FcεRI by immunohistochemistry (Tunon-De-Lara et al., 1996).

First, we examined FcεRI expression in human lung BDCA1$^+$ DCs by flow cytometry. We found that lung BDCA1$^+$ DCs bound an anti-FcεRI antibody significantly more than an isotype control antibody, indicating that these cells indeed express FcεRI (Fig. 2A). Next, we examined how stably FcεRI is expressed in BDCA1$^+$ DCs using our cohort of healthy and diseased lungs. We found that FcεRI was expressed in BDCA1$^+$ lung DCs throughout cohort to varying degrees (Fig. 2B), which is consistent with previous reports of varying FcεRI levels in BDCA1$^+$ DCs in the blood (Dehlink et al., 2010; Foster et al., 2003; Vasudev et al., 2012). Despite this variation, surface FcεRI was detectable for all donors examined (Fig. 2B), and the

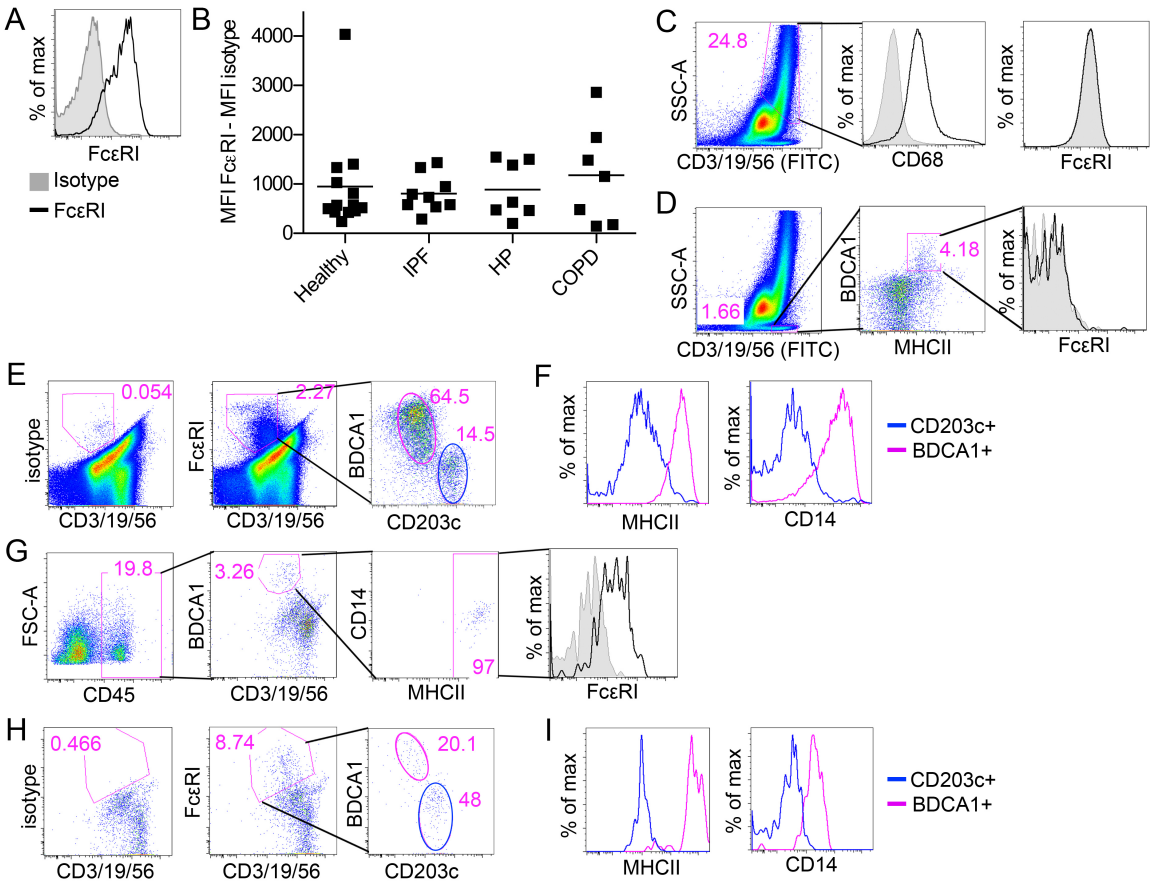

Figure 2: Lung BDCA1$^+$ DCs express FcεRI. (A) Lung BDCA1$^+$ DCs from one representative donor were gated using the strategy as described in Figure 1 and examined for expression of FcεRI. Isotype control is shown in grey and anti-FcεRI antibody is in black. **(B)** Mean fluorescence intensity of FcεRI signal minus isotype control intensity in lung BDCA1$^+$ DCs was plotted for each donor tested in Figure 1. Bar represents mean. There are no significant differences between subject groups as measured by unpaired, two-tailed Student's t-test. **(C)** Macrophages were gated based on high autofluorescence reflected to the FITC channel and high side scatter and tested for their expression of CD68 and FcεRI. Isotype control is shown in grey and anti-CD68 antibody or FcεRI antibody is in black. (D) B-cells were gated based on lymphocyte marker (CD3/19/56)$^+$SSC^{lo}MHCII$^+$BDCA1$^+$ cells and tested for their expression of FcεRI. Isotype control is shown in grey and anti-FcεRI antibody is in black. **(E)** FcεRI$^+$ cells from lung parenchyma were identified by comparing isotype control antibody-stained PMCs (left panel) to FcεRI antibody-stained PMCs (middle panel). FcεRI$^+$ cells from the middle panel were examined for the expression of the DC marker BDCA1 and the mast cell/basophil marker CD203c (right panel). **(F)** Cell populations gated in Fig. 2E (right panel) were compared for expression of MHCII and CD14; only BDCA1$^+$ cells (pink line) are MHCII$^+$CD14$^+$. (G) BDCA1$^+$ DCs were identified from airway biopsies and were examined for FcεRI expression. Among CD45$^+$ cells, BDCA1$^+$ DCs were gated as described in Fig. 1C and examined for the expression of FcεRI$^+$. Isotype control is shown in grey and anti-FcεRI antibody is in black. (H) FcεRI$^+$ cells from airway biopsies were identified and analyzed for the expression of BDCA1

and CD203 as described in Fig. 2E. (I) Cell populations gated in Fig. 2H (right panel) were compared for expression of MHCII and CD14; only $BDCA1^+$cells (pink line) are $MHCII^+CD14^+$.

average expression levels did not significantly differ between health and disease (Fig. 2B). This finding indicates that FcεRI is stably expressed in BDCA1$^+$ DCs in the lungs.

We also examined whether FcεRI was expressed in cell types other than BDCA1$^+$ DCs in the lungs. First, macrophages were examined. These cells were gated by high autofluorescence and high side scatter (Fig. 2C). This gating strategy has been previously used by others (Demedts et al., 2005), and further validated by us confirming the expression of human macrophage markers such as CD68 in gated cells (Fig. 2C). No FcεRI expression was detected in these cells (Fig. 2C, right panel). Secondly, B-cells were examined. They were identified as [CD3/19/56 antibody mixtures]$^+$MHCII$^+$ BDCA1$^+$ (Fig. 2D). No FcεRI expression was detected in these cells, either (Fig. 2D, right panel). Lastly, to get a more comprehensive view of FcεRI-expressing cells present in the lungs, we gated all PMCs that were non-autofluorescent but labeled by FcεRI antibody (Fig. 2E), and determined which cells are included in this gating. Expression of CD203c, a specific marker of human mast cells and basophils, and BDCA1 were examined. We found that FcεRI+ PMCs were segregated into two distinct populations that were either CD203$^+$ or BDCA1$^+$ (Fig. 2E, right panel). This finding suggested to us that the FcεRI$^+$ PMCs are almost entirely composed of basophils/mast cells and BDCA1$^+$ DCs. Further examination of the BDCA1$^+$ cells showed that they were MHCII$^+$CD14$^+$ (Fig. 2F), confirming they were indeed BDCA1$^+$ DCs. Conversely, CD203c$^+$ cells had very low levels of MHCII and CD14 (Fig. 2F). This finding indicates that mast cells/basophils and BDCA1$^+$ DCs are the only significant cell types expressing FcεRI in human lungs.

To expand our examination of FcεRI expression in BDCA1$^+$ DCs in lung parenchyma, we also examined BDCA1$^+$ DCs in airways. Two airway bronchial biopsies were obtained from a patient with asthma and the whole biopsy digests, rather than PMCs, were analyzed by flow

cytometry to maximize the recovery of cells. By looking only at $CD45^+$ cells, we found a distinct population of cells that express BDCA1 but not lymphocyte markers (Fig. 2G). A majority of these cells were $MHCII^+$ (Fig. 2G), confirming that they are $BDCA1^+$DCs. Furthermore, we found that these DCs expressed FcεRI similar to what we found with parenchymal DCs (Fig. 2G, right panel). We also analyzed the composition of $FcεRI^+$ cells in the airway by taking the same approach described above for parenchymal tissues. We found that like in the parenchyma, $FcεRI^+$ cells in the airways were segregated into either $BDCA1^+$ or $CD203c^+$ cells (Fig. 2H), and that $BDCA1^+$ cells but not $CD203^+$ cells expressed MHCII and CD14 (Fig. 2I). Thus, mast cells/basophils and $BDCA1^+$ DCs are the only cell types expressing FcεRI in the airways. These findings indicate that FcεRI can be used as a marker of $BDCA1^+$ DCs in human lungs and airways when combined with either BDCA1, MHCII, or CD14.

$BDCA1^+$ DCs increase in the epithelium of Th2-high but not Th2-low asthmatic airways.
Having found that $BDCA1^+$ DCs in human airways can be identified by their co-expression of FcεRI and MHCII, we examined the prevalence of $BDCA1^+$ DCs in asthma by employing immunofluorescence microscopy of bronchial biopsies using FcεRI and MHCII antibodies. Recent studies have illustrated that human asthma is heterogeneous and can be segregated into two distinct subsets based on differential expression of Th2-responsive genes in the airway epithelium (Woodruff et al., 2009). “Th2-high” asthma is characterized by high levels of Th2-responsive gene expression and it is associated with mast cell infiltration, sub-epithelial fibrosis, and eosinophilia (Woodruff et al., 2009). In comparison, “Th2-low” asthma is characterized by the usual airflow dysfunction but relative absence of mast cell infiltration, sub-epithelial fibrosis, and eosinophilia (Woodruff et al., 2009).

		Asthmatic	
	Healthy	**Th2-low**	**Th2-high**
Number	7	8	8
Age ± SD	36 ± 12	30 ± 12	41 ± 8
Gender M:F (% F)	5:2 (29%)	6:2 (25%)	5:3 (38%)
Ethnicity			
White	5	5	6
African American	0	1	1
Asian	1	1	1
Pacific Islander	0	0	0
Hispanic	1	1	0
FEV1 % predicted ± SD	92% ± 7	91% ± 10	79% ± 10

Table 2: Subject data for asthmatic cohort. Healthy or asthmatic subjects were enrolled and tested for airway hyper-responsiveness as measured by airway hyper-responsiveness upon methacholine challenge and allergies as measured by serum IgE. Th2 skew was categorized by methods from Woodruff et al. (Woodruff et al., 2009). Age and FEV % predicted are shown as mean ± SD.

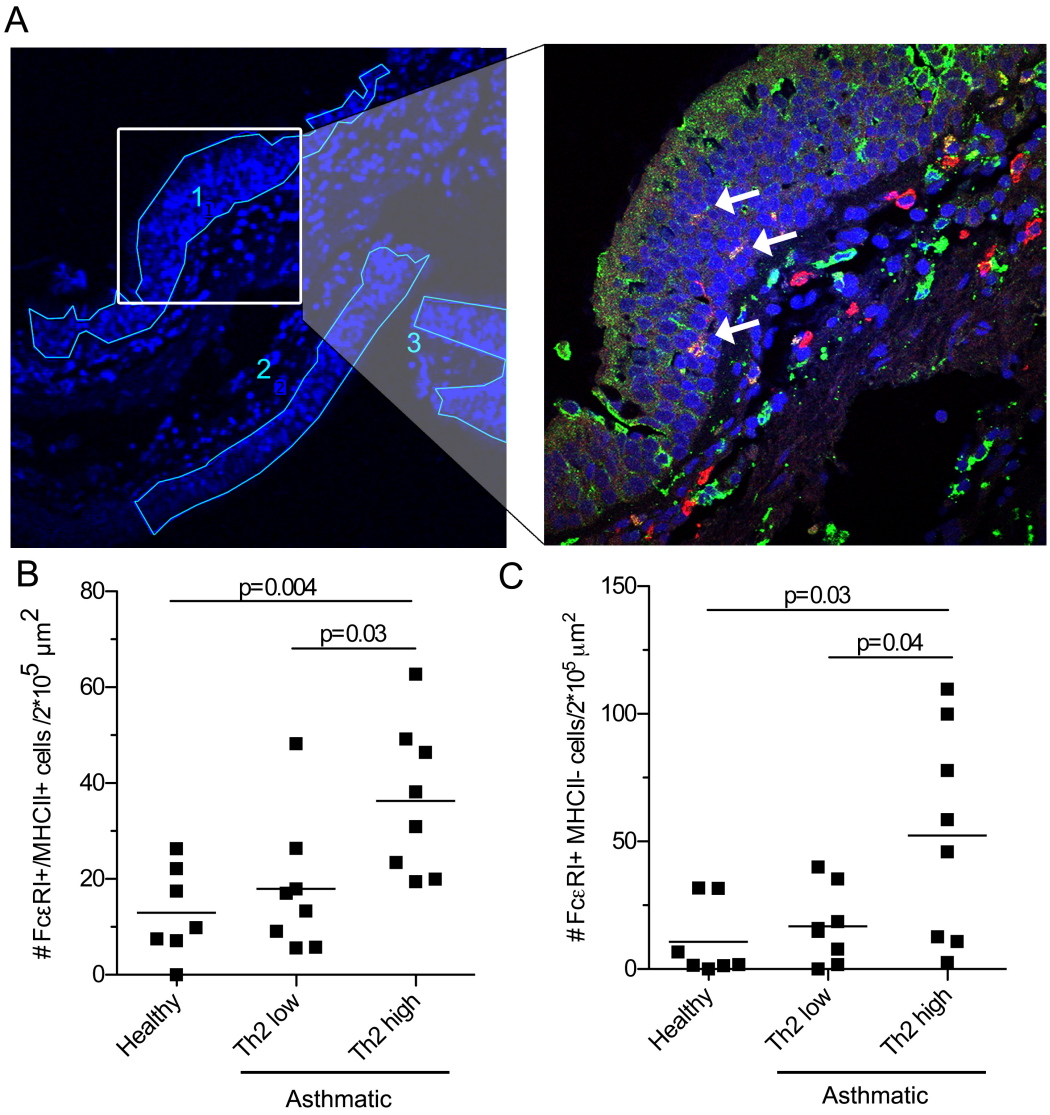

Figure 3: BDCA1$^+$ DCs increase in the epithelium of Th2-high but not Th2-low asthmatic airways. (A) Left panel, representative image of bronchial airway biopsy showing DAPI staining in blue (other channels are omitted for clarity). Lumen is located at the image peripheries and sub-epithelial tissues are centrally located. Images were obtained by a Nikon C1si microscope at 20x magnification. Areas of airway epithelium (numbered) were manually outlined using FIJI software and area was calculated for each individual biopsy. Region highlighted in left was imaged at 60x as shown in right panel (lumen is located to the top left); DCs were identified based on expression of MHCII (green) and FcεRI (red) and presence of significant cell body. Double-positive DCs in epithelium are indicated with arrows. **(B-C)** FcεRI$^+$ MHCII$^+$ double positive cells (B) and FcεRI$^+$ single positive cells (C) in the epithelium were counted for each biopsy. Number of cells per 2 X 10^5 µm^2 epithelium was calculated. Bar represents mean. Statistical significance (p values) was determined via two-tailed, unpaired Student's t-test.

We examined bronchial biopsies of eight Th2-low asthmatics and eight Th2-high asthmatics compared to seven healthy controls (Table 2). 3mm-thick biopsy sections were stained with FcεRI and MHCII antibodies and subsequently with fluorophore-conjugated secondary antibodies. Images of each section were taken by confocal microscopy at 20x magnification and the area of epithelium was manually marked with Fiji software (Fig. 3A, left panel), which was then quantified as μm^2/biopsy. The same section was then imaged at 60x and the number of $FcεRI^+MHCII^+$ cells in the epithelium was counted. (Fig. 3A, right panel). The reason that we analyzed DCs only within the epithelium is that Th2-low and -high asthma had been characterized based on gene expression profiles of the airway epithelium (Woodruff et al., 2007). In addition, recent mouse studies suggest a significant contribution of epithelial cell-to-DC crosstalk to asthma pathology (Lambrecht and Hammad, 2012), implicating the important role of epithelial DCs in this disease. We found that the number of $FcεRI^+MHCII^+$ cells was significantly increased in the epithelium of Th2-high asthmatics compared to Th2-low asthmatics and healthy controls (Fig. 3B). Thus, $BDCA1^+$ DCs accumulate in the airway epithelium of Th2-high asthma but not Th2-low. We also counted the number of epithelial $FcεRI^+MHCII^-$ cells - presumably mast cells or basophils - and found that these cells also significantly increased in Th2-high asthmatics but not in Th2-low asthmatics (Fig. 3C), consistent with previous findings (Dougherty et al., 2010).

Discussion

Our study demonstrates that IPF is associated with a robust (5-6 fold) increase in $BDCA1^+$ DCs in the parenchyma. By quantifying DCs as a percentage of PMCs, we show a specific increase in DCs as opposed to overall increase in hematopoietic cells. Others have

shown a significant increase in DCs in bronchoalveolar lavage from IPF patients (Tsoumakidou et al., 2009), consistent with our finding. We also found that HP is associated with a dramatic increase in BDCA1$^+$ DCs in lung parenchyma. To our knowledge, this is the first study that examined DC frequency in HP in a quantitative manner. We also found a significant increase in the frequency of DCs in the lungs of COPD, but to a much lesser degree (~ 2 fold) than IPF or HP. Others have examined the frequency of the same interstitial lung DC subsets in COPD and found a slight increase (up to ~ 2 fold) in the median DC frequency compared to healthy controls (Freeman et al., 2009), although it did not reach significance in their statistical analysis. Notably, this study had significant patient variation and used a different method of statistical analysis, which may have lowered their threshold for statistical significance.

Our finding that DCs dramatically increased in IPF and HP implicates a potential contribution of DCs to these two diseases. As DCs are potent activators of T-cells, they may contribute to lymphocyte-mediated inflammation in these diseases. Consistent with this hypothesis, chemokines that favor recruitment and accumulation of DCs and lymphocytes have been shown to significantly increase in the bronchoalveolar lavage fluid and lungs of IPF or HP patients (Burkhardt et al., 2012; Marchal-Sommé et al., 2007; Pisabarro et al., 2006). While there are notable infiltrates of mixed lymphocytic populations in IPF lungs (Scadding and Hinson, 1967), the major pathology associated with IPF is progressive, extensive fibrosis of the lungs (Noble et al., 2012), and the ineffectiveness of immunosuppressant treatment such as corticosteroids (Richeldi et al., 2003) has led to the belief that immune cells are not likely involved in the disease process. Like IPF, late stage, chronic HP is also associated with severe fibrosis in the lungs (Hanak et al., 2007), though there is also a significant contribution of lymphocytic inflammation especially at early stages of the disease (Selman et al., 2012).

Alternatively, or in addition to their role in activating inflammatory T-cells, DCs may significantly contribute to fibrosis in these diseases. In a mouse model of bleomycin-induced lung fibrosis, pharmacological inactivation of DCs resulted in a marked reduction in fibrosis (Bantsimba-Malanda et al., 2010). Furthermore, DCs in IPF accumulate in fibrotic foci (Marchal-Sommé et al., 2007) and recent evidence indicates that they can regulate the vasculature and function of fibroblast-type cells (Chyou et al., 2011). Indeed, DCs can express TGF-β1, which could potentially act directly on mesenchymal cells to modulate their behavior to be more profibrotic (Gruschwitz and Hornstein, 1992; Zhou and Tedder, 1995).

Our studies indicate that FcεRI can serve as a useful marker of BDCA1$^+$ lung DCs. FcεRI expression by antigen presenting cells in human lungs has been previously examined by immunohistochemistry, which showed that some CD1a$^+$ cells were FcεRI$^+$ (Tunon-De-Lara et al., 1996). However, the extent to which FcεRI is expressed in a more comprehensive lung DC subset has not been examined. Our study indicates that FcεRI is constitutively expressed in BDCA1$^+$ lung DCs, similarly to what had been found in blood, tonsil (Foster et al., 2003), and intestine (Bannert et al., 2012). Furthermore, a recent study revealed that FcεRI is expressed in BDCA1$^+$ DCs in human arthritic synovial fluid and malignant tumor ascites (Segura et al., 2013), and additionally demonstrated that FcεRI is a useful marker that distinguishes DCs from macrophages, which do not express FcεRI but do express mannose receptors and DC-SIGN (Segura et al., 2013). Collectively, these and our findings suggest that FcεRI can serve as a useful marker for BDCA1$^+$ DCs in the lungs and possibly many other human tissues.

We found that the majority of BDCA1$^+$ DCs in the lungs express CD14 while those in the blood do not. A previous study has shown that BDCA1$^+$ DCs in tissues of inflammatory conditions express CD14 and suggested that CD14 expression is a specific feature of

inflammatory DCs (Segura et al., 2013). However, this study did not analyze DCs from any non-inflammatory tissue environment. Therefore, it was unclear whether CD14 expression is linked to the inflammatory environment or due to the distinct property of tissue DCs versus blood DCs. When we examined BDCA1$^+$ DCs in healthy lungs, we found that most express CD14. Thus, we suggest that CD14 expression is not a marker of inflammatory DCs, but a marker of tissue DCs as opposed to circulating blood DCs. Interestingly, a minor fraction of BDCA1$^+$ DCs in the blood also express CD14 along with FcεRI; these cells may represent DCs that are initiating or are in the process of migrating into peripheral tissue sites.

By employing dual-antibody immunofluorescence microscopy using FcεRI and MHCII antibodies, we found that BDCA1$^+$ DCs are enriched specifically in Th2-high asthmatic airway epithelium. One might suspect that DCs of healthy and Th2-low asthmatics were undercounted in association with their FcεRI expression levels compared to Th2-high asthmatics, as Th2-associated conditions such as high serum IgE tend to correlate with surface FcεRI expression (Dehlink et al., 2010). However, we consider this unlikely because we were able to readily localize rare FcεRI^{low} cells by using a high laser power and a high magnification optic (60X), and thus were able to clearly distinguish them from FcεRI$^-$ cells. One might also argue that basophils or mast cells contaminated our DC identification strategy because of potential MHCII expression in these cells. Besides remaining controversy surrounding MHCII expression levels in lung mast cells or basophils (Galli et al., 2005; Hammad et al., 2010), we found their MHCII expression levels to be 10-100-fold lower than DCs (Fig. 2F), even in Th2-high asthmatic airways (Fig. 2I). This large difference was readily distinguishable with fluorescence microscopy by keeping the laser power within a non-saturating range of MHCII levels. Thus, it is unlikely that mast cells or basophils were included in our MHCII$^+$FcεRI$^+$ cell counting.

Our finding of BDCA1$^+$ DC accumulation in Th2-high asthma is consistent with data in mouse models of asthma, and the functional role of BDCA1$^+$ DCs in asthma can be predicted from these studies. Depletion of mouse lung DCs or inhibition of their function has been shown to abrogate asthma-like pathology in mouse models (Idzko et al., 2006; van Rijt et al., 2005). DCs also have been found to play a critical role in inducing and maintaining Th2 immunity in mouse lungs (Idzko et al., 2006; Sallmann et al., 2011). Similar to DCs in mice, BDCA1$^+$ DCs in humans may play a crucial role in generating and maintaining Th2 immunity in asthma. Notably, Th2-high asthma typically responds well to steroid therapy (Woodruff et al., 2009), and steroid treatment has been shown to lower the number of DCs in airways (Nelson et al., 1995). DC accumulation may be tightly connected to disease exacerbation; however, the specific mechanism by which airway epithelial DCs accumulate remains to be determined. Epithelial cells in Th2-high asthma express a number of cytokines and chemokines (Woodruff et al., 2007) which may recruit DCs to the epithelium. Alternatively, DCs may proliferate in the epithelium of Th2-high asthmatics; interestingly, a recent study has shown that airway epithelial DCs but not interstitial DCs proliferate in a Th2 mouse asthma model (Veres et al., 2013). Given the central role of DCs in inducing and perpetuating Th2 inflammation in mouse and possibly human asthma, understanding the mechanism by which DCs increase in the airway epithelium may provide a novel therapeutic approach to control this disease.

In conclusion, by using flow cytometry and dual-color immunofluorescence microscopy we found that IPF, HP, COPD, and Th2-high asthma are associated with the accumulation of BDCA1$^+$ DCs at the sites of disease pathology. Further biochemical and functional analyses of this DC subset are expected to improve our understanding of the pathogenesis of these diseases.

Acknowledgements

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CHAPTER III: FcεRI EXPRESSION IN HUMAN DENDRITIC CELLS AND MONOCYTES MEDIATES CELLULAR ENTRY OF CIRCULATING IgE, CONTRIBUTING TO SERUM IgE CLEARANCE

The following is in press at *The Journal of Clinical Investigation* as "A role of human FcεRI in serum IgE turnover".

Abstract

The high affinity IgE receptor, FcεRI, is constitutively expressed in mast cells and basophils and plays an important role in transmitting stimulatory signals upon engagement of IgE-bound allergens. FcεRI is also constitutively expressed in dendritic cells (DCs) and monocytes in humans. However, the specific functions of FcεRI expressed by these cells are not completely understood. Here, we report that FcεRI expressed by human BDCA1$^+$ DCs and monocytes but not basophils traffics to endolysosomal compartments in the steady state. IgE bound to FcεRI on BDCA1$^+$ DCs but not basophils was rapidly endocytosed, transported to the lysosomes, and degraded in vitro. IgE injected into human FcεRIα-Tg mice was also endocytosed by conventional DCs and monocytes, and this endocytosis accompanied rapid clearance of circulating IgE from these mice. Importantly, this rapid IgE clearance was dependent on monocytes or DCs but not basophils. These findings strongly suggest that constitutive internalization of human FcεRI by DCs and monocytes distinctively contributes to serum IgE clearance.

Introduction

FcεRI is the high affinity IgE receptor best known for its role in mediating allergic reactions. It is assembled from multiple protein subunits: an IgE-binding α subunit, two immunoreceptor tyrosine-based activation motif (ITAM)-containing, signal-transducing γ subunits, and an ITAM-containing, signal-amplifying β subunit (Dombrowicz et al., 1998; Donnadieu et al., 2000; Lin et al., 1996)(Gould and Sutton, 2008). The α subunit associates with the β and/or γ subunits in the endoplasmic reticulum (ER), which is required for ER exit and subsequent transport to the plasma membranes (Letourneur et al., 1995a).

FcεRI is highly expressed in mast cells and basophils. When crosslinked by cognate allergens, IgE/FcεRI complexes on these cells initiate a signaling cascade that induces degranulation, which results in the release of inflammatory mediators such as histamine and creates the typical symptoms of acute allergic reaction (Gould and Sutton, 2008). In addition to basophils and mast cells, humans, but not rodents, additionally express FcεRI in dendritic cells (DCs: $BDCA1^+$ DCs, plasmacytoid DCs, and Langerhans cells) and monocytes in the steady state (Foster et al., 2003; Maurer et al., 1994; Takenaka et al., 1995). In cases of inflammation, such as viral infection, mice express FcεRI in some DCs (Grayson et al., 2007; Hammad et al., 2010; Plantinga et al., 2013). Unlike mast cells and basophils, DCs and monocytes lack FcεRIβ and thus express FcεRI in its trimeric form (αγγ) (Maurer et al., 1996). Previous studies have shown that when crosslinked by multivalent antigens, antigen:IgE:FcεRI complexes are rapidly endocytosed by $BDCA1^+$ DCs and monocytes, and the antigens are subsequently presented to T cells (Maurer et al., 1995; 1998). This antigen presentation has been suggested to significantly contribute to Th2 inflammation associated with allergic diseases (Maurer et al., 1995; 1998; Sallmann et al., 2011).

IgE binding to FcεRI has been shown to stabilize FcεRI expression at the cell surface in vitro (Borkowski et al., 2001). Consistent with cell surface stabilization, mast cell and basophil FcεRI surface levels increase as serum IgE concentration increases both in humans and mice (Conroy et al., 1977; Foster et al., 2003; Saini et al., 2001; Yamaguchi et al., 1997). This presumably enhances the ability of mast cells and basophils to sense and react to allergens during allergic responses. However, whether FcεRI on DCs and monocytes is also stabilized by IgE binding is not clearly established. Some studies have shown that surface FcεRI of human blood BDCA1$^+$ DCs and monocytes correlates positively with serum IgE levels (Sihra et al., 1997; Vasudev et al., 2012). However, other studies have shown a lack of correlation between IgE levels in blood and FcεRI levels on BDCA1$^+$ DCs or monocytes among individuals with normal ranges of serum IgE levels (Dehlink et al., 2010; Foster et al., 2003). These findings raise a possibility that FcεRI surface expression in DCs and monocytes may be regulated uniquely from mast cells and basophils, and perhaps independently of IgE.

In this study, we compared human blood basophils and BDCA1$^+$ DCs for their ability to regulate surface FcεRI expression in response to serum IgE. We also examined FcεRI intracellular trafficking in these cells as well as monocytes, and how FcεRI trafficking influences the fate of IgE. From these and additional studies using human FcεRI-transgenic mice, we reveal that FcεRI expressed in DCs and monocytes distinctively traffics to lysosomes, and uniquely participates in serum IgE clearance.

Materials and Methods

Mice

hFcεRIα transgenic mice have been previously described (Dombrowicz et al., 1996). Neither transgenic nor littermate Tg^- control mice express mFcεRIα. In all experiments, mice were between 4 and 8 weeks old, except bone marrow chimeras, which were 8-12 weeks old. All mice were housed in the UCSF animal facility.

Antibodies

Human IgE was obtained from Abcam and BioFrontTech and was extensively dialyzed against PBS prior to in vivo experiments. Unlabeled antibodies for confocal microscopy included rabbit polyclonal anti-human/anti-mouse Calnexin (Abcam), rabbit polyclonal anti-human TGN46 (Abcam), rabbit polyclonal anti-human EEA1 (Abcam), rabbit polyclonal anti-human HLA-DR (DRAB2, obtained from Yale), mouse monoclonal CRA-1 (anti-human FcεRI α subunit, eBioscience), and goat anti-human IgE (Vector). Labeled antibodies for confocal microscopy included Alexa 488-H4A3 (mouse IgG1 anti-human Lamp1, eBioscience), Alexa 647-H4A3, and Alexa 488-1D4B (rat IgG2a anti-mouse Lamp1, Biolegend). Labeled secondary antibodies for confocal microscopy included Invitrogen goat anti-mouse IgG2b-Alexa 568, goat anti-rabbit-Alexa 647, and rabbit anti-goat Alexa 568. Labeled anti-human monoclonal antibodies for flow cytometry included Biolegend Percp/Cy5.5-BDCA1 (L161, mouse IgG1), Pacific Blue-HLA-DR (L243, mouse IgG2a), PE/Cy7-CD14 (HCD14, mouse IgG1) and CD123 (6H6, mouse IgG1), APC- FcεRIα, PE-FcεRIα (both mouse IgG2b CRA-1), and FITC-CD3 (HIT3A, mouse IgG2a), CD19 (HIB19, mouse IgG1), and CD56 (MEM-188, mouse IgG2a). Labeled anti-mouse antibodies for flow cytometry included Biolegend PE/Cy7-CD11c (N418, Armenian hamster Ig), biotin-CD115 (AFS98, rat IgG2a), Percp/Cy5.5-CD11b (M1/70, rat IgG2b), Pacific Blue-MHCII (M5/114.15.2, rat IgG2b), Alexa 700-Gr1 (RB6-8C5, rat IgG2b),

FITC-CD49b (DX5, rat IgM), APC-ckit (ACK2, rat IgG2b), and biotin-anti-human IgE (MHE-18, mouse IgG1). PE-CD131 (rat IgG1) and biotin-CD45RA (14.8, rat IgG2b) were purchased from BD Biosciences. Antibodies for FcεRIα immunoprecipitation and Western blot included a rabbit polyclonal anti-FcεRIα (Upstate) and TrueBlot HRP-conjugated, light-chain specific mouse anti-rabbit IgG (eBioscience). Antibodies for human IgE ELISA included unlabeled G7-18 anti-human IgE (mouse IgG2a, BD Biosciences Pharmingen), and biotinylated MHE-18 anti-human IgE (mouse IgG1, Biolegend). Rat IgG1 anti-mouse IL3 antibody (MP2-8F8) for basophil enrichment was purchased from Biolegend.

Blood and lung donors

Blood was collected from reportedly healthy individuals who have no history of allergic rhinitis or asthma. Human lung tissues were obtained from lungs originally designated for transplantation by the Northern California Transplant Donor Network but ultimately not used for various reasons or through lungs resected from patients with lung disease.

Isolation of human DCs, basophils, and monocytes

PBMCs were isolated using Ficoll Plaque Plus (GE Healthcare). $BDCA1^+$ DCs were isolated using the Miltenyi Biotec human DC isolation kit. Non-DCs were saved and from this, basophils were isolated using the Miltenyi Biotec basophil isolation kit. Cells were checked for purity via flow cytometry; $BDCA1^+$ DCs were 96.4 % ± 0.3 % SEM pure and were the only FcεRI-expressing cells in the isolates; basophils were approximately 69.8 % ± 3.5 % SEM pure and were the only FcεRI-expressing cells in the isolates. Monocytes were isolated from PBMCs with the Miltenyi Biotec $CD14^+$ isolation kit followed by flow cytometric sorting for $BDCA1^-$

cells, yielding extremely pure monocytes (> 99%). Lung BDCA1$^+$ DCs were isolated as following: pieces of lung parenchyma were minced and placed in digestion buffer (HBSS containing 10 mg collagenase D and 2 mg DNAse I) for 45 min at 37 °C while rocking. The generated single cell suspension was run through Ficoll gradient centrifugation to isolate mononuclear cells. BDCA1$^+$ DCs were isolated from these mononuclear cells using the Miltenyi Biotec human DC isolation kit followed by flow cytometry cell sorting (> 99 % pure).

Isolation of mouse blood monocytes and basophils, peritoneal mast cells, and lung DCs, and CD11b$^+$ BMDCs.

To isolate blood monocytes and basophils, PBMCs were isolated from blood with Ficoll Paque Plus. Cells were then stained using an antibody cocktail designed to identify monocytes (CD11b and CD115), and basophils (CD131 and DX5) in 5% BSA/PBS. Each cell type was sorted using a FacsAria3 and FACSDiva into 50 % cold FCS. To isolate peritoneal mast cells, mice were peritoneally lavaged with 3 mL of PBS. Lavaged cells were stained using a c-kit antibody and c-kit$^+$SSC^{hi} cells were sorted by FACS. To isolate lung DCs, mouse lungs were perfused with cold PBS, then excised and digested using the GentleMacs (Miltenyi Biotec) dissociator and digestion buffer (HBSS with 10 mg collagenase D and 2 mg DNAse I) and incubated for 30 min at 37 °C to generate a single cell suspension. Cells were stained with anti-MHCII and anti-CD11c antibodies and CD11c$^+$MHCII^{hi} cells were sorted by FACS. To isolate CD11b$^+$ BMDCs, BMDCs were cultured using Flt3L as described (Naik et al., 2005). Cells were stained using anti-CD11c, anti-CD45RA, and anti-CD11b antibodies and CD11c$^+$CD45RA$^-$ CD11b$^+$ cells were sorted by flow cytometry.

Generation of bone marrow chimeras

Bone marrow was isolated from femurs and tibias of donor mice, pooled into either Tg^+ or Tg^- groups, and placed on ice until injection. Recipient Tg^- mice were irradiated with 550 rads given in two doses, three hrs apart, then were reconstituted with 10^7 bone marrow cells within 12 hrs of the second irradiation. Mice were housed with antibiotic tablets and their health checked daily for 4 weeks following irradiation.

Administration of Fl3L-producing melanoma or IL3/anti-IL3 antibody complexes in mice

Murine Flt3L-producing B16 melanoma cells (Mach et al., 2000) were cultured in DMEM supplemented with 10% FBS, penicillin/streptomycin and L-glutamine before harvesting for injection. 10^6 cells were injected subcutaneously into the backs of anesthetized mice. Mice were analyzed 14 days post injection. Murine IL3 was purchased from Peprotech and was stored at 1 μg/μL at -20 °C until use. Just before injection, 10 μg of IL3 was thawed and mixed at room temperature with 2 μg of anti-IL3 antibody for 1 min. After 1 minute, the mixture was diluted with PBS to 100 μL total. Mice were anesthetized and, injected i.v. with the IL3/antibody mixture, and analyzed 5 days post-injection.

Cell culture

Bone marrow-derived DCs (BMDCs) were generated by culturing hFcεRIα-Tg mouse bone marrow for 6 days in DMEM media supplemented with 10 % FBS, L-glutamine, penicillin/streptomycin, and 100 ng/mL recombinant mouse Flt3L. At day 6, cells were stained and sorted for $CD11b^+$ cells. Bone marrow-derived mast cells (BMMCs) were generated by isolating bone marrow from an hFcεRIα-Tg mouse and culturing the cells for three weeks in

RPMI media supplemented with 10% FBS, L-glutamine, penicillin/streptomycin, IL3 (5 ng/ml), and SCF (5 ng/ml). Media and culture flasks were changed every week. Purity of the cells was determined by flow cytometry at the end of three-week culture; over 95 % of cells were c-kit^{+}hFcεRI^{+}.

Hexosaminidase release assay

The colorimetric hexosaminidase release assay was employed as described (Shin et al., 2006b). Briefly, BMMCs equilibrated with Tyroid buffer were mixed with human IgE or anti-hFcεRI Ab:anti-mouse IgG Ab complexes in a 96 well plate. After incubating at 37 °C for 1 hr, the plate was centrifuged, supernatant was collected, and cell pellet was lysed with 0.1 % Triton X-100. The supernatant and cell lysates were mixed with the hexosaminidase substrate, p-nitrophenyl-N-acetyl-b-D-glucosaminide (1 mM). After incubating at 37 °C for 1 hr, 0.1 M sodium acetate buffer was added to stop the enzyme reaction and absorbance was read at 400 nm.

Confocal microscopy

Single-cell suspensions layered on Alcian-blue treated coverslips were fixed with 4 % paraformaldehyde, permeabilized with 0.05 % saponin in 10 % of goat serum or 5 % BSA, and stained with specific primary antibodies or isotype control antibodies followed by fluorophore-conjugated secondary antibodies. Confocal microscopy was performed with a Nikon C1si spectral confocal microscope with a 60x plan Apo oil-immersion objective (Nikon) at the Biological Imaging Developmental Center at UCSF or using a Leica SP5 spectral confocal microscope with a 63x plan Apo oil-immersion objective (Leica).

Immunoprecipitation and Western blot

7.5 X 10^5 human blood basophils and 6.4 X 10^6 blood BDCA1$^+$ DCs were isolated from apheresis tubing of an anonymous donor. Cells were lysed in 75 and 640 μL of cold lysis buffer containing 1% Triton-X, 10 mM Tris, 100 mM NaCl, 5 mM $MgCl_2$ (pH 7.6) with 20 mM N-ethylmaleimide (NEM, Sigma) and protease inhibitor cocktail (Thermo) freshly added. FcεRIα was immunoprecipitated with a rabbit polyclonal anti-FcεRIα antisera (Upstate) and protein G sepharose beads (GE Healthcare). The immunoprecipitates were split into two samples. One sample had sample buffer added and was boiled for 10 min, and the other sample was treated with EndoH (New England Biolabs) for 2 hrs at 37 °C before sample buffer was added. Samples were run on SDS-PAGE and Western blot analysis was performed using FcεRIα-specific rabbit antisera and HRP-conjugated anti-rabbit antibody.

Quantification of fluorescence intensity from confocal micrographs

Single-cell images from human or mouse were obtained at a distance of 3 μm from the bottom of the cell via confocal microscopy using controlled laser intensity and gain for each cell type. For each image, the intracellular portion and cell membrane ('extracellular') were identified and the mean fluorescence intensity/area was determined by using FIJI software. For human cells, 30 BDCA1$^+$ DCs and 30 basophils were analyzed and plotted as intracellular/extracellular mean fluorescence intensity (MFI)/area. For mouse cells, 10 monocytes and 10 basophils from Tg$^-$ mice following IgE injection had their intracellular and extracellular IgE MFI determined and averaged to control for autofluorescence. Then, 30 monocytes and basophils from Tg$^+$ mice injected with IgE were analyzed and corrected for

autofluorescence by subtracting the Tg^- averages, then plotted as intracellular/extracellular IgE MFI.

Acid stripping of human DCs and basophils

Human PBMCs were isolated as described above and put into two samples. One sample was incubated with ice-cold acetic acid buffer (0.2M acetic acid, 0.15M NaCl, pH 2.5) for 5 min on ice, and the other sample was incubated with PBS. After 5 min, samples were spun at 1300 rpm for 5 min without adding any media. After spinning, the supernatant was aspirated and cells were resuspended in 5% BSA in PBS and spun again. All samples were then fixed and permeabilized using the BD Fixation/Permeabilization kit with manufacturer's instructions before staining with antibodies against cell-specific markers and IgE or an isotype control. Parallel samples of non-fixed and non-permeabilized cells were also stripped, stained, and analyzed as controls for the efficiency of acid-stripping.

Flow cytometry

All samples were run using FACSDiva on a BD LSRII cytometer at low or medium speed, and all experiments were analyzed using FlowJo software (Treestar). For all experiments excluding intracellular staining, live (PI-negative) singlet cells were used for final data analysis.

Human IgE ELISA

For serum IgE ELISA experiments, 2.5 μg of human IgE in 100 μL PBS was injected retro-orbitally into isoflurane-anesthetized mice. 0 hr time point serum was taken at least one day before the experiment. At 1, 2, 4, 8, and 24 hrs post-hIgE injection, a small amount of blood was

collected into PCR strip tubes via submandibular bleed and serum was collected for ELISA. Samples were diluted to 1:40 in 1 % BSA for ELISA, and human IgE from the same lot was also diluted for a standard curve. A standard ELISA protocol was used with G7-18 and biotinylated MHE-18 anti-IgE antibodies. Plates were read using an X-Flour plate reader and data analysis was performed with Excel and Prism software.

Statistical analysis

For all experiments, statistical significance was determined via unpaired, two-tailed T-tests. P values of < 0.05 were considered significant.

Study approval

All animal experiments and procedures were performed according to protocols approved by the UCSF Institutional Animal Ethics Committee. Human blood donors gave written and informed consent for flow cytometric analysis of blood cells and clinical testing of serum IgE levels. Experiments using human blood and lung samples were performed under the UCSF CHR approved protocols #11-07039, #10-02596, and #10-00198.

Results

The surface level of FcεRI is tightly regulated in BDCA1$^+$ conventional DCs compared to basophils.

We recruited 11 adult healthy blood donors (Supplementary Table 1) and examined the correlation between serum IgE levels and surface FcεRI levels in basophils and BDCA1$^+$ DCs (hereafter referred to in the Results as 'DCs'). Serum IgE concentration was determined by

Variable		Healthy non-asthmatics
Subjects (n)		11
Age, mean ±SD		29.55 ±3.47
Gender		
	Male, n (%)	7 (64)
	Female, n (%)	4 (36)
Race (n)		
	Caucasian	6
	Asian	5

Supplemental Table 1: Human blood donors

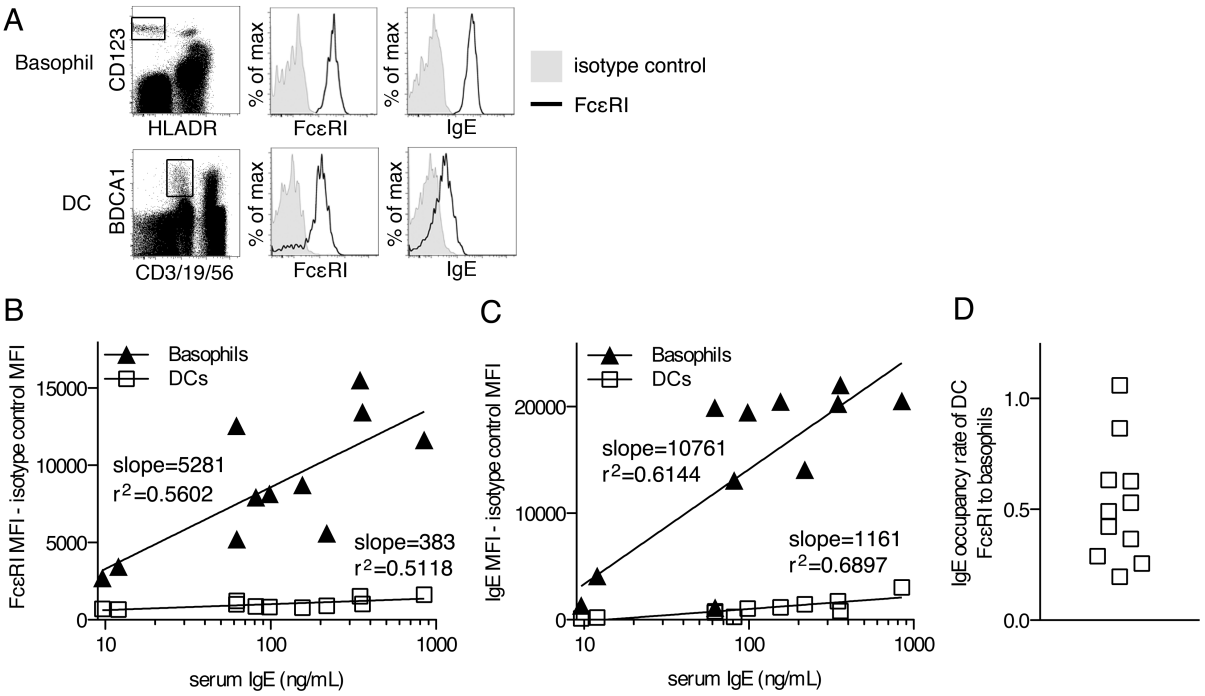


Figure 1. DCs regulate FcεRI surface expression more tightly than basophils. (**A**) Gating strategy of human basophils and DCs, and histograms of surface FcεRI expression and surface IgE bound to the cells. Anti-hFcεRIα antibody (CRA-1) and anti-hIgE staining is shown in black, and isotype control antibody staining is shown in grey. (**B**) Surface FcεRI levels of human blood basophils and DCs in healthy donors. PBMCs were analyzed for FcεRI expression by flow cytometry and serum was analyzed for IgE concentration by ELISA. Isotype control stain MFI was subtracted from anti-hFcεRIα antibody stain MFI. Each donor was plotted according to their serum IgE level. Lines of best fit were calculated and drawn; slope and r^2 values are shown. (**C**) Surface IgE levels for the same donors as in (B) with similarly calculated lines of fit. (**D**) IgE occupancy rate of FcεRI in DCs as compared to basophils. The MFI of IgE was divided by the MFI of FcεRI for DCs and basophils after subtracting appropriate isotype control MFI values to generate an 'occupancy index'. DC occupancy index was divided by basophil occupancy index to generate an 'occupancy rate' of DCs compared to basophils.

ELISA. FcεRI surface levels were determined by flow cytometry using the antibody CRA-1, which binds to FcεRI irrespective of its binding to IgE (Figure 1A). IgE surface levels were also determined by flow cytometry using an anti-IgE antibody (Figure 1A). We found that FcεRI surface levels increased with serum IgE concentration both in basophils and DCs, but to a much lesser degree in DCs; when a line of best fit was calculated for each cell type, the slope for basophils was more than an order of magnitude higher than the slope for DCs (Figure 1B). The same trend was seen with surface IgE levels, which rose rapidly in basophils with serum IgE but much slower in DCs (Figure 1C). Thus, surface FcεRI levels in DCs correlate with serum IgE to a much lesser degree than those in basophils, indicating comparatively tight regulation of surface FcεRI in DCs.

Previous studies have shown that DCs lack FcεRIβ (Maurer et al., 1996) and that FcεRIβ promotes FcεRIα transport from the ER to the plasma membranes, thus enhancing FcεRI surface expression (Donnadieu et al., 2000). Consistent with this idea, we found that DCs had much lower FcεRI surface levels than basophils in every donor examined (Figure. 1B). IgE surface levels were also lower in DCs (Figure 1C). Interestingly however, the degree of difference appeared bigger for IgE than for FcεRI (See the histograms in Figure 1A as an example), suggesting that FcεRI on DCs might be occupied by IgE to a lesser degree than that on basophils. To quantitatively assess FcεRI occupancy in DCs relative to that of basophils, we divided the mean fluorescence intensity (MFI) of IgE by the MFI of FcεRI for DCs and basophils from each donor, and normalized the value of DCs to the value of basophils. Remarkably, we found that FcεRI occupancy in DCs was lower than that in basophils in 10 out of 11 donors examined (Figure 1D). This was unexpected considering that these cells were exposed to the same IgE pool in the blood and because the IgE-binding portion of FcεRI, the α-chain (Hakimi et al., 1990), is

present in both cell types. The relatively low occupancy of FcεRI in DCs raised the possibility that FcεRI on the surfaces of these cells may turn over in an active manner irrespective of IgE binding. Alternatively, DCs may turn over in the blood much faster than basophils. We sought to address the former possibility by comparatively examining FcεRI trafficking in DCs and basophils.

FcεRI is specifically localized in endolysosomes of DCs and monocytes but not of basophils.
To determine the trafficking patterns of FcεRI, we examined FcεRI localization in blood basophils and BDCA1$^+$ DCs by confocal microscopy. In basophils, FcεRIα was localized in mesh-like structures (Figure 2A) whereas in DCs it was localized in vesicular compartments (Figure 2B). The FcεRI$^+$ mesh-like structures in basophils were extensively labeled with Calnexin, a marker of the ER, but not with Lamp1, a lysosome marker, indicating that basophil FcεRI is specifically localized in the ER (Figure 2A). In contrast, FcεRI$^+$ compartments in DCs were labeled by neither Calnexin nor TGN46 (a marker of Golgi), but partially labeled with EEA1 (an early endosome marker) and extensively labeled with Lamp1 and HLA-DR (lysosome markers) (Figure 2B), indicating that DC FcεRI is specifically localized in endolysosomal compartments. To further determine FcεRI endolysosomal localization in DCs, we incubated DCs with FITC-conjugated ovalbumin (Ova) for 30 min at 37°C, washed extensively, and incubated for another 30 min. This method has been commonly used to label functional endolysosomes (Garrett et al., 2000). We found that the anti-FcεRIα antibody colocalized strongly with FITC (Figure 2B), confirming that FcεRIα is localized in endolysosomes in DCs. To determine whether FcεRI lysosomal localization is restricted to blood DCs or a general feature of DCs regardless of tissue origin, we performed a similar microscopic analysis using

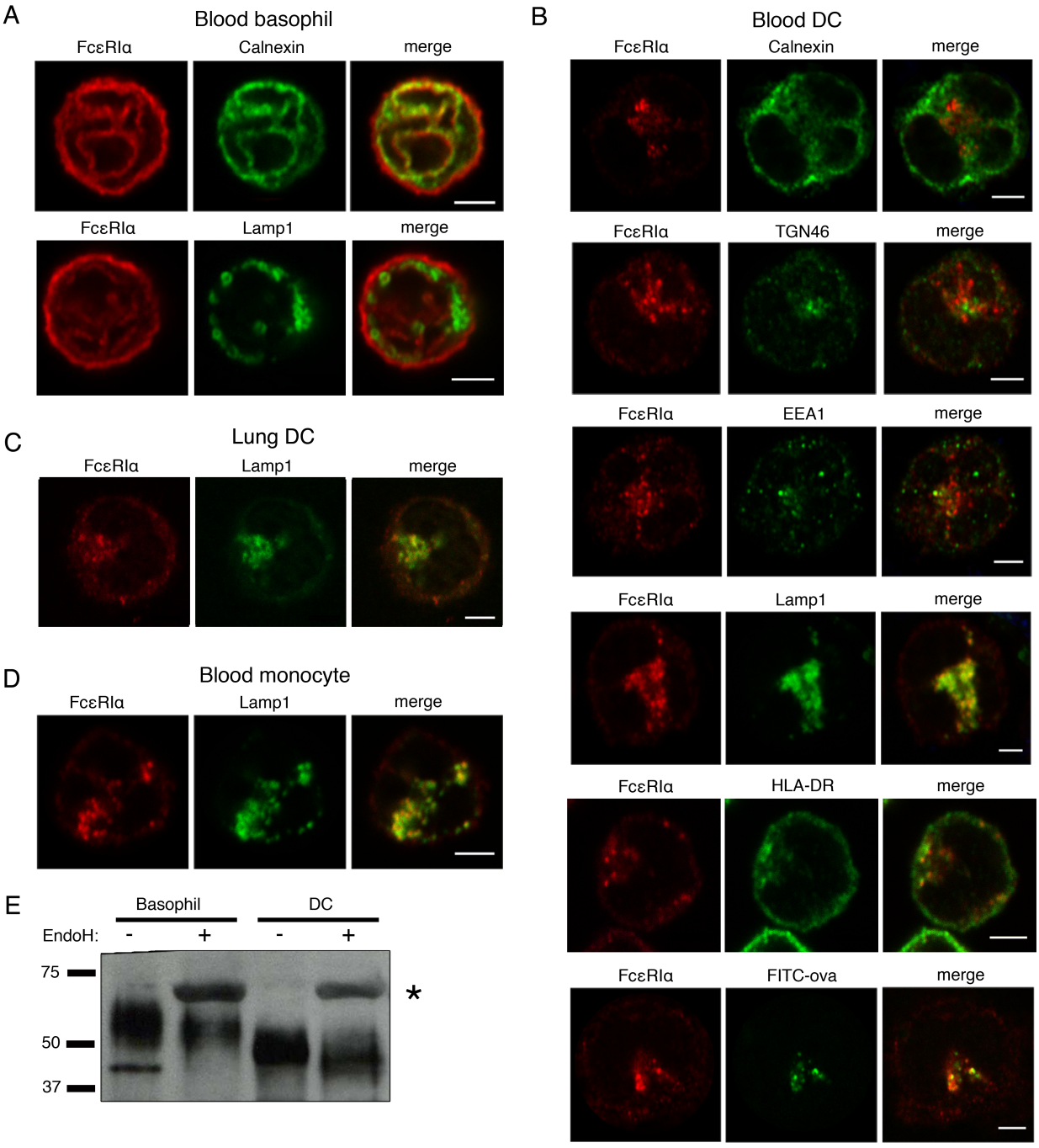


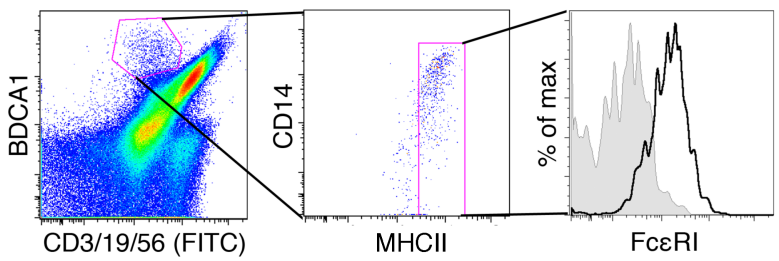
Figure 2. FcεRI is localized in the lysosomes of DCs and monocytes. (A-D) Intracellular localization of hFcεRI in blood basophils (A), blood BDCA1^{+} DCs (B), lung BDCA1^{+} DCs (C), and blood monocytes (D). Each cell type was isolated as described in Methods, stained using indicated antibodies, and examined by confocal microscopy. Basophil images are representative of at least 48 recorded images from at least 7 unique and representative donors. Blood DC images are representative of at least 25 recorded images from 2 - 6 unique and representative

donors. Lung DC image is representative of 10 images from 4 unique and representative donors. Monocyte image is representative of 26 recorded images from 3 unique and representative donors. For all confocal images, magnification is 60x, size bars represent 2.5 μm, and negligible staining by isotype control antibodies was confirmed (Supplemental Figure 2). **(E)** FcεRIα maturation state in basophils and DCs. FcεRIα was immunoprecipitated from blood basophil and blood $BDCA1^{+}$ DC lysates. Half of the immunoprecipitates were treated with EndoH. Resulting samples were run on SDS-PAGE, transferred, and blotted with an FcεRIα antibody. * indicates EndoH that cross-reacted with FcεRIα-antisera.

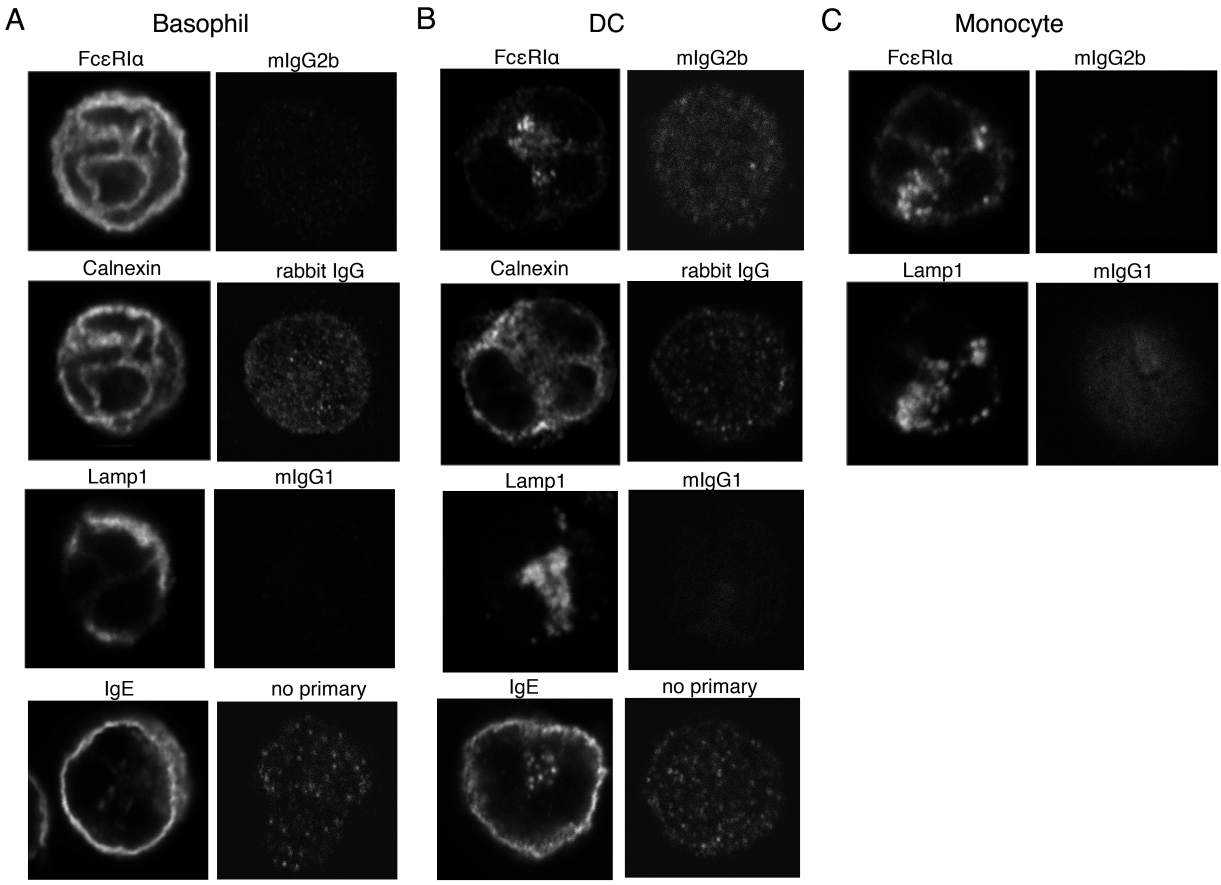
BDCA1$^+$ DCs isolated from the lungs. FcεRI expression in these lung DCs was confirmed by flow cytometry prior to microscopy (Supplemental Figure 1). Similar to blood DCs, lung DCs also localized FcεRI in Lamp1$^+$ lysosomes (Figure 2C). Lastly, we examined FcεRI localization in monocytes isolated from blood and found that it was also localized in lysosomes labeled by Lamp1 (Figure 2D). These findings indicate that FcεRIα is distinctively localized in endolysosomes in human DCs and monocytes.

FcεRI in DCs is mature.

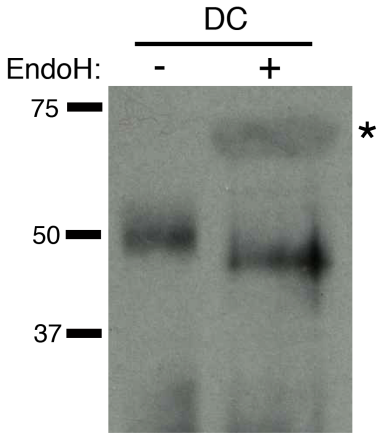
Our confocal microscopy indicated that FcεRIα in basophils is localized in the ER whereas FcεRIα in DCs and monocytes is mostly excluded from the ER. This finding suggests that a significant portion of FcεRIα is immature in basophils whereas a majority of FcεRIα in DCs is mature. To confirm this, we directly examined the maturation status of FcεRIα in basophils and DCs by determining its sensitivity to endoglycosidase H (EndoH). EndoH cleaves the high mannose residues that are attached to immature FcεRIα in the ER; mature α-chain has trimmed glycosylated residues that are resistant to EndoH cleavage (Albrecht et al., 2000). In FcεRIα immunoprecipitates from basophils, we found a sharp band at 45 kD and a smear of bands between 50 and 75 kD (Figure 2E), consistent with a previous report (Saini et al., 2001). The 45 kD band completely disappeared after EndoH treatment reflecting EndoH-sensitive immature FcεRIα whereas the bands between 50 and 75 kD were resistant to EndoH reflecting mature FcεRIα. When the same experiments were performed with DCs, we saw a smear of bands between 40-55 kD, and no extra band was observed. Furthermore, the smear did not disappear after EndoH treatment. This experiment was repeated with cells from another independent donor, and the result was similar (Supplemental Figure 3). This finding suggests that basophils contain



Supplemental Figure 1: Expression of FcεRI in human lung BDCA1$^+$ DCs. Live, singlet cells were stained with a cocktail of antibodies. BDCA1$^+$ DCs were gated by selecting BDCA1$^+$[CD3/19/56]$^-$ cells, then further selecting MHCII$^+$ cells. BDCA1$^+$ DCs are FcεRI$^+$, as shown in the histogram on the right. Anti-FcεRIα antibody (CRA-1) is black, and isotype control is shown in grey. Data shown are from one representative donor of 4 tested.



Supplemental Figure 2: Isotype control stains for confocal microscopy of human cells. Basophils **(A)**, DCs **(B)**, and monocytes **(C)** were isolated as per Methods and were stained with antibodies indicated (left panels) or matched isotype control antibodies (right panels). Note that for basophils and DCs, a 'no primary' antibody control was used for IgE.



Supplemental Figure 3: Second representative Western blot of FcεRIα. DCs were isolated according to Methods and some immunoprecipitated FcεRIα was treated with EndoH. The star indicates EndoH band which cross-reacts with FcεRIα antibody.

both immature and mature FcεRIα, whereas DCs contain FcεRIα mainly in its mature form with distinct carbohydrate moieties attached.

IgE bound to FcεRI on DCs is efficiently internalized, delivered to lysosomes, and degraded.

Our finding that FcεRIα is localized in endolysosomal compartments in DCs raised a possibility that FcεRI might be constitutively endocytosed from the plasma membrane in these cells. In this event, IgE bound to FcεRI at the DC surface would also be endocytosed, and if so, it would be detected inside DCs. To determine the presence of intracellular IgE, we fixed and permeabilized freshly isolated blood basophils and BDCA1^{+} DCs and stained them using an anti-IgE antibody. We found that the anti-IgE antibody mainly labeled the plasma membranes of basophils while it additionally labeled intracellular compartments in DCs (Figure 3A). To quantitatively determine intracellular IgE, used flow cytometry to measure the fraction of total IgE left after surface IgE was stripped by an acid-wash. We found that ~ 6 % of total IgE remained after the stripping of basophils while ~12 % was left in DCs (Figure 3B, left panel). To examine the possibility that this acid-resistant IgE simply represents surface IgE that was not completely stripped, we determined the efficiency of the acid-wash by measuring the fraction of surface IgE remaining after acid treatment. We found that ~ 3 % and 4 % of surface IgE was not stripped in basophils and DCs, respectively (Figure 3B, right panel), indicating that the acid-wash could not completely remove surface IgE. Nevertheless, acid-resistant fraction of total IgE (left panel of Figure 3B) was significantly higher than that acid-resistant fraction of surface IgE (right panel) in both basophils and DCs, indicating that both cell types have intracellular IgE although DCs have significantly more.

To more directly compare the ability of FcεRI on DCs and basophils to mediate IgE

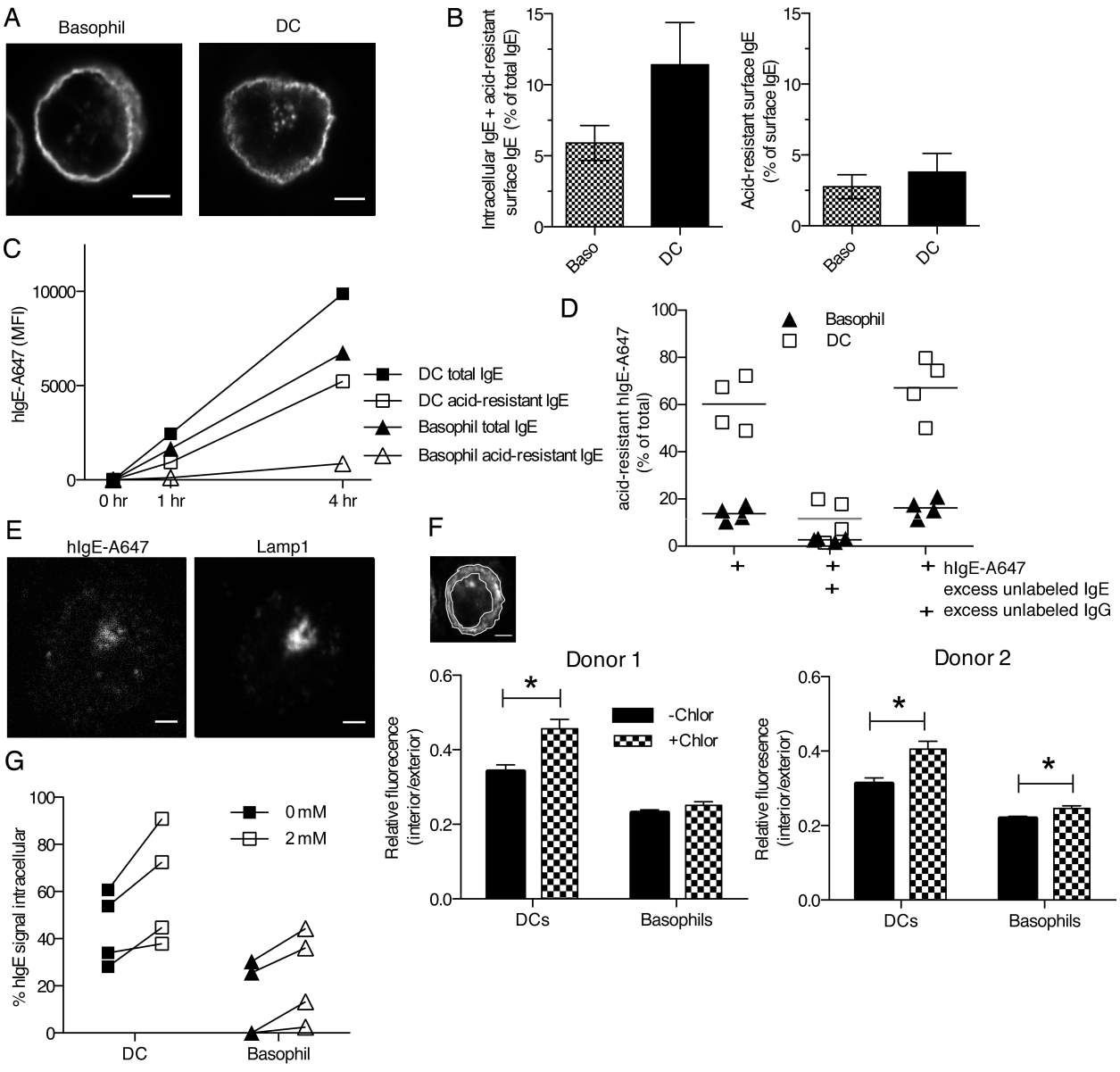
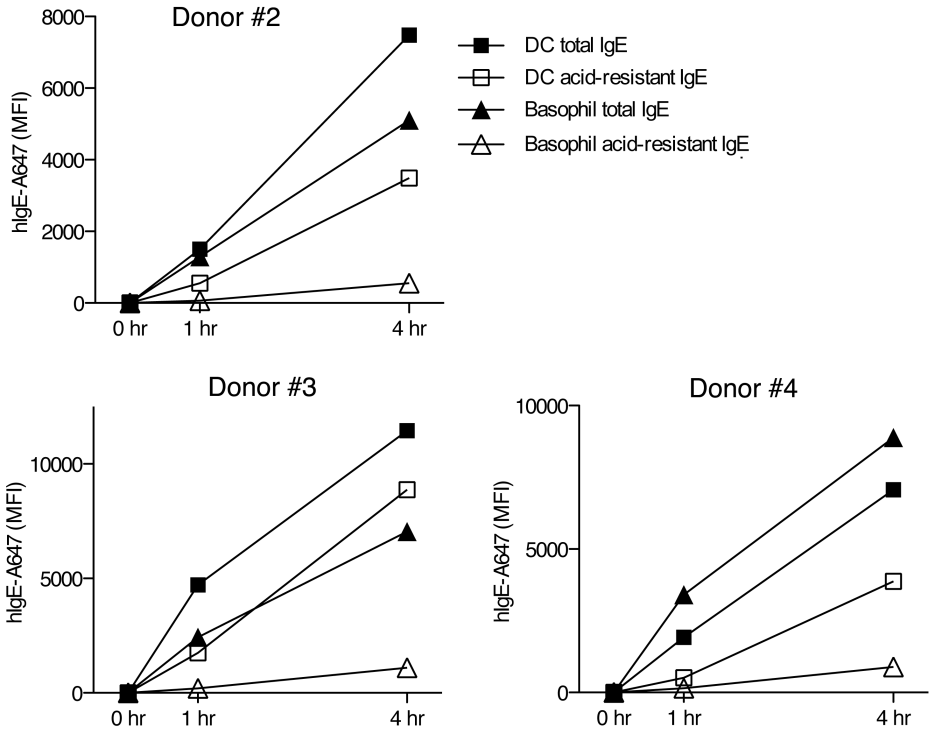


Figure 3. IgE is efficiently internalized and degraded by DCs. (A) Intracellular IgE in basophils and DCs. Basophil image is representative of 83 recorded images from 7 unique and representative donors, and DC image is representative of 121 recorded images from 9 unique and representative donors. Scale bars are 2.5 µm. **(B)** Intracellular IgE was quantified by flow cytometry. Isolated basophils and DCs were washed with acid (see Methods for detail) or PBS. To determine the acid-resistant fraction of total IgE, cells were permeabilized, stained with an anti-IgE antibody, and the MFI of acid-washed cells was divided by that of unwashed cells (left panel). To determine acid-resistant fraction of surface IgE, cells were stained with anti-IgE without permeabilization, and the MFI of acid-washed cells was divided by that of unwashed cells (right panel). Shown are 8 representative donors with mean ± SEM. (**C)** Entry of hIgE-A647 into basophils and DCs of one representative donor. hIgE-A647 (0.5 µg/mL) was added to PBMCs. At each indicated time point, cells were treated with acid or PBS before permeabilization and analysis by flow cytometry. Scale bars are 2.5 µm. **(D)** IgE entry to

basophils and DCs is IgE receptor-mediated. PBMCs were incubated for 4 hrs with hIgE-A647 (0.5 μg/mL) alone (left), with excess unlabeled IgE (middle), or IgG (40 μg/mL) (right). Cells incubated with hIgE-A647 alone were washed or unwashed with acid. Cells incubated together with excess IgE or IgG were all washed with acid. Then, the A647 MFI of acid-washed cells was divided by that of unwashed cells to comparatively determine intracellular IgE content between conditions. Shown are 4 representative donors with mean ± SEM. **(E)** IgE traffics to lysosomes after entering DCs. DCs were incubated with 0.5 μg/mL hIgE-A647 for 4 hrs before preparation for confocal microscopy. Data are representative of 17 images from two unique and representative donors. **(F)** Effect of 0.5 μM chloroquine (Chlor) on the intracellular IgE pool determined by confocal microscopy. On each confocal micrograph, intracellular and cell-membrane ('extracellular') regions were identified (left, scale bar is 2.5 μm), fluorescence density was measured, and the signal ratio of intracellular to extracellular regions was determined. Shown are summarized data from 30 cells of each type in each condition for two donors. Data represent mean ± SEM and *P<0.005. **(G)** Effect of 2 mM chloroquine on intracellular IgE pool determined by flow cytometry. The acid-resistant IgE fraction of basophils and DCs was determined by staining cells with anti-IgE after permeabilization, and dividing MFI of acid-washed cells by MFI of unwashed cells as described in (B). Shown are 4 representative donors.

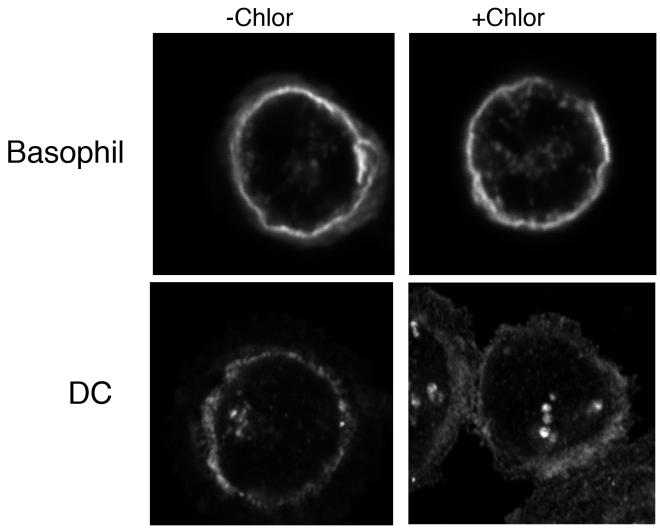
internalization, we incubated PBMCs with human IgE conjugated to the fluorophore Alexa 647 at 37 °C for 1 or 4 hr. At each time point, the level of total and acid-resistant hIgE-Alexa 647 associated with DCs and basophils were determined by flow cytometry. We found that total IgE increased over time both in DCs and basophils (Figure 3C and Supplemental Figure 4). Remarkably, the amounts of IgE associated with DCs were comparable to or higher than those associated with basophils (Figure 3C and Supplemental Figure 4) despite FcεRI surface expression being much lower in DCs (Figure 1B), which is consistent with our earlier observation that FcεRI is less occupied by IgE in DCs than in basophils (Figure 1D). We found that acid-resistant IgE also increased over time in both cell types, but to a much higher level in DCs (Figure 3C and Supplemental Figure 4). In fact, up to 60 % of total IgE in DCs was acid-resistant at 4 hrs incubation while a maximum of 10 % was acid-resistant in basophils (Figure 3D), indicating that DCs internalize IgE more efficiently than basophils do. To verify that internalization is mediated by FcεRI, we performed the same experiment in the presence of excess amounts of unlabeled IgE or IgG. Unlabeled IgE but not IgG markedly inhibited entry of hIgE-Alexa 647 to both DCs and basophils (Figure 3D), indicating that the entry was indeed IgE receptor-mediated. Since the low affinity IgE receptor FcεRII is not expressed in blood DCs or basophils in the steady state (Krauss et al., 1993; MacGlashan et al., 1999), it is FcεRI that mediates internalization of IgE in these cells.

Next, we determined the fate of the internalized IgE in DCs and basophils. First, we identified the subcellular localization of hIgE-Alexa 647 that had entered DCs or basophils by confocal microscopy. While IgE is readily denatured at a pH of 5 and below (Demarest et al., 2006), Alexa 647 is stable and fluorescent in acidic environments such as lysosomes. We found that Alexa 647 colocalized with Lamp1 in DCs (Figure 3E), which indicates that IgE had been



Supplemental Figure 4: hIgE-A647 binding and internalization by DCs and basophils of three additional healthy blood donors. Experiments were run in parallel with that shown in Figure 3C.

delivered to the lysosomes. No Alexa 647 was detected inside basophils (data not shown), consistent with limited entry of IgE into these cells. Secondly, we determined whether IgE internalized by DCs was degraded in the lysosomes. DCs were isolated from PBMCs and cultured in the presence or absence of chloroquine, an inhibitor of lysosomal acidification (Mellman et al., 1986). After 8 hrs of culture, cells were fixed, stained using anti-IgE antibody, and examined by microscopy. We found that chloroquine treatment enlarged the size of the anti-IgE-labeled intracellular compartments specifically in DCs (Supplemental Figure 5). To quantitatively determine the effect of chloroquine on the intracellular IgE pool, chloroquine-treated and untreated DCs and basophils were imaged by confocal microscopy. On the micrographs of each individual cells imaged, the regions of plasma membrane and cytosol were manually drawn (Figure 3F). The anti-IgE antibody signal density in each region was quantitated, and the ratio of cytosolic ('intracellular') to plasma membrane region ('extracellular') signal density was determined. We found that this ratio was significantly higher in chloroquine-treated DCs compared to untreated cells (Figure 3F), indicating chloroquine treatment increased intracellular IgE in DCs. For comparison, the same experiment was performed using basophils isolated from the same donors. We found that chloroquine treatment did not increase or only slightly increased the intracellular IgE fraction (Figure 3F). Lastly, we employed flow cytometry to better quantitate the effect of chloroquine on the amount of intracellular IgE. We found that chloroquine treatment consistently increased the fraction of intracellular IgE in DCs (Figure 3G). Notably, intracellular IgE in basophils also increased (Figure 3G), indicating that they too contained some IgE in lysosomes, although significantly less than DCs.



Supplemental Figure 5: The effect of chloroquine on IgE-containing intracellular compartments. Basophils and DCs were incubated for 8 hrs at 37° C with or without 0.5 μM chloroquine (Chlor) and stained with an anti-IgE antibody for confocal microscopy. The same laser intensity was employed for chloroquine-treated and untreated cells. Images are representative from 2 unique donors.

Human FcεRIα is specifically localized in the lysosomes of hFcεRIα-transgenic mouse conventional DCs (cDCs) and monocytes.

To obtain evidence that IgE entered DCs via FcεRI in vivo, we utilized the human FcεRIα-transgenic (Tg) mice that lack mouse FcεRIα (mFcεRIα) but express human FcεRIα (hFcεRIα) under transcriptional control of the human promoter (Dombrowicz et al., 1996). We found that these mice express hFcεRI on blood cDCs, monocytes, and basophils whereas B cells and other lymphocytes did not express it, similarly to humans (Figure 4A and not shown). hFcεRI in blood basophils of these mice did not colocalize with Lamp1 but did colocalize with Calnexin (Figure 4B), similar to observations with human basophils (Figure 2A). We also found that hFcεRIα in blood monocytes of these mice colocalized strongly with Lamp1 (Figure 4B). hFcεRIα in blood cDCs of these mice could not be visualized by microscopy due to relatively low expression of FcεRIα. Instead, we imaged $CD11b^+$ cDCs (the mouse counterpart of human $BDCA1^+$ DCs (Robbins et al., 2008)) cultured from bone marrow of these mice using Flt3L (Naik et al., 2005)(Supplemental Figure 6). hFcεRI in these BMDCs colocalized strongly with Lamp1 (Figure 4C). Thus the cell-type specific expression and trafficking of human FcεRIα was recapitulated in these hFcεRIα-Tg mice.

IgE bound to hFcεRI on monocytes and cDCs is internalized in hFcεRIα-Tg mice.

Having found that hFcεRIα-Tg mice express and localize hFcεRI in the lysosomes of cDCs and monocytes similarly to humans, we used these mice to examine whether hFcεRI mediates cellular internalization of IgE in vivo. First, Tg^+ and Tg^- mice were i.v. injected with

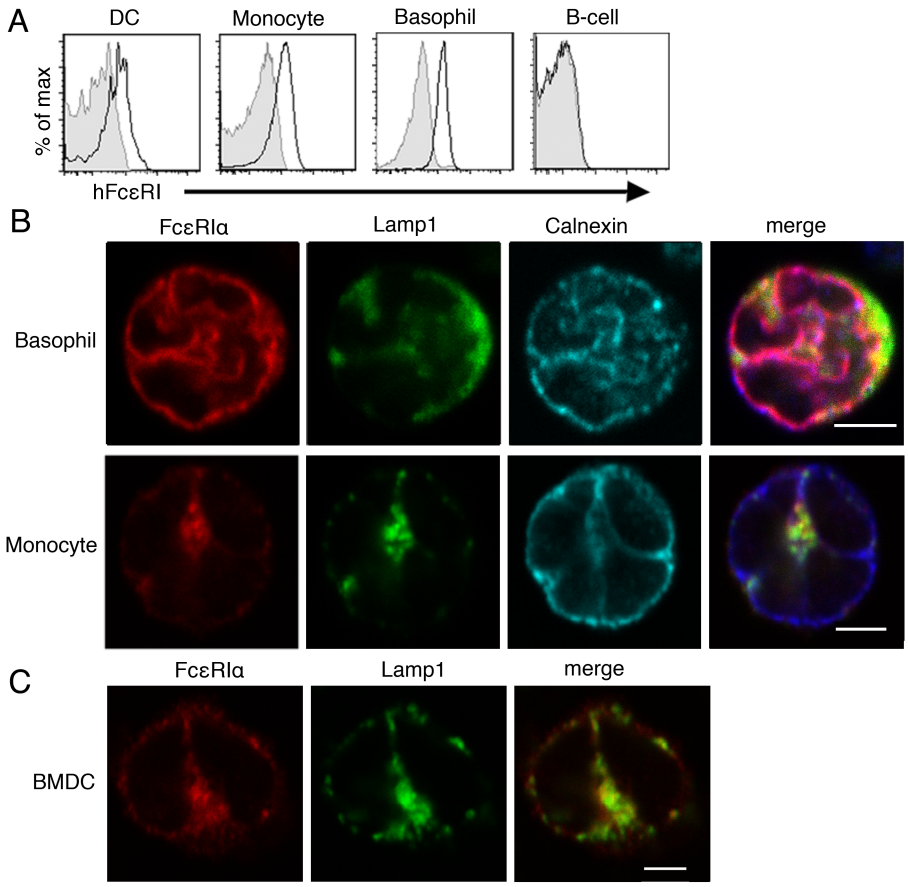
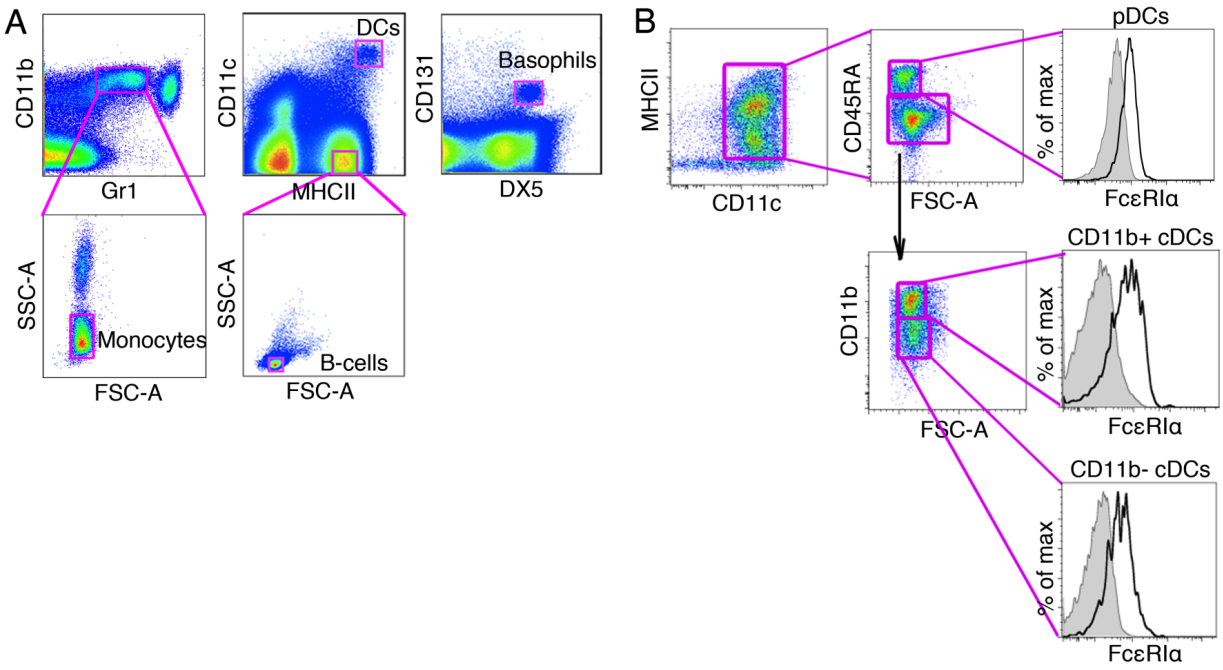
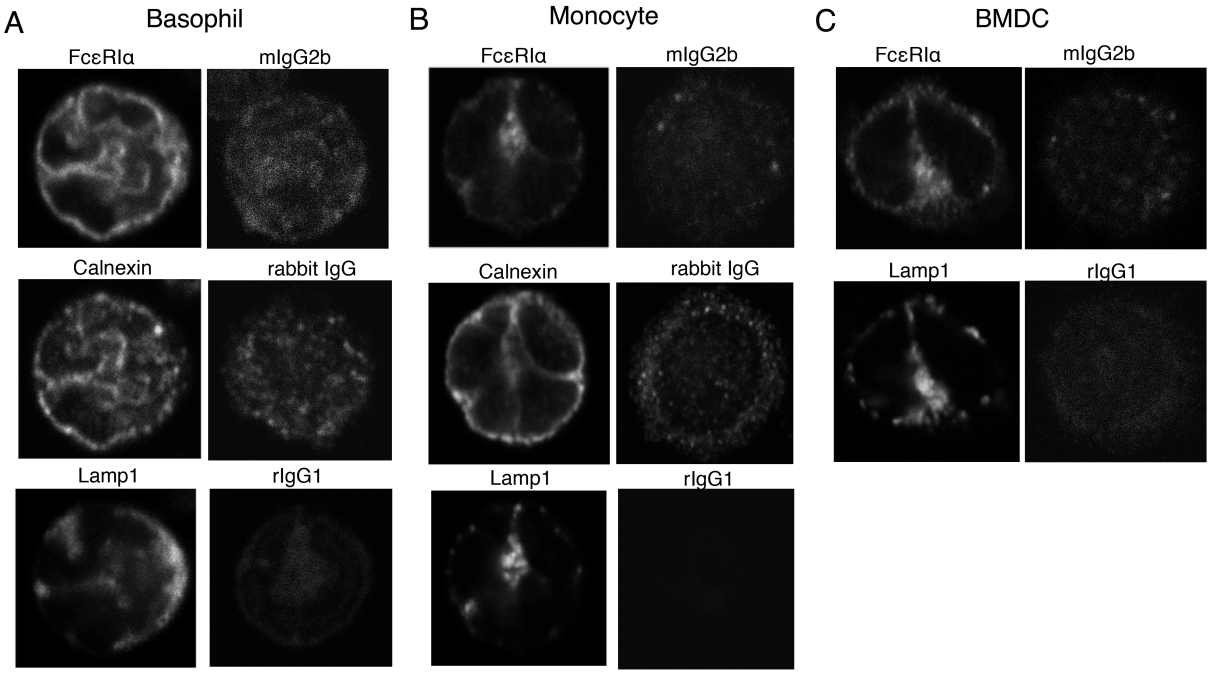


Figure 4. hFcεRIα-Tg mice express and localize hFcεRI in a similar manner to humans. (**A**) Surface expression of hFcεRI in hFcεRIα-Tg mouse blood leukocytes. Blood from adult hFcεRIα(+) transgenic (Tg^+, black) and hFcεRIα(-) (Tg^-, grey shaded) littermates was collected and stained with an anti-hFcεRIα antibody and cell type-specific antibodies after red blood cell lysis. Gating strategies are shown in Supplemental Figure 6A (**B**) Intracellular localization of hFcεRI in Tg^+ mouse blood basophils and monocytes. Sorted blood basophils and monocytes were stained for confocal microscopy as described in Figure 2. Images are representative of results from 3 independent experiments. Note that the Calnexin signal has been switched from blue to cyan for ease of visualization in single-stain format, while it has not been altered for merged images. (**C**) Intracellular localization of hFcεRI in Tg^+ mouse $CD11b^+$ BMDCs. BMDCs were cultured using Flt3L, sorted for $CD11b^+$ cDCs as described in Supplemental Figure 6B, and stained as in (B). Images are representative results from 3 independent experiments. For all confocal images, scale bars are 2.5 μm and negligible staining by isotype control antibodies was confirmed (Supplemental Figure 7).



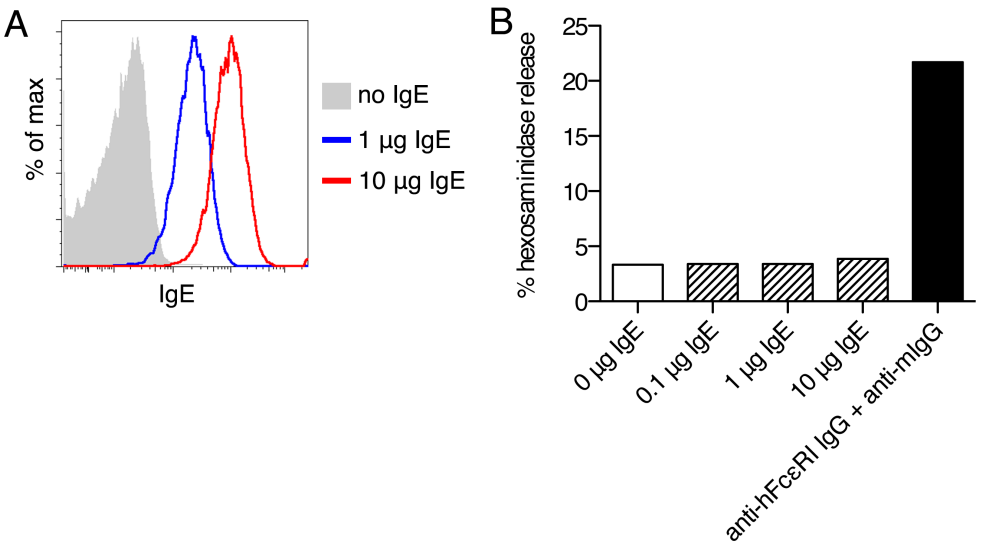
Supplemental Figure 6: Gating strategy for blood leukocytes (A) and CD11b^{+} BMDCs (B). **(A)** Flow cytometric gating strategy used for the experiment described in Figure 4A. **(B)** Bone marrows were isolated from hFcεRIα-Tg mice and cultured for 6 days in the presence of 100 ng/mL Flt3L. Generated cells were stained using a cocktail of antibodies and each DC subset was gated as shown. Expression of FcεRI (black) was also determined compared to isotype control (grey).



Supplemental Figure 7: Isotype controls for confocal microscopy of mouse blood cells and BMDCs. Blood basophils **(A)**, monocytes **(B)**, and CD11b^{+} Flt3L-derived BMDCs **(C)** were isolated as described in Methods and were stained with antibodies indicated (left columns) or with the corresponding isotype controls (right columns). Images were taken with the same laser intensity and gain for the antibodies indicated compared to isotype controls.

human IgE. It is noteworthy that the injected human IgE had been pre-tested and confirmed for the absence of aggregates that can potentially activate hFcεRI-expressing cells by crosslinking hFcεRI. Briefly, we added the human IgE preparation to mast cells cultured from hFcεRIα-Tg mouse bone marrow, and measured degranulation of these cells by a hexosaminidase release assay (Shin et al., 2006b). The addition of up to 10 μg/mL of IgE to the cells did not cause any release of hexosaminidase above the level of spontaneous release (Supplemental Figure 8). In contrast, addition of anti-hFcεRI antibody/ secondary antibody complexes resulted in 20 % of hexosaminidase released. This data indicated that the human IgE that we injected to mice did not contain a significant level of aggregates and would not cause hFcεRI crosslinking in vivo.

After injection of human IgE into Tg^+ and Tg^- mice, we bled the mice at multiple time points and determined the amount of human IgE bound to hFcεRI-expressing cells by flow cytometry. We found very little IgE bound to monocytes and basophils of Tg^- mice at all time points examined (Figure 5A). In contrast, surface IgE levels increased both for basophils and monocytes in Tg^+ mice, reached their maximum ~4 hrs post-injection, and remained at least for next 4 hrs at that level (Figure 5A). At 24 hrs post-injection however, monocytes had lost ~80 % of IgE while basophils retained ~100 % (Figure 5A). We also monitored IgE on the surface of cDCs in the blood. Since cDCs are scarce in blood, we euthanized individual mice at each time point and performed flow cytometry using whole blood. Similarly to monocytes, cDCs lost most of their surface IgE at 24 hrs post-injection (Figure 5B). In addition to the blood cells, we also examined monocytes and DCs in the lungs. They also captured significant amounts of IgE but lost most of it as early as 12 hrs post-injection (Figure 5C). Lastly, we examined mast cells in the peritoneal cavity. Unlike monocytes and cDCs, these cells retained 100 % of the peak surface



Supplemental Figure 8: Purified human IgE (Abcam) does not degranulate hFcεRI-expressing bone-marrow derived mast cells (BMMCs). BMMCs cultured from hFcεRIα-transgenic mice were incubated with varying concentrations of human IgE (Abcam) or anti-hFcεRI IgG/anti-mouse IgG Ab complex. **(A)** Binding of IgE was determined by flow cytometry. **(B)** Degranulation was determined by hexosaminidase assay as described in Methods.

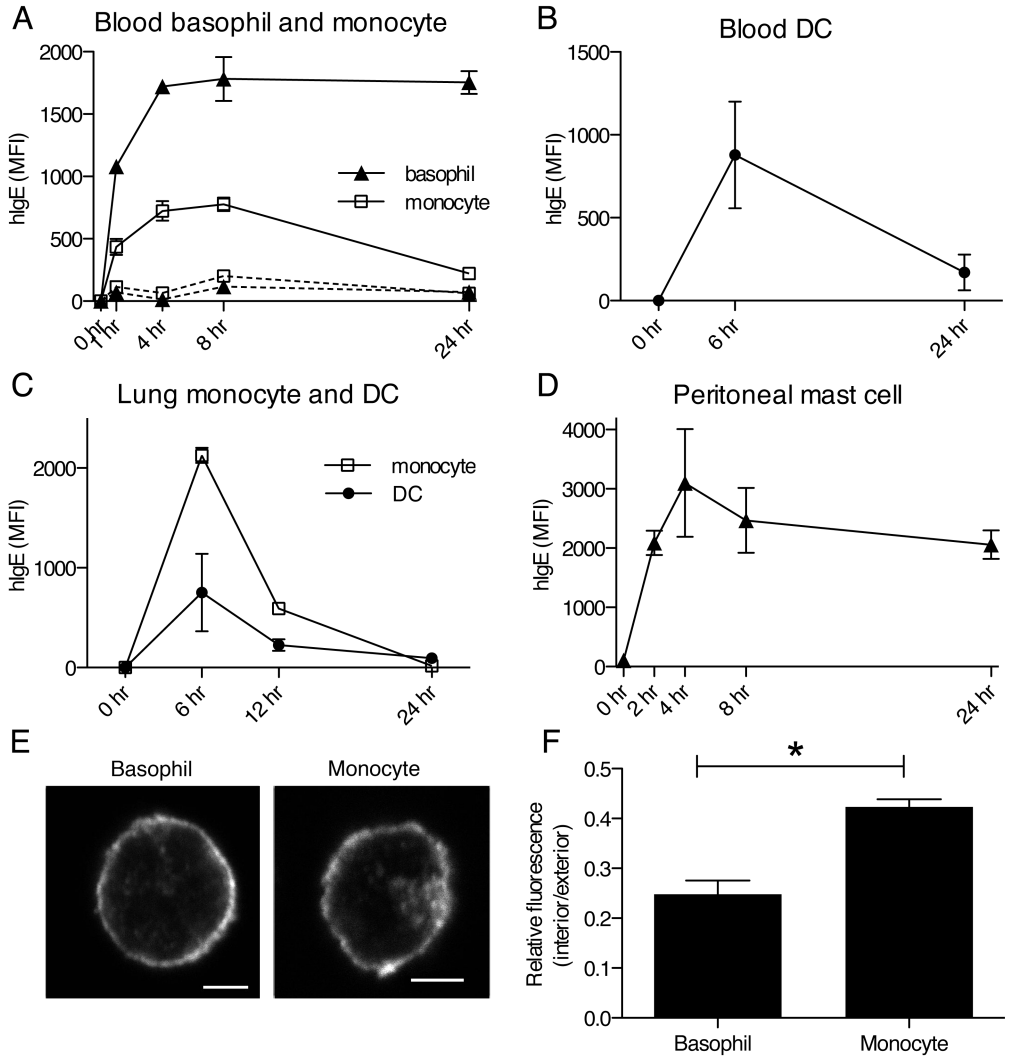


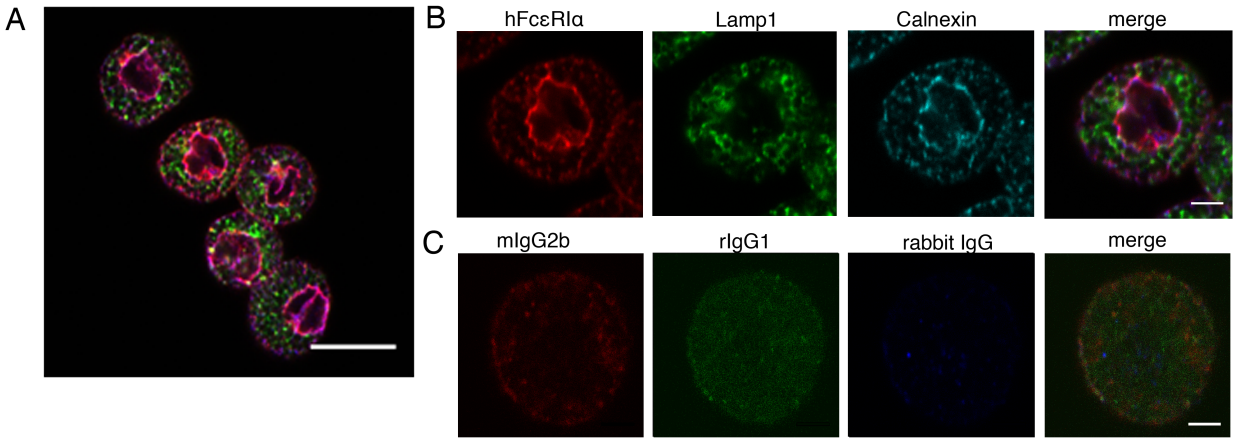
Figure 5. Human IgE injected into hFcεRIα-Tg mice is internalized by cDCs and monocytes in the steady state. (A) Surface IgE levels on blood basophils and monocytes following human IgE injection. Before, and at 1, 4, 8, and 24 hrs post-hIgE injection, Tg^+ and Tg^- mice were bled for flow cytometric analysis of surface IgE levels in basophils and monocytes. Tg^+ mice are shown in solid lines, and Tg^- mice are shown in dotted lines. Data of 3 mice from one representative experiment of 3 are presented with mean ± SEM. **(B-D)** Surface IgE levels on blood cDCs (B), lung DCs and monocytes (C), and peritoneal mast cells (D) of Tg^+ mice following human IgE injection. At each time point following hIgE injection, mice were sacrificed, whole blood was collected and analyzed by flow cytometry. Data of 9 mice (B), 8 mice (C), or 14 mice (D) from one representative experiment of 2 are presented with mean ± SEM. **(E)** Intracellular localization of human IgE in basophils and monocytes of hFcεRIα-Tg mice injected with hIgE. At 6 hrs post-injection, basophils and monocytes were isolated and examined for intracellular human IgE by confocal microscopy as described in Figure 3A. Scale bars are 2.5 μm. On the right, intracellular IgE levels are quantified as in Figure 3F. 30 images of Tg^+ monocytes and basophils were analyzed. Resulting values are presented with mean ± SEM.

IgE at 24 hrs post-injection, similarly to basophils (Figure 5D). Notably, hFcεRI was not found in the lysosomes of mast cells but was found on the plasma membranes and ER, similarly to basophils (Supplemental Figure 9). These findings indicate that human IgE injected into hFcεRIα-Tg mice initially binds to all hFcεRI-expressing cells, but gradually disappears from monocytes and cDCs.

We reasoned that IgE bound to monocytes and cDCs of Tg^+ mice could be subsequently internalized, which might explain the gradual loss of IgE from surface of these cells while the IgE bound to basophils and mast cells remained. Therefore, we looked for intracellular hIgE in blood monocytes and basophils of hIgE-injected Tg^+ mice by isolating each cell population at 6 hrs post-IgE injection, staining with an anti-human IgE antibody after permeabilization, and examining by confocal microscopy. We found that the human IgE was mostly associated with the plasma membranes in basophils, whereas it was detected both at the plasma membranes and in intracellular compartments in monocytes (Figure 5E). Quantification of micrograph images showed that monocytes had a significantly higher proportion of IgE intracellularly compared to basophils (Figure 5F). Taken together, these findings suggest that FcεRI expressed in cDCs and monocytes actively mediates IgE internalization *in vivo*.

Human FcεRI expressed by cDCs or monocytes contributes to serum IgE clearance in FcεRIα-Tg mice.

Previous studies have demonstrated that wild type mice and mFcεRIα-deficient mice clear serum IgE at similar rates, indicating that mFcεRI is not involved in IgE clearance in mice (Cheng et al., 2010; Dombrowicz et al., 1993). Nevertheless, we hypothesized that human FcεRI could be involved in IgE clearance based on our finding that human FcεRI expressed in



Supplemental Figure 9: FcεRIα in peritoneal mast cells of hFcεRI-Tg mice is located in the ER, not in the lysosome. Peritoneal mast cells were isolated from peritoneal lavage cells of hFcεRIα-transgenic mice by flow-cytometric sorting based on expression of c-kit and side-scatter properties. Sorted cells were placed on coverslips and stained as described in Methods for hFcεRIα, Lamp1, and Calnexin, then imaged by confocal microscopy at 60x. **(A)** A group of mast cells. hFcεRIα is in red, Lamp1 is in green, and Calnexin is in blue. Size bar is 10 um. **(B)** One mast cell imaged at higher magnification. Note that Calnexin is changed to cyan in single-color format for ease of visualization. Size bar is 2.5 um. **(C)** Isotype controls for stains shown in (B). Images were taken with the same laser intensity and gain for the antibodies indicated compared to isotype controls.

monocytes and cDCs mediated cellular internalization of IgE in a constitutive manner. To test this hypothesis, we injected human IgE into Tg^+ and Tg^- mice, bled them at various hrs post-injection, and determined serum concentrations of human IgE by ELISA. We found that serum concentrations of human IgE were reduced over time in both strains of mice, but at a markedly accelerated rate in Tg^+ mice (Figure 6A). A quantitative analysis showed that the half-life of human IgE in the Tg^+ mice was approximately 4-fold shorter than in Tg^- mice (Figure 6B) suggesting hFcεRI significantly contributes to serum IgE clearance.

To verify that the rapid serum IgE clearance observed in Tg^+ mice is directly attributed to FcεRI-expressing cells and not to some other features associated with genetic alteration of Tg^+ mice, we reconstituted the hematopoietic compartment of Tg^- mice with bone marrow that had been isolated from Tg^- or Tg^+ mice and mixed at specific ratios of Tg^+:Tg^-. We found that mice reconstituted with Tg^+ bone marrow cleared serum IgE at much faster kinetics than those reconstituted with Tg^- bone marrow (Figure 6C). We also found that mixed chimeric mice cleared serum IgE in rates proportional to the percentage of Tg^+ bone marrow used (Figure 6C). Interestingly, however, the half life of serum IgE in 50 % Tg^+ chimeric mice was comparable to that in 100 % Tg^+ chimeric mice (Figure 6D), suggesting that in this experimental setting, IgE is sufficiently cleared by 50 % of the hematopoietic, hFcεRI-expressing cell compartment. Nevertheless, this study demonstrates that it was indeed hFcεRI-expressing cells that are responsible for the rapid serum IgE clearance in hFcεRIα-Tg mice.

Next, we examined the specific contribution of cDCs and monocytes vs. basophils to serum IgE clearance; the former cells internalize and degrade IgE while the latter retain IgE at the cell surface. Since we found the rate of IgE clearance increased in proportion to the number of hFcεRI-cells only when these cells were present in limited numbers (Figure 6D), we increased

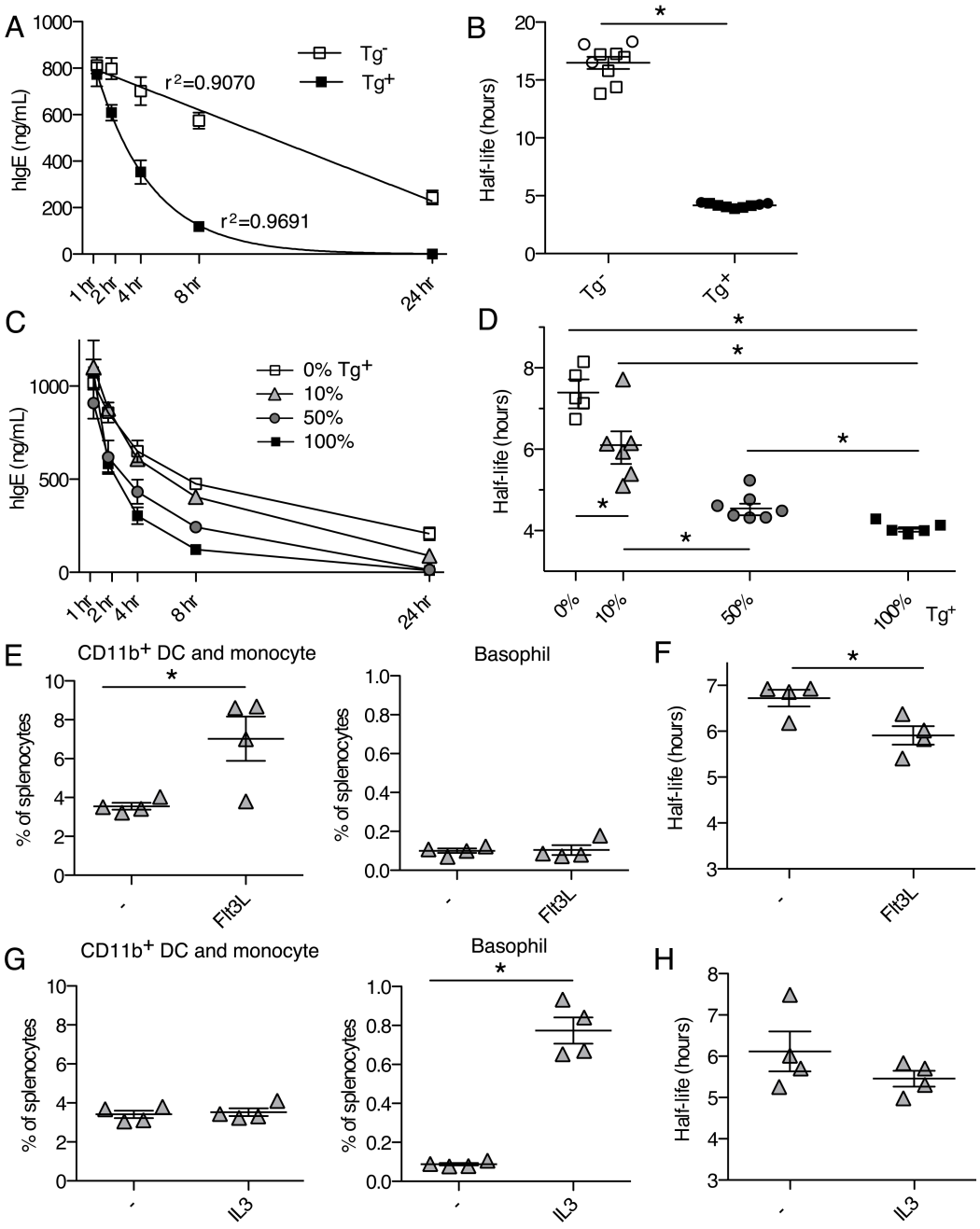
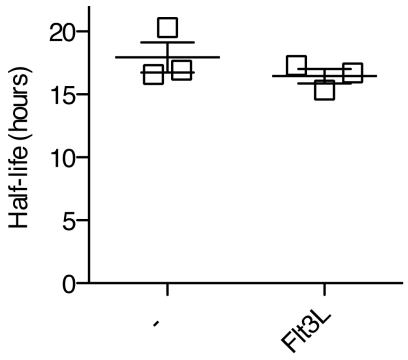


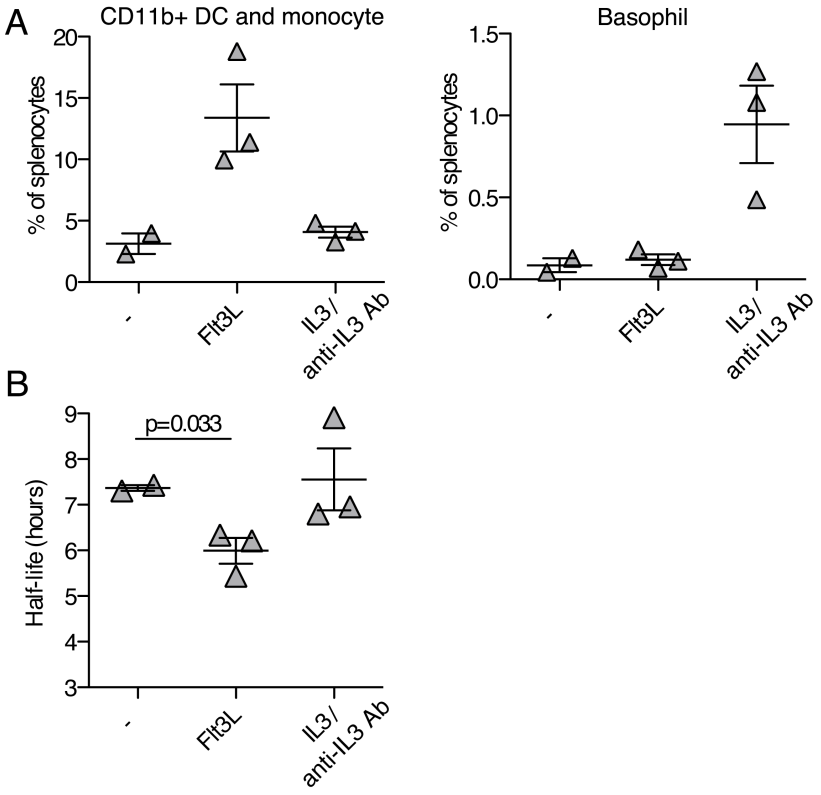
Figure 6. Human FcεRI expressed by DCs or monocytes significantly contributes to serum IgE clearance in hFcεRIα-Tg mice. (**A**) Kinetics of serum IgE clearance in Tg^+ and Tg^- littermates. 2.5 μg of purified human IgE was i.v. injected into Tg^+ and Tg^- mice, blood was collected at different time points following injection, and human IgE concentration was determined by ELISA. Figure is representative of 3 independent experiments using 3 mice per strain. For each mean, a line of best fit with indicated R^2 values was calculated. (**B**) Serum half-life of human IgE in Tg^+ and Tg^- mice. IgE half-life was calculated as the time for peak serum IgE to halve from lines of best fit calculated as in (A). Data are plotted as individual mice; squares represent mice injected with 2.5 μg and circles with 5 μg. *$P < 1.5 \times 10^{-6}$. **(C-D)** hFcεRI-expressing hematopoietic cells are responsible for serum IgE clearance. Mixed bone

marrow chimeras were made on Tg^- recipient hosts. *P<0.05. **(E, G)** Flt3L increases the frequency of DCs/monocytes, while IL3 increases basophils. 10:90 (Tg^+:Tg^-) mixed chimeric mice were (E) implanted with Flt3L-expressing tumors or (G) injected with IL3/anti-IL-3 Ab complexes. The percentage of [$CD11b^+$ DCs + monocytes] or basophils in spleen was determined by flow cytometry. **(F, H)** Serum hIgE clearance is accelerated by an increase in DCs/monocytes (*P=0.024 (F)) but not by an increase in basophils. Data in (C-H) are representative of 2 independent experiments. The second experiment of (E-G) is shown in Supplemental Figure 11.

the number of cDCs and monocytes or basophils in 10:90 (Tg^+:Tg^-) mixed bone marrow chimeric mice by implanting them with a melanoma cell line producing Flt3L. Flt3L stimulates proliferation and differentiation of the monocyte/DC common progenitor in mice (Liu et al., 2009; Waskow et al., 2008). Accordingly, injection of Flt3L or implantation with Flt3L-producing melanoma has been shown to markedly increase the number of monocytes and cDCs in mice (Liu et al., 2009; Mach et al., 2000; Waskow et al., 2008). Consistent with this previous finding, Flt3L-melanomas significantly increased the frequency of monocyte and $CD11b^+$ cDC populations, but not basophils (Figure 6E). Note that we did not include $CD8^+$ cDCs in this analysis because these DCs hardly expressed hFcεRI in hFcεRIα-Tg mice (data not shown), and are thus irrelevant to our study. Remarkably, Flt3L melanoma-implanted mice cleared serum IgE at a significantly faster rate than untreated mice (Figure 6F). When the same experiment was performed using Tg^- mice however, no difference was observed (Supplemental Figure 10). This finding indicates that neither Flt3L, the melanoma, nor the increase in DCs and monocytes accelerates serum IgE clearance independently of hFcεRI. Next, we increased the frequency of basophils in a separate group of 10:90 (Tg^+:Tg^-) mixed chimeric mice by injecting IL3/anti-IL3 antibody complexes. As shown in previous studies (Finkelman et al., 1993; Ohmori et al., 2009), this treatment increased the frequency of basophils by ~8 fold (Figure 6G, right panel) but did not increase the frequency of monocytes or DCs (Figure 6G, left panel). Furthermore, the rate of serum IgE clearance was not accelerated by injection of IL3/anti-IL3 antibody complexes (Figure 6H). Taken together, these findings indicate that hFcεRI-expressing cDCs or monocytes, but not basophils, significantly contribute to serum hIgE clearance in hFcεRIα transgenic mice.



Supplemental Figure 10: Flt3L does not accelerate serum IgE clearance in Tg$^-$ mice. Half-lives of human IgE in Tg$^-$ mice or Tg$^-$ mice implanted with Flt3L-transfected B16 melanomas. Half-lives are not significantly different as measured by Student's T-test.



Supplemental Figure 11: Flt3L, but not IL3, accelerates serum IgE clearance in 10:90 (Tg^+:Tg^-) mixed chimeric mice. (A) The percentage of [$CD11b^+$ DCs + monocytes] or basophils in spleen was determined by flow cytometry. Data are plotted as mean ± SEM. **(B)** Serum human IgE half-lives were determined as described in Figure 6. Significance was determined by Student's T-test.

Discussion

The present study addressed the question of whether FcεRI expressed in human BDCA1$^+$ DCs and monocytes traffics and functions uniquely from that expressed by basophils and mast cells. We found that FcεRI is constitutively endocytosed in these DCs and monocytes, mediating cellular entry of circulating IgE and thus contributing to serum IgE clearance.

Three independent lines of investigation provide evidence that FcεRI is constitutively endocytosed in human BDCA1$^+$ DCs and monocytes. First, FcεRI was found in the endocytic compartments of these DCs and monocytes in human blood. It was surprising to find FcεRI in the endolysosomes in cells freshly isolated from blood because previous studies have suggested that FcεRI is endocytosed and transported to the lysosomes only when it is crosslinked by IgE/antigen complexes (Maurer et al., 1998). We find it unlikely that the cells examined had all been engaged by IgE/antigen complexes because they were isolated from non-allergic healthy blood donors. Furthermore, basophils isolated from the same donors did not show any sign of FcεRI-crosslinking such as degranulation; Lamp1, the granular membrane-associated protein, was all sequestered intracellularly. In addition, cDCs cultured from the bone marrows of hFcεRIα-Tg mice also localized hFcεRI in the lysosomes. These DCs had not been exposed to any agents capable of interacting with or crosslinking hFcεRI. Thus, FcεRI endolysosomal localization in DCs and monocytes does not seem to be due to FcεRI-crosslinking, but attributed to constitutive endolysosomal trafficking of FcεRI in these cells.

Secondly, BDCA1$^+$ DCs in human blood contained an appreciable amount of IgE intracellularly. In addition, fluorescently labeled IgE, when added to these DCs in vitro, was rapidly internalized and delivered to the lysosomes. This internalization was inhibited by excess amounts of unlabeled IgE but not IgG, indicating the internalization was mediated by IgE

receptor(s). Since FcεRI is the only IgE receptor DCs express, this finding supports for constitutive endocytosis of FcεRI in these cells. Thirdly, human FcεRI expressed in cDCs and monocytes of FcεRIα-Tg mice was also localized in the lysosomes. Monomeric human IgE injected into these mice was detected at the surface of these cells; however, the IgE soon disappeared from surface but appeared in the intracellular compartments of monocytes. This spontaneous entry of IgE to these cells strongly suggests constitutive endocytosis of FcεRI.

The specific mechanism underlying FcεRI endolysosomal trafficking remains to be determined. We could speculate that FcεRIβ stabilizes FcεRI at cell surface and that its presence in basophils helps to retain FcεRI at cell surface, whereas its absence in DCs and monocytes drives FcεRI to the lysosomes. However, genetic ablation of mFcεRIβ in hFcεRIα-Tg mice did not appear to reduce FcεRI surface levels in basophils (Dombrowicz et al., 1998). In addition, the trimeric form of FcεRI (αγγ) expressed in U937 cells was not localized in the lysosomes (data not shown). These data indicate that receptor makeup alone is not sufficient to determine receptor trafficking. Instead, there appear to be cell-intrinsic factors that constitutively mediate FcεRI endocytosis in DCs and monocytes. One potential mechanism is its association with an accessory molecule capable of driving endocytic pathways. The cytoplasmic domain of FcεRI may associate with an adaptor molecule only present in DCs and monocytes that recruits endocytosis protein machineries. Alternatively, FcεRI may interact with an endocytic receptor that facilitates constitutive internalization in cells that express both receptors. For example, DCs and monocytes express many carbohydrate-binding receptors, some of which are constitutively endocytosed (Higashi et al., 2002; Mahnke et al., 2000). One of these may laterally interact with FcεRI through carbohydrate moieties attached to the FcεRIα extracellular domain, thus driving FcεRI to endocytic pathways. Glycosylation of G-protein-coupled receptors has been shown to

influence the kinetics or routing of endocytosis (Cho et al., 2012; Tansky et al., 2007). Notably, FcεRI in BDCA1$^+$ DCs appears to be glycosylated distinctly from FcεRI in basophils, as the hFcεRI imunoprecipitated from these DC lysates mobilized by SDS-PAGE at a slightly faster rate.

Previous studies have shown that IgE binding inhibits endocytosis of FcεRI in human basophils and hFcεRI-transfected cell lines, thereby increasing FcεRI surface levels in these cells (MacGlashan et al., 2001). However, FcεRI surface levels in human blood BDCA1$^+$ DCs did not increase with increasing serum IgE as much as those in basophils, suggesting that IgE binding may not stabilize FcεRI on the DC surface. Furthermore, IgE bound to human BDCA1$^+$ DCs or bound to hFcεRI-expressing mouse monocytes was rapidly internalized in vitro and in vivo, implicating IgE endocytosis after binding to FcεRI. Thus, the mechanism that mediates FcεRI endocytosis in DCs and monocytes does not appear to be negatively affected by IgE binding.

It is well established that IgE is unique among immunoglobulins in its short serum half life: it is lost from human serum at a rate 10 times faster than the loss of IgG, resulting in a half-life of roughly two days (Dreskin et al., 1987; Iio et al., 1978). Modeling studies have repeatedly predicted that IgE, distinct from other immunoglobulins, is degraded by two catabolic pathways and that the secondary pathway could involve internalization and degradation of IgE by FcεRI-expressing cells (Iio et al., 1978). However, this prediction has been largely unsupported by the literature. Mice deficient in FcεRI or mast cells were shown to clear serum IgE as fast as wild type mice (Cheng et al., 2010; Dombrowicz et al., 1993; Watanabe et al., 1986). Furthermore, mice with high levels of IgE due to IgE-producing hybridomas also cleared serum IgE at an equal rate to normal mice (Haba et al., 1985), supporting the hypothesis that IgE receptor-bearing cells do not play a significant role in serum IgE loss. While these studies indicate that

mouse FcεRI does not significantly contribute to IgE clearance, no studies have examined the role of human FcεRI.

Using human FcεRIα-Tg mice, our studies showed that human FcεRI significantly contributes to serum IgE clearance. By selectively manipulating the number of DCs and monocytes vs. basophils in these mice, we further showed that the IgE-clearing role of FcεRI is attributed to its expression in DCs and monocytes, but not basophils. Although underlying mechanisms remain to be determined, the constitutive endocytosis of FcεRI in cDCs and monocytes and consequent entry of IgE into these cells implicates the role of FcεRI-mediated cellular internalization of IgE in serum IgE clearance. It has been shown recently that mouse CD23, the low affinity IgE receptor, mediates internalization of IgE by B cells, but does not contribute to the rate of serum IgE clearance (Cheng et al., 2010). Interestingly, some of Fcγ receptors, including FcγRII, III, and IV (Mancardi et al., 2008; Takizawa et al., 1992) also bind IgE in mice. Therefore, it is plausible that IgG receptors instead of IgE receptors may play a role in clearing IgE in mice.

In summary, we have shown that human FcεRI is constitutively endocytosed and transported to the lysosomes in DCs and monocytes, and that this mediates cellular entry of circulating IgE and contributes to serum IgE clearance. We speculate that diseases associated with high serum IgE may involve alteration in FcεRI endolysosomal trafficking in DCs or monocytes, which would result in an accumulation of IgE in circulation and/or an increase in IgE available to bind to mast cells or basophils. Interestingly, individuals with high IgE – either from atopic disease, the genetic disease Hyper-IgE Syndrome, or IgE myelomas – clear serum IgE at significantly slower rate than normal individuals (Dreskin et al., 1987; Waldmann et al., 1976) . Examination of FcεRI trafficking in people with these diseases may reveal new mechanisms of

disease pathogenesis, and identification of specific mechanisms directing endocytosis of FcεRI in DCs and monocytes may lead to novel allergy therapeutics.

Acknowledgements

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CHAPTER IV. CONCLUSIONS AND FUTURE DIRECTIONS

Summary

Human DCs have been implicated in numerous inflammatory diseases, but a lack of consensus regarding proper DC markers has hindered the study of these important cells. We were interested in determining the prevalence of $BDCA1^+$ DCs in human lungs of both healthy and diseased patients to better characterize the potential role of these cells in the inflammatory process. To do this, we stained pulmonary mononuclear cells with a panel of antibodies designed to thoroughly identify $BDCA1^+$ DCs and exclude contaminating monocytes, macrophages, and B-cells. We found that $BDCA1^+$ DCs were present in the parenchyma of healthy individuals but were significantly more prevalent in the fibrotic interstitial lung diseases of IPF and HP. Because we wanted to expand our studies, we identified FcεRIα as an additional $BDCA1^+$ DC-specific marker when used in concert with MHCII or BDCA1 for fluorescence microscopy. By using MHCII along with FcεRI, we quantified the numbers of epithelial DCs and mast cells in a cohort of healthy or asthmatic bronchial biopsies. Interestingly, only Th2 high asthmatics had significantly more epithelial DCs compared to healthy individuals, insinuating the Th2 milieu as correlated with DC infiltration.

Beyond simply being a useful marker for human DCs, FcεRI is also the way cells sense the presence of allergens, and little is known about the role of FcεRI on human DCs in allergy. We found that DCs constitutively traffic FcεRI from the cell surface to terminal lysosomes with or without IgE bound, in stark contrast to basophils and mast cells. This lysosomal trafficking resulted in internalization and degradation of human IgE over time and a loss in surface IgE. To confirm these studies with an in vivo model, we utilized hFcεRIα-transgenic

mice developed by the Kinet laboratory (Dombrowicz et al., 1996) and found that these mice recapitulated the FcεRI and hIgE trafficking patterns seen in humans. Injected human IgE rapidly bound to FcεRI-expressing cells in the blood and in the periphery, but was quickly lost from the surfaces of DCs and monocytes. Intracellular IgE content increased in monocytes after human IgE injection in an FcεRI-dependent fashion, indicating that bound IgE was internalized. Furthermore, serum IgE catabolism was accelerated in hFcεRIα^{+} transgenic mice, indicating that the receptor plays a significant role in serum IgE loss, contrary to previous belief. Hematopoietic, FcεRI-expressing bone marrow was sufficient to cause this acceleration in serum IgE loss; furthermore, DCs and monocytes, but not basophils, contribute significantly to this phenomenon. In conclusion, we identified a novel steady-state form of FcεRI trafficking in human myeloid cells that plays a role in serum IgE clearance.

Future directions

Role of DCs in fibrotic disease

BDCA1^{+} DCs accumulate in remarkable numbers in the lungs of patients with fibrotic lung disease. These are an important finding for the field of ILD because it implicates DCs in the disease process and may represent a novel drug target for future studies. Two major paths of research lie ahead: first, to characterize the phenotype of infiltrating lung DCs in health and disease, and second to identify the function of these cells with in vitro testing and mouse models of disease. In healthy individuals, DCs internalize antigen and migrate to local lymph nodes, even in the absence of pathogen stimulus (Wilson et al., 2008). Therefore, lung DCs have the capacity to activate both pathogen-specific effector T-cells in the presence of activating stimulus,

and harmless-antigen-specific tolerogenic T-regulatory cells in the absence of stimulus. As such, it is possible that the higher DC numbers observed in ILD may not reflect any pathological function of the DCs, as they may not encounter any special inflammatory stimulus. For example, hormone treatment with recombinant human GM-CSF (molgromastin) significantly increases the number of circulating DCs but doesn't cause obvious autoimmune inflammation (Lane et al., 1995). However, cDCs in the airways of COPD patients express more maturation markers, indicating that there may in fact be a more inflammatory environment in the afflicted lung that could cause DCs to promote pathological T-cell activation (Freeman et al., 2009), which could also be the case for IPF and HP. Therefore, it will be important to determine more conclusively the phenotype of the parenchymal BDCA1$^+$ DCs found in fibrotic lungs compared to healthy lungs.

DCs from each disease condition should be tested by flow cytometry for a wide array of costimulatory molecules such as CD40, CD80, CD83, and CD86, and MHCII in order to assess the basic level of DC maturation in these samples, similar to (Freeman et al., 2009). Further phenotyping could be done by isolating BDCA1$^+$ DCs from one representative donor of each disease type and performing microarray analysis on isolated RNA, focusing on genes for costimulatory molecules, chemokines, and cytokines. Microarray analysis has been performed with success on whole-lung mRNA isolates as well as isolated fibroblast RNA from fibrotic and healthy lungs (reviewed in (Studer and Kaminski, 2007)), but lung DCs have never been specifically examined. Differentially regulated genes identified by microarray can be validated by testing additional lung samples for those markers of interest by flow cytometry.

To test the functional consequences of these differences, isolated BDCA1$^+$ DCs could be cocultured in the presence of autologous CD4+ T-cells incubated in the presence of tritiated

thymidine, similarly to the method in (Demedts et al., 2006). After 5 days of coculture, the cells will be tested for the incorporation of thymidine to assess the extent of T-cell activation and subsequent division. Autologous T-cells can be acquired from lymph nodes taken from the same patient as the parenchymal tissue; these lymph nodes are commonly available to us currently through collaboration with Dr. Paul Wolters (UCSF) and should be a rich source of $CD4^+$ T-cells. Coculture experiments can further isolate the potential of $BDCA1^+$ DCs to skew T-cell responses by extending the coculture period in a method similar to (Ito et al., 2005) and performing intracellular FACS to identify what cytokines the activated T-cells are producing. One potential limitation of this study is that differences in T-cell activation and skew upon coculture with healthy or diseased DCs may be very slight; previous studies have compared coculture experiments using different cell types as APCs (Demedts et al., 2006) which may provide the largest opportunity for observing differences in resulting T-cell activation.

Repeated failures of immunotherapy to successfully treat IPF have led to the consensus that the disease is not directed by immune cell-mediated inflammation (Noble et al., 2012). Furthermore, neither IPF nor late-stage HP is associated with significant lymphocytic involvement. However, while immune dysregulation may not play a causative role in the pathogenesis of this disease, our data shows significant $BDCA1^+$ DC recruitment into the lungs. One way to test the relevance of these findings is to examine the bleomycin mouse model of IPF, which has been used in the past to show a crucial role for DCs in the development of the disease (Bantsimba-Malanda et al., 2010). These studies suffered from the caveat that whole-body inactivation of DCs can cause many off-target defects that could influence the development of lung disease. To better address the role of lung DCs in IPF, intranasal or intra-tracheal diphtheria-toxin (DT) treatment of CD11c-DTR chimeric mice could be used during the

bleomycin IPF development period to specifically ablate lung DCs (GeurtsvanKessel et al., 2008). If the DT-treated mice develop attenuated fibrosis, lung DCs significantly contribute to the development of the disease. Another approach would be to administer pertussis toxin intranasally to prevent DC migration from the lungs to local lymph nodes (Jakubzick et al., 2008a), to identify whether lung DC function in lung-draining lymph nodes contributes to disease. While pertussis toxin will also inhibit migration of other immune cells, limitation of pertussis usage to the initial bleomycin treatment period may help to isolate the role of immune cell migration in activating T-cells or other immune cells in the development of the disease, which can later be linked to DCs by combining with CD11c-DTR models.

To tease apart the specific function of lung DCs in the disease process, mouse knockouts can be used with the same bleomycin model. Endogenous CD11c-DTR DCs can be ablated with intra-tracheal diphtheria toxin (DT) treatment and monocytes can be injected intravenously, where they will home to the lung and mature into DCs (Jakubzick et al., 2008b; Landsman et al., 2007) before the bleomycin treatment period. To see whether DC activation of T-cells causes the exacerbation of disease, injected wild-type monocyte-derived DCs (moDCs) could be compared with MHCII knockout moDCs. Results from the human DC microarray could also be used to identify other knockout moDCs to test.

If DCs are so central to the disease process in IPF, why is it recalcitrant to treatment with corticosteroids? Some studies have shown that different subtypes or maturation states of human DCs are differentially affected by corticosteroid treatment (Hackstein and Thomson, 2004; Matyszak et al., 2000). While maturation of DCs is potently suppressed by steroid treatment, activities of already-mature DCs may not be affected; this way, tissues with large numbers of mature DCs may not be significantly influenced by corticosteroid treatment. Experimental

treatment of human and mouse bleomycin-induced IPF respond to treatment with tacrolimus, a different type of immunosuppressant that may have different effects on DC function (Horita et al., 2011; Nagano et al., 2006), though there is no clear consensus on the specific effects of various immunosuppressants on DC function (Hackstein and Thomson, 2004).

While allergic asthma is not considered a strictly fibrotic disease, increased sub-epithelial fibrosis is a hallmark of Th2-high asthma in particular (Woodruff et al., 2009) and it is possible that DCs could contribute to the fibrosis in addition to other pathological functions. It will be interesting to compare the phenotype of allergic asthmatic epithelial DCs to healthy epithelial DCs and healthy parenchymal DCs in order to better characterize their potential for inflammatory cytokine production and T-cell activation, especially in the context of significant surface FcεRI expression.

Mechanism of FcεRI trafficking in DCs

It remains a mystery what mechanism controls differential FcεRI trafficking in DCs and monocytes versus basophils and mast cells of humans and transgenic mice. We first hypothesized that the FcεRI β-chain controlled FcεRI complex trafficking, likely through β-chain dependent linking with partner proteins that could target the trimeric or tetrameric receptor to different fates. Alternatively, the β-chain could mask a cytoplasmic endocytosis motif present on the alpha or gamma chains, leading to differential FcεRI trafficking. However, preliminary studies in the lab do not seem to support this hypothesis.

Early studies in our lab utilizing the human monocytic FcεRI-expressing U937 cell line did not recapitulate FcεRI trafficking patterns seen in primary cells; namely, U937 cell lines expressing either of the tetrameric or trimeric forms of the receptor both had FcεRI protein

localized largely on the surface and in intracellular compartments excluding the lysosome (data not shown). This led us to believe that there are additional factors that dictate its trafficking patterns in the cell beyond simply receptor makeup. It seemed likely that primary human and transgenic mouse DCs and monocytes express unique regulatory factors that influence FcεRI trafficking that are not found in basophils, mast cells, or the U937 cell line.

To overcome this initial limitation, we devised a method of forcing murine FcεRIβ expression in all hematopoietic cells of the hFcεRIα-transgenic mouse to see whether expression of FcεRIβ in vivo changed FcεRIα trafficking patterns in transgenic monocytes. Mouse bone marrow stem cells were infected ex vivo with an FcεRIβ-IRES-GFP construct using a lentiviral vector and were transferred into a lethally irradiated Tg^- host as a mixture of GFP^+ and GFP^- (uninfected) cells. As a control, we also generated mixed bone marrow chimeras with empty GFP-expressing vectors mixed with uninfected cells. 4 weeks post-transfer, we found a significant number of GFP^+ cells, but no differences in surface FcεRIα levels between GFP^+ and negative monocytes or basophils (data not shown). This indicated to us that FcεRIβ does not influence surface levels in vivo as it does in U937 cells. Unfortunately, there were some early caveats to this work. First, we did not test the cells by confocal microscopy for the intracellular localization of FcεRI complexes. It is possible that while surface FcεRI levels were not affected by the expression of FcεRIβ, that intracellular trafficking was altered. Second, it is possible that the transfection did not generate a stable FcεRIβ protein that was able to complex with FcεRIα and FcεRIγ. Further studies infecting hFcεRIα Tg^+ BMDCs with FcεRIβ-GFP and then performing co-immunoprecipitation for hFcεRIα to detect the presence of complexed FcεRIβ along with FcεRIα and γ would need to be done to ensure that the construct can complex with the other chains of FcεRI properly.

Receptor trafficking within the cell often comes under the control of ubiquitination machinery; MHCII, for example, is constitutively endocytosed in immature DCs due to expression of the E3 ubiquitin ligases of the March family, which bind to MHCII and target it for endocytosis and degradation. Upon maturation of the DC, expression of March proteins decreases, resulting in an overwhelming increase in surface MHCII levels (Shin et al., 2006a). In fact, previous studies have shown that endocytosis, endosomal sorting, and degradation of crosslinked FcεRI requires ubiquitination via Cbl family E3 ligases (Fattakhova et al., 2009; Molfetta et al., 2010). Is it possible that this also occurs in the absence of crosslinking stimulus? Future work in the lab will be dedicated to investigating this possibility. First, western blotting for ubiquitin moieties on FcεRIα isolated from healthy human DCs versus basophils will help identify whether ubiquitin is bound to FcεRIα of DCs in the steady state. This could be compared to IgE- and antigen-crosslinked DCs and basophils as positive controls for FcεRI ubiquitination and endocytosis. In addition, co-immunoprecipitation studies using an anti-FcεRI α-chain antibody as bait could detect α-chain binding partners that might be involved in FcεRI trafficking, such as Cbl family proteins. By performing mass spectroscopy analysis of proteins pulled down with FcεRIα in DCs but not in basophils, we may be able to identify previously unknown regulators of FcεRI surface levels. Western blots for Cbl, Rabex, and related ubiquitination machinery could also easily be performed with FcεRIα co-immunoprecipitates to check for proteins previously found to be associated with FcεRI only during receptor crosslinking (Fattakhova et al., 2009; Molfetta et al., 2010). Finally, in an ideal scenario, cytoplasmic lysine residues on FcεRIα could be mutated to see if trafficking patterns of the receptor change when lysine residues are gone; however, previous difficulty with generating an in vitro cell line model of FcεRI trafficking has so far precluded this possibility.

Alternatively, it is possible that glycosylation of FcεRIα plays a role in targeting the receptor complex for endocytosis and degradation. Ours and other labs have found that the α-chain is glycosylated differently in primary human blood basophils and DCs (Maurer et al., 1996). This glycosylation is not thought to influence IgE binding to the receptor, as previous work has shown that alteration or impairment of FcεRIα glycosylation patterns does not impair IgE binding (Albrecht et al., 2000; Garman et al., 1999; Kraft et al., 1998). Instead, specific glycosylation could promote the receptor's interaction with a chaperone protein that targets the receptor for endocytosis. For example, MUC1 receptor mutants lacking proper O-linked glycosylation end up accumulating intracellularly, potentially due to an increase in endocytosis from the cell surface (Altschuler et al., 2000). Other studies have seen an increase in endocytosis of multiple receptors upon inhibition of N-linked glycosylation (Vagin et al., 2009), thereby effectively decreasing apical surface receptor levels. Interestingly, this phenomenon could explain why U937 cells fail to recapitulate the FcεRI trafficking patterns seen in primary human cells; both trimeric and tetrameric-expressing U937 cell lines show identical band patterns of FcεRIα by western blot (Donnadieu et al., 2000), indicating that both cell lines show similar FcεRIα glycosylation unlike primary human basophils and dendritic cells. Perhaps other cell lines more accurately recapitulate FcεRI glycosylation patterns seen in human primary cells. For example, rat basophilic leukemia (RBL) cells incubated with glucosidase I inhibitors have normal FcεRI-IgE binding affinities, yet total IgE bound to the cell is decreased – leading to the hypothesis that receptor localization on the cell surface may be impaired (Gavériaux and Loor, 1987). In fact, incubation with glucosidase inhibitors caused a decrease in the molecular weight of FcεRIα by western blot that was resistant to treatment with EndoH, similar to what we saw with primary human DCs compared to basophils (LaCroix and Froese, 1993). Other groups,

however, did not see a similar trend with FcεRI-expressing CHO cells (Albrecht et al., 2000); however, CHO cells appear to have altered glycosylation in a similar pattern to primary blood DCs (Maurer et al., 1996), which would render glucosidase inhibitors useless. A simple way to test the relevance of these findings would be to incubate both tetrameric and trimeric U937 cells in the presence of a variety of inhibitors of glycosylation (see (Varki et al., 2009) for a summary of useful inhibitors) and compare intracellular FcεRI localization with band patterns by Western blot. If treatment with an inhibitor changes the localization of FcεRI and induces an Endo-H resistant shift in kD, FcεRIα glycosylation is crucial for receptor localization and half-life on the cell surface. Alternatively, mass spectroscopy of primary human DC FcεRIα versus basophil FcεRIα and U937-expressed FcεRIα could comprehensively identify differences in glycosylation between cell types. Then, U937 cells can be used in concert with glycosylation inhibitors to reproduce identified patterns of FcεRI glycosylation found in blood DCs to look for changes in FcεRI localization.

Effects of FcεRI trafficking on DC function

FcεRI trafficking may significantly influence the DC's response to IgE. For example, IgE endocytosis could limit a DC's capacity to respond to the presence of allergen by temporally restricting the amount of time that any given clone of IgE is present on the surface of the cell and able to be crosslinked with allergen. Mouse studies in our laboratory showed that IgE is present on basophils and mast cells for a long period of time (>24 hrs, and likely much longer, according to previous work (Kubo et al., 2003)) whereas on DCs and monocytes it is greatly diminished even by 12 hrs post-injection and is essentially gone by 24 hrs (Chapter III, Figure 5). Therefore, the ability of a DC or monocyte to respond to the presence of allergen is limited to a window

during which the IgE is bound and the allergen could be present. Because DCs are particularly potent at promoting T-cell mediated responses, this phenomenon might especially limit the propensity for T-cell dependent allergic responses in humans. This could be tested in a number of ways using human and mouse models. For example, isolated human DCs and basophils could be loaded with human IgE, incubated for various timepoints, and activated via the addition of crosslinking antigen. Then, functional tests could be done with the cells, including T-cell co-culture with the activated DCs, and analysis of histamine release by basophils. The main limitation of this study, unfortunately, is that isolated primary human DCs rapidly downregulate FcεRI in culture (Kraft et al., 1998), which complicates any comparison with basophils cultured in vitro. Further development of proper human DC in vitro culture systems that do not influence FcεRI expression levels would significantly help with this aim, though there remain significant obstacles to this goal. Many of these limitations can be overcome with the use of the human FcεRIα transgenic mouse. BMDCs cultured for 0 or 12 hrs after being loaded with TNP-specific IgE can be compared for maturation and FcεRI crosslinking upon incubation with TNP-ova; DCs can then be incubated with ova-specific OTII cells to compare their ability to activate and skew T-cell responses. Based on our work, we would hypothesize that DCs let to rest for 12 hrs after IgE loading will lose surface IgE over time and will respond less to FcεRI crosslinking with cognate antigen. Basophils, however, should show no changes in the strength of FcεRI crosslinking over a 12 hr period.

Sallmann et al. (2011) used CD11c-FcεRIα transgenic mice to show that FcεRI expressed by DCs exacerbates allergy. It would be interesting to repeat these studies using the human FcεRIα transgenic mouse (Dombrowicz et al., 1996) and compare mice that had been exposed to allergen immediately after injection with human IgE with mice that are exposed to allergen 24

hrs after IgE injection. Presumably, this should also illustrate the contribution of DC and monocyte FcεRI to allergic exacerbation while highlighting the temporal limitations of FcεRI's function on these cells.

FcεRI trafficking in atopy

Our current understanding of IgE regulation and allergy is complicated by the differential trafficking patterns of FcεRI seen in human DCs and basophils. Given the degradation of IgE in the lysosomes of human DCs and the rapid loss of serum IgE in an FcεRI-dependent manner in transgenic mice, it appears that DCs are potent at destroying IgE and therefore could play a tolerogenic role in the development of allergy. At first glance, this may not seem particularly effective given the remarkably high affinity of FcεRI for IgE, the long half-life of IgE on FcεRI, and the half-lives of basophils and mast cells in blood and tissues. Despite decreased serum IgE, basophils and mast cells will still be heavily saturated with IgE and will retain it for a as much as weeks at a time in vivo (Kubo et al., 2003). Despite these limitations, it remains possible (and even likely) that constitutive IgE endocytosis by monocytes and DCs could serve to limit the possibility for FcεRI crosslinking by limiting the relative abundance of any given clone of IgE circulating in the body. IgE endocytosis could also limit systemic allergic responses by effectively lowering surface FcεRI on mast cells and basophils. Omalizumab is a humanized, monoclonal antibody that binds to IgE and prevents it binding to FcεRI in vivo (Presta et al., 1993), causing effective serum IgE levels to decrease precipitously. One effect of omalizumab treatment is that with less IgE bound, surface FcεRI levels on mast cells (Beck et al., 2004) and even DCs (Prussin et al., 2003) also precipitously decline. Like omalizumab, DCs may decrease the concentration of serum IgE by endocytosing and degrading it. With less serum IgE, there is

less IgE-mediated FcεRI stabilization on mast cells and basophils, which effectively lowers surface FcεRI levels.

Do individuals with high IgE have defective FcεRI trafficking in their DCs and monocytes? It is possible that aberrant FcεRI trafficking in atopic patients may contribute to serum IgE accumulation because of a lack of constitutive IgE endocytosis in DCs and monocytes. Initial tests with freshly isolated DCs from human donors with allergic asthma with high IgE found no gross differences in FcεRI localization compared to healthy donors (data not shown), although there may have been significant differences in the relative proportions of lysosomal to cell-surface FcεRI. It will be important to compare the rates of IgE internalization by healthy and allergic DCs by incubating cells for 4 hrs with 0.5 μg/mL human IgE-Alexa 647 and quantifying the relative amounts of surface-bound and intracellular IgE following incubation. If allergic DCs internalize significantly different amounts of IgE compared to healthy DCs, it may be worthwhile to do comparative microarrays on healthy and allergic DCs to help identify genes that may be involved in the differential trafficking of FcεRI, as a parallel to biochemical methods listed in the previous sections.

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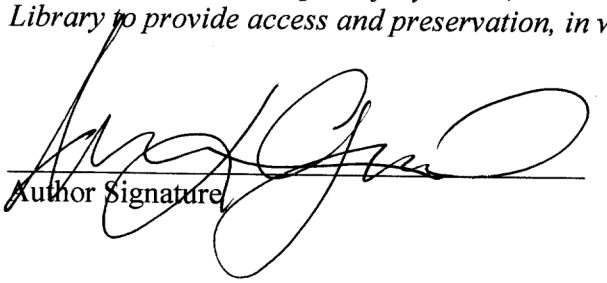

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