REGULATION OF HUMAN MONOCYTE FUNCTION BY

CROSS-LINKING OF IMMUNOGLOBULIN E

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DEDICATION

As I complete this journey through graduate school I cannot help but think of all those who helped me along the way. I would not have made it this far without their support, so I would like to dedicate this dissertation:

To Dr. Michelle Gill, my wonderful mentor and my friend. You taught me when I needed to learn, you pushed me when I needed motivation, and you listened to me when I had ideas. Your enthusiasm for science made every meeting exciting. Your constant belief in me made me believe in myself even when I was ready to give up. You showed me that it's possible to be a brilliant scientist, a caring physician, and a great parent at the same time. You have taught me so much in our years together and I consider myself lucky to have had you as a mentor.

To my thesis committee – Dr. David Farrar, Dr. Becky Gruchalla, Dr. Felix Yarovinsky, and Dr. Jeffrey Kahn. Your guidance and challenges helped to guide this project and develop me into a better scientist.

To Dr. Victoria Yang, Dr. Regina Rowe, and Ms. Lorene Sandifer. Your assistance during your rotations helped me to accomplish much more than I could have alone. You worked tirelessly along side me through nights and weekends when you didn't have to. It was a pleasure working with each one of you.

To Gagan, Zheng, Baomei, Nan, and Caroline. Each of you taught me a lot during my time in the lab. Your discussions of science and chats about life made the Gill lab a fun place to work.

To Sarah, Navin, Jon, Didem, Leo, Kristan, and Dr. Farrar. Your insightful questions and suggestions during lab meetings helped me troubleshoot more experiments than I can count. You were always ready to lend reagents, talk science, and just have fun.

To Harmony, Stephanie, and Robin. Your hard work in making the Immunology Program and Medical Scientist Training Program run smoothly has made my life easier on many occasions. I greatly appreciate each one of you.

To Mom, Dad, Jon, and Bekah. Your continuous love and support has been a joy during the best of times and helped me endure the worst of times. I love you all.

To Alaina, my amazing wife. You have shared the emotions of graduate school with me through the good times and the bad, the failed experiments and the new discoveries. You have always pushed me to be better at everything I do. You supported me through the late night experiments, the weekends at lab, and the weeks of writing this document. I love you very much.

To Alexander, my little miracle. You are my inspiration. Your constant excitement and joy never fails to make me smile. I love you buddy.

REGULATION OF HUMAN MONOCYTE FUNCTION BY CROSS-LINKING OF IMMUNOGLOBULIN E

By

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DISSERTATION

Presented to the Faculty of the Graduate School of Biomedical Sciences

The University of Texas Southwestern Medical Center at Dallas

In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

The University of Texas Southwestern Medical Center at Dallas

Dallas, Texas

May, 2013

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REGULATION OF HUMAN MONOCYTE FUNCTION BY

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Cross-linking of IgE by allergen triggers many cellular processes that drive allergic disease. While the role of IgE in mediating allergic responses is best described on basophils and mast cells, expression of the high-affinity IgE receptor on other innate immune cells, including monocytes, suggests that it may impact the function of these cells in allergic environments. Exacerbations of allergic disease have been associated with allergen exposure as well as viral and bacterial infection, but the mechanisms of these phenomena are not well understood. Monocytes are recruited to sites of inflammation in both allergic disease and infection, serving a number of important functions, including cytokine secretion, phagocytosis, and stimulation of

adaptive immune responses. However, the impact of IgE cross-linking on monocyte functions is poorly understood.

To determine how IgE cross-linking affects monocyte phenotype and function I isolated primary human monocytes from blood samples and stimulated them in the presence or absence of a cross-linking anti-IgE antibody. My studies reveal that IgE cross-linking induces up regulation of CD14, an important component of inflammatory responses. IgE cross-linking on monocytes also induces secretion of inflammatory cytokines – including tumor necrosis factor α, and interleukin-1, -6, and -23 – as well as autoregulatory interleukin-10. These inflammatory responses to IgE cross-linking are enhanced in monocytes from individuals with elevated serum IgE concentration, compared to monocytes from individuals with normal IgE concentration. In contrast, IgE cross-linking suppresses expression of an important mediator of phagocytosis, CD64. Indeed, IgE cross-linking specifically impairs monocyte phagocytic function without disrupting the capacity of monocytes to kill intracellular bacteria. IgE cross-linking also reduces expression of several surface molecules associated with antigen presentation and inhibits virus-induced up regulation of these molecules. Furthermore, IgE cross-linking during virus exposure suppresses monocyte-driven differentiation of type 1 helper T cells.

My findings suggest that IgE cross-linking on monocytes may contribute to allergic disease as well as pathogen-associated exacerbations of disease by three mechanisms: 1) enhancing detrimental inflammatory responses in a serum IgE-dependent manner, 2) concomitantly crippling phagocytosis, a primary mechanism utilized by these cells to resolve inflammation, and 3) suppressing stimulation of appropriate T cell responses in response to infection.

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LIST OF ABBREVIATIONS

° C – Degrees Celsius

APC – Allophycocyanin

BAL – Broncho-alveolar lavage

cAMP – Cyclic adenosine monophosphate

CCL – C-C motif chemokine ligand

CCR – C-C motif chemokine receptor

cDMEM – Complete DMEM medium

CDP – Common dendritic cell progenitor cell

CFSE – Carboxyfluorescein succinimidyl ester

CFU – Colony-forming units

cGMP – Cyclic guanosine monophosphate

COAST – Childhood Origins of Asthma

COPD – Chronic obstructive pulmonary disease

cPBS – Complete PBS

cRPMI – Complete RPMI medium

CTLA4 – Cytotoxic T lymphocyte antigen 4

CXCL – C-X-C motif chemokine ligand

DC – Dendritic cell

DIC – Differential interference contrast

DMEM – Dulbecco's Modified Eagle Medium

ED – Emergency Department

EDTA – Ethylenediaminetetraacetic acid

ELISA – Enzyme-linked immunosorbent assay

FBS – Fetal bovine serum

FcγRI – CD64; high-affinity IgG receptor

FcγRIII – CD16; low-affinity IgG receptor

FcεRI – High-affinity IgE receptor

FITC – Fluorescein isothiocyanate

Flu – Influenza virus

GM-CSF – Granulocyte/macrophage-colony stimulating factor

HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HLA – Human leukocyte antigen

hRV – Human rhinovirus

IFN – Interferon

Ig – Immunoglobulin

IgG – Rabbit whole IgG antibody

IL – Interleukin

IL-1RA – IL-1 receptor antagonist

IP-10 – CXCL10, Interferon-inducible protein 10

ISG – Interferon-stimulated genes

ITAM – Immunoreceptor tyrosine-based activation motif

KC – CXCL1

LAT – Linker for activation of T cells

LPS – Lipopolysaccharide

M-CSF – Macrophage-colony stimulating factor

MAPK – Mitogen-activated protein kinase

MCP-1 – CCL2; monocyte chemoattractant protein 1

mDC – Myeloid dendritic cell

MDP – Monocyte, macrophage, and dendritic cell progenitor cell

MEM – Minimum Essential Medium

MESF – Mean equivalent standard fluorescence

MHC – Major histocompatibility complex

MIP-1α – CCL3; macrophage inflammatory protein 1 α

MLR – Mixed leukocyte reaction

MOI – Multiplicity of infection (infectious units per cell)

NFκB – Nuclear factor κ B

OVA – Ovalbumin

PAMP – Pathogen-associated molecular pattern

PBMC – Peripheral blood mononuclear cells

PBS – Phosphate-buffered saline

pDC – Plasmacytoid dendritic cell

PE – Phycoerythrin

PerCP – Peridinin chlorophyll

PFU – Plaque-forming units

PI3K – Phosphoinositide 3-kinase

PLCγ – Phospholipase C γ

PMA – Phorbol 12-myristate 13-acetate

RANTES – CCL5; regulated on activation, normal T cell expressed and secreted

rh – Recombinant human

RPMI – Roswell Park Memorial Institute Medium 1640

RSV – Respiratory syncytial virus

sCD14 – Soluble CD14

SHIP – SH2-domain-containing inositol 5' phosphatase 1

SOCS – Suppressor of cytokine signaling

TGFβ – Transforming growth factor β

Th1 – Type 1 helper T cell

Th17 – Type 17 helper T cell

Th2 – Type 2 helper T cell

TLR – Toll-like receptor

TNFα – Tumor necrosis factor α

TTP – Tristetraprolin

U – Units

US – United States

αIgE – Rabbit anti-human IgE antibody

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

Introduction to Allergic Disease

Allergy is defined by the World Allergy Organization as "a hypersensitivity reaction initiated by immunologic mechanisms" (Johansson 2004). Though allergies can be mediated by a number of immunological mechanisms, many common allergic diseases are mediated by immunoglobulin (Ig) E (Jones 2008). Atopy indicates a tendency for an individual to become sensitized to an allergen and produce IgE antibodies against that allergen (Johansson 2004). Through complex interactions between genetic predisposition and environmental factors, atopic individuals may develop symptoms of atopic diseases such as allergic asthma, allergic rhinitis, and atopic dermatitis (Jones 2008).

Immunologically, atopic diseases are mediated by a complex network of cells and cytokines, involving both the innate and adaptive immune systems. B cells produce specific IgE antibodies against sensitized allergens (Hamilton 2010). IgE binds strongly to the high-affinity IgE receptor (FcεRI), which is expressed on a variety of innate immune cells (Novak 2001b). IgE bound to FcεRI can be cross-linked by multivalent allergens and mediate cell-type specific responses in these cells (Novak 2003; Wu 2011a). During allergic sensitization, naïve $CD4^+$ T cells are activated and induced to become type 2 helper T (Th2) cells (Holgate 2012). Both dendritic cells (Maurer 1998; Schroeder 2010) and basophils (Sokol 2009) have been implicated in presenting allergens to naïve $CD4^+$ T cells and promoting Th2 differentiation. Activated Th2

cells secrete cytokines such as interleukin (IL)-4, IL-5, and IL-13, which promote IgE production from B cells (Del Prete 1988; Pene 1988) and stimulate effector responses in macrophages (Hart 1999), eosinophils (Kita 1992), and epithelial cells (Zhu 1999).

Healthcare Burden of Atopic Disease

Atopic diseases represent an ever-increasing healthcare burden for pediatric and adult populations in the United States (US): Between 1980 and 1996, childhood asthma period prevalence increased by an average of 4.6% per year (Akinbami 2009). In 2009, asthma prevalence in the US was 8.2%, with approximately 17.5 million adults and 7.1 million children being affected by this disease (Lafeuille 2012). Atopic dermatitis has been reported in several studies to have a lifetime prevalence of between 5 and 20% of children worldwide (Carroll 2005). Thirteen percent of US children and 14% of adults have been formally diagnosed with allergic rhinitis, though several studies have estimated the true prevalence to be as high as 40% in children and 30% in adults (Meltzer 2009).

Despite advances in the understanding of disease pathogenesis and several therapeutic regimens, atopic disease is still represents a large burden on the healthcare system. In 2006, asthma was responsible for 593,000 Emergency Department (ED) visits and 155,000 hospitalizations in children, accounting for 2.3% of all pediatric ED visits and 5.6% of all pediatric hospitalizations (Akinbami 2009). In 2007, asthma accounted for 1.8 million ED visits and 456,000 hospitalizations among children and adults. Atopic dermatitis also represents a significant healthcare burden: a 1993 study estimated an annual cost of $364 million for physician and ED visits, while another study in 2002 estimated total direct costs of $0.9-3.8

billion per year (Carroll 2005; Ellis 2002; Mancini 2008). In addition to the financial burden presented, atopic disease also results in significant social burden. In a recent survey, parents of children with allergic rhinitis report significantly less happiness and productivity for their children as compared to children without allergic rhinitis (Meltzer 2009). Children and adults with atopic dermatitis also report lower quality of life compared to their unaffected peers (Carroll 2005).

Contribution of Viral Infection to Atopic Disease

An important feature of allergic asthma is disease exacerbations, in which patients experience acute worsening of their asthma symptoms and frequently require hospitalization. Exacerbations of asthma are associated with a number of factors, including allergen exposure and environmental irritants (Singh 2006). Since the early 1970's, respiratory virus infections have also been recognized as an important cause of acute exacerbations of asthma (Minor 1974). In a 1995 study of asthmatic schoolchildren by Johnston et al., respiratory virus infection was detected in greater than 80% of asthma exacerbation episodes. In this and other studies, human rhinovirus (hRV) accounted for 60-65% of viruses detected. Also detected were influenza virus, parainfluenza virus, respiratory syncytial virus (RSV), human metapneumovirus, and coronaviruses (Johnston 1995; Khetsuriani 2007). A study of adults with asthma compared the concurrence of subjective asthma exacerbation with symptoms of the common cold: 80% of patients complaining of asthma exacerbations had concurrent cold symptoms and 89% of symptomatic colds presented with worsening asthma symptoms (Nicholson 1993). Confirming the relevance of these epidemiological associations, two studies observed more severe illness and

reduced respiratory function in asthmatic, but not control, human subjects after experimental infection with hRV (Message 2008; Zambrano 2003). During hRV infection of subjects with high serum IgE concentration, Zambrano et al. also observed a marked increase in nasal eosinophil cationic protein and expired nitric oxide, two clinical markers of asthma exacerbations. In addition to reduced respiratory function, Message et al. observed increased infiltration of lymphocytes, neutrophils, and eosinophils into the lung of hRV-infected asthmatic subjects as compared to non-asthmatic controls. The authors also observed a greater Th2 response to hRV in asthmatic subjects; this response was correlated with more severe disease. These studies extended the earlier reports of associations between hRV detection and asthma exacerbations by providing evidence of a causative effect for hRV infection on the induction of asthma exacerbations.

Interestingly, several studies have observed that atopy and respiratory virus infection act in a synergistic manner to increase the risk of asthma exacerbations. In a study by Green et al., the combination of exposure to a sensitized allergen with virus infection increased the risk of hospitalization higher than either factor alone (Green 2002). Another (Rakes 1999) reported that the risk of wheezing in children was more than 3 times greater in individuals with both hRV infection and elevated allergen-specific IgE level than in individuals with only one of those factors. The synergistic relationship between viral infection and allergen sensitization and exposure suggests an interaction between viral and allergic immune responses.

The role of respiratory virus infection at the onset of allergic disease has also been an area of great interest and controversy. The “hygiene hypothesis”, put forth by Strachan in 1989, suggests that early childhood infections are protective against the development of atopic disease (Strachan 1989). However, viral bronchiolitis in early childhood has been associated with

increased risk of subsequent atopic disease (Sigurs 2000; Wu 2008). Several birth cohort studies have provided evidence supporting a role for viral infection in the onset of allergic asthma (Jackson 2008; Kusel 2007; Wu 2008). The Childhood Origins of Asthma (COAST) study observed that wheezing associated with RSV or hRV during the first 3 years of life conferred a significantly higher risk of asthma at age 6 (Jackson 2008). The authors found that hRV-associated wheezing during the 3rd year of life was the strongest independent predictor of asthma diagnosis, with an odds ratio of 25.6, representing an even greater risk than allergen sensitization. In a second cohort, Kusel et al. found similar associations for hRV and RSV wheezing illnesses as risk factors for later asthma diagnosis (Kusel 2007). A large cohort study by Wu et al. reported that children born approximately 4 months before the winter respiratory virus season had a higher incidence of asthma than their peers born at different times (Wu 2008). Long term follow up of children who were hospitalized for RSV bronchiolitis before age 1 found that the increased risk of asthma persisted into early adulthood (Sigurs 2010).

Despite the clear association of these viruses with the risk of developing asthma, these studies do not prove causation. In fact, given the widespread prevalence of respiratory viral infections (Hall 2009) it is clear that not all children who experience hRV or RSV infections will develop asthma. Several theories have been posited concerning this issue. Some have suggested that viral infections may not cause asthma, but rather that certain genetic factors predispose children to both asthma and severe viral bronchiolitis (Singh 2007; Thomsen 2009). The authors of the COAST study suggest that prior sensitization to aeroallergens may actually lead to more severe viral infection (Jackson 2012). Others have suggested that certain viral strains are more likely than others to be associated with asthma (Kistler 2007; Miller 2009). Taken together, the

data suggest that many factors – genetic, environmental, and infectious – work in concert to contribute to the development of allergic asthma.

Though the role of viral infection in the development and exacerbation of atopic disease has been most studied in regard to allergic asthma, evidence for the involvement of infection in other atopic diseases has also been observed. Individuals with atopic dermatitis experience skin infection with viruses and bacteria more frequently than their unaffected peers (Boguniewicz 2010). A particularly severe form of atopic dermatitis, called eczema herpeticum, can occur with patients acquire disseminated infection with herpes simplex virus (Wollenberg 2003). Individuals with atopic dermatitis have also been observed to acquire disseminated infection from the live attenuated smallpox vaccine (Reed 2012). The propensity of patients with atopic dermatitis to develop complicating skin infections has been attributed to loss of skin barrier integrity (Broccardo 2011) as well as abnormal immune responses (Leung 2011). Allergic rhinitis has been associated with greater incidence and severity of otitis media with effusion (middle ear infection) in young children (Skoner 2000). A study of more than 200 children observed a much higher prevalence of allergic rhinitis among those with chronic otitis media (89%) than in the general population (20%), further supporting a strong relationship between these conditions (Alles 2001). Though the exact mechanisms of interaction are not fully understood, it is clear that atopic disease and microbial infection are intrinsically linked.

IgE Plays a Central Role in Atopic Disease

The association of IgE with allergic responses was first described in a series of publications by Ishizaka and Ishizaka in 1966 (Ishizaka 1966a). They determined that the serum

protein associated with hypersensitivity reactions, then termed “reaginic antibody” was in fact a new class of Ig distinct from the previously described IgG, IgA, IgD or IgM (Ishizaka 1966b). Since that time, the clinical immunology field has evolved to include total and allergen-specific IgE concentrations as primary biomarkers to reflect allergen sensitization (Hamilton 2010; Kjaer 2009; Matsui 2010). Exemplifying the importance of IgE in atopic disease is the finding that significant correlations exist between total and allergen-specific serum IgE concentration and disease severity in allergic asthma and atopic dermatitis (Kovac 2007; Laske 2004; Matsui 2010).

The clear association between IgE and atopic disease has led to profound efforts to develop IgE-reducing clinical therapies. The monoclonal anti-IgE antibody omalizumab has achieved remarkable success in clinical trials (Busse 2001). Omalizumab binds to IgE only when it is not occupying its receptor, effectively lowering free IgE in serum without activating FcεRI (Presta 1993) The inability of Omalizumab to cross-link receptor-bound IgE prevents it from inducing anaphylaxis. Omalizumab has been shown to significantly improve symptoms of allergic asthma while also reducing dependence on corticosteroids and reducing asthma-related ED visits and hospitalizations (Busse 2011; Lafeuille 2012). Omalizumab treatment also reduced symptom severity and antihistamine use in patients with allergic rhinitis (Casale 2001). Limited case studies have also shown efficacy for omalizumab in reducing disease severity in patients with atopic dermatitis (Kim 2012; Thaiwat 2011).

Expression and Regulation of FcεRI

FcεRI consists of three subunits: α, β, and γ. The α subunit is responsible for binding IgE at the cell surface (Baniyash 1987), while the β and γ domains contain immunoreceptor tyrosine-based activation motifs (ITAM) and associate with FcεRIα at the cell surface (Wu 2011a). The cell types classically associated with allergic responses, mast cells and basophils, express FcεRI as an αβγγ heterotetramer (MacGlashan 2008). Antigen presenting cells, such as dendritic cells (DC) and monocytes, have also been shown to express FcεRI (**Figure 1.1**), though at a lower level than basophils (Foster 2003; Sihra 1997). On antigen presenting cells, the β subunit is absent, leaving an αγγ trimeric receptor (Novak 2003). The trimeric receptor is functional and responds to cross-linking of IgE with intracellular signaling (Lin 1996), implying that the β subunit is not required for signaling. While the exact role of the β subunit is not clear, several findings point to an amplifying role: the αβγγ isoform of FcεRI displays 5-7 fold higher signal strength upon IgE cross-linking (Lin 1996) and 4-6 fold higher surface expression (Donnadieu 2000) as compared to the αγγ isoform.

Though many factors can influence the surface expression of FcεRI, IgE itself plays an important role in dictating the availability of its own receptor (Borkowski 2001; MacGlashan 2005). In basophils, IgE enhances surface FcεRI expression by increasing its stability at the cell surface, but does not affect transcription or translation (MacGlashan 2001). When not bound to IgE, FcεRI regularly undergoes endocytosis and can be either recycled to the cell surface or degraded in the lysosome (MacGlashan 2007).

The role of IgE in regulating FcεRI expression was first observed in the late 1970's when Malveaux et al reported a correlation between serum IgE concentration and the number of surface FcεRI molecules on basophils (Malveaux 1978). Since that time, serum IgE

concentration has also been correlated with FcεRI expression on DCs (Foster 2003) and monocytes (Cheng 2006; Pyle 2013). Given the association of elevated IgE with worsening atopic disease, it follows that FcεRI expression should also reflect disease severity. Indeed, numerous studies have observed that individuals with atopic disease have elevated FcεRI expression on basophils, DCs, and monocytes (Beeren 2005; Cheng 2006; Foster 2003; Gill 2010; Sihra 1997). Lowering IgE with omalizumab treatment has been shown to reduce FcεRI expression on basophils, mast cells, DCs, and monocytes (Beck 2004; Cheng 2006; MacGlashan 1997; Prussin 2003). Additionally, omalizumab therapy resulted in reduced basophil and mast cell responses to sensitized allergens (Eckman 2010).

IgE-Mediated Signaling Through FcεRI

The current understanding of FcεRI signaling in mast cells was recently summarized by Wu (Wu 2011a). When FcεRI is aggregated on the cell surface via cross-linking of receptor-bound IgE, the Src family kinase Lyn phosphorylates the ITAMs on the β and γ subunits. Another Src kinase, Syk, is recruited to the phosphorylated β and γ subunits and is itself phosphorylated by Lyn. Syk recruits and activates the adaptor proteins Linker for Activation of T cells (LAT) and LAT2. These adaptor proteins activate a variety of downstream signaling pathways, including phospholipase C γ (PLCγ) and Calcium, phosphoinositide 3-kinase (PI3K) and lipid mediators, mitogen-activated protein kinases (MAPK) – including p38, Jnk, and Erk – and Akt, which collectively promote cell survival and mediate effector functions. In mast cells and basophils, cross-linking of receptor-bound IgE induces release of granules containing preformed inflammatory mediators including histamine (Baniyash 1987), prostaglandins (Ugajin

2011), and cytokines such as IL-4 and IL-13 (Amin 2012; Redrup 1998) (**Figure 1.1**). Binding of monomeric IgE to the FcεRIα subunit enhances mast cell survival but does not induce degranulation. This is thought to result from a low level of signaling through monomeric IgE, which does not reach the necessary threshold for degranulation.

Over the last 2 decades, the importance of FcεRI on dendritic cells, critical antigen presenting cells (**Figure 1.1**), has gained recognition (Novak 2003). Two primary subtypes of dendritic cells are found in human blood: myeloid (mDC) and plasmacytoid (pDC). Myeloid DCs, also called conventional DCs, serve as professional antigen presenting cells and secrete cytokines to regulate adaptive immune responses (de Heer 2005). Plasmacytoid DCs, also known as professional interferon (IFN) producing cells, play a critical role in anti-viral responses by secreting type I IFN (IFNα and IFNβ) in response to virus exposure or toll-like receptor (TLR) stimulation (Guiducci 2008). IgE cross-linking on both mDCs and pDCs induces secretion of tumor necrosis factor α (TNFα) and IL-10 (Le 2009; Schroeder 2008). Both mDCs and pDCs from allergic individuals were able to induce proliferation and Th2 polarization of naïve $CD4^+$ T cells when exposed to relevant allergen, but this effect was reduced after treatment with omalizumab (Schroeder 2010). An interesting cross-regulation between anti-viral responses and allergic responses has been observed in pDCs: IgE cross-linking reduced expression of the virus responsive TLRs 7 and 9, while TLR7 and TLR9 agonists and influenza reduced FcεRI expression (Gill 2010; Schroeder 2005). Individuals with atopy exhibit reduced pDC IFNα secretion upon stimulation with TLR7 and TLR9 agonists or influenza (Gill 2010; Tversky 2008). Confirming the interactions between virus and allergic responses in pDCs, influenza-induced IFNα correlated inversely with both serum IgE concentration and pDC FcεRI expression (Gill 2010). IFNα from pDCs is also able to augment FcεRI-mediated auto-regulatory IL-10

secretion from mDCs (Le 2009), suggesting that virus-induced IFNα from pDCs may play a role in modulating allergic responses in other cell types. Indeed, IFNα has been shown to inhibit differentiation of naïve $CD4^+$ T cells into Th2 cells by reducing the expression and activity of the Th2 master transcription factor GATA-3 (Huber 2010). IFNα also reversed the Th2 commitment of previously differentiated $CD4^+$ T cells (Huber 2010), highlighting the importance of type I interferon in the regulation of allergic responses and the potential role IgE-mediated effects on the secretion of this cytokine in the pathogenesis of atopic disease.

Monocyte Recruitment and Differentiation

The development of monocytes, macrophages, and dendritic cells was recently reviewed by Geissmann et al. (Geissmann 2010). Monocytes are derived from the myeloid lineage through a committed progenitor cell called a monocyte, macrophage and dendritic cell progenitor cell (MDP). After leaving the bone marrow, newly formed monocytes circulate through the blood stream before exiting into tissues where they can differentiate into macrophages (van Furth 1968). Myeloid dendritic cells can be derived from monocytes as well as a common dendritic cell progenitor cell (CDP), which is also derived from the MDP precursor. Monocyte development in the bone marrow is highly dependent on the growth factor macrophage-colony stimulating factor (M-CSF) (Wiktor-Jedrzejczak 1996). Another myeloid growth factor, granulocyte/macrophage-colony stimulating factor (GM-CSF), is not required for monocyte development (Stanley 1994), but is capable of stimulating monocyte differentiation into macrophages (Fleetwood 2007) or dendritic cells (Conti 2008).

A number of studies have documented the recruitment of monocytes to tissues under a variety of conditions (**Figure 1.2**). By labeling dividing monocyte precursors with [^{3}H]Thymidine, van Furth and colleagues found that monocytes contribute to macrophage populations in the lung, liver, spleen and peritoneum during both steady-state and inflammatory conditions (van Furth 1985). Upon depletion of lung myeloid cells in mice, monocytes can replenish both parenchymal and alveolar macrophages in the lung (Landsman 2007a). In humans, monocytes have been observed in broncho-alveolar lavage (BAL) fluid after lipopolysaccharide (LPS) inhalation (Schaumann 2008) and in nasal secretions during influenza infection (Gill 2008). The mechanisms of monocyte recruitment during inflammation have also been widely studied. Blockade of monocyte chemoattractant protein-1 (MCP-1), also known as C-C motif chemokine ligand (CCL)2, in mice prevented the normal accumulation of macrophages in the lung after influenza infection (Narasaraju 2010). Similarly, monocytes deficient for the MCP-1 receptor, C-C motif chemokine receptor (CCR)2, were unable to migrate to the lung during influenza infection (Herold 2006). In a murine model of pneumococcal pneumonia, three chemokines – MCP-1, macrophage inflammatory protein 1 α (MIP-1α; CCL3), and regulated on activation, normal T cell expressed and secreted (RANTES; CCL5) – were responsible for monocyte recruitment to the lung (Fillion 2001). The myeloid growth factors M-CSF and GM-CSF have also been implicated in monocyte recruitment. One study found that both M-CSF and GM-CSF were involved in monocyte recruitment to mouse lung and peritoneum during inflammation induced by inhaled LPS or intraperitoneal thioglycolate, while only M-CSF was involved in steady-state macrophage accumulation (Lenzo 2012).

Monocytes are also an important precursor of dendritic cells under both steady-state and inflammatory conditions (Leon 2008b) (**Figure 1.2**). Monocytes adoptively transferred into

naïve mice migrated to the lung and acquired a dendritic cell phenotype (Landsman 2007b). A study of *Cryptococcus neoformans* infection revealed that monocytes migrated into infected lung in a CCR2 –dependent manner and differentiated into dendritic cells. A recent report from Cao et al observed that in vitro infection of human monocytes with influenza induces rapid differentiation into mDCs. The same study showed that monocyte trafficking to the lung during in vivo influenza infection in mice was required for both accumulation of mDCs in the lung and survival of infection (Cao 2012). This study highlights the crucial role of monocytes as a source for DCs during infection. Monocytes also serve as dendritic cell precursors in the skin. Langerhans cells, the only type of dendritic cell present in the epidermis, are capable of self-renewal in situ under steady-state conditions (Merad 2002). However, during skin inflammation induced by ultraviolet light, monocytes infiltrated the inflamed skin and differentiated into Langerhans cells (Ginhoux 2006). In a mouse model of *Leishmania* infection, adoptively transferred monocytes trafficked to inflamed dermis and there differentiated to dendritic cells (Leon 2007). Monocytes are recruited to a wide variety of tissues under both steady-state and inflammatory conditions, providing an important source of both macrophages and dendritic cells in these tissues.

Classification of Monocyte Subtypes

Monocytes are a heterogeneous population of cells that can be divided into several subtypes. Historically, monocytes have been categorized by many criteria, including size, density, and surface expression of CD14, CD16, and CD64. A recent nomenclature convention defined 3 primary subtypes: classical, intermediate, and non-classical monocytes (Ziegler-

Heitbrock 2010). Using this nomenclature, classical monocytes are defined as $CD14^{high}$ $CD64^{+}$ $CD16^{-}$ cells. This population comprises 80-90% of the total monocyte population in human blood and is commonly referenced simply as "monocytes". It is this classical monocyte subset that expresses FcεRI (Cheng 2006). Non-classical monocytes, which constitute 5-10% of monocytes, are defined as $CD14^{low}$ $CD16^{+}$ $CD64^{-}$ while intermediate monocytes express all three markers. In mice, classical monocytes are defined as $Ly6C^{high}$ $CD43^{low}$, non-classical monocytes are $Ly6C^{low}$ $CD43^{high}$, and intermediate monocytes are $Ly6C^{high}$ $CD43^{high}$ (Ziegler-Heitbrock 2010). The classification of "inflammatory monocytes" frequently seen in the literature is misleading due to discrepancies in the definition of the term "inflammatory". Some groups refer to classical monocytes as inflammatory because they are more readily recruited to tissues under inflammatory conditions (Geissmann 2003), while others apply this term to non-classical monocytes based on their more robust secretion of inflammatory cytokines in response to LPS (Ziegler-Heitbrock 2007).

Early reports on monocyte subsets showed that $CD14^{low}$ $CD16^{+}$ monocytes can be derived in vitro from $CD14^{high}$ $CD16^{-}$ monocytes, suggesting that non-classical monocytes are a more mature form of monocytes (Ziegler-Heitbrock 1993). Intermediate monocytes are thus thought to be a transition state between the classical and non-classical subsets (Ziegler-Heitbrock 2010). Other reports have shown differential recruitment of monocyte subsets. In one study, non-classical monocytes migrated into a number of different tissues at baseline, while classical monocytes were preferentially recruited to inflamed tissue (Geissmann 2003). This finding is consistent with an early description of distinct "marginating" and "circulating" pools of monocytes (van Furth 1986). Another study showed that classical monocytes respond preferentially to MCP-1, while non-classical monocytes respond to MIP-1α (Weber 2000). Both

major subsets of monocytes can differentiate into dendritic cells in vitro (Sanchez-Torres 2001), though functional studies have suggested that classical monocytes are more macrophage-like (Grage-Griebenow 2000), while intermediate and non-classical monocytes are thought to be more DC-like (Almeida 2001; Grage-Griebenow 2001). A number of studies have found differences in inflammatory cytokine production between subsets as well, though these results are often contradictory. One study found higher IL-1, IL-6, and TNFα in classical monocytes after stimulation with LPS (Ziegler-Heitbrock 1992), while another reported no difference in these cytokines (Frankenberger 1996), and a third reported increased TNFα in non-classical monocytes (Belge 2002). The precise differences between monocyte subsets with regard to recruitment, differentiation, and function are not completely understood and require more in depth study.

Monocyte Function

In addition to its usefulness for classifying monocyte subsets, expression of surface antigens also reflects functional properties of monocytes. CD64 (FcγRI) is the high-affinity IgG receptor and has a fundamental role in antibody-mediated phagocytosis (Huang 2011). CD64-expressing monocytes have increased phagocytic function and oxidative burst compared to $CD64^-$ monocytes (Almeida 2001; Grage-Griebenow 2000). In fact, CD64 expression level has been correlated to phagocytosis in lung macrophages (Alexis 2001). CD16 (FcγRIII) is a low-affinity IgG receptor which has been implicated in phagocytosis of immune complexes for antigen presentation (Dobel 2013). $CD16^+$ monocytes display higher antigen presenting function and accessory stimulatory capacity than their $CD16^-$ counterparts (Grage-Griebenow 2001).

CD14 contributes to TLR2 (Yang 1998) and TLR4 (da Silva Correia 2001) signaling and is crucial for innate immune responses to bacteria (Brekke 2008). CD14 has been shown to mediate responses to lipophilic pathogen-associated molecules from gram-negative, gram-positive, and acid-fast bacteria, including LPS and Lipoarabinomannan (Pugin 1994). CD14 has also been reported to be intricately involved in apoptosis, both as an antagonist of monocyte and macrophage apoptosis and as a receptor for phagocytosis of apoptotic cells (Heidenreich 1999).

Monocytes, macrophages and mDCs serve many important functions during inflammation. Monocytes express several receptors for pathogen-associated molecular patterns (PAMPs), such as TLRs, which recognize conserved motifs on pathogens. In response to activation of these receptors, monocytes, macrophages, and DCs secrete inflammatory cytokines including TNFα, IL-1β, IL-6, IL-12, and IL-23, as well as regulatory cytokines such as IL-10 (Makela 2009; Rossol 2011). These cytokines interact with other cell types, such as vascular endothelium (Ward 2009), and T and B cells (Shirota 2012; Sutton 2006) to augment inflammatory responses. Upon stimulation with virus, monocytes have been shown to secrete chemokines such as C-X-C motif chemokine ligand 1 (CXCL1; KC), Interferon-inducible protein 10 (IP-10; CXCL10), MCP-1, and RANTES (Gotera 2012; Korpi-Steiner 2006; Narasaraju 2010). LPS stimulation has also been shown to induce production of KC and IP-10 in monocytes (Srivastava 2005). These chemokines recruit a wide variety of other immune cells, including T cells, NK cells, neutrophils, and additional monocytes.

Monocyte and macrophage phagocytosis is important in both inflammatory and homeostatic states. Deficiency in phagocytosis and killing of bacteria by murine macrophages has been associated with increased bacterial sepsis (Buckley 2011). Diseases associated with increased susceptibility to bacterial infection, such as cystic fibrosis and chronic obstructive

pulmonary disease (COPD), often display impaired macrophage phagocytosis (Taylor 2010; Thomassen 1979). Phagocytosis of apoptotic cellular debris is a crucial step in the resolution of inflammation (Vandivier 2006). Monocytes and macrophages have been shown to be crucial for resolution of inflammation after influenza and RSV infection (Narasaraju 2010; Reed 2008). Even under steady-state conditions, cells throughout the body regularly undergo apoptosis and require phagocytosis for clearance (Park 2011).

Another important function of monocytes and their progeny is presentation of antigens to T cells and direction of $CD4^+$ T cell differentiation in a variety of inflammatory conditions (Randolph 2008). Nakano et al. found that following influenza infection or immunization with ovalbumin (OVA), monocytes were recruited to lymph nodes and differentiated into inflammatory DCs. These DCs produced IL-12 and stimulated type 1 helper T cell (Th1) responses (Nakano 2009). Monocytes-derived DCs are also required for stimulation of Th1 responses during infection with intracellular bacteria, such as Salmonella species (Flores-Langarica 2011). In a study of house dust mite asthma in mice, monocyte-derived DCs were able to induce Th2 cells upon sensitization and present allergen to T cells during inhaled allergen challenge (Plantinga 2013). Additionally, Human monocytes from sites of inflammation in patients with active rheumatoid arthritis have been shown to induce type 17 helper T cell (Th17) differentiation from naïve $CD4^+$ T cells (Evans 2009).

Role of Monocytes in Allergic Disease

Though monocytes are not among the traditional group of cells thought to mediate atopic disease, several studies have suggested important roles for monocytes and monocyte-derived

cells in allergic inflammation. When individuals with allergic asthma were challenged with inhaled allergens, post-challenge BAL fluid macrophage counts were increased compared to pre-challenge numbers, suggesting allergen-induced recruitment of monocytes from the blood to the respiratory mucosa (Lensmar 2006). A similar study found that the combination of allergen and LPS increased recruitment of monocytes and mDCs to the lung compared with allergen alone (Schaumann 2008). In a mouse model of allergic asthma, monocytes were recruited to the lung in an MCP-1-dependent manner after allergen challenge (Gonzalo 1998). Monocyte recruitment in allergic disease has also been observed in other tissues. Compared to non-atopic controls, monocytes from patients with atopic dermatitis express higher levels of P-selectin and L-selectin (Jockers 2006), two molecules known to be involved in monocyte recruitment to inflamed skin (Leon 2008a). Taken together, these findings suggest that monocytes are recruited to sites of allergic inflammation.

Several studies have interrogated the phenotype and function of monocyte-derived cells in the context of allergic asthma. Alveolar macrophages reside in alveolar spaces and typically serve homeostatic and anti-inflammatory functions (Soroosh 2013). Two studies have identified enhanced expression of Major Histocompatibility Complex (MHC) class I and II (Viksman 1997) as well as CD86 (Lensmar 2006) in alveolar macrophages after allergen challenge, suggesting that allergen exposure promotes a more mature, dendritic cell phenotype of these cells. Alveolar macrophages from OVA-sensitized rats secreted more TNFα and less IL-10 than cells from unsensitized rats (Careau 2006), suggesting an inflammatory, rather than homeostatic, phenotype. Despite the increased secretion of TNFα, allergen sensitization led to reduced phagocytic capacity of the alveolar macrophages. Reduced macrophage phagocytosis in allergic disease has also been observed in humans: compared to healthy controls, BAL macrophages

from individuals with severe asthma displayed reduced phagocytosis of bacteria (Fitzpatrick 2008) as well as apoptotic cells (Huynh 2005).

A deficiency in monocyte and macrophage phagocytosis could have a profound impact on lung immune responses. In asthma, like other inflammatory diseases, phagocytosis of apoptotic cells is necessary for resolution of inflammation (Vandivier 2006). A study by Kulkarni et al. examined eosinophil protein in airway macrophages from subjects with severe allergic asthma (Kulkarni 2010). The presence of eosinophil remnants in macrophages was associated with ongoing inflammation. This and other studies have shown that treatment of asthma symptoms with corticosteroids increased macrophage phagocytosis of apoptotic cells (Huynh 2005; Kulkarni 2010), indicating that clearance of apoptotic debris is important for resolution of inflammation. Another group found that reduced sputum macrophage phagocytosis correlated with reduced lung function in asthmatic subjects (Alexis 2001). In apparent contrast to the indication that reduced phagocytosis leads to poor lung function, a report by Lay et al. found that monocytes and macrophages from individuals with mild asthma demonstrated increased phagocytosis of small particles compared to cells from healthy controls (Lay 2009). One possible explanation of these contradictory findings is the possibility that alveolar macrophages from asthmatic individuals preferentially phagocytose small particles in order to present antigen to infiltrating T cells. This theory is supported by the observation that alveolar macrophages efficiently bind and phagocytose allergen-containing pollen granules (Currie 2000). While the exact regulation of phagocytosis during allergic inflammation is not fully understood, this process is important during inflammation and likely plays a role in atopic disease.

Another area of investigation concerning monocytes in allergic disease involves CD14, a major monocyte lineage marker. In addition to its function as a multifunctional cell surface

receptor, CD14 can also be released in a soluble form (Bufler 1995). One important study showed that monocytes from asthmatic patients increased surface CD14 expression as well as soluble CD14 (sCD14) after exposure to allergen (Monteseirin 2003). The authors also observed that this allergen-induced increase in surface CD14 was ablated by allergen immunotherapy while sCD14 secretion was actually increased suggesting that sCD14 may be important for amelioration of allergic disease. Providing a possible mechanism for this finding, Arias et al. found that sCD14 can suppress IgE production from human B cells while increasing IgG1 production (Arias 2000). The human CD14 gene contains a C/T polymorphism that may influence atopic disease: individuals with the CC genotype were reported to have lower serum levels of sCD14, but significantly higher serum IgE concentration than individuals with CT or TT genotypes (Baldini 1999). Taken together, these findings suggest that sCD14 may serve a regulatory function in atopic disease by modulating IgE production in B cells.

Considered together, the findings described here suggest that monocytes are present at sites of allergic inflammation and have the potential to influence the course of atopic disease. However, the regulation of monocyte function during allergic disease and the impact that may have on disease progression are not well understood.

Role of IgE Cross-Linking on Monocytes

The first evidence that IgE could interact with cells of the monocyte/macrophage lineage came in 1975 when Capron et al. found that macrophage binding to *Schistosoma masoni* parasites required the presence of IgE (Capron 1975). In the subsequent years expression of the low affinity IgE receptor, CD23, was described on monocytes and macrophages after stimulation

with IL-4, GM-CSF, IFNα, or IFNγ and at baseline in atopic individuals (Williams 1992). In 1994, the expression of functional FcεRI on human monocytes was first reported (Maurer 1994). Unlike CD23, FcεRI is expressed on monocytes of non-atopic individuals (Reischl 1996), with elevated expression in atopic subjects (Cheng 2006; Sihra 1997). Like other FcεRI-expressing cells, monocyte FcεRI correlates to serum IgE concentration (Cheng 2006). FcεRI expression on monocytes is also enhanced by IL-4 but inhibited by IFNγ (Reischl 2000). FcεRI (Sihra 1997), but not CD23 (Borish 1991), is occupied by IgE in monocytes isolated from blood. Though IgE is bound to FcεRI in freshly isolated monocyte, in vitro addition of IgE increases the level of surface bound IgE, indicating that FcεRI is not saturated on monocytes in vivo (Von Bubnoff 2002).

The precise consequences of IgE cross-linking on monocytes are not fully known. Cross-linking of CD23 on IL-4-primed monocytes has been shown to increase intracellular cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) concentrations (Paul-Eugene 1995). Another study observed secretion of TNFα and MIP-1α after CD23 cross-linking in IL-4-primed monocytes (Ezeamuzie 2009). When Maurer et al. reported the expression of FcεRI on monocytes, they showed that cross-linking of the receptor leads to increased intracellular calcium. Cross-linking of FcεRI on monocytes has also been observed to induce activation of nuclear factor κ B (NFκB) in monocytes and monocyte-derived mDCs (Kraft 2002). This activation of NFκB led to secretion of TNFα and MCP-1. Another study revealed that IgE cross-linking also induces production of IL-6 and MIP-1β (Von Bubnoff 2002). FcεRI ligation has also been shown to prevent monocyte apoptosis in a TNFα-dependent manner (Katoh 2000). Novak et al. determined that IgE cross-linking affects in vitro monocyte differentiation into DCs: when monocytes were exposed to DC-inducing cytokines after FcεRI

cross-linking, the monocytes failed to become DCs, and instead acquired a macrophage-like phenotype (Novak 2001a). This effect was shown to be dependent on FcεRI-induced IL-10 secretion from the monocytes.

Despite the potential importance of monocytes and their progeny in allergic disease, the effect of IgE cross-linking on these cells and its consequences for allergic disease are poorly understood (**Figure 1.1**). Determination of the functional consequences of IgE cross-linking on monocytes is important for understanding the contribution of this cell to the perpetuation and exacerbation of atopic disease. Thus, I sought to define the impact of IgE cross-linking on the phenotype and function of human monocytes.

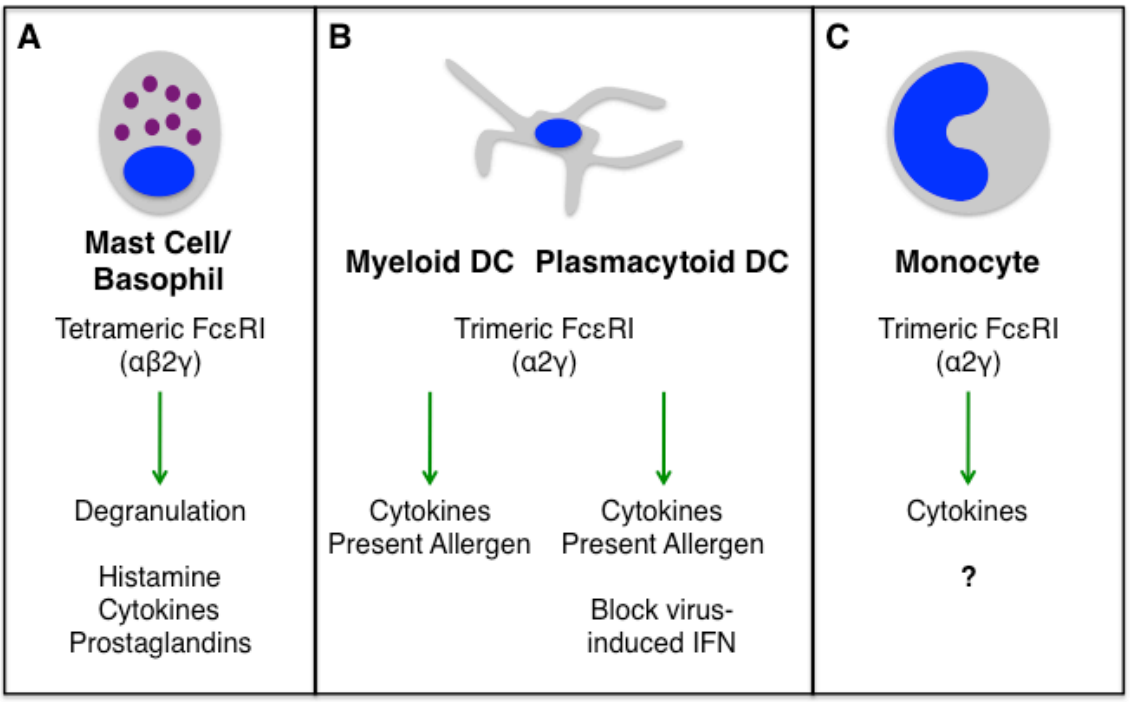


Figure 1.1 – FcεRI Has Cell Type-Specific Actions

Several cell types express FcεRI, including mast cells basophils, myeloid and plasmacytoid DC, and monocytes. Mast cells and basophils (A) express a tetrameric FcεRI with one α subunit, one β subunit, and two γ subunits. Activation of FcεRI induces degranulation to release histamine, cytokines and prostaglandins. Myeloid and plasmacytoid DCs (B) express a trimeric FcεRI, lacking the β subunit. Activation of FcεRI on these cells induces secretion of inflammatory cytokines and presentation of allergens to T cells. On plasmacytoid DCs, FcεRI activation blocks virus-induced secretion of type I interferon (IFNα/β). Monocytes (C) express the trimeric isoform of FcεRI. Activation of FcεRI on monocytes induces cytokine secretion but the functional effects on this cell type are not known.

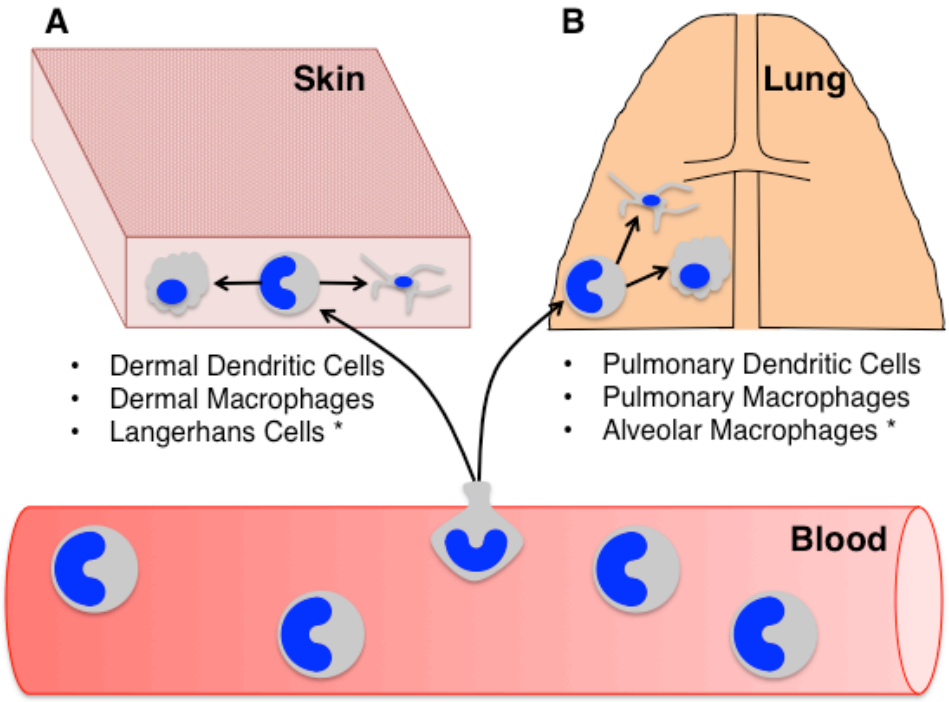


Figure 1.2 – Monocyte-Derived Cells in Skin and Lung

Monocytes contribute to macrophage and dendritic cell populations throughout the body. In the skin (A), monocytes can differentiate into dermal macrophages and dendritic cells as well as Langerhans cells (epidermal dendritic cells). In the lung (B), monocytes are precursors for pulmonary (interstitial) macrophages and dendritic cells and can also contribute to alveolar macrophage populations. * indicates a population that is also capable of self-renewal under homeostatic conditions.

CHAPTER TWO

MATERIALS AND METHODS

Human Subjects

Leukocyte-enriched blood samples were obtained from a local blood bank. For some experiments, peripheral blood samples were obtained from healthy volunteers by venipuncture and collected in vacutainer tubes with Acid Citrate Dextrose Solution A (BD, Franklin Lakes, NJ). Individuals with a history of serum IgE concentration >100 U/ml were recruited for select experiments. Individuals with lower IgE were recruited as controls. Participants underwent skin testing (MultiTest II; Lincoln Diagnostics, Decatur, Ill) with the following nine allergens: dust mite (*Dermatophygoides farinae* and *Dermatophygoides pteronyssinus*), cockroach (German and American mix), rat, mouse, mold (*Alternaria* and *Aspergillus*), cat, and dog (mixed breeds). Cockroach extract was ordered from Bayer Corp. (Spokane, Wash), and all other extracts were ordered from Greer Laboratories (Lenoir, NC). Positive result was defined as observation of a wheal of diameter at least 3 mm greater than saline control. Each recruited participant in both groups had positive skin test to ≥1 indoor allergen. Serum IgE levels were determined for each participant as follows: whole blood was collected into silicone-coated vacutainer tubes (BD) containing no additives and centrifuged at 1500xg for 15 min. Serum was collected and stored at -80 degrees Celsius (° C). Total IgE was measured using a fluorescent-based enzyme immunoassay performed on the ImmunoCAP 250 (Phadia, Kalamazoo, MI) at the Johns Hopkins University Dermatology Allergy and Clinical Immunology Reference Laboratory. These studies

were approved by the University of Texas Southwestern institutional review board (Study # STU 122010-139). Written informed consent and assent were obtained.

Reagents and Media

Phosphate Buffered Saline (cPBS) was supplemented 1X PBS with 2% heat-inactivated fetal bovine serum (FBS), and 2mM Ethylenediaminetetraacetic acid (EDTA) to make complete PBS (cPBS). Complete RPMI medium (cRPMI) was derived by supplementing Roswell Park Memorial Institute Medium 1640 (RPMI) with 10% FBS, 1% GlutaMAX-I, 1X minimum essential medium (MEM) non-essential amino acids, 1mM Na pyruvate, 10mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer solution, 1X penicillin-streptomycin solution, and 100 μM β-mercaptoethanol. Complete DMEM media (cDMEM) was derived by supplementing Dulbecco's Modified Eagle Medium (DMEM) media with 10% FBS, 1X MEM non-essential amino acids, 1mM Na pyruvate, 1X penicillin-streptomycin solution, and 100 μM β-mercaptoethanol. FBS was purchased from Atlanta Biologicals (Lawrenceville, GA) and EDTA was purchased from Invitrogen™ (Life Technologies, Grand Island, NY). PBS, medias and other supplements were purchased from GIBCO® (Life Technologies, Grand Island, NY). Rabbit anti-human IgE (αIgE) and Rabbit whole IgG (IgG) antibodies were purchased from Bethyl Laboratories (Montgomery, TX), and dialyzed to remove sodium azide preservative. Dialysis was performed using a D-Tube Dialyzer Midi, 3.5kDa (Novagen, San Diego, CA). Azide-free Rabbit whole IgG was purchased from Jackson ImmunoResearch (Westgrove, PA). F(ab)'$_2$ fragments of αIgE and IgG antibodies were derived by GenScript (Piscataway, NJ). Unless otherwise specified, all recombinant human (rh) cytokines, anti-human cytokine

antibodies, and anti-human cytokine receptor antibodies were purchased from R&D Systems (Minneapolis, MN).

Purification of Immune Cells from Blood

Blood samples were diluted 1:1 (vol/vol) with cPBS and peripheral blood mononuclear cells (PBMC) were isolated by centrifugation with Ficoll-Paque (GE Healthcare, Uppsala, Sweden). Monocytes were purified using the EasySep™ Negative Selection Human Monocyte Enrichment Kit (STEMCELL Technologies Inc., Vancouver, Canada) according to manufacturer recommendations. Purity of enriched monocyte samples was assessed by flow cytometry for $CD14^+$ $CD16^-$ cells and ranged from 85%-95%. Naïve $CD4^+$ T cells were obtained by using the EasySep™ Negative Selection Human Naïve $CD4^+$ T Cell Enrichment Kit (STEMCELL Technologies Inc.) according to manufacturer recommendations. Purity of enriched naïve $CD4^+$ T cells was assessed by flow cytometry for $CD3^+$ $CD4^+$ $CD45RO^-$ cells and ranged from 90-95 %.

Monocyte Culture

Isolated monocytes were cultured in cRPMI at a concentration of $1x10^6$ monocytes/ml. αIgE or control IgG antibodies (10 μg/ml) were added to monocyte cultures as indicated. For select experiments, $F(ab)'_2$ fragments from αIgE and IgG antibodies were added at 10 μg/ml. For cytokine neutralization experiments, mouse anti-human antibodies against IL-10, IL-6, TNFα, IL-10Rα, IL-6R, or TNFRI and IgG1 or IgG2b isotype controls were added to monocyte cultures

at 10 μg/ml (anti-TNFα, IgG1) or 5 μg/ml (others). Cultures were incubated at 37° C for the indicated times and cells and supernatants were harvested for analysis.

Monocyte Culture with Viruses

Isolated monocytes were cultured in cRPMI in the presence of rhM-CSF (1ng/ml) at a concentration of $1x10^6$ cells/ml. αIgE (10 μg/ml), control IgG (10 μg/ml), influenza (Influenza A/PR/8/34 H1N1; 0.1 multiplicity of infection (MOI), Charles River Laboratories International, Inc.), or hRV (Human Rhinovirus 16; 10^6 plaque-forming-units(pfu)/ml, a generous gift from Dr. James E. Gern, University of Wisconsin, Madison, WI) were added simultaneously to monocyte cultures as indicated. Cultures were incubated at 37° C for indicated times and cells and supernatants were subsequently harvested for analysis.

Monocyte-T Cell Co-Culture

Isolated monocytes were cultured in cRPMI with rhM-CSF (1ng/ml) in the presence or absence of αIgE (10 μg/ml), control IgG (10 μg/ml), influenza (0.1 MOI), or hRV (10^6 pfu/ml) at 37° C for 24 hours. Pretreated monocytes were then mixed at a 1:1 ratio with naïve $CD4^+$ T cells from a different human donor. For control T lymphocyte conditions, naïve $CD4^+$ T cells were cultured without monocytes on culture plates coated with mouse anti-human CD3 (OKT3, a generous gift of Dr. David Farrar, UT Southwestern Medical Center, Dallas, TX) and mouse anti-human CD28 (BioLegend, San Diego, CA). Cells were plated at a concentration of $2x10^6$ cells/ml. rhIL-2 was added to all T cell cultures at 50 Units (U)/ml. For in vitro polarization of

$CD4^+$ T cells the following cytokines and antibodies were added: for Th1 polarization, rhIL-12 (10 ng/ml) and mouse anti-human IL-4 (2 μg/ml); for Th2 polarization, rhIL-4 (20 ng/ml), anti-human IL-12 (5 μg/ml, Dr. David Farrar), and anti-human IFNγ (5 μg/ml, Dr. David Farrar); for Th17 polarization, rhIL-6 (20 ng/ml), rhIL-1β (10 ng/ml), rhIL-23 (20 ng/ml), rh transforming growth factor β (TGFβ; 1 ng/ml), anti-human IL-4 (2 μg/ml), anti-human IL-12 (5 μg/ml), and anti-human IFNγ (5 μg/ml); for neutralized conditions, anti-human IL-4 (2 μg/ml), anti-human IL-12 (5 μg/ml), and anti-human IFNγ (5 μg/ml). After 3 days, T cells and monocyte-T cell co-cultures were diluted 1:10 in fresh cRPMI containing 50 U/ml of recombinant human IL-2. After an additional 4 days, T cells were washed and rested overnight at 37° C in fresh cRPMI. Cells were then re-stimulated for 6 hours with phorbol 12-myristate 13-acetate (PMA, 50 ng/ml, Sigma, St. Louis, MO), Ionomycin (1μM, Invitrogen™), and Monensin (1X, BioLegend) for intracellular cytokine staining. A small aliquot of cells from each treatment condition received only Monensin. A schematic of the experimental design is depicted in **Figure 2.1**.

Flow Cytometry Analysis of Surface Antigens

The following fluorochrome-conjugated anti-human antibodies were used: CD3-AlexaFluor®700, CD4-allophycocyanin (APC), CD8-Phycoerythrin (PE), CD14-V450, CD45RO-FITC CD64-Fluorescein isothiocyanate (FITC), CD64-PE, CD80-FITC, CD86-PE-Cy™5, FcεRI-PE, Human Leukocyte Antigen (HLA)-A,B,C-APC, HLA-DR-APC-Cy™7, HLA-DR-Peridinin chlorophyll (PerCP), HLA-DR-PE, (BD Pharmigen, San Diego, CA). For some experiments, cells were rinsed with cPBS and stored in Streck Cell Preservative (Streck, Omaha, NE) at 4° C prior to staining. Fresh or preserved samples were washed and incubated with the

appropriate antibodies diluted in cPBS for 30 min at 4° C. Cells were then washed and resuspended in 1% paraformaldehyde. Samples were subsequently acquired on a BD LSR II flow cytometer (BD Biosciences, San Diego, CA) and analyzed with FlowJo software (Tree Star, Inc., Ashland, OR). Compensation between fluorescent markers was performed in FlowJo using samples stained with individual antibodies. Live cells were gated using forward and side scatter characteristics. For some analyses, cells were further gated for CD14 expression. Mean fluorescence intensity for each antigen was determined and subsequently converted to mean equivalent standard fluorescence (MESF), using Ultra Rainbow Calibration Particles (Spherotech, Lake Forest, IL) and FlowJo.

Flow Cytometry Analysis of Intracellular Cytokines

The following fluorochrome-conjugated anti-human antibodies were used: IFNγ-PE-Cy7, IL-4-PE (BD Biosciences), and IL-17-Alexa Fluor®488 (eBioscience, San Diego, CA). Fresh samples were washed and fixed in 4% paraformaldehyde and permeabilized with 0.1% saponin in cPBS. Samples were then incubated with the appropriate antibodies diluted in 0.1% saponin in cPBS for 30 min at room temperature. Cells were then washed and resuspended in cPBS. Samples were subsequently acquired on a BD LSR II flow cytometer (BD Biosciences) and analyzed with FlowJo. Compensation between fluorescent markers was performed in FlowJo using samples stained with individual antibodies. Gates for cytokine$^+$ cells were set using samples that did not undergo restimulation. Percentages of cytokine$^+$ cells were obtained.

Cytokine Secretion Analysis

To obtain supernatants for cytokine secretion analysis, cell culture plates were centrifuged at 1500 rpm for 10 minutes. Supernatants were removed and stored at -80° C until use. The following Enzyme-linked Immunosorbent Assay (ELISA) kits were used according to manufacturer recommendations: Legend Max Human ELISA kits (BioLegend) for TNFα and IL-10; READY-SET-GO! Human ELISA kits (eBioscience) for TNFα, IL-10, IL-6, IL-1β, IL-23, IL-12p70, IFNγ, IL-4, and IL-17; Human IL-12+p40 Cytoset (Invitrogen™) for IL-12p40. Absorbance was measured on a POLARSTAR Omega microplate reader (BMG Labtech, Ortenberg, Germany) and analyzed in GraphPad Prism Versions 5 and 6.

Analysis of Monocyte Phagocytosis of Bacteria

BODIPY FL-conjugated E. coli BioParticles (Molecular Probes, Eugene, OR) were opsonized with E. coli Opsonizing Reagent (Molecular Probes) according to manufacturer instructions, added to monocyte cultures at a ratio of 10 bacteria/monocyte and incubated at 37° C for 2 hours. For microscopy, monocytes were washed and mounted onto slides with a Cytospin 4 Centrifuge (Thermo Scientific, Waltham, MA). Slides were fixed with methanol and cover slipped with Vectashield Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA). Differential interference contrast (DIC) and fluorescence (BODIPY and DAPI) images were acquired on a Deltavision Deconvolution Microscope (Applied Precision, Issaquah, WA) for 50 cells per sample. Internalized particles were counted in ImageJ (Freeware available from the National Institutes of Health at http://rsb.info.nih.gov/ij/) using macros I wrote. For flow

cytometry, monocytes were washed, stained for CD14-V450, and acquired on the LSR II. This experimental design is portrayed in **Figure 2.2 A**.

Analysis of Monocyte Phagocytosis of Apoptotic Cells

HEp2 cells were grown in cDMEM media and passaged as needed. Cells were incubated with 5μM carboxyfluorescein succinimidyl ester (CFSE) (Molecular Probes) for 10 minutes, washed extensively with media, and incubated for 24 hours with 1μg/ml Actinomycin D (Sigma) to induce apoptosis. Apoptotic cells were washed, opsonized with 100 μg/ml whole human IgG (Bethyl Laboratories, Montgomery, TX) and added to monocyte cultures at a 1:1 ratio. After 4 hours, cells were washed and stained for CD14 for flow cytometry analysis. In some experiments, $CD14^+$ monocytes were sorted on a BD FACSAria flow cytometer (BD Biosciences, San Diego, CA) based on CFSE fluorescence and mounted on slides for fluorescence microscopy analysis. This experimental design is portrayed in **Figure 2.2 B**.

Quantification of Bacterial Killing

Escherichia coli (DH5α strain, Dr. David Farrar) were grown in LB media (Sigma) and added to monocyte cultures at 10 colony-forming units (CFU)/monocyte for 45 min. Gentamicin (Amresco, Solon, OH) was added (100 μg/ml) and monocytes were harvested immediately (0 hours) or after 16 hours. Monocytes were washed extensively, counted, and lysed in sterile deionized H2O. Lysates were plated on LB agar (Sigma) overnight and colonies were counted. CFU/Cell was determined for each harvest and % of bacteria killed was calculated as %Killed =

$(CFU/Cell_{0hr} - CFU/Cell_{16hr}) \div CFU/Cell_{0hr} \times 100$. For the purpose of this calculation, "0hr" refers to the end of the 45 min internalization period and "16hr" refers to 16 hours subsequent to that time. The experimental design for this assay is depicted in **Figure 2.3**.

Data Analysis and Statistics

Data are presented as means ± SEM. For all data sets comparing >8 individual human donors, Grubb's test for outliers was applied with α=0.0001 and outliers were removed from analysis. Where indicated, data were normalized to the media condition for each experiment. For experiments containing 3 or more conditions, one-way repeated measures ANOVA and pairwise Tukey's post hoc comparisons were performed for each time point. For comparison of 2 conditions, paired or unpaired t tests were performed with Holm-Sidak correction where appropriate. For experiments comparing responses of monocytes purified from individuals with high vs. low IgE, Pearson correlations between experimental results and log of serum IgE concentration were performed. $p<.05$ was considered significant. All statistical analyses were performed with GraphPad Prism versions 5 and 6.

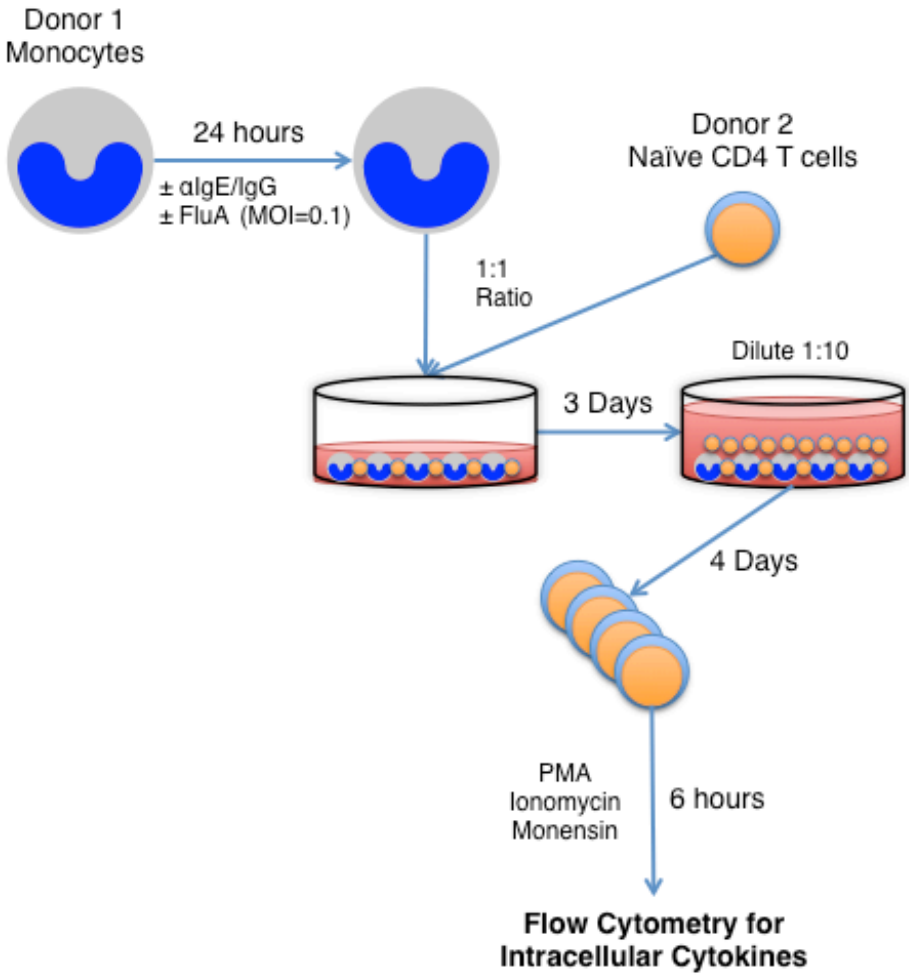


Figure 2.1 – Schematic of Monocyte-T cell Co-culture Experiments

Experimental design for co-culture of monocytes with T cells to determine T cell polarization.

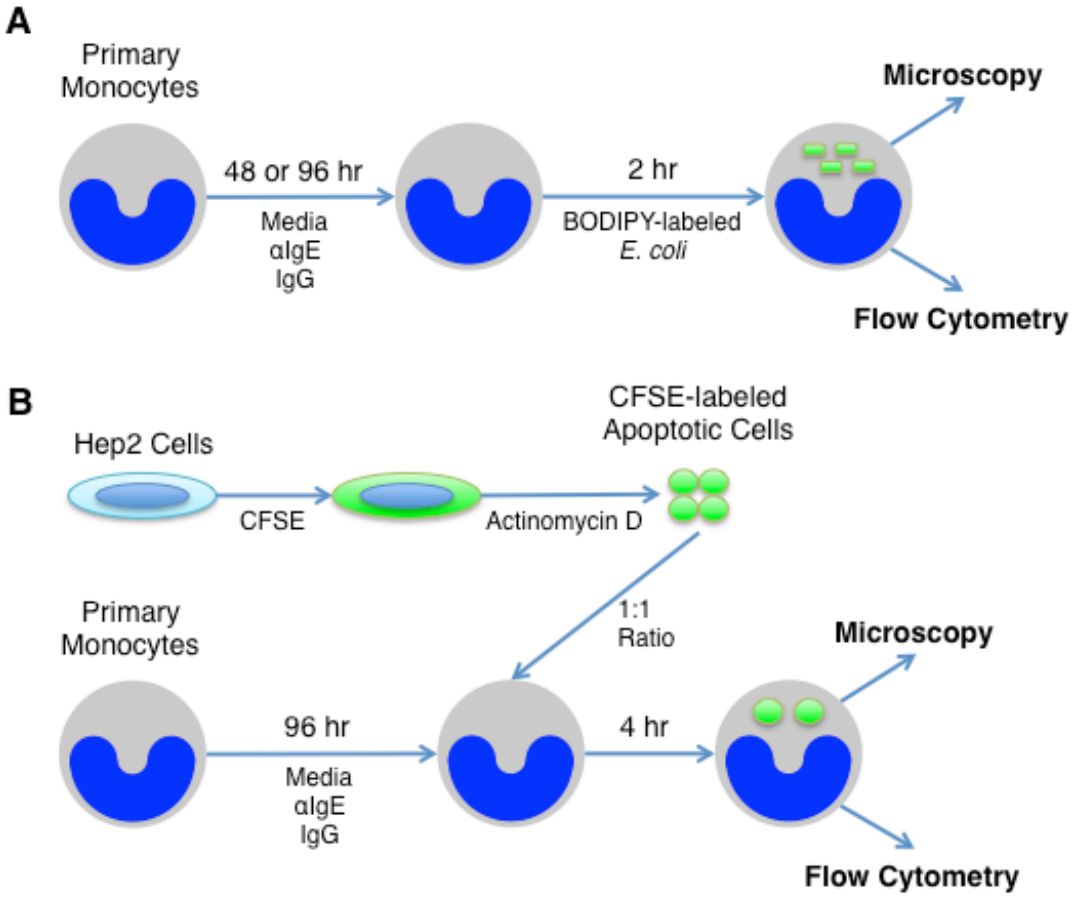
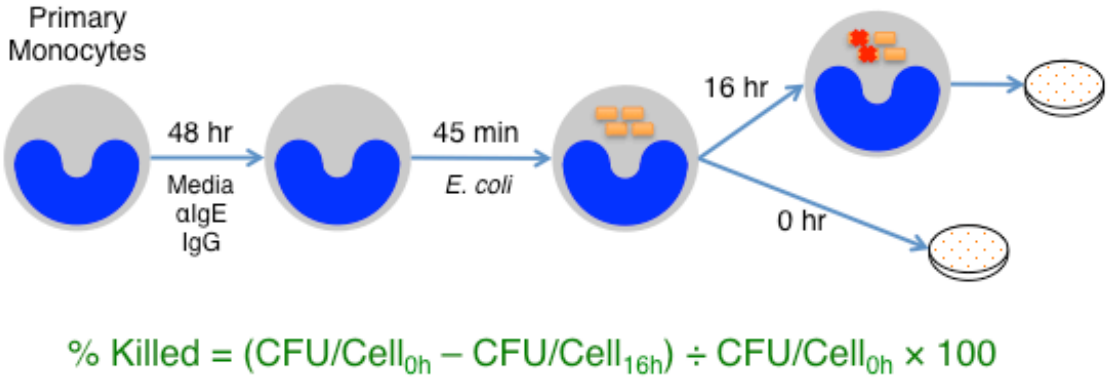


Figure 2.2 – Schematic of Monocyte Phagocytosis Experiments

Experimental design for analysis of monocyte phagocytosis of bacteria (A) and apoptotic cells (B).



$$\% \text{ Killed} = (\text{CFU/Cell}_{0h} - \text{CFU/Cell}_{16h}) \div \text{CFU/Cell}_{0h} \times 100$$

Figure 2.3 – Schematic of Bacterial Killing Experiments

Experimental design for assays to quantify killing of intracellular bacteria by monocytes. Equation used to calculate percentage of bacteria killed is depicted in green text.

CHAPTER THREE

IGE CROSS-LINKING CRITICALLY IMPAIRS HUMAN MONOCYTE FUNCTION BY BLOCKING PHAGOCYTOSIS

Much of the work in this chapter has been published in the Journal of Allergy and Clinical Immunology, volume 131, pages 491-500 (Pyle 2013). This work is reproduced with the permission of the Journal of Allergy and Clinical Immunology (license number 3125370240289). Copyright 2013 The American Association of Asthma, Allergy, and Immunology. Experiments were performed by David M. Pyle unless otherwise noted in the figure legends.

Introduction

Despite the significant healthcare burden of atopic disease in the US (Akinbami 2009; Mancini 2008; Meltzer 2009), the mechanisms underlying pathogenesis are incompletely understood. IgE plays a critical role in mediating atopic disease: significant correlations between serum IgE concentration and disease have been demonstrated in allergic asthma and atopic dermatitis (Kovac 2007; Laske 2004). Indeed, serum IgE concentration represents a diagnostic criterion for these conditions (Kjaer 2009; Matsui 2010). Therapies that reduce serum IgE concentration, such as omalizumab, result in clinical improvement in patients with severe atopic disease (Busse 2011; Thaiwat 2011).

IgE exerts its effect on atopic disease via the high-affinity IgE receptor, FcεRI (Wu 2011a). Upon cross-linking of allergen-specific IgE by a multivalent allergen the receptor is activated, resulting in intracellular signaling and cell-type specific effects (Wu 2011a). Surface FcεRI expression on several immune cells, including basophils and dendritic cells, is increased in individuals with atopic disease and correlates with serum IgE concentration (Foster 2003; Sihra 1997).

FcεRI mediates IgE-dependent pathways in several cell types, and its role is best characterized in basophils and mast cells. In these cells, IgE cross-linking induces release of inflammatory mediators including histamine, prostaglandins, and cytokines (Sawaguchi 2012; Ugajin 2011). FcεRI plays an important role on mDC and pDC as well (Foster 2003); IgE cross-linking on these cells induces pro-inflammatory cytokine secretion (Le 2009; Schroeder 2008). In pDCs, IgE-mediated and TLR9-mediated pathways have been shown to oppose one other (Schroeder 2008); allergic stimulation via this pathway also interferes with in vitro pDC antiviral responses (Gill 2010).

FcεRI is also expressed on monocytes and its expression on these cells is increased in individuals with atopic diseases (Cheng 2006; Liang 2012; Sihra 1997). Present in high numbers at mucosal surfaces and in the skin both during steady state and inflammatory conditions, such as allergen exposure, monocytes and their progeny are poised to influence allergic responses (Gill 2008; Ginhoux 2006; Landsman 2007b; Lensmar 2006; Lenzo 2012; Leon 2008a). Monocytes play many important roles during inflammatory processes, including regulating immune responses through the release of cytokines (Makela 2009), and resolving inflammation though phagocytosis of cellular debris (Mahdavian Delavary 2011; Narasaraju 2010; Reed 2008). Expression of specific surface molecules can also reflect functional properties of monocytes.

CD14 contributes to TLR4 signaling and is thus important for immune responses to LPS (Brekke 2008). CD64, the high affinity IgG receptor, contributes to phagocytosis; its expression reflects monocyte phagocytic function (Alexis 2001; Grage-Griebenow 2000).

Despite the expression of FcεRI on monocytes from both atopic and non-atopic individuals (Cheng 2006; Liang 2012; Sihra 1997) and the importance of these cells in inflammatory processes, the consequences of FcεRI activation on monocytes remain incompletely characterized. Stimulation of FcεRI has been shown to induce activation of NFκB and secretion of TNFα, IL-6, and MCP-1 in human monocytes (Kraft 2002; Von Bubnoff 2002). In addition, FcεRI cross-linking of GM-CSF and IL-4 treated monocytes in vitro has been shown to promote IL-10 secretion and differentiation into macrophages (Novak 2001a).

I set out to define the impact of IgE cross-linking on the function of human monocytes and to determine whether serum IgE concentration impacts the magnitude of these responses. Monocytes, by virtue of their expression of FcεRI, inflammatory capacity, and prevalence in mucosal tissues, have the potential to significantly influence allergic inflammation. Determining how IgE cross-linking impacts monocyte function will lead to a better understanding of the role of this important cell type in allergic processes and may reveal critical pathways that contribute to the pathogenesis of allergic disease.

Results

Participant Information

The majority of the assays reported hereafter were performed on monocytes from healthy human blood donors. To establish potential clinical impact, I performed certain assays on monocytes obtained from participants with either low (<100 U/ml) or high (>100 U/ml) serum IgE levels. Participant information is reported in **Table 3.1**. The majority of participants in both groups had a history of asthma and all tested positive for at least one environmental allergen by skin test. There was no statistically significant difference in age or demographic characteristics between groups. As our lab has reported previously for pDCs from individuals with elevated IgE levels (Gill 2010), monocyte expression of FcεRI was significantly elevated in the high IgE group. Data from these experiments were compared for correlation with serum IgE concentration; these analyses are summarized in **Table 3.2**.

IgE Cross-Linking Alters Monocyte Surface Marker Expression

To determine the effect of IgE-mediated stimulation on functionally relevant monocyte surface markers, I analyzed the impact of IgE cross-linking on the surface expression of CD14 and CD64.

IgE cross-linking resulted in significant up regulation of CD14 expression at both time points measured (**Figure 3.1 A**). In contrast, surface expression of CD64 was significantly diminished by IgE cross-linking (**Figure 3.1 B**). An F(ab)'2 fragment of the IgE cross-linking

antibody induced similar up regulation of CD14, indicating that the effects of the whole antibody were not mediated by Fcγ receptors (**Figure 3.3 A**).

Additionally, CD14 up regulation after IgE cross-linking was greater in individuals with elevated serum IgE (**Figure 3.1 C, left**) at both time points. Moreover, the expression of CD14 after IgE cross-linking was positively correlated with serum IgE levels (**Figure 3.1 C, right**) at 96 hours.

IgE Cross-Linking Induces Secretion of Inflammatory Cytokines

To determine the temporal patterns and interactions of cytokines induced by IgE cross-linking, I analyzed three cytokines commonly secreted by monocytes: TNFα, IL-6, and IL-10.

TNFα secretion was significantly increased by IgE cross-linking at all time points measured (**Figure 3.2 A**). The concentration of TNFα induced by IgE cross-linking was greatest after 4 hours and diminished significantly by 24 hours. This reduction may reflect degradation of TNFα between 4 and 24 hours. IgE cross-linking induced robust IL-6 secretion at 4 and 24 hours; in contrast to TNFα, IL-6 levels were maintained at 48 hours (**Figure 3.2 B**). The F(ab)'2 fragment of the IgE cross-linking antibody induced similar secretion of IL-6 after 48 hours (**Figure 3.3 B**), confirming that effects of αIgE on monocytes are not Fcγ-mediated.

IL-10 Suppresses IgE-Mediated Cytokine Secretion

IgE cross-linking also induced significant IL-10 secretion (**Figure 3.2 C**). Notably, the greatest IL-10 concentrations were observed after 24 hours of IgE cross-linking and

corresponded with lower TNFα concentrations. To evaluate a potential regulatory role of IL-10 on TNFα production, I used neutralizing antibodies against both IL-10 and its receptor in the presence of IgE cross-linking. The neutralizing antibodies were chosen such that they did not interfere with cytokine detection by ELISA. IL-10 blockade prevented the reduction in IgE-mediated TNFα secretion over time (**Figure 3.4 A, left**), suggesting an autoregulatory role for IL-10. Furthermore, IL-10 blockade led to a dramatic increase in IgE-mediated secretion of IL-6, as well as IL-10 itself (**Figure 3.4 A, middle and right**). However, neutralization of TNFα and IL-6 did not affect IL-10 levels (**Figure 3.4, B and C**), suggesting that the induction of IL-10 by IgE cross-linking is not mediated by TNFα or IL-6.

This autoregulatory IL-10 secretion was increased in monocytes from individuals with elevated serum IgE (**Figure 3.5 A, left**); in fact, IL-10 secretion after IgE cross-linking significantly correlated with serum IgE concentration (**Figure 3.5 A, right**). While the increased autoregulatory IL-10 response in individuals with elevated serum IgE might predict reduced pro-inflammatory cytokine secretion, this was not the case. TNFα secretion upon IgE cross-linking was actually increased and IL-6 secretion was 4-fold higher in participants with elevated IgE (**Figure 3.5 B**).

IgE Cross-Linking Induces Secretion of IL-1β and IL-23

To further explore the cytokine profile induced by IgE cross-linking in monocytes, I next analyzed three additional pro-inflammatory cytokines: IL-1β, IL-12, and IL-23. IgE cross-linking induced secretion of IL-1β as early as 4 hours and levels of IL-1β were maintained to at least 48 hours (**Fig 3.6 A**). Secretion of IL-12p40 was also induced by IgE cross-linking, with

maximal secretion occurring by 24 hours (**Fig 3.6 B**). As IL-12p40 is a component of both IL-12 and IL-23 (Oppmann 2000), I examined each of these cytokines individually. Whereas no IL-12p70 was observed (**Fig 3.6 C, left**), IgE cross-linking did induce secretion of IL-23 in a similar time course to that of IL-12p40 (**Fig 3.6 C, right**).

Effects of IgE Cross-Linking Are Concentration Dependent

I next examined the effects of αIgE concentration on IgE-mediated surface marker expression and cytokine secretion. IgE cross-linking induced concentration dependent up regulation of CD14, down regulation of CD64, and secretion of IL-6, and IL-10, while TNFα secretion was similar at both concentrations of αIgE (**Figure 3.7**). Subsequent experiments were performed using 10 μg/ml, as this concentration of αIgE induced the maximum effect.

IgE Cross-Linking Impairs Monocyte Phagocytosis

Given the inflammatory nature of IgE-mediated monocyte cytokine secretion, I next explored the impact of IgE cross-linking on a critical monocyte function: phagocytosis.

Utilizing microscopy to quantitate internalized bacteria, I determined that IgE cross-linking significantly impairs monocyte phagocytosis. Monocytes exposed to IgE cross-linking internalized fewer killed, opsonized bacteria compared to monocytes cultured in control conditions (**Figure 3.8 A**). Quantitation of internalized bacteria revealed a significant reduction in phagocytosis after IgE cross-linking (**Figure 3.8 B**). To extend these findings to multiple time points and assess the role of specific cytokines in phagocytosis, I utilized a higher throughput

flow cytometry assay to similarly measure phagocytosis; this revealed a significant reduction in monocyte phagocytosis at both 48 and 96 hours after IgE cross-linking (**Figure 3.8 C**). The impairment of phagocytosis induced by IgE cross-linking was not altered by neutralization of TNFα, IL-6, or IL-10 (**Figure 3.9**), suggesting that this effect of IgE cross-linking is independent of IgE-mediated cytokine secretion and not subject to autoregulation by IL-10.

One important function of monocytes and their progeny is the clearing of apoptotic debris after infection or inflammation (Mahdavian Delavary 2011; Narasaraju 2010; Reed 2008). I next determined the effect of IgE cross-linking on phagocytosis of apoptotic cells. After exposure of monocytes to CFSE-labeled apoptotic cells, monocyte CFSE fluorescence was diminished in the IgE cross-linking condition, indicating diminished phagocytosis of apoptotic cells (**Figure 3.10 A and B**). With regard to CFSE fluorescence, two distinct populations of monocytes were observed: $CFSE^{low}$, which contained small apoptotic debris (**Figure 3.10 C**), and $CFSE^{high}$, which contained large apoptotic cell remnants (**Figure 3.10 D**). IgE cross-linking significantly reduced monocyte phagocytosis of small debris ($CFSE^{low}$ monocytes; **Figure 3.10 E**) as well as the percentage of monocytes that phagocytosed large apoptotic cells ($CFSE^{high}$ monocytes; **Figure 3.10 F**). In combination, these two measures reflect diminished phagocytosis of apoptotic cells by monocytes exposed to IgE cross-linking.

Unlike the pro-inflammatory effects of IgE cross-linking, the impairment of monocyte phagocytosis was not dependent on serum IgE concentration. Monocytes from participants with elevated IgE levels showed similar levels of phagocytosis after 48 and 96 hours of IgE cross-linking (**Figure 3.11 A**). Moreover, there was no significant correlation between serum IgE and phagocytosis after IgE cross-linking (**Figure 3.11 B**), again suggesting that IgE-mediated inhibition of phagocytosis is independent of the pro-inflammatory effects of IgE cross-linking.

IgE Cross-Linking Does Not Affect Intracellular Killing of Bacteria

To assess the extent of the functional impairment resulting from IgE cross-linking, I next investigated whether bacterial killing was altered in monocytes that had already engulfed bacteria. By comparing the number of live, internalized bacteria immediately after phagocytosis and after a 16-hour period, I was able to determine the ability of monocytes to kill internalized bacteria even when different numbers of bacteria were initially engulfed. Confirming my findings with killed, opsonized bacteria (**Figure 3.7**), monocytes exposed to 48 hours of IgE cross-linking internalized fewer live, unopsonized bacteria than monocytes in control conditions (**Figure 3.12 A**). Surprisingly, IgE cross-linking did not affect the killing of internalized bacteria after a further 16-hour incubation (**Figure 3.11 B**), suggesting that the IgE-mediated functional deficit on monocytes is specific to phagocytosis.

Discussion

In this chapter, I demonstrate that IgE cross-linking impairs the function of human monocytes. Despite the inflammatory phenotype induced by IgE cross-linking, the phagocytic function of these cells is concomitantly crippled (**Figure 3.13**). In addition, this study demonstrates the impact of serum IgE concentration, a biomarker of allergic disease, on the magnitude of IgE-mediated monocyte responses.

IgE-mediated induction of CD14 represents one potential mechanism by which monocytes may contribute to allergic inflammation. My finding that IgE-mediated CD14 expression correlates with serum IgE concentration may explain the clinical observation that allergen exposure up regulates CD14 on monocytes from sensitized individuals (Monteseirin 2003). Since CD14 is essential for LPS responses, one potential consequence of increased CD14 expression is enhancement of this response. This is relevant to allergic disease considering that individuals with allergic asthma have increased bronchial reactivity to inhaled LPS (Michel 1989), which itself contributes to airway inflammation in mouse models of allergic asthma (Tsuchiya 2012). My results suggest a potential link between increased CD14 expression and allergic airway disease and a role for IgE in this process.

Another prominent finding in my study was the rapid and robust secretion of the inflammatory cytokines TNFα and IL-6. Several studies have implicated TNFα in the pathogenesis of allergic disease, where it has been shown to impact airway inflammation (Hutchison 2008; Lee 2012; Widegren 2008). Additionally, IL-6 sputum concentrations correlate inversely with respiratory function in asthma patients (Morjaria 2011). The results of my study

suggest that IgE cross-linking on monocytes could thus contribute to allergic disease via the induction of TNFα and IL-6 secretion.

The gradual rise in IL-10 secretion was in marked contrast to the kinetics of TNFα induced by IgE cross-linking. The ability of IL-10 neutralization to reverse the TNFα decline and dramatically augment secretion of IL-6, and even IL-10 itself, suggests that IL-10 acts in an autocrine fashion to limit IgE-mediated cytokine secretion and possibly induce degradation of existing TNFα. In fact, IL-10 is proposed to play a suppressive role in allergic asthma (Nayyar 2012). Since IL-10 has been shown to suppress T cell and monocyte/macrophage responses to pathogens (Brooks 2010; Lee 2011), excess IL-10 could potentially disrupt these immune responses. Given the importance of pathogen-associated exacerbations of allergic diseases (Jackson 2010), this potential effect of IgE-mediated monocyte IL-10 secretion represents an exciting direction for future studies.

I have demonstrated that IgE cross-linking specifically disrupts monocyte phagocytosis without affecting bacterial killing. The apparent discrepancy between IgE-mediated impairment of phagocytosis and induction of a pro-inflammatory program – including TNFα, a cytokine known to promote phagocytosis (Mosser 2008) – suggests potential activation of divergent pathways by IgE cross-linking. One possible mechanism is impairment of TNFα responsiveness after IgE cross-linking, as increased TNFα concentration after IL-10 neutralization did not rescue the IgE-mediated repression of phagocytosis. However, TNFα unresponsiveness cannot completely account for IgE-mediated effects on monocyte function, as bacterial killing, another TNFα-responsive process (Mosser 2008), remained intact. CD64 is also involved in phagocytosis and its expression is known to reflect monocyte phagocytic ability (Alexis 2001;

Grage-Griebenow 2000). The down regulation of CD64 induced by IgE cross-linking represents another potential mechanism contributing to impaired phagocytosis.

Another key regulator of phagocytosis is SH2-domain-containing inositol 5' phosphatase 1 (SHIP), which inhibits macrophage phagocytosis through its action on membrane phospholipids (Tamura 2009). SHIP has also been reported to augment TLR-induced pro-inflammatory cytokine secretion (Fang 2004) and to mediate formation of reactive oxygen intermediates, which promote killing after phagocytosis (Kamen 2008). In addition to its roles in macrophages, SHIP is a negative regulator of allergic signaling in basophils; upon activation by IgE cross-linking it limits degranulation (Gibbs 2006). Activation of SHIP by IgE cross-linking in monocytes could potentially explain my observation of impaired phagocytosis despite secretion of pro-inflammatory cytokines and intact bacterial killing.

The relevance of IgE-mediated disruption of phagocytosis to allergic disease is evidenced by studies demonstrating that alveolar macrophage phagocytosis of bacteria and apoptotic cells is impaired in individuals with severe allergic asthma (Fitzpatrick 2008; Huynh 2005). Indeed, reduced macrophage phagocytosis has been correlated with increased sputum eosinophils and reduced respiratory function in individuals with allergic asthma (Alexis 2001). Furthermore, macrophage ingestion of apoptotic granulocytes has been shown to reflect the resolution of asthma symptoms (Kulkarni 2010), underscoring the importance of this process in allergic disease. IgE-mediated impairment of monocyte and macrophage phagocytosis in vivo could thus lead to reduced clearance of apoptotic debris and delayed resolution of inflammation.

In summary, I have demonstrated that IgE cross-linking drives select functions in human monocytes including the secretion of both pro-inflammatory and autoregulatory cytokines, which is enhanced in monocytes from individuals with elevated serum IgE levels. In contrast, the

ability of monocytes to engulf bacteria or cellular debris is significantly impaired by IgE cross-linking. This suggests that while allergic stimulation of monocytes promotes some aspects of inflammation, it concomitantly impairs the ability of these cells to resolve inflammation via phagocytosis. For individuals with elevated serum IgE concentration, the inflammation induced by allergic stimulation may be more pronounced considering their enhanced secretion of TNFα and IL-6. Yet, their ability to resolve such inflammation is blocked by IgE-mediated signaling. The ability of IL-10 to modulate IgE-mediated pro-inflammatory cytokine secretion without impacting the impairment of phagocytosis suggests that divergent pathways are induced by IgE cross-linking (**Figure 3.13**). Further, the discordant regulation of cytokine secretion and phagocytosis may perpetuate inflammation that occurs during infection in the context of allergic stimulation. Future studies designed to delineate how IgE-mediated signaling leads to these disparate functional effects will further elucidate the consequences of IgE cross-linking on human monocytes and provide a foundation for understanding the role of this important cell type in IgE-mediated allergic disease.

Table 3.1 – Participant Information

	Low IgE	**High IgE**	**P value**
Number	6	6	n/a
Age	23.0 (18-27)	21.2 (14-44)	0.71
Gender	3 Male, 3 Female	4 Male, 2 Female	1.00
Ethnicity	2 Black, 2 White, 2 Hispanic	5 Black, 1 White	0.16
Atopy			
-Asthma	4	6	0.45
-Allergic Rhinitis	3	6	0.18
-Atopic Dermatitis	0	1	1.00
-Skin Test (≥1 Positive)	6	6	n/a
-Skin Test (# of Positive)	3.5 (1-7)	5.8 (3-9)	0.11
Serum IgE (U/mL)	50.2 (25-99)	477.0 (134-1017)	**0.01**
Monocyte FcεRI (MESF)	2951 (1592-4582)	5171 (2011-8065)	**0.04**

Demographic information, atopic status, serum IgE concentration, and monocyte FcεRI expression are presented for enrolled participants. P values for categorical values were calculated by Chi-square or Fisher's Exact Test. Mean (range) and t test are shown for quantitative values.

Table 3.2 – Correlation of Serum IgE Concentration with the Effect of IgE Cross-Linking on Monocyte Phenotype and Function

Monocytes after IgE cross-linking		**Vs. Log Serum IgE**	
		Pearson R	**P Value**
CD14 Expression	48hr	0.55	0.061
	96hr	**0.69**	**0.019**
Phagocytosis	48hr	-0.34	0.274
	96hr	-0.18	0.588
TNFα Secretion		0.34	0.341
IL-6 Secretion		0.49	0.099
IL-10 Secretion		**0.72**	**0.007**

Correlations (Pearson R and P value) are presented for measurements obtained from monocytes after IgE cross-linking and log of serum IgE concentration.

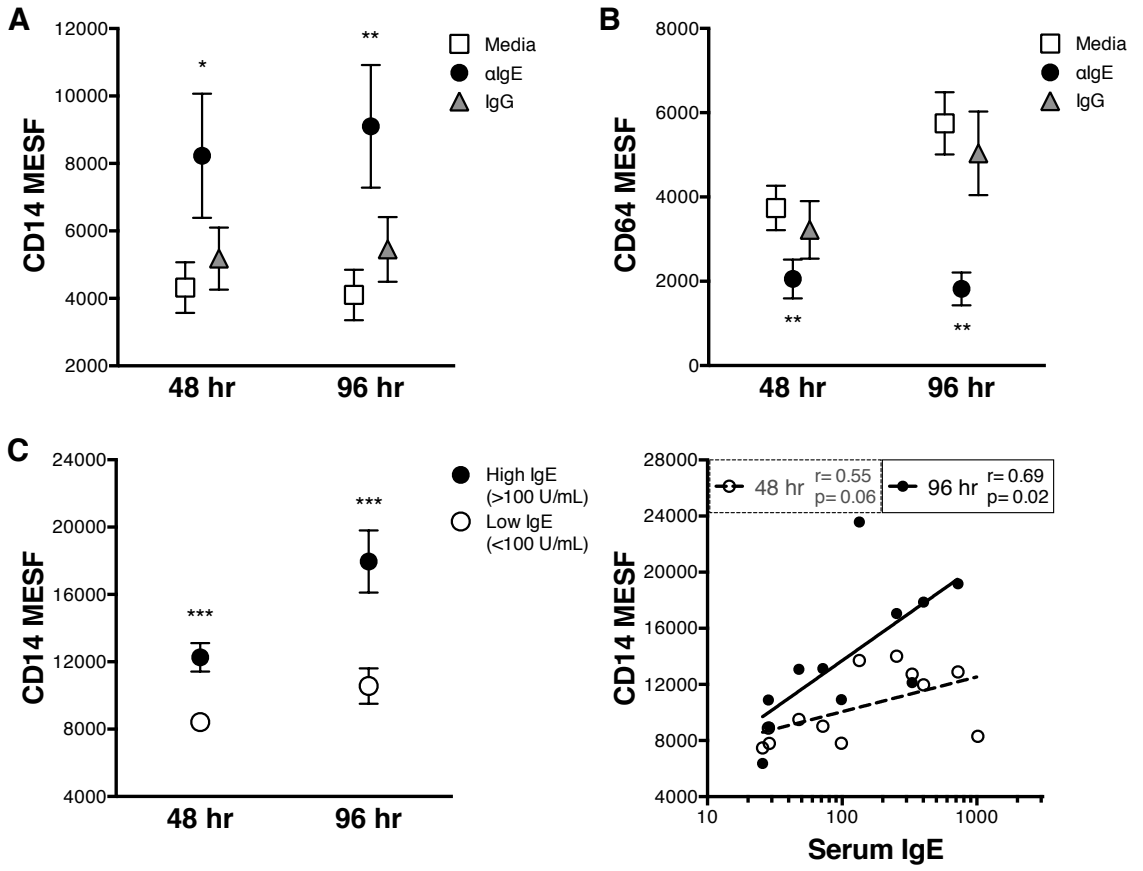


Figure 3.1 – IgE Cross-Linking Up Regulates Surface CD14 and Down Regulates CD64

Monocyte CD14 (A) and CD64 (B) expression after 48- or 96-hour culture in media alone (unfilled squares), αIgE (black circles), or control IgG (gray triangles) is depicted as mean equivalent standard fluorescence (N≥9). CD14 expression after IgE cross-linking on monocytes from individuals with low or high serum IgE (C, left); Pearson correlation between CD14 expression and serum IgE (C, right; N≥11). * p<.05, ** p<.01, and *** p<.001 for αIgE vs. media and IgG within time points (A and B) or high IgE vs. low IgE (C). The experiments in panel C were performed with the assistance of Dr. Victoria Yang.

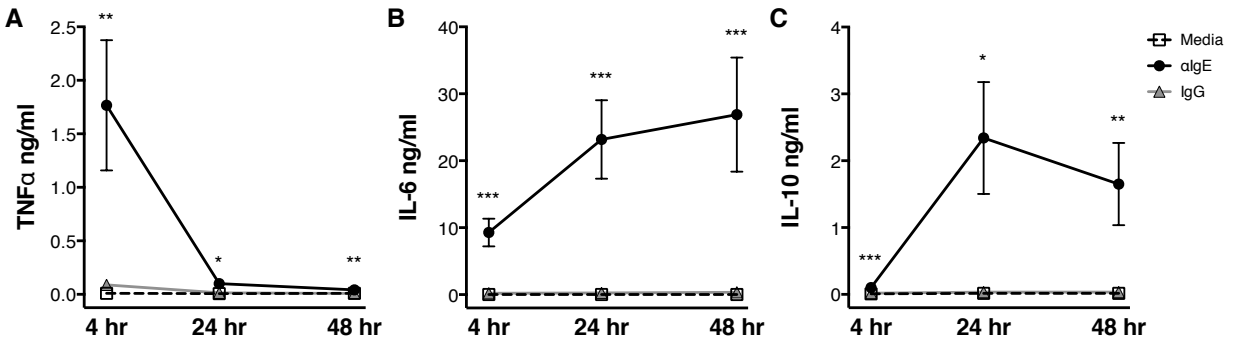


Figure 3.2 – IgE Cross-Linking Induces Monocyte Secretion of TNFα, IL-6, and IL-10

TNFα (A), IL-6 (B), and IL-10 (C) concentrations in monocyte supernatants after 4-, 24-, or 48-hour culture in media alone (unfilled squares), αIgE (black circles), or control IgG (gray triangles). Data are expressed as means ± SEM for N≥9 experiments. * p<.05, ** p< 01, *** p<.001 for αIgE vs. media and IgG.

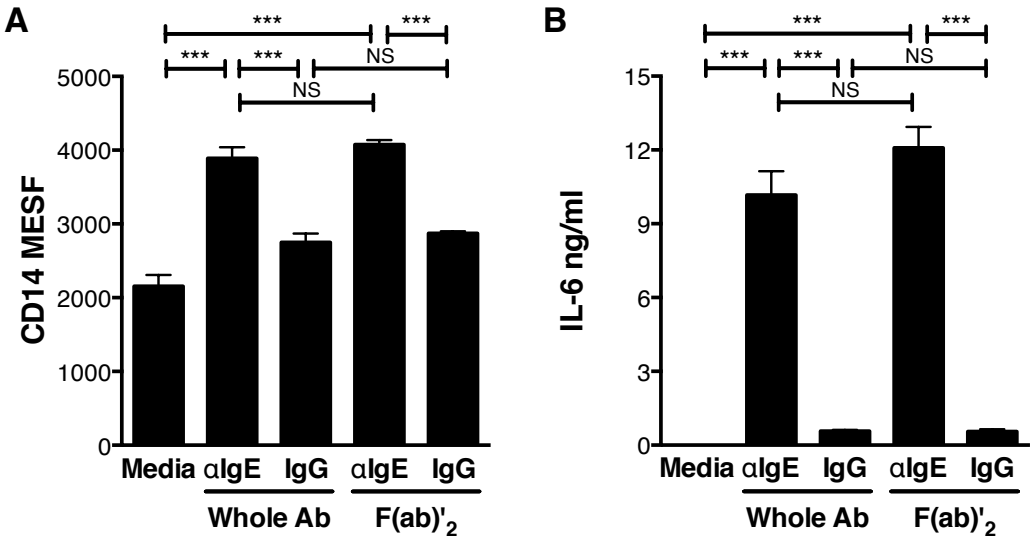


Figure 3.3 – The Effects of αIgE are not Fc-Mediated

Expression of CD14 (A) and secretion of IL-6 (B) are depicted for monocytes cultured in the following: media alone, αIgE, control IgG, αIgE F(ab)'2 fragment, or IgG F(ab)'2 fragment. Data are expressed as triplicate mean ± SEM for 1 of 4 independent experiments. *** $p<.001$, NS $p>.05$ for indicated comparisons.

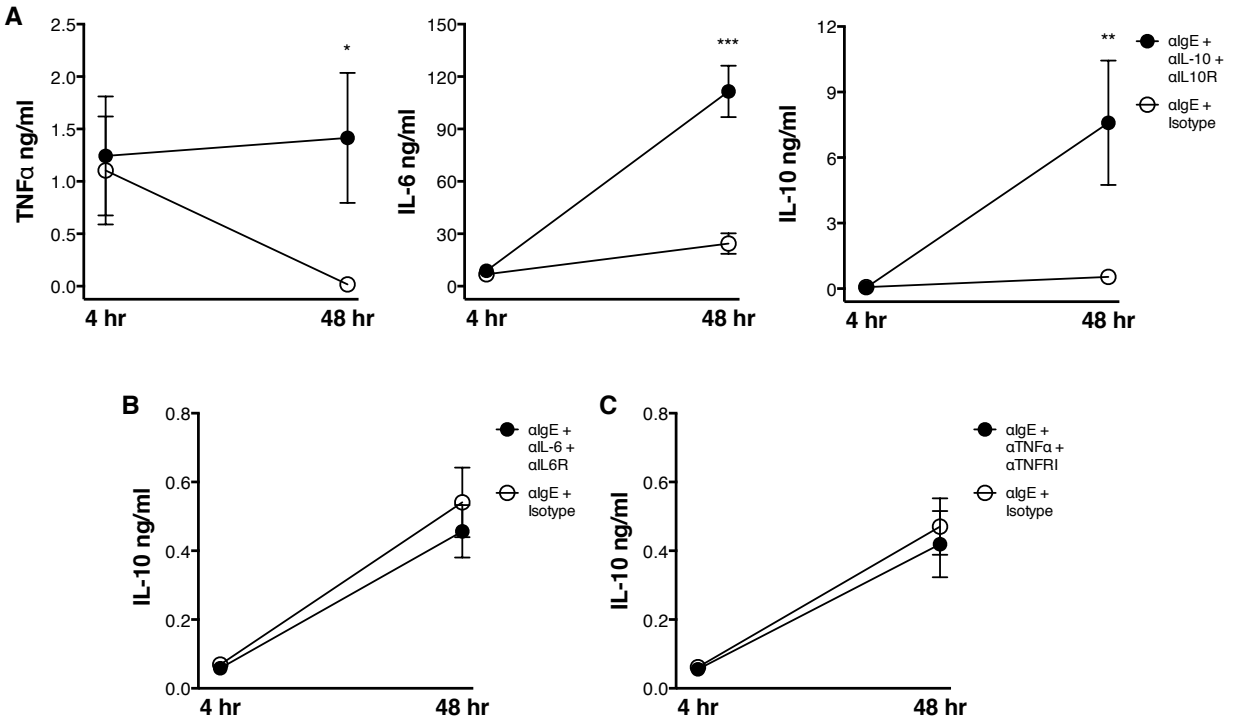


Figure 3.4 – IL-10 Suppresses IgE-Mediated Cytokine Secretion

Concentration of TNFα (A, left), IL-6 (A, middle), and IL-10 (A, right) from supernatants of monocytes cultured with αIgE for 4 or 48 hours in the presence of IL-10 and IL-10R neutralizing antibodies or isotype control (N=3). Concentration of IL-10 secreted by monocytes cultured with αIgE in the presence of neutralizing antibodies for TNFα and TNFRI (B), IL-6 and IL-6R (C), or appropriate isotype controls (N=3). * p<.05, ** p< 01, *** p<.001 for neutralizing antibodies vs. isotype.

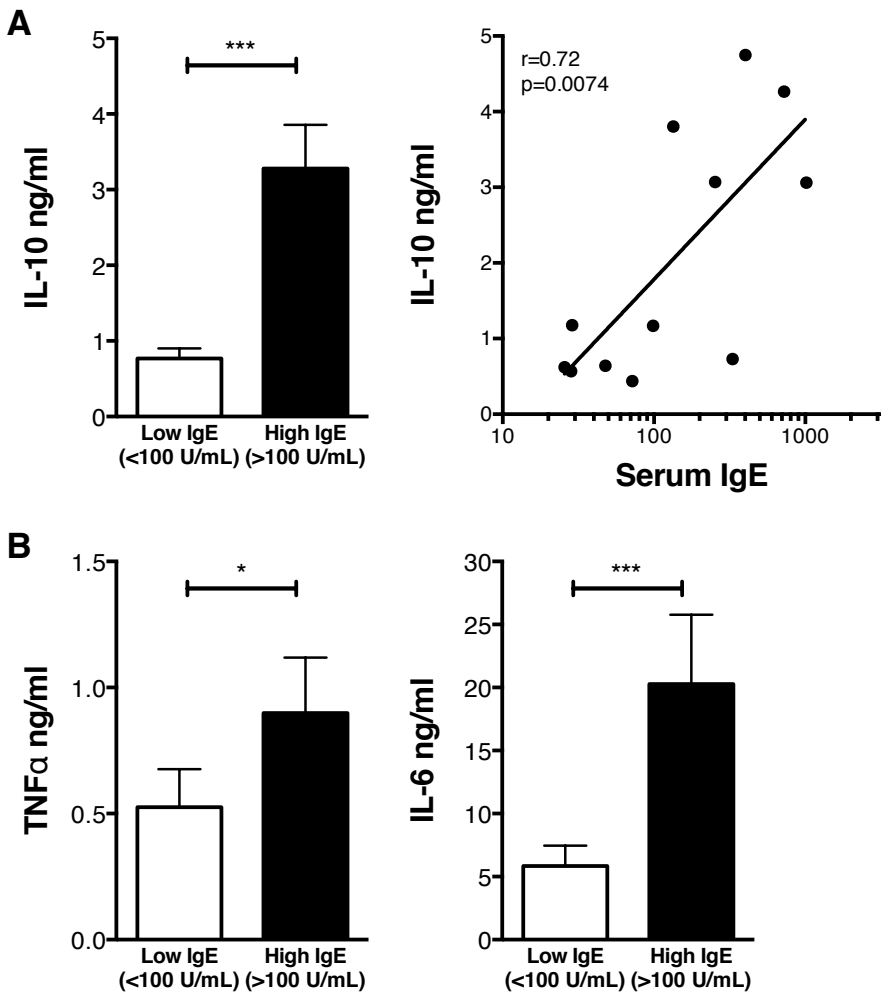


Figure 3.5 – IgE-Mediated Cytokine Secretion is Dependent on Serum IgE Concentration

IL-10 concentration (A, left) after 48 hours of IgE cross-linking on monocytes from individuals with low (<100 U/mL) or high (>100 U/mL) serum IgE and corresponding Pearson correlation (A right; N=12). Concentration of TNFα after 4 hours (B, left) and IL-6 after 48 hours (B, right) of IgE cross-linking on monocytes from individuals with low vs. high serum IgE (N≥10). * $p<.05$, *** $p<.001$ for high IgE vs. low IgE. The experiments in this figure were performed with the assistance of Dr. Victoria Yang.

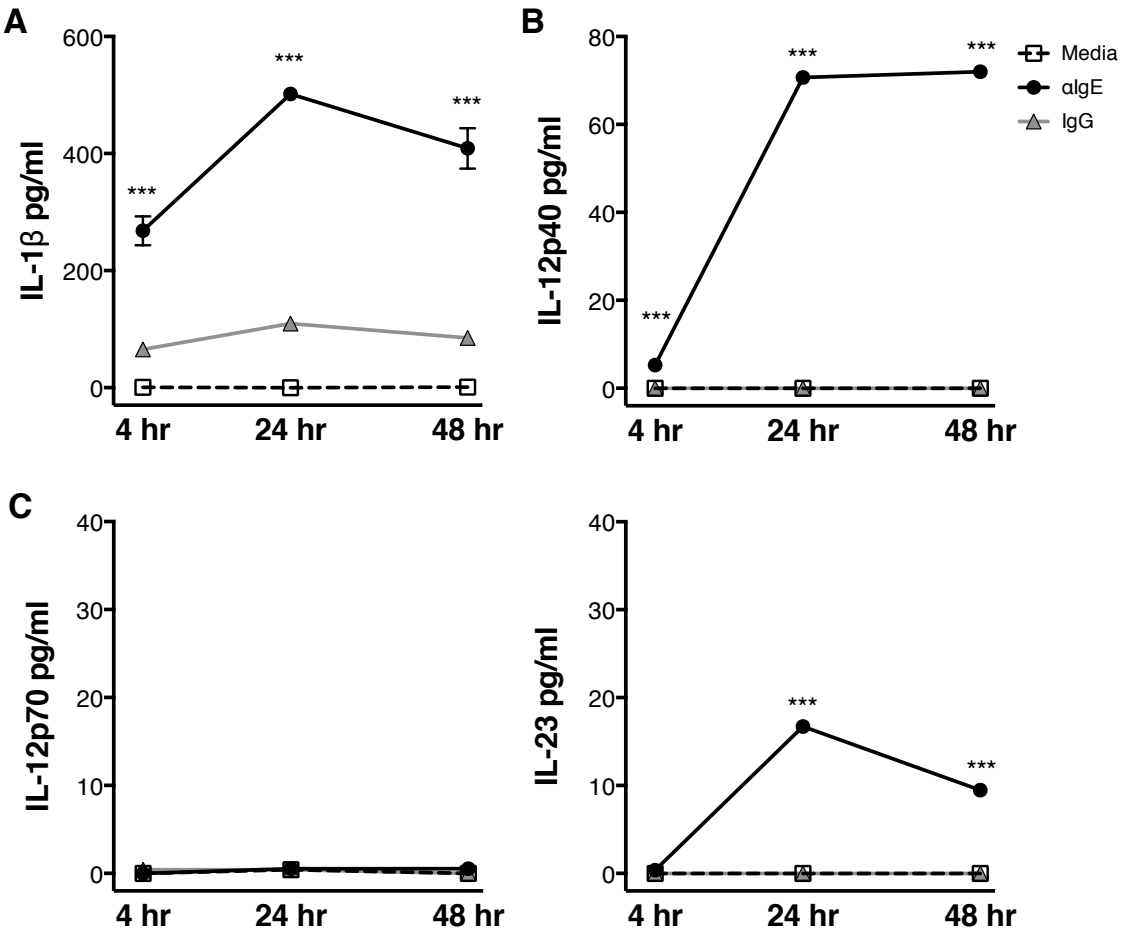


Figure 3.6 – IgE Cross-Linking Induces Secretion of IL-1β and IL-23

IL-β (A), IL-12p40 (B), IL-12p70 (C, left), and IL-23 (C, right) concentrations in supernatants from monocytes cultured for 4, 24, or 48 hours in media alone, αIgE, or control IgG. Data are expressed as triplicate mean ± SEM for 1 of 4-8 independent experiments. *** $p<.001$ for αIgE vs. media and IgG. The experiments in panels A and C were performed with the assistance of Ms. Lorene Sandifer.

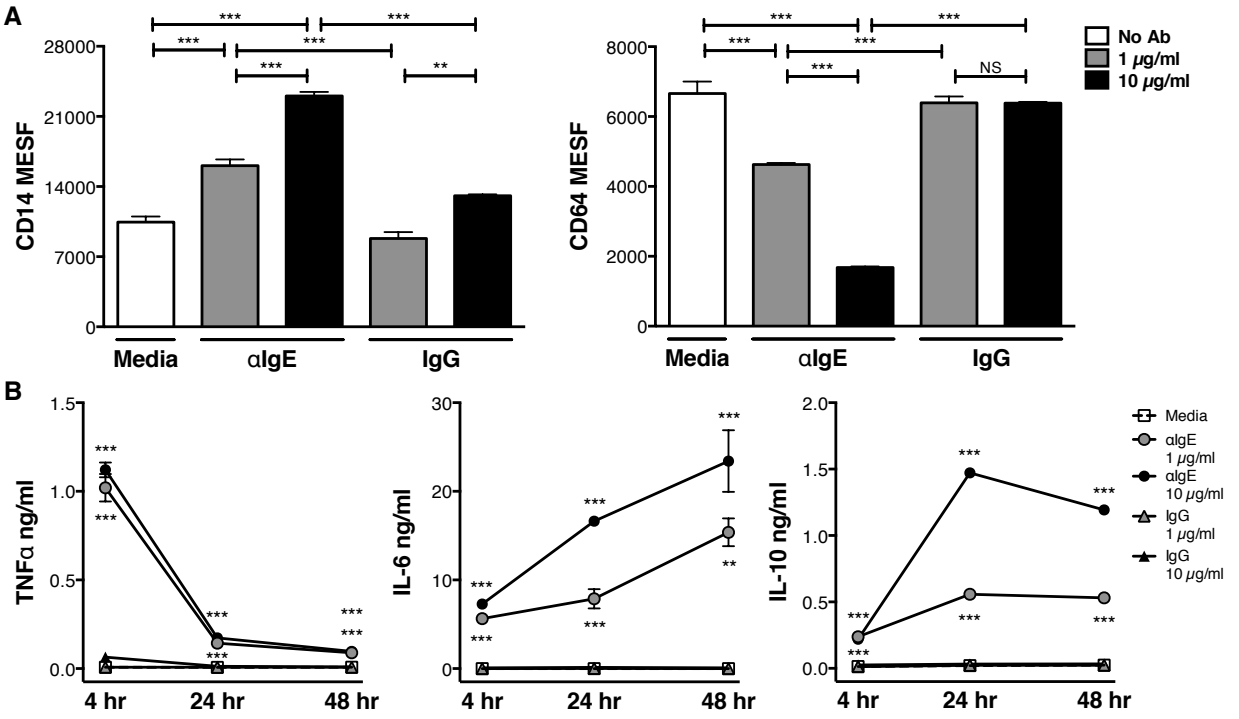


Figure 3.7 – Effects of IgE Cross-Linking are Concentration Dependent

Expression of CD14 (A, left) and CD64 (A, right) is depicted for monocytes cultured for 96 hours in the following: media alone, αIgE (1 or 10 mg/ mL), or control IgG (1 or 10 mg/mL). Concentration of TNFα (B, left), IL-6 (B, middle), and IL-10 (B, right) secreted by monocytes cultured as in A. ** p<.01, *** p<.001, NS p>.05 for indicated comparisons (A) or for αIgE (1 or 10 mg/mL) vs. media and IgG (1 or 10 mg/mL, respectively) (B). Data are expressed as triplicate means ± SEM for 1 of 5 independent experiments.

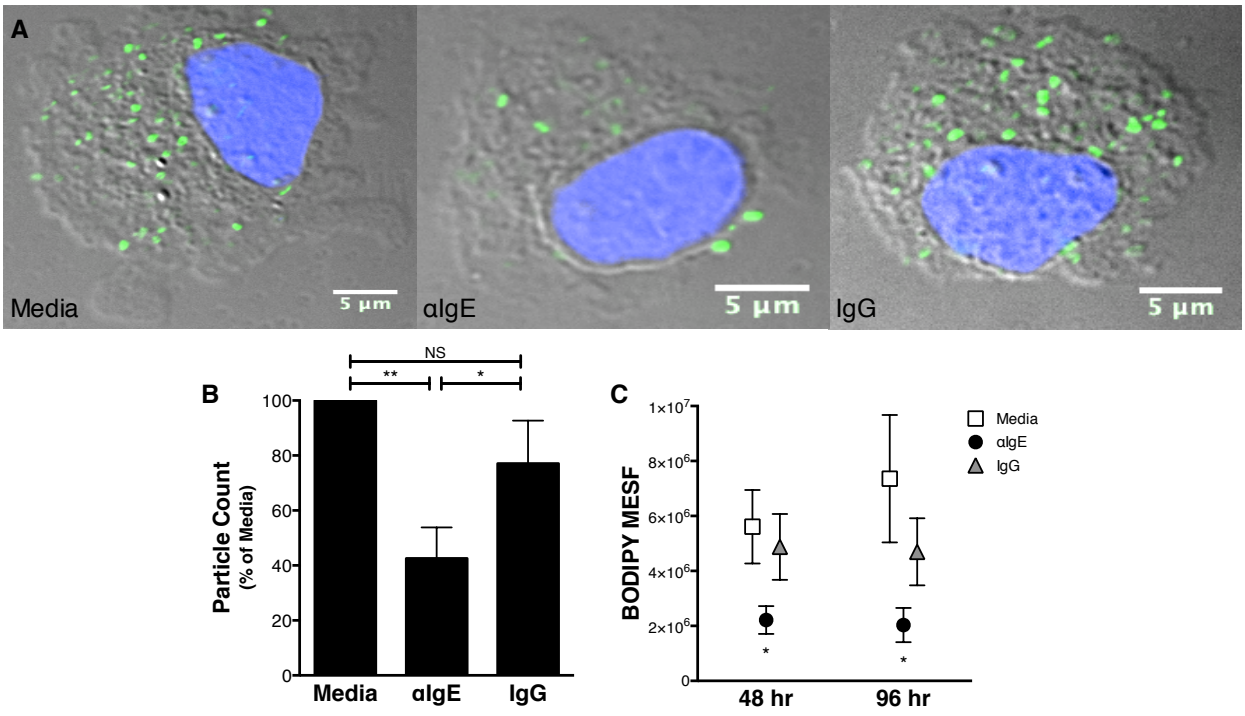


Figure 3.8 – IgE Cross-Linking Impairs Monocyte Phagocytic Function

Photomicrographs (A; gray=DIC, blue=DAPI, green=BODIPY-FL) and particle count per cell (B) of BODIPY-labeled bacteria phagocytosed by monocytes after 96-hour culture in indicated conditions (N=5). Fluorescence of internalized bacteria (C) determined by flow cytometry for indicated conditions (N≥9). * $p<.05$, ** $p<.01$, NS $p>.05$ for indicated comparisons (B) or αIgE vs. media and IgG (C).

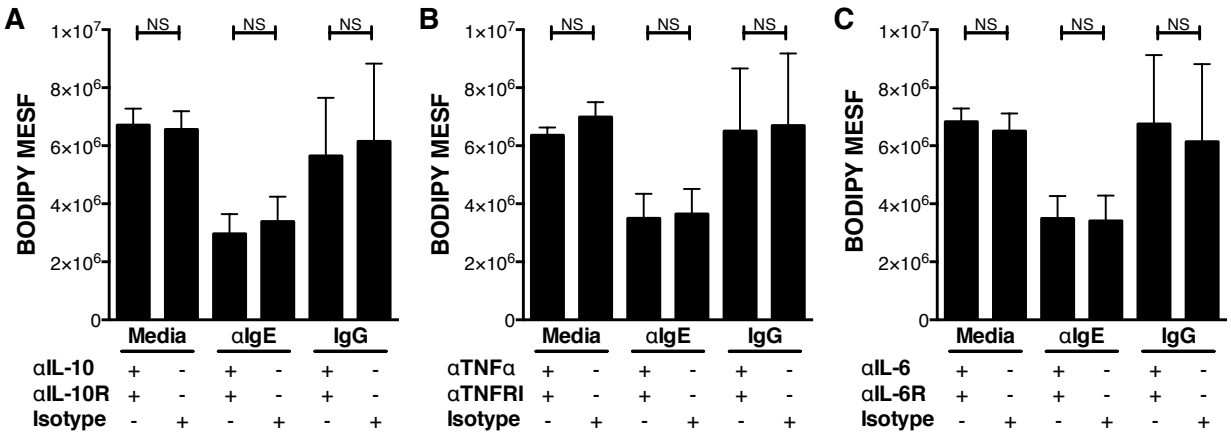


Figure 3.9 – IgE-Mediated Suppression of Phagocytosis is Independent of IL-10, TNFα, or IL-6

Fluorescence of internalized bacteria in monocytes cultured for 48 hours in media alone, IgE cross-linking antibody, or control IgG in the presence of neutralizing antibody for IL-10 and IL-10R (A), TNFα and TNFRI (B), IL-6 and IL-6R (C), or appropriate isotype controls (N=3). NS $p>.05$ for indicated comparisons.

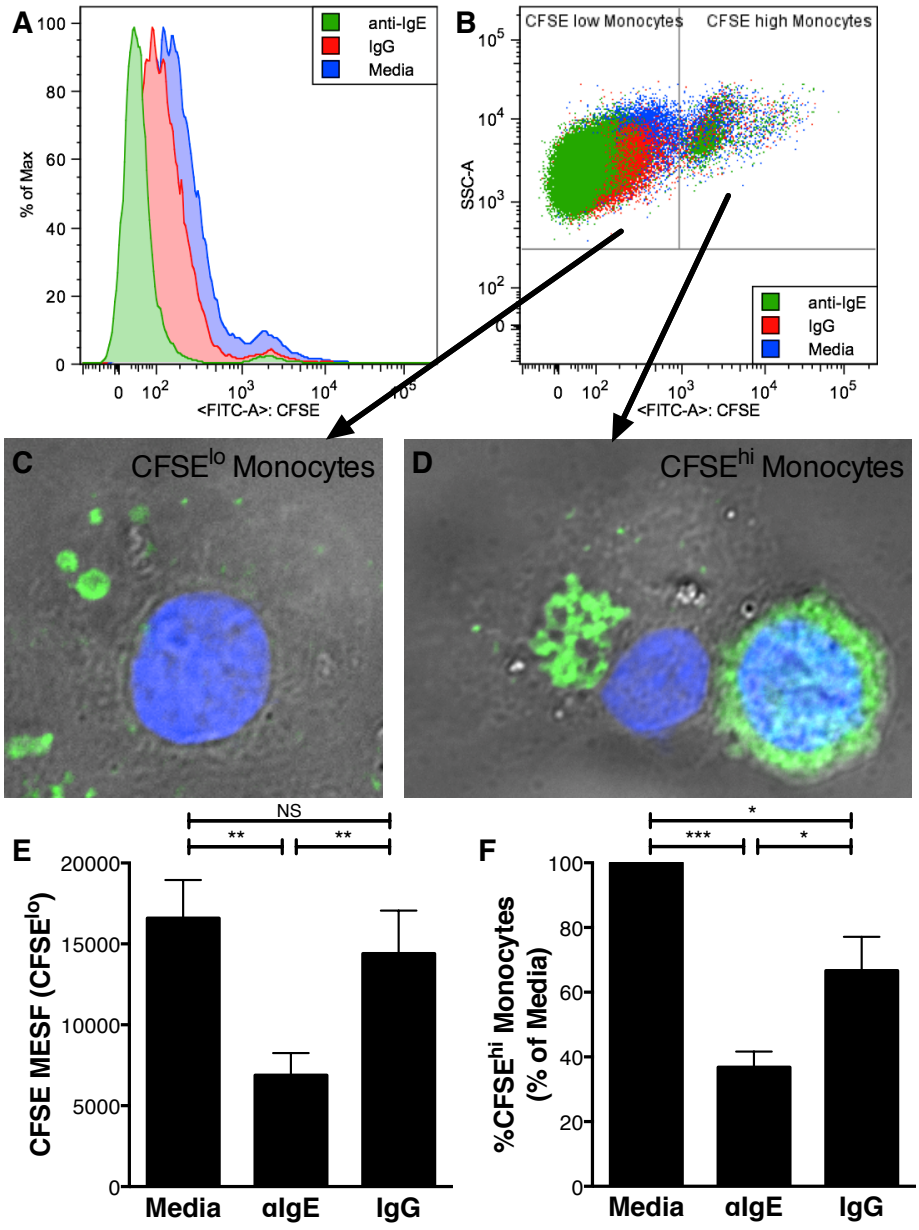


Figure 3.10 – IgE Cross-Linking Inhibits Phagocytosis of Apoptotic Cells

CFSE fluorescence (A) in monocytes (gated on $CD14^{+}$) after phagocytosing CFSE-labeled apoptotic cells. Gating strategy (B) for $CFSE^{low}$ and $CFSE^{high}$ monocytes. Photomicrographs (gray=DIC; blue=DAPI; green=CFSE) of sorted $CFSE^{low}$ monocytes (C) and $CFSE^{high}$ monocytes (D). CFSE fluorescence of $CFSE^{low}$ monocytes (E) and percentage of $CFSE^{high}$ of total monocytes (F) for indicated culture conditions (N=4). * $p<.05$, ** $p<.01$, *** $p<.001$, NS $p>.05$ for indicated comparisons (E and F).

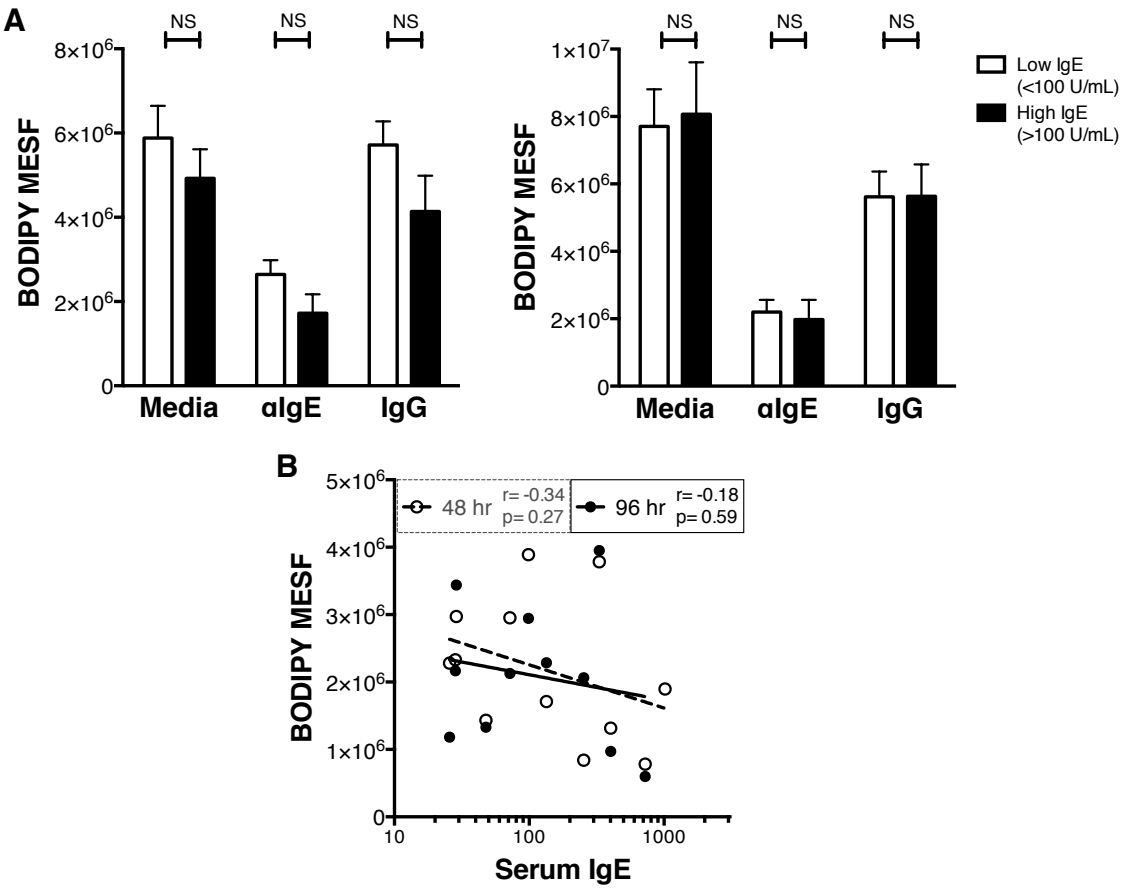


Figure 3.11 – Inhibition of Phagocytosis by IgE Cross-Linking is Independent of Serum IgE Concentration

Fluorescence of internalized bacteria in monocytes from individuals with low (<100 U/mL) or high (>100 U/mL) serum IgE, cultured for 48 (A, left) or 96 (A, right) hours in indicated conditions; corresponding Pearson correlation (B) for αIgE condition (N=12). NS p>.05 for indicated comparisons. The experiments in this figure were performed with the assistance of Dr. Victoria Yang.

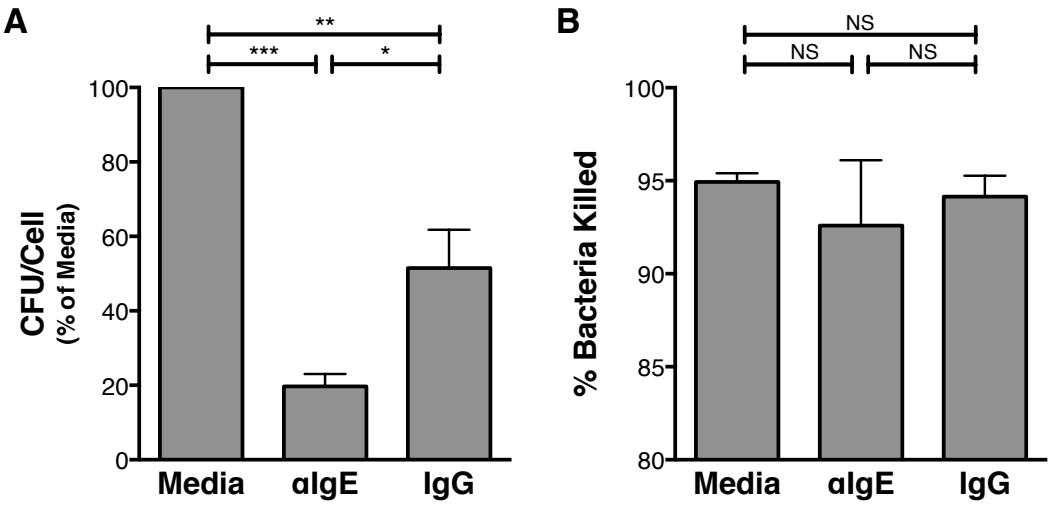


Figure 3.12 – IgE Cross-Linking does not Affect Killing of Internalized Bacteria

Internalization (A; CFU/cell) of live bacteria by monocytes cultured in media alone, αIgE, or IgG control for 48 hours, then exposed to *E. coli* for 45 minutes. Percentage of internalized bacteria killed (B) by monocytes 16 hours after internalization (N=4). %Killed = ($CFU/Cell_{0hr}$ - $CFU/Cell_{16hr}$) ÷ $CFU/Cell_{0hr}$ × 100. * $p<.05$, ** $p<.01$, *** $p<.001$, NS $p>.05$ for indicated comparisons. The experiments in this figure were performed with the assistance of Dr. Victoria Yang.

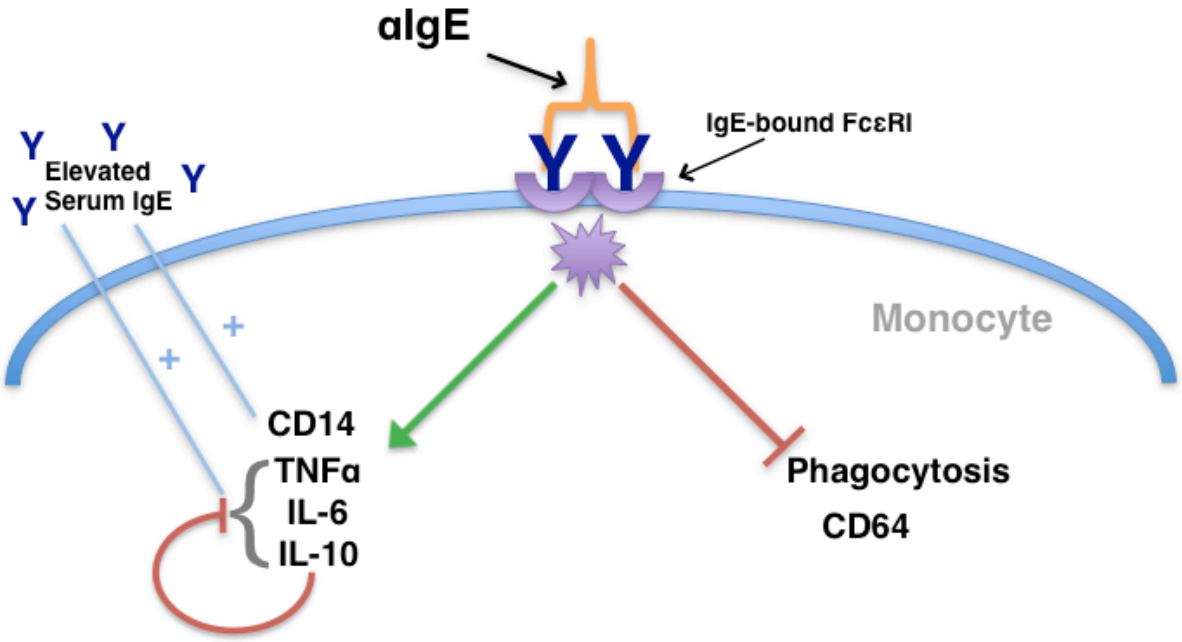


Figure 3.13 – Proposed Model of IgE Cross-Linking on Human Monocytes

IgE cross-linking drives (green arrow) increased CD14 expression and secretion of both pro-inflammatory cytokines (TNFα and IL-6) and autoregulatory IL-10. These effects are enhanced in individuals with elevated serum IgE (blue lines). In contrast, phagocytosis and CD64 expression are suppressed by IgE cross-linking (red line). The IgE-mediated impairment of phagocytosis is independent of regulation by IL-10, suggesting that divergent pathways are activated by IgE cross-linking on monocytes.

CHAPTER FOUR

IGE CROSS-LINKING MODULATES MONOCYTE RESPONSE TO RESPIRATORY VIRUSES

Introduction

Monocytes are an important component of immune responses to respiratory viral infection. During influenza infection, monocytes are recruited to the lung and nasal mucosa (Gill 2008; Herold 2006). Monocytes and monocyte-derived cells in the lung have been shown to be important for resolution of influenza (Cao 2012; Narasaraju 2010) as well as RSV infection (Shirey 2010). Several respiratory viruses, including hRV, influenza, and RSV can infect monocytes (Cao 2012; Laza-Stanca 2006; Villani 1991).

Monocytes serve a number of significant roles during viral infection. One such role entails clearing apoptotic and necrotic debris by phagocytosis to resolve inflammation (Narasaraju 2010; Reed 2008). In addition to their role in resolving inflammation, monocytes secrete a number of factors that help to direct the immune response. Virus-exposed monocytes also secrete chemokines to recruit other immune cells to the site of infection. Exposure of monocytes to influenza or hRV results in significant secretion of IP-10 and MCP-1, which recruit memory T cells, NK cells, and monocytes (Cao 2012; Hall 2005; Korpi-Steiner 2006). RSV infection induces monocyte secretion of RANTES and MCP-1, which recruit a wide variety of cell types (Gotera 2012). Another important aspect of virus response in monocytes is secretion

of type I interferon. Monocytes and monocyte-derived dendritic cells have been shown to produce type I interferon in response to both hRV and influenza (Cao 2012; Schrauf 2009). Type I interferon, including IFNα and IFNβ, are potent anti-viral cytokines. Activation of the type I interferon receptor on several cell types induces a multitude of gene products, termed interferon-stimulated genes (ISG), which mediate anti-viral activity (Der 1998).

In response to virus infection, monocytes play an important role in stimulating T cells. Monocytes and monocyte-derived cells present antigens to $CD4^+$ and $CD8^+$ T cells via MHC class I and class II molecules, respectively (Geppert 1986). While memory T cells can be activated simply with antigen-bound MHC molecules, activation of naïve T cells also requires co-stimulation with surface molecules such as CD40, CD80, and CD86 (Croft 1994). As such, the expression of these MHC and co-stimulatory molecules plays a critical role in the capacity of monocytes to promote T cells responses during viral exposure. In response to inflammatory stimuli, including virus and bacterial infection (Wu 2011b), monocytes and monocyte-derived DCs increase expression of MHC molecules and co-stimulatory molecules to increase their ability to stimulate T cell responses (Trombetta 2005).

Upon stimulation with antigen, naïve $CD4^+$ T cells can differentiate into several subtypes defined by the cytokines secreted by the T cells. IL-12 directs naïve $CD4^+$ T cells to become Th1 cells, producing IFNγ and TNFα, while IL-4 drives a Th2 phenotype, producing IL-4, IL-5, and IL-13 (Murphy 2000). Combinations of IL-6, IL-1β, TGFβ, and IL-23 induce development of Th17 cells, which produce IL-17A and IL-17F (Ganjalikhani Hakemi 2011). In addition to the cytokines required for differentiation of T cell subtypes, other cytokines can directly suppress or even reverse T cell commitment. IL-4 has been shown to suppress Th1 development in naïve $CD4^+$ T cells and can also reverse the commitment of previously differentiated Th1 cells

(Ouyang 1998). Similarly, type I interferon can suppress both Th2 and Th17 commitment (Huber 2010; Moschen 2008). Type I interferon can also stimulate Th1 development in mice, though this is not true for human T cells (Ramos 2007).

In normal circumstances, respiratory viral infections promote the development of IFNγ-secreting Th1 cells (Xing 2000). In atopic diseases including allergic asthma, Th2 cells – not Th1 cells – are the primary effector T cell subtype (Holgate 2012). Given the association between respiratory viral infections and exacerbations of asthma, it follows that respiratory viral infections in asthmatics may result in altered T lymphocyte responses (from Th1 to Th2), thus contributing to disease pathogenesis. Indeed, during hRV infection in asthmatic patients, reduced Th1 responses and increased Th2 responses have been observed (Message 2008). While greater Th1 response was associated with protection, higher levels of the Th2 cytokines IL-4, IL-5, and IL-13 were associated with more severe symptoms.

Epidemiological evidence for an interaction of viral and allergic immune responses has also been observed. Respiratory viral infection is associated with increased risk of asthma exacerbations (Johnston 1995). The risk for asthma exacerbation is synergistically increased if exposure to a sensitized allergen and virus infection are present (Green 2002; Rakes 1999). Furthermore, a number of prospective cohort studies have observed an intricate relationship between allergic sensitization, severe viral infections in early childhood, and subsequent development of allergic asthma (Jackson 2012; Sigurs 2010).

Viral and allergic responses have been shown to interact in several cell types. In $CD4^+$ T cells, the Th2 cytokine IL-4 suppresses Th1 commitment, while the anti-viral type I interferon suppress Th2 development. Plasmacytoid DCs down regulate the high-affinity IgE receptor upon exposure to viruses or TLR agonists (Gill 2010; Schroeder 2005). Conversely, stimulation of

FcεRI by IgE cross-linking suppresses expression of the virus detecting receptors TLR7 and TLR9. IgE cross-linking also suppressed virus-induced type I interferon in pDCs (Gill 2010).

Monocytes serve many important functions during viral infection, but little is known about the potential regulation of these responses by allergic stimulation. Thus, I decided to examine monocyte responses to virus exposure in the context of IgE cross-linking. Understanding how IgE cross-linking impacts monocyte maturation and T cell stimulatory capacity will provide important insight into potential mechanisms of viral exacerbations of allergic asthma, and will also provide better understanding of the mechanisms by which opposing immune responses regulate each other.

Results

IgE Cross-Linking Down Regulates Monocyte Maturation Markers

I previously determined that IgE cross-linking induces secretion of pro-inflammatory cytokines but suppresses phagocytic function in monocytes (Pyle 2013). Antigen presenting function in monocytes and monocyte-derived cells is enhanced by up regulating MHC molecules and co-stimulatory molecules during maturation (Leon 2007). To investigate a potential role for IgE cross-linking in monocyte maturation, I analyzed monocyte expression of several surface markers: the MHC class I (HLA-A,B,C), MHC class II (HLA-DR), and the co-stimulatory molecules CD80 and CD86.

Monocyte expression of both MHC class I and MHC class II was significantly reduced 48 hours after IgE cross-linking (**Figure 4.1 A and B**), though MHC class II expression was more robustly impacted. Similarly, CD86 expression was significantly reduced by αIgE compared to control conditions (**Figure 4.1 C**). In contrast, CD80 expression was actually increased by IgE cross-linking (**Figure 4.1 D**).

IgE Cross-Linking Suppresses Virus-Induced Monocyte Maturation

Because IgE cross-linking reduced expression of several monocyte maturation markers, I next decided to examine whether IgE cross-linking is capable of altering monocyte maturation in the setting of a known maturation stimulus, such as virus exposure.

Upon exposure to influenza virus for 48 hours, monocytes significantly up regulated MHC class I, class II, CD80, and CD86 (**Figure 4.2 A-D**). IgE cross-linking suppressed the influenza-induced up regulation of MHC class I, class II and CD86 (**Figure 4.3 A-C**), and in the case of MHC class II and CD86, expression was reduced to control levels. In contrast with the other maturation markers evaluated, influenza-induced up regulation of monocyte CD80 expression was not impacted by IgE cross-linking (**Figure 4.3 D**).

I next wanted to determine whether IgE cross-linking could also impact maturation with a different viral stimulus: human rhinovirus. Preliminary experiments demonstrate that while MHC class II and CD80 were not significantly affected by hRV stimulation (**Figure 4.4 A and D**), monocytes exposed to hRV for 48 hours expressed significantly higher MHC class I and CD86 compared with mock-treated monocytes (**Figure 4.4 B and C**). In the setting of hRV exposure, IgE cross-linking again suppressed surface expression of MHC class I, class II, and CD86 (**Figure 4.5 A-C**). Similar to its effect on uninfected cells, IgE cross-linking increased CD80 expression (**Figure 4.5 D**).

The pattern of αIgE effect on virus-stimulated and unstimulated monocytes indicates that it preferentially suppresses MHC class I and class II expression even in the face of a strong inducing stimulus. IgE cross-linking had differential effects on two similar co-stimulatory molecules, suppressing CD86, while inducing CD80.

IgE Cross-Linking Suppresses Virus-Induced Th1 Differentiation

The ability of IgE cross-linking to down regulate monocyte expression of MHC molecules and CD86 led me to explore its potential impact on monocytes interactions with T

cells. Because of the importance of monocytes and monocyte-derived DCs in directing T cell polarization and the recognized clinical association between viral exposure and exacerbations of allergic asthma, I wanted to examine how IgE cross-linking affects the ability of monocytes to drive T cell polarization in response to virus.

Utilizing a mixed-leukocyte reaction (MLR), I cultured allogeneic monocytes and naïve $CD4^+$ T cells together to induce T cell activation. I then determined which cytokines the T cells produced upon re-stimulation as an indicator of polarization. To ensure that the T cells were being activated, I cultured control T cells on plate-bound αCD3 and αCD28 with defined cytokine conditions for Th1 or Th2 polarization, or with neutralizing antibodies to prevent polarization (**Figure 4.6 A**). Significantly more T cells produced IFNγ after culture under Th1-promoting conditions than in neutralized or Th2 conditions (**Figure 4.6 B**). Similarly, exposure to Th2-promoting conditions induced more IL-4-producing T cells (**Figure 4.6 D**). In addition to the IFNγ-producing Th1 and IL-4-producing Th2 populations, a distinct population of T cells produced both IFNγ and IL-4. This double positive population was not significantly enriched in any of the polarizing conditions (**Figure 4.6 C**).

Monocytes primed for 24 hours in the presence of influenza, IgE cross-linking, or control conditions induced development of IFNγ-producing (Th1), IL-4-producing (Th2), and IFN/IL-4 co-producing T cells upon co-culture with allogeneic naïve $CD4^+$ T cells (**Figure 4.7 A**). As expected, $CD4^+$ T cells stimulated with influenza-treated monocytes developed greater numbers of Th1 cells compared with $CD4^+$ T cells co-cultured with untreated (Mock) monocytes, indicating that influenza increased monocyte-driven Th1 differentiation in these co-cultures (**Figure 4.7 B, "Media"**). Remarkably, IgE cross-linking in influenza-treated monocytes considerably suppressed the influenza-induced increase in monocyte-driven Th1 polarization,

while control IgG had no effect (**Figure 4.7B, filled bars**). Influenza-exposed monocytes also induced significantly more IFNγ/IL-4 double positive T cells (**Figure 4.7 C, filled bars**), an effect which was also suppressed by αIgE. While influenza-treated monocytes did not significantly alter the percentage of IL-4 positive T cells compared to mock-treated monocytes (**Figure 4.7 D, filled bars**), monocytes exposed to both influenza and IgE cross-linking induced more IL-4^+ Th2 cells compared to influenza alone, though this effect has not yet reached statistical significance, suggesting that IgE cross-linking may be transforming a Th1-inducing stimulus, influenza, into a Th2-inducing stimulus. IgE cross-linking in the absence of influenza induced a slight, but non-significant increase in all three populations (**Figure 4.7 B-D, unfilled bars**).

To determine whether the effect of IgE cross-linking on Th1 polarization was specific to influenza-stimulated monocytes, I examined T cell polarization by monocytes primed with human hRV (**Figure 4.8 A**). Similar to the effect seen in influenza-stimulated monocytes, initial experiments demonstrate that IgE cross-linking leads to a significant reduction in hRV-induced Th1 polarization by monocytes (**Figure 4.8 B**). In contrast, IgE cross-linking had no affect on the ability of hRV-primed monocytes to promote the development of IFNγ/IL-4 co-producing T cells (**Figure 4.8 C**). In these initial experiments, IgE cross-linking on monocytes increased hRV-associated Th2 polarization (**Figure 4.8 D**), though this effect was not statistically significant.

I have previously shown that IgE cross-linking stimulates secretion of IL-1β, IL-6, and IL-23 from monocytes. As these cytokines have all been linked to the induction and activation of Th17 cells, I wanted to determine whether IgE cross-linking affected Th17 polarization in the monocyte-T cell co-culture system. In T cells cultured with αCD3 and αCD28 in the presence of

defined polarizing cytokine conditions, a small number of T cells acquired the ability to produce IL-17 (**Figure 4.9 A**). However, Th17 polarization was not significantly increased by Th17 polarizing conditions compared to neutralized conditions (**Figure 4.9 B**). Monocytes co-cultured with T cells did not induce measurable Th17 polarization, regardless of monocyte stimulation conditions (**Figure 4.9 C and D**).

Th1 polarization by virus-primed monocytes was consistently suppressed by IgE cross-linking. Conversely, IgE cross-linking increased virus-induced Th2 polarization, but this effect needs further investigation to achieve statistical significance. Taken together, these data suggest that IgE may mediate important effects on virus-induced $CD4^+$ T cell differentiation *in vivo*.

Discussion

In this chapter, I have shown preliminary results indicating that IgE cross-linking inhibits expression of important monocyte surface markers related to T cell stimulation. This suppression was observed with IgE cross-linking alone as well as in the context of viral stimulation. I also demonstrated that IgE cross-linking during virus exposure suppresses monocyte-driven Th1 differentiation and may simultaneously increase Th2 development.

IgE-mediated impairment of MHC class I and MHC class II expression on monocytes represents a potentially important mechanism by which allergic stimulation may regulate other immune responses. Up regulation of MHC molecules by virus is important for monocytes and monocyte-derived cells to efficiently present antigens to $CD4^+$ and $CD8^+$ T cells to stimulate adaptive immune responses (Trombetta 2005). In combination with the finding that IgE cross-linking reduces phagocytosis (reported in Chapter 3, Figure 3.8), IgE-mediated suppression of MHC expression after viral stimulation suggests that allergic stimulation may suppresses the ability of monocytes to acquire and present viral antigens. Future studies defining how IgE cross-linking affects the ability of monocytes to present antigens to memory $CD4^+$ and $CD8^+$ T cells will provide further insight on the practical effects of diminished MHC class I and MHC class II following IgE cross-linking.

Co-stimulatory molecules such as CD80 and CD86 are required for activation of naïve T cells, but are expendable for re-activation of memory T cells (Croft 1994). The differential effect of IgE cross-linking on monocyte expression of CD80 and CD86 is intriguing. Temporal differences in the expression of CD80 and CD86 have been observed in monocytes during in vitro culture (Fleischer 1996). As I have only examined one time point in this study, it is possible

that the differential effect of IgE cross-linking on these molecules is due to differences in the timing of expression. For example, as CD80 expression increases more slowly than CD86 (Fleischer 1996), it follows that down regulation of CD80 may also occur more slowly. Examination of earlier or later time points may reveal additional information about the regulation of these molecules by IgE cross-linking. Despite the fact that they share the same T cell receptors in CD28 and cytotoxic T lymphocyte antigen 4 (CTLA4), differential functions of CD80 and CD86 have been reported (Ellis 1996). However, the conclusions of these reports have varied: CD80 (Fleischer 1996) and CD86 (Bashian 1997) have each been reported as the primary molecule responsible for T cell proliferation in human PBMC cultures. Both molecules have been implicated in presentation of allergen (Jaffar 1999) as well as viral stimuli (Lumsden 2000). CD80 has also been implicated in inhibitory interactions with CTLA4 on memory T cells (Manzotti 2006). Though the global effect of IgE-mediated increase in CD80 expression and reduction in CD86 is not clear, simultaneous reduction of MHC molecules suggests an overall negative effect of IgE cross-linking on antigen presenting function. Reduction in antigen presentation and T cell proliferation after allergic stimulation could lead to enhanced severity of viral infection in atopic individuals. Future studies assessing monocyte-induced proliferation of naïve $CD4^+$ and $CD8^+$ T cells will provide better understanding of IgE-mediated regulation of monocyte co-stimulatory function.

A key finding of this study is the ability of IgE cross-linking to alter the capacity of virus-stimulated monocytes to drive Th1 polarization of naïve $CD4^+$ T cells. Though more experiments are needed, IgE cross-linking on monocytes appears to switch a Th1-inducing stimulus into a Th2-inducing stimulus. There are a number of possible mechanisms by which this could occur. As monocytes have not been observed to secrete IL-12 in response to hRV and influenza (Cao

2012; Schrauf 2009), it is unlikely that the IgE-mediated reduction in virus-stimulated Th1 commitment is simply due to suppression of IL-12 by IgE cross-linking, though this possibility does need to be investigated. Cytokines induced by IgE cross-linking on monocytes may also mediate this effect. One such cytokine, IL-6 (reported in Chapter 3, Figure 3.3), has been shown to suppress Th1 differentiation (Diehl 2000). IL-6 has also been shown to drive Th2 commitment by inducing autocrine IL-4 secretion from naïve $CD4^+$ T cells (Rincon 1997). Another possibility is that IgE cross-linking of monocytes indirectly suppresses the Th1 phenotype by promoting Th2 commitment. Exposure of DCs to IL-10 has been shown to preferentially induce Th2 polarization upon co-culture of DCs with naïve $CD4^+$ T cells (Liu 1998). IL-10 secreted by monocytes after IgE cross-linking (reported in Chapter 3, Figure 3.3) may act in an autocrine manner to induce monocytes to drive Th2, rather than Th1, phenotype in $CD4^+$ T cells. Monocytes are also known to secrete type I interferon, IFNα and IFNβ, after stimulation with hRV or influenza (Cao 2012; Schrauf 2009). IFNα and IFNβ have been shown to negatively regulate Th2 development (Huber 2010), potentially allowing for enhanced Th1 polarization by suppressing the Th2 programs. IgE cross-linking on pDCs suppresses IFNα and IFNβ secretion after virus exposure (Gill 2010). If a similar suppression exists in monocytes, reduced type I interferon production could indirectly suppress Th1 commitment by relieving inhibition on Th2 commitment.

My finding of IgE-mediated impairment of monocyte-driven Th1 polarization and enhancement Th2 polarization after virus may represent a basic mechanism underlying the clinical phenomenon of virus-associated exacerbations of allergic disease. Exposure to allergen could potentially cross-link allergen-specific IgE on monocytes and prevent monocytes and monocyte-derived cells from inducing protective Th1 response upon viral infection. This process

could lead to not only greater duration and severity of viral illnesses, but also to exacerbation of allergic responses by promoting the development of additional Th2 cells. Further study in this area to determine the mechanism of IgE-mediated suppression of virus-induced Th1 response will provide important understanding of how virus infection contributes to asthma exacerbations and potentially provide novel targets for therapeutic intervention.

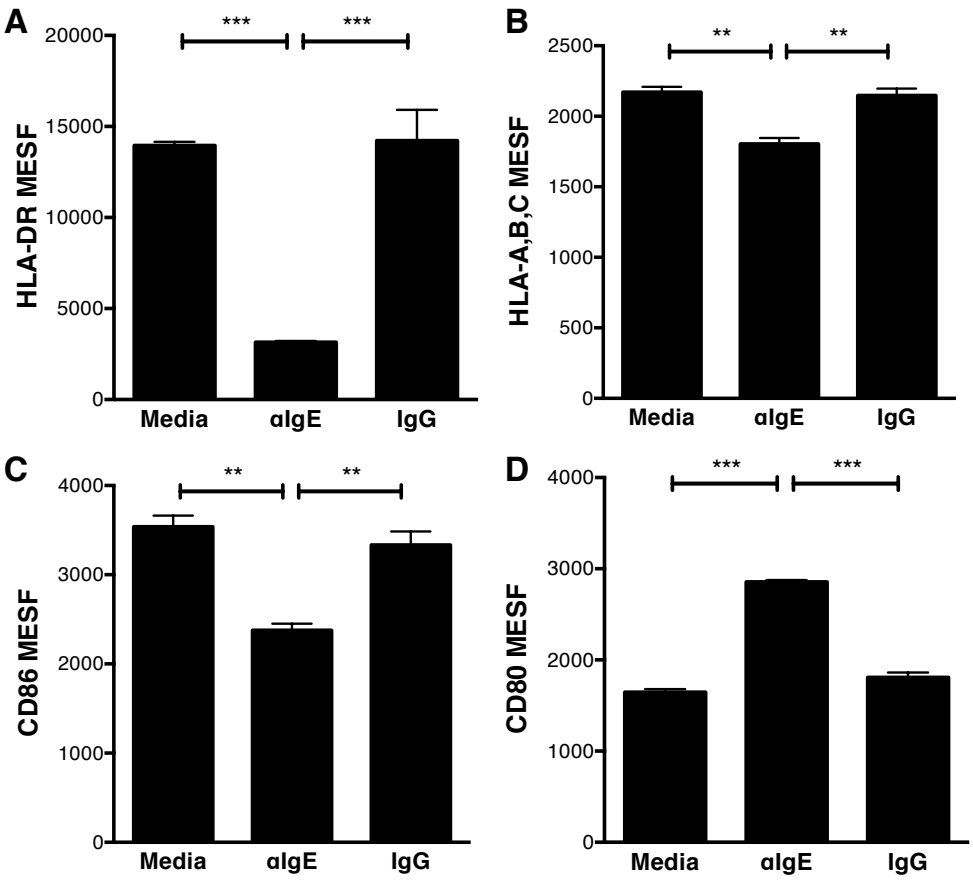


Figure 4.1 – IgE Cross-Linking Alters Expression of Monocyte Maturation Markers

Surface expression of HLA-DR (A), HLA-A,B,C (B), CD86 (C), and CD80 (D) on monocytes after 48 hour culture in media alone, IgE cross-linking antibody, or control IgG. Surface expression of each molecule is depicted as mean equivalent standard fluorescence. Data are expressed as triplicate means ± SEM for one representative of three independent experiments. ** $p<.01$, *** $p<.001$ for indicated comparisons. Some of the experiments represented in this figure were performed with the assistance of Dr. Regina Rowe.

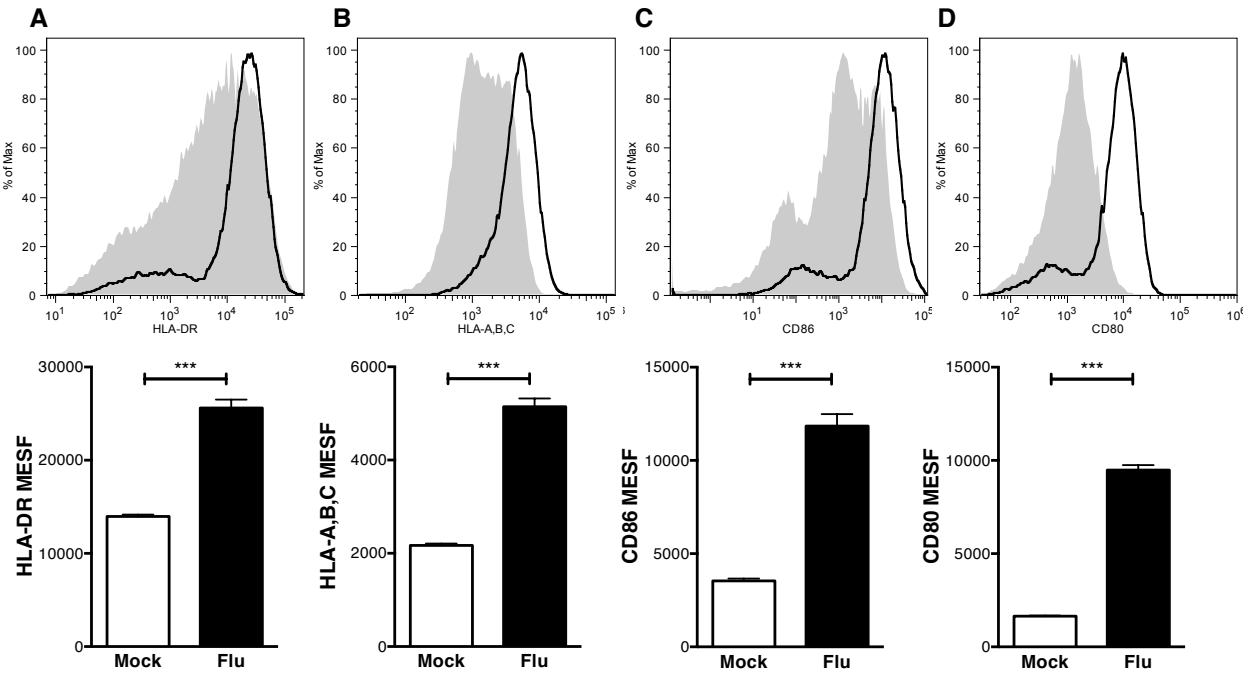


Figure 4.2 – Influenza Virus Induces Monocyte Maturation

Expression of HLA-DR (A), HLA-A,B,C (B), CD86 (C), and CD80 (D) on monocytes cultured with media only ("Mock", gray filled histograms) or influenza virus ("Flu", black line histograms) for 48 hours. Top panels depict sample histograms for each condition. Bottom panels depict triplicate means ± SEM for one representative of three independent experiments. *** $p<.001$ for indicated comparisons. Some of the experiments represented in this figure were performed with the assistance of Dr. Regina Rowe.

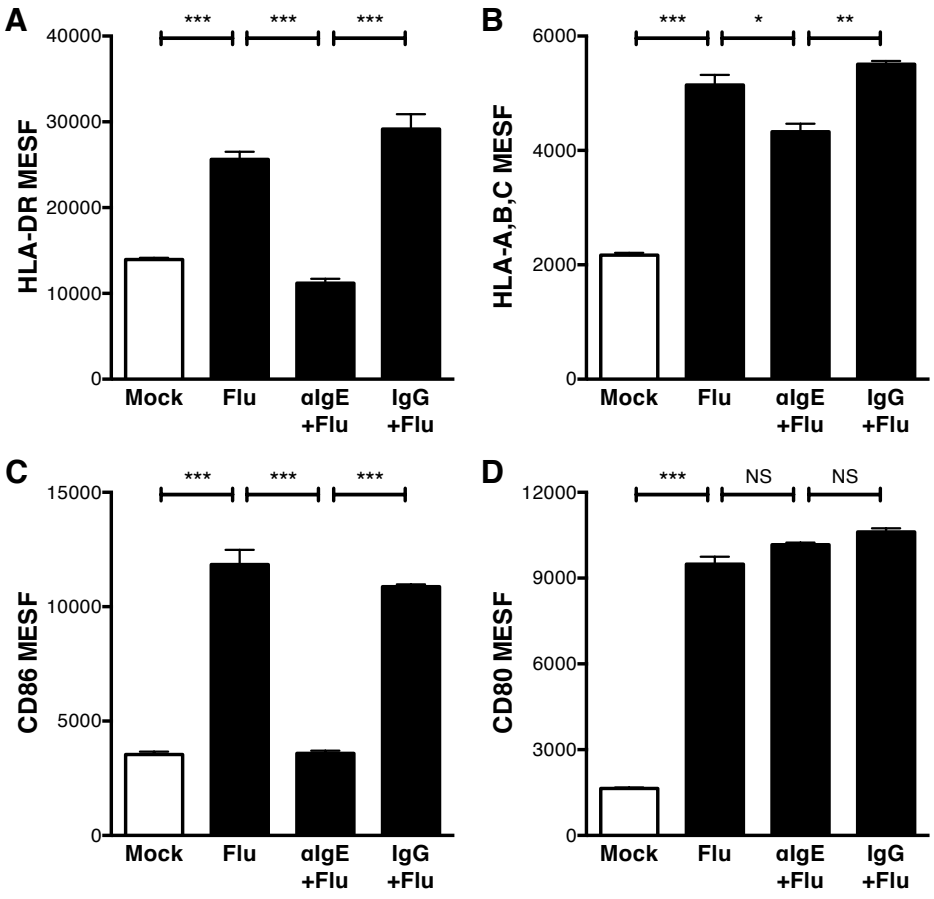


Figure 4.3 – IgE Cross-Linking Suppresses Influenza-Induced Monocyte Maturation

Monocyte Expression of HLA-DR (A), HLA-A,B,C (B), CD86 (C), and CD80 (D) after culture for 48 hours in media only ("Mock", white bars) or with influenza ("Flu", black bars) in the presence or absence of αIgE or control IgG. Data are expressed as triplicate means ± SEM for one representative of three independent experiments. * $p<.05$, ** $p<.01$, *** $p<.001$ for indicated comparisons. Some of the experiments represented in this figure were performed with the assistance of Dr. Regina Rowe.

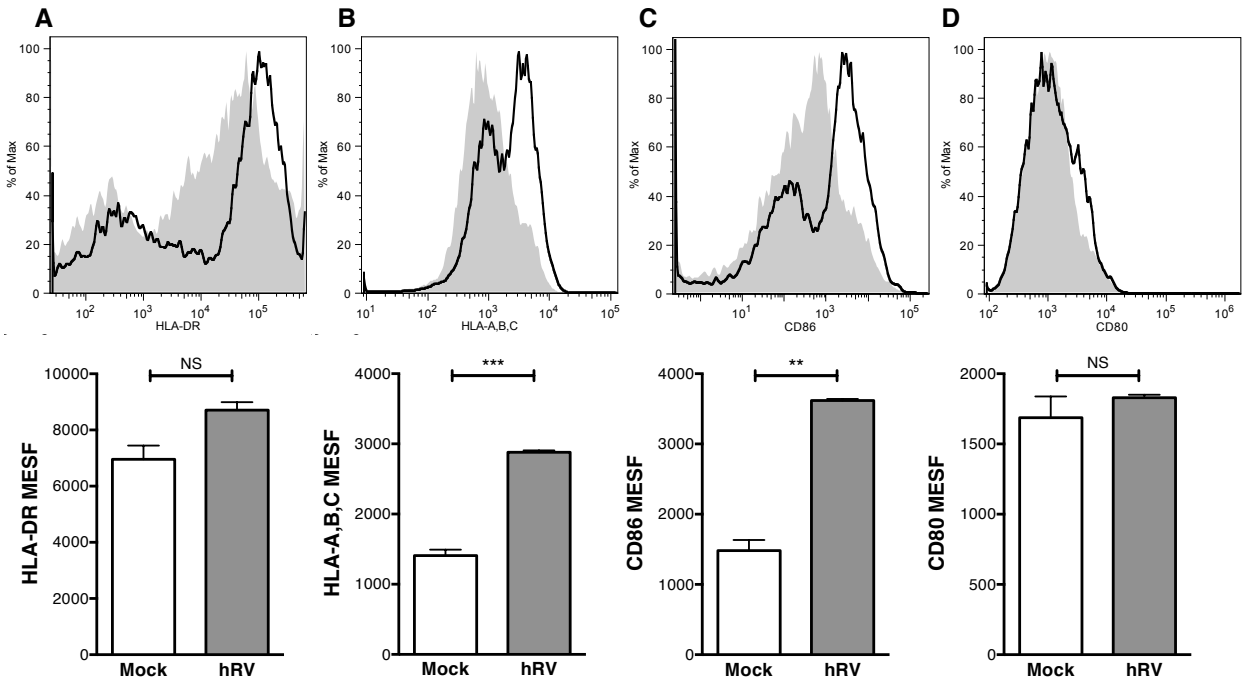


Figure 4.4 – Human Rhinovirus Induces Monocyte Maturation

Monocyte surface expression of HLA-DR (A), HLA-A,B,C (B), CD86 (C), and CD80 (D) after 48-hour culture in the presence or absence of human rhinovirus. Top panels depict sample histograms for mock treated (gray filled) or hRV treated (black line) conditions. Bottom panels depict triplicate means ± SEM for replicates within one preliminary experiment. ** $p<.01$, *** $p<.001$, NS $p>.05$ for indicated comparisons. Some of the experiments represented in this figure were performed with the assistance of Dr. Regina Rowe.

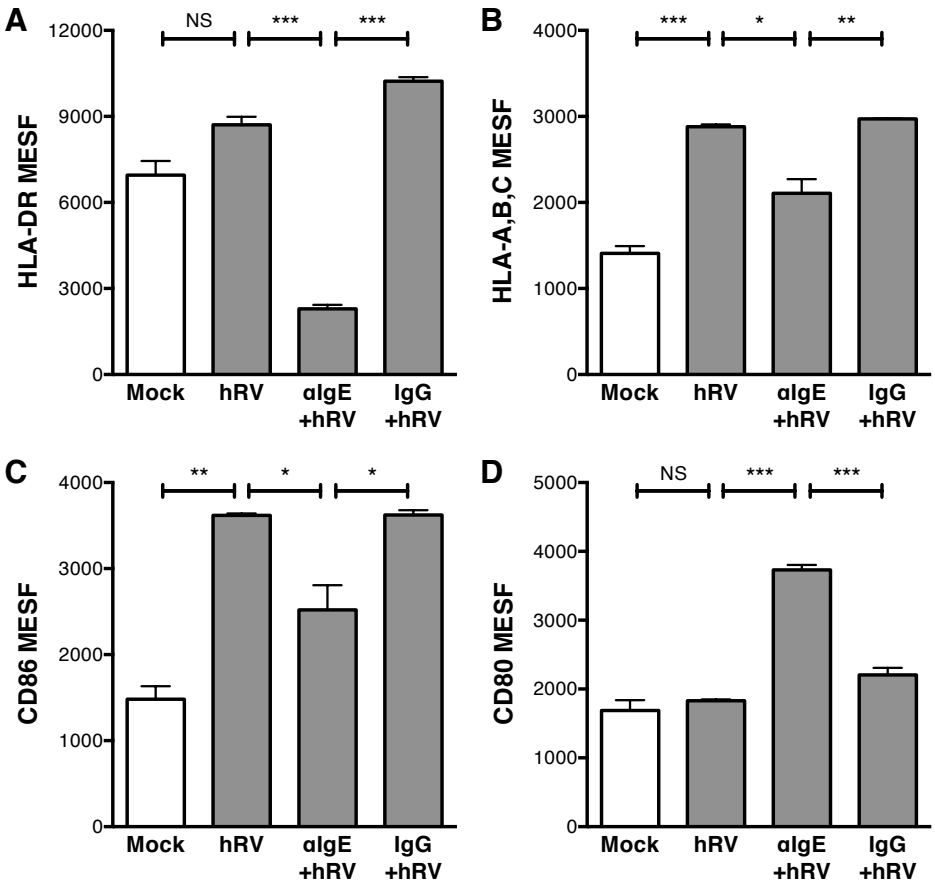


Figure 4.5 – IgE Cross-Linking Inhibits Rhinovirus-Induced Monocyte Maturation

Relative expression of HLA-DR (A), HLA-A,B,C (B), CD86 (C), and CD80 (D) on monocytes cultured in media alone (white bars) or with hRV (gray bars) for 48 hours in the presence or absence of αIgE or control IgG. Data are expressed as triplicate means ± SEM for one preliminary experiment. * $p<.05$, ** $p<.01$, *** $p<.001$ for indicated comparisons. Some of the experiments represented in this figure were performed with the assistance of Dr. Regina Rowe.

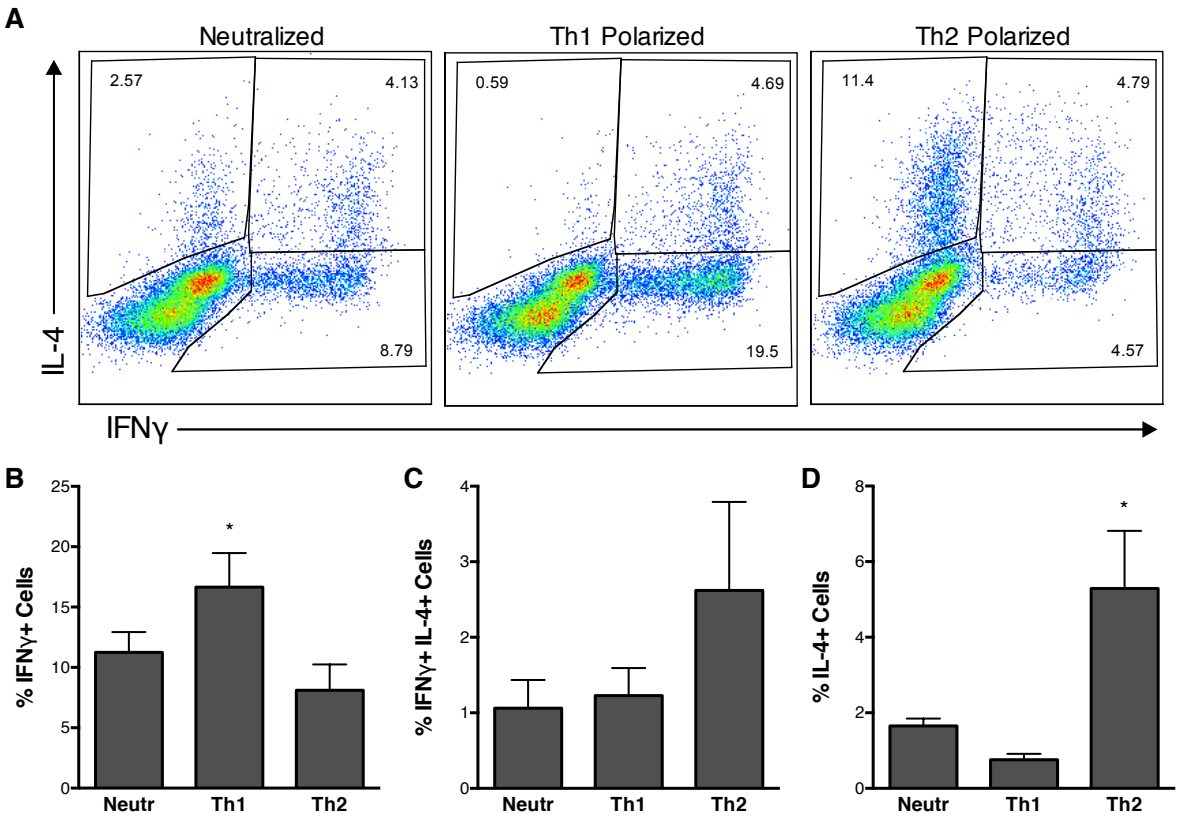


Figure 4.6 – Polarized CD4⁺ T Cells Produce IFNγ, IL-4, or Both

Representative plots of intracellular cytokine staining (A) in T cells cultured for 7 days on plate-bound anti-CD3 and anti-CD28 under defined polarization conditions. Percent of T cells producing IFNγ only (B), both IFNγ and IL-4 (C), or IL-4 only (D) for indicated conditions. N=5. * $p<.05$ for indicated condition vs. Neutralized. Some of the experiments represented in this figure were performed with the assistance of Dr. Regina Rowe and Zheng Hu.

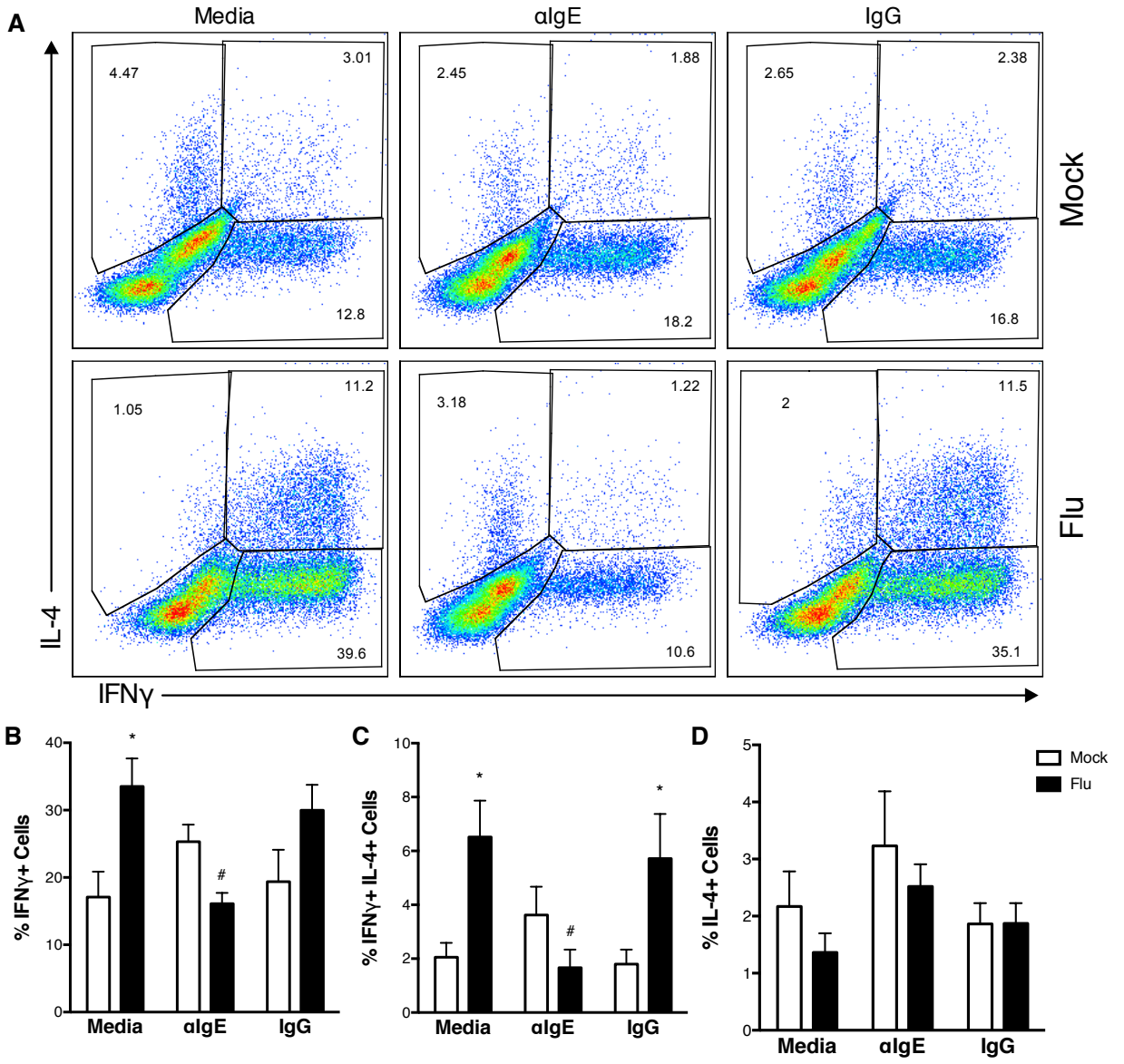


Figure 4.7 – IgE Cross-Linking Suppresses the Ability of Monocytes to Drive Th1 Response to Influenza

Representative plots of cytokine production in $CD4^+$ T cells after co-culture with allogeneic monocytes pretreated with media alone, αIgE, or IgG control in the presence ("Flu") or absence ("Mock") of influenza. Percent of T cells producing IFNγ only (B), both IFNγ and IL-4 (C), or IL-4 only (D) after culture with monocytes from indicated pretreatment conditions. N=5. * $p<.05$ for Mock vs. Flu, # $p<.05$ for αIgE vs. Media and IgG. Some of the experiments represented in this figure were performed with the assistance of Dr. Regina Rowe and Zheng Hu.

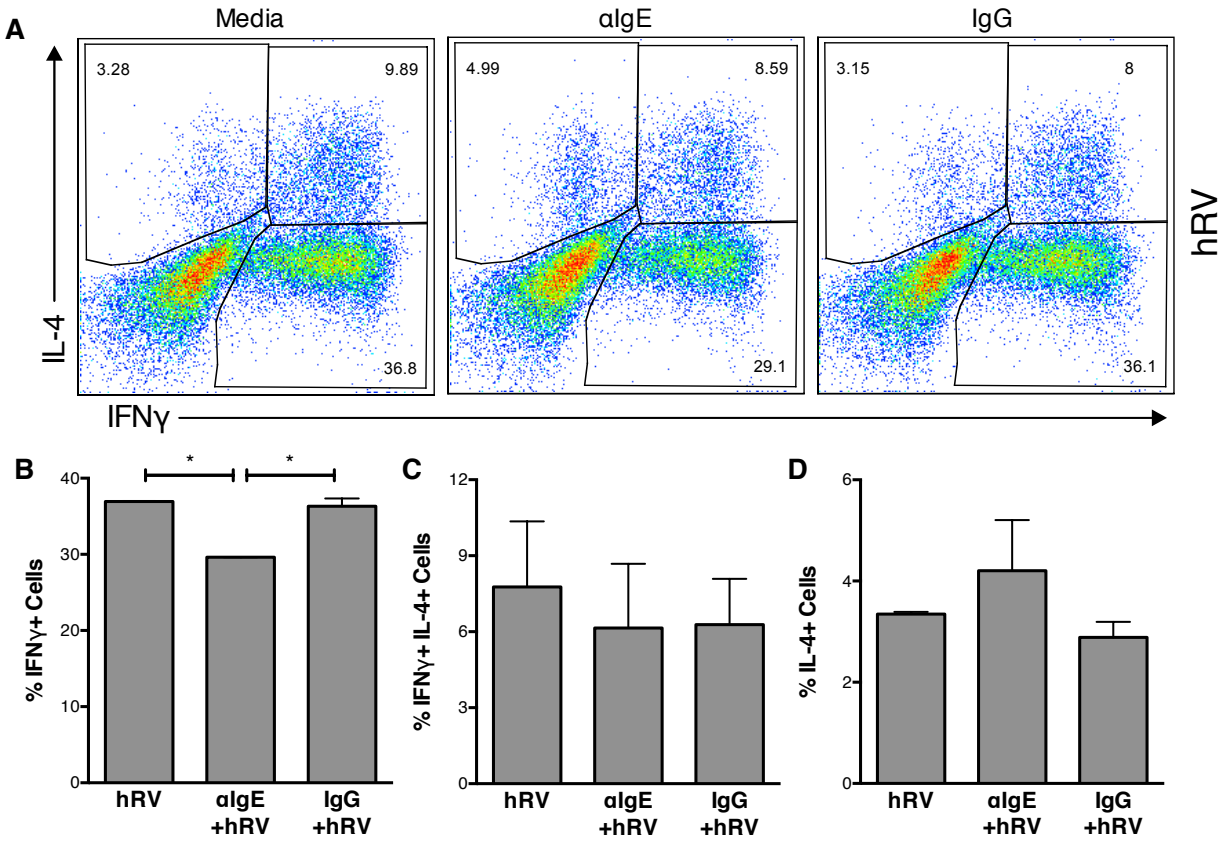


Figure 4.8 – IgE Cross-Linking Suppresses the Ability of Monocytes to Drive Th1 Response to Rhinovirus

Representative plots of cytokine production (A) in T cells after co-culture for 7 days with allogeneic monocytes pretreated with media alone, αIgE, or IgG control in the presence of hRV. Percent of cytokine positive T cells for IFNγ only (B), both IFNγ and IL-4 (C), or IL-4 only (D) after co-culture with indicated monocyte conditions. N=2. * $p<.05$ for indicated comparisons. Some of the experiments represented in this figure were performed with the assistance of Dr. Regina Rowe.

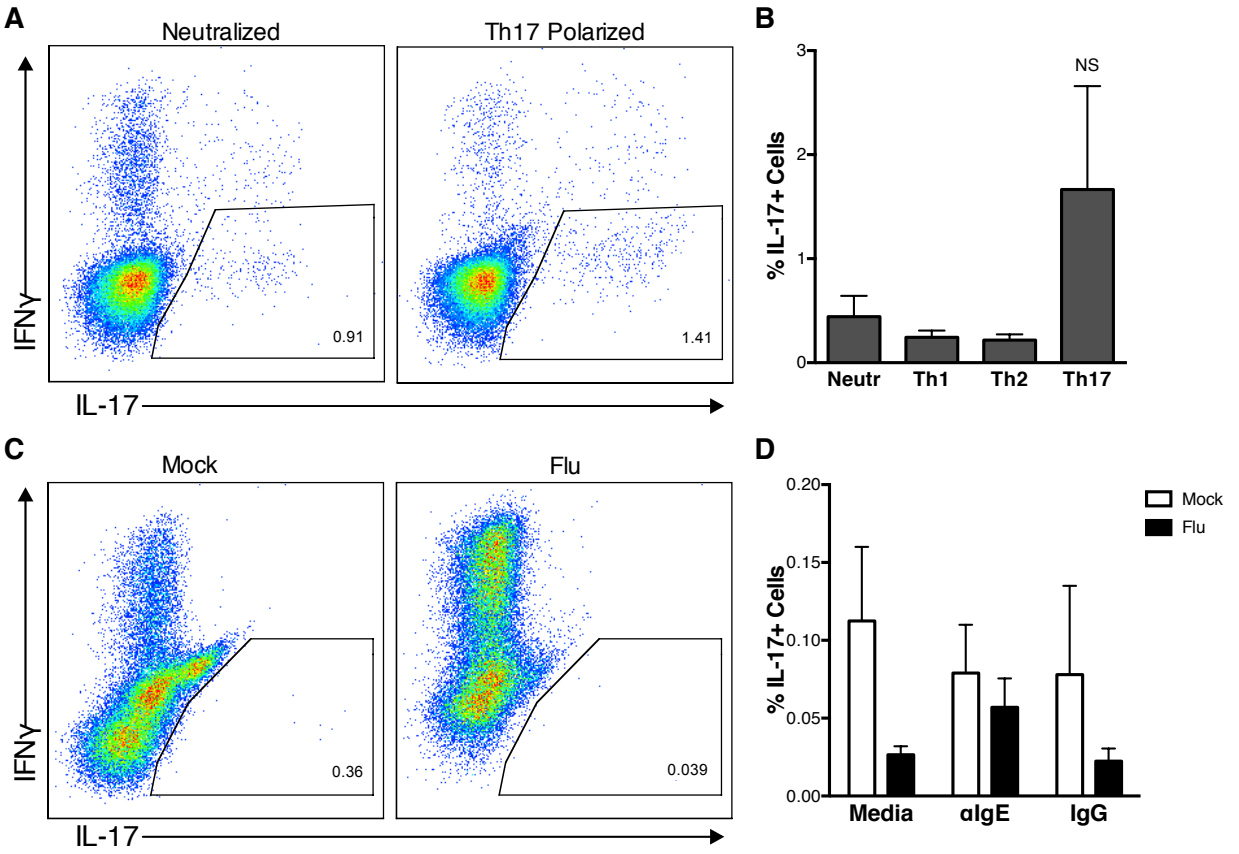


Figure 4.9 – Monocytes Do Not Induce Th17 Polarization *in vitro*

Representative plots of cytokine production in T cells cultured with anti-CD3 and anti-CD28 in the presence of defined polarizing conditions (A) or with monocytes (C). Percent of T cells producing IL-17 after culture in indicated polarizing conditions (B) or after co-culture with allogeneic monocytes pretreated with the indicated conditions (D). Data are expressed as means ± SEM of 2 (C, D) or 3 (A, B) independent experiments. NS $p>.05$ for Th17 polarizing condition vs. Neutralized (B).

CHAPTER FIVE

DISCUSSION AND CONCLUSIONS

Summary of Results

My research has explored the phenotypic and functional consequences of allergic stimulation on human monocytes. Monocytes and monocyte-derived cells serve a number of important roles during immune responses. Though they are present during allergic inflammation and express the high-affinity IgE receptor, little is understood about the consequences of IgE cross-linking on monocytes and in the potential impact on the pathogenesis of allergic disease. The data presented here describe modification of monocyte surface antigen expression, cytokine secretion, phagocytic function, virus response, and T cell stimulation by IgE cross-linking.

IgE cross-linking affected expression of several monocyte surface antigens. Exposure of monocytes to a cross-linking αIgE antibody increased CD14 expression in a dose-dependent manner. The magnitude of this effect was enhanced in individuals with elevated serum IgE concentration, suggesting *in vivo* relevance of my observations. Conversely, the high-affinity IgG receptor CD64 was down regulated by αIgE in a dose-dependent manner. IgE cross-linking also affected expression of molecules associated with antigen presentation on monocytes. Expression of both class I and class II MHC molecules was reduced by IgE cross-linking. One co-stimulatory molecule, CD86, was down regulated by αIgE exposure, while another, CD80, was up regulated. IgE cross-linking was able to suppress expression of MHC Class I, Class II, and CD86 even in the presence of strong activating stimuli such as influenza and hRV.

IgE cross-linking also induced production of a variety of inflammatory cytokines from monocytes. TNFα was secreted by monocytes at high levels rapidly after cross-linking, but was subsequently decreased. αIgE also promoted rapid secretion of IL-6 and IL-1β from monocytes, which persisted over time. I was unable to detect IL-12 production from monocytes after IgE cross-linking, but IL-23, which shares a subunit with IL-12, was secreted at later time points. IL-10 secretion induced by IgE cross-linking mediated the decrease in TNFα level over time in addition to suppressing IL-6, as evidenced by robust increases in TNFα and IL-6 levels in the presence of IL-10 neutralizing antibodies. IL-10 was also found to regulate its own secretion after IgE cross-linking. Neutralizing antibodies to IL-6 and TNFα, however, had no effect on IgE-mediated IL-10 secretion, indicating that IL-10 was not induced in response to these pro-inflammatory cytokines. Demonstrating the relevance of monocyte cytokine secretion to atopic disease, induction of TNFα, IL-6, and IL-10 by αIgE was more robust in subjects with elevated serum IgE concentration.

IgE cross-linking critically impaired monocyte phagocytosis of bacteria, as measured by fluorescence microscopy, flow cytometry, and culture of cell lysates. The inhibition of phagocytosis by αIgE was not dependent on TNFα, IL-6, or IL-10, as neutralizing antibodies to each of these cytokines had no effect on this IgE-mediated suppression. Unlike the effects on monocyte CD14 expression and cytokine secretion, where significant correlations with serum IgE were observed, the IgE cross-linking-induced suppression of phagocytosis did not correlate with serum IgE concentration. This suggests that IgE cross-linking may activate divergent signaling mechanisms in these cells. In addition to the IgE-mediated suppression of monocyte phagocytosis of bacteria, αIgE exposure also significantly impaired monocyte phagocytosis of both small and large apoptotic debris. Despite the significant reduction of phagocytosis after IgE

cross-linking, the ability of monocytes to kill internalized bacteria – another critical function of these cells – was unaffected.

Monocytes co-cultured with naïve $CD4^+$ T cells induced polarization of the T cells into IFNγ-producing Th1 cells, IL-4-producing Th2 cells, or IFNγ/IL-4-co-producing T cells. Exposure of monocytes to influenza before co-culture dramatically increased the induction of Th1 cells and IFNγ/IL-4-co-producing T cells. However, IgE cross-linking suppressed this influenza-induced increase in Th1 commitment as well as the increase in IFNγ/IL-4-co-producing cells. IgE cross-linking on influenza-stimulated monocytes simultaneously increased Th2 commitment. A similar effect was observed with another virus: exposure of monocytes to αIgE and hRV induced fewer Th1 cells and more Th2 cells, though the IFNγ/IL-4 co-producing T cell population was unaffected.

To my knowledge, this is the first report to describe inhibition of human monocyte phagocytic function by IgE cross-linking. Previous reports concerning IgE and FcεRI cross-linking on monocytes identified inflammatory cytokine secretion, but this is the first to identify a relationship between the inflammatory effects of IgE cross-linking and serum IgE concentration. The ability of monocyte-derived cells to up regulate antigen presenting and co-stimulatory molecules in response to virus and induce Th1 polarization from naïve $CD4^+$ T cells has been documented (Nakano 2009; Wu 2011b). However, this report is the first to describe suppression of virus-stimulated monocyte maturation and regulation of virus-induced T cell polarization by IgE cross-linking on monocytes.

When monocytes are recruited to tissues in either homeostatic or inflammatory conditions, they undergo differentiation into either dendritic cells or macrophages. Monocyte fate can be governed by cytokines such as M-CSF, GM-CSF, IFNγ, and IL-4 (Conti 2008; Fleetwood 2007; Popova 2011), or by interaction with pathogens (Cao 2012; Krutzik 2005). Differentiated macrophages and DCs express lower levels of CD14, but higher MHC Class II, than monocytes (Hargett 2010). My findings of increased monocyte CD14 and reduced MHC class II expression, as well as reduced phagocytosis after IgE cross-linking could suggest that allergic stimulation suppresses monocyte differentiation. In contrast, however, the intact killing of intracellular bacteria and the macrophage-like morphology of αIgE-treated monocytes (see photomicrographs in Figure 3.8) indicate that these monocytes are, in fact, differentiating. Novak *et al*. determined that IgE cross-linking on monocytes prevents *in vitro* differentiation of monocyte-derived dendritic cells and instead induces a macrophage phenotype (Novak 2001a). More recent work from the same group has shown that IgE cross-linking induces monocyte differentiation into histamine-responsive macrophages (Novak 2013). Considered together, my data and the findings of others suggest that rather than suppressing monocyte differentiation, IgE cross-linking induces differentiation of monocytes into macrophages with altered phenotype and function.

During infection, a key role of monocytes is differentiation into mDCs, which can then travel to lymph nodes to present antigens to T cells (Nakano 2009). The importance of monocyte differentiation into dendritic cells for appropriate immune responses has been demonstrated for several pathogens, including influenza (Cao 2012), *Leishmania major* (Leon 2007), and *Mycobacterium leprae* (Krutzik 2005). In order to effectively stimulate T cells, monocyte-

derived dendritic cells must undergo maturation, which involves up regulating MHC class I and class II molecules as well as co-stimulatory molecules like CD80, CD86, and CD40 (Castiello 2011). The finding that IgE cross-linking on monocytes prevents virus-induced up regulation of MHC class I, class II, and CD86 suggests that IgE cross-linking may also suppress antigen presentation by monocyte-derived dendritic cells. In the setting of atopic disease, allergen-mediated disruption of differentiation into dendritic cells and subsequent maturation during infection could severely impact adaptive immune responses to secondary infections. Another effect that may actually be beneficial could be inhibition of allergen presentation to T cells. In support of my observation that IgE cross-linking increased CD80 but decreased CD86, a study by Balbo *et al*. found that CD80, but not CD86 was elevated in alveolar macrophages of asthmatic individuals. However, after allergen challenge, CD86 was more effective at stimulating Th2 responses, suggesting that IgE-mediated down regulation of CD86 on monocytes could be a compensatory mechanism to suppress allergen presentation (Balbo 2001). In addition to potential effects on infection, alteration of monocyte differentiation and antigen presentation could also impact immune responses to vaccinations. In murine models, monocyte-derived dendritic cells are important for both $CD4^+$ (Nakano 2009) and $CD8^+$ (Le Borgne 2006) responses to subcutaneous immunization with OVA and adjuvant. While the possibility has not been investigated, it would follow that immunization concurrent with allergic stimulation may interrupt effective induction of adaptive immune responses, particularly if the allergic stimulation is present at the site of immunization.

Monocyte Cytokines in Allergic Inflammation

The cytokines produced by monocytes after IgE cross-linking may contribute to allergic disease in several ways (**Figure 5.1**). Monocyte TNFα can impact allergic inflammatory responses at multiple levels. Inhaled TNFα challenge induces both airway hypersensitivity and neutrophil influx in healthy and asthmatic individuals (Thomas 1995; Thomas 2002). Additionally, intranasal TNFα challenge promotes neutrophil recruitment and activation in healthy subjects and those with allergic rhinitis (Widegren 2008). These studies suggest that TNFα can exert similar effects on allergic inflammation regardless of atopic status. The correlation of IgE-mediated TNFα secretion with serum IgE level suggests that the presence or absence of allergen-specific IgE on monocytes (in atopic individuals or healthy individuals, respectively) may be an important determinant of the severity of allergic inflammation. Supporting this possibility, TNFα production has been observed after topical allergen application in individuals with atopic dermatitis, but not healthy individuals (Junghans 1998).

Secretion of IL-6, IL-1β, and IL-23 after IgE cross-linking on monocytes may also be important in allergic disease. Higher concentrations of IL-6 in sputum have been linked to reduced respiratory function in asthmatic patients, suggesting a role for this cytokine in airway inflammation (Morjaria 2011). Airway hypersensitivity during OVA-induced asthma is reduced in mice deficient for IL-1β, but enhanced in IL-1 receptor antagonist (IL-1RA)-deficient mice (Nakae 2003). My observations, coupled with the finding that airway macrophages from asthmatic, but not control, subjects secret IL-1β (Borish 1992), suggest that IL-1β secretion after IgE cross-linking on monocytes may contribute to airway hypersensitivity in asthma. Like TNFα, IL-1β has also been detected in the skin of atopic dermatitis patients after allergen exposure (Junghans 1998). IL-23, though not typically associated with allergic responses, is

produced in the lung of allergen-challenged mice (Wakashin 2008). A neutralizing antibody against IL-23 suppressed allergen-mediated eosinophil recruitment, while over-expression of IL-23 in the lung enhanced eosinophil recruitment and airway hypersensitivity. Taken together, these findings suggest that monocyte secretion of TNFα, IL-6, IL-1β, and IL-23 after IgE cross-linking may contribute to inflammation and airway hypersensitivity during allergic asthma.

IgE-mediated up regulation of CD14 on monocytes could also influence monocyte cytokine secretion from secondary stimuli. A recent report from Lee *et al*. found that PBMC secretion of TNFα and IL-6 after TLR2, TLR4, and TLR7/8 stimulation is greater during exacerbations of allergic asthma compared to convalescence in the same subjects (Lee 2012). Though the authors did not determine the specific cell type or types responsible for this effect, it is possible that IgE cross-linking could enhance monocyte cytokine secretion in response to TLR2 and TLR 4 ligands by inducing CD14 expression.

Monocyte Cytokines in B and T Cell Responses

Cytokines secreted by monocytes after IgE cross-linking may also feed back to enhance IgE secretion from B cells. Both IL-6 and IL-10 act as stimulatory factors for B cells, inducing differentiation and antibody secretion (Kobayashi 2002; Urashima 1996). Additionally, both IL-6 and IL-10 have been shown to enhance IgE production in the presence of IL-4 and CD40 ligand stimulation (Kobayashi 2002; Vercelli 1989). IL-1β may also influence IgE production: IL-1β-deficient mice have reduced total and antigen-specific IgE during OVA-induced asthma (Nakae 2003). Though I did not interrogate IL-1-β during my studies of individuals with elevated serum IgE concentrations, my observation of enhanced αIgE-mediated secretion of IL-6 and IL-10 in

monocytes from individuals with elevated IgE suggests a positive feedback loop between B cell IgE production and monocyte IgE-mediated cytokine secretion (**Figure 5.1**).

Monocyte secretion of inflammatory cytokines may also influence T cell differentiation in the context of allergic disease (**Figure 5.1**). By inducing IL-4 production in from naïve $CD4^+$ T cells, IL-6 indirectly induces Th2 differentiation (Diehl 2002; Rincon 1997). IL-6 has also been determined to inhibit Th1 differentiation by inducing suppressor of cytokine signaling (SOCS) 1 (Diehl 2000). In the context of viral infection during allergic disease, IL-6 from monocytes may mediate suppression of virus-induced Th1 responses and instead support increased Th2 responses. IL-1β has been shown to be important for Th1 (Nambu 2006), Th2 (Nakae 2003), and Th17 (Lalor 2011) responses. IL-1β from monocytes in allergic disease may serve as a general stimulatory factor, enhancing, but not altering polarization. While the importance of Th2 cells in atopic disease is well established, elevated Th17 responses have recently been observed in allergic asthma (Zhao 2010), which have been proposed to enhance Th2-mediated allergic inflammation (Wakashin 2008). IL-6, IL-1β, IL-23, and TGFβ in various combinations can induce naïve $CD4^+$ T cells to become Th17 cells (Ganjalikhani Hakemi 2011). Though monocytes did not directly induce Th17 differentiation in my studies, the possibility remains that monocyte derived IL-1β, IL-6, and IL-23 could contribute to *in vivo* Th17 differentiation. In addition to its important role in the development of Th17 cells (McGeachy 2009), IL-23 has also been found to augment OVA-induced Th2 differentiation *in vitro* (Wakashin 2008), suggesting an important role for IL-23 in generation of both Th2 and Th17 responses in allergic disease.

The role of IL-10 in T cell polarization and allergic disease is less clear than other monocyte-derived cytokines. One study used IL-10 to prime murine dendritic cells for several

hours and observed increased Th2 differentiation upon co-culture with naïve T cells (Liu 1998). However, long-term exposure of dendritic cells to IL-10 during differentiation from monocytes can induce a tolerogenic phenotype (Lu 2011; Nayyar 2012). Adoptive transfer of these tolerogenic DC actually suppresses Th2 responses and ameliorates disease in mouse models of allergic asthma. IL-10 secreted after IgE cross-linking may thus play a duel role in developing Th2 responses, initially promoting Th2 differentiation while simultaneously inducing tolerogenic DCs to later modulate those responses.

Monocytes are also known to secrete type I IFN during viral infection (Cao 2012; Schrauf 2009). The work of Huber *et al*. recently demonstrated that IFNα/β signaling can suppress human Th2 development by suppressing the Th2 master transcription factor GATA3 (Huber 2010). These findings suggest that type I IFN may be an important mechanism for indirectly supporting Th1 development by suppressing Th2 differentiation programs. Though not evaluated in my studies, IgE cross-linking may suppress type I IFN production from monocytes in a similar manner to that shown in pDCs (Gill 2010). Suppression of monocyte type I IFN by IgE cross-linking would relieve inhibition of Th2 differentiation and could thus indirectly suppress virus-induced Th1 development.

Balancing Inflammation and Resolution: A Role for IL-10

Monocytes from individuals with allergic asthma (Prasse 2007) and atopic dermatitis (Aiba 2003) produce more IL-10 upon LPS stimulation than monocytes from non-atopic individuals, suggesting that this cytokine may have important functions in atopic disease. IL-10 is a pleiotropic cytokine that can regulate many aspects of immune responses. Many of the roles

of IL-10 are anti-inflammatory in nature, serving to suppress a variety of cell types and inflammatory processes. I have described one such regulatory role for IL-10 in allergic responses: suppression of monocyte inflammatory cytokine secretion. Though it has not been tested in the context of allergic disease, auto-regulatory effects of IL-10 on LPS-induced cytokines have been attributed to induction of tristetraprolin (TTP). TTP binds and degrades AU-rich mRNAs, which include transcripts for the inflammatory cytokines TNFα, IL-6, IL-12, and IL-23, as well as IL-10 itself (Gaba 2012). While the suppressive actions of IL-10 are necessary to prevent excessive inflammation, induced by allergen exposure could subsequently suppress crucial immune responses to viruses and other pathogens. IL-10 has been shown to suppress monocyte secretion of inflammatory cytokines after RSV exposure (Panuska 1995). Additionally, the capacity of monocytes to stimulate T cells in response to hRV (Stockl 1999) and Epstein-Barr virus (de Waal Malefyt 1991) is suppressed by IL-10. Murine macrophages are unable to kill *Salmonella typhimurium* after treatment with IL-10, instead allowing the bacteria to replicate inside phagosomes (Lee 2011). T cell responses can also be affected: IL-10 induced in response to murine lymphocytic choriomeningitis virus acts directly on virus-specific T cells to suppress Th1 responses, leading to chronic infection (Brooks 2006).

IL-10 production by monocytes may also play a role in virus-associated exacerbations of asthma. In addition to IgE cross-linking, some respiratory viruses, including RSV (Panuska 1995) and hRV (Stockl 1999) have been observed to induce IL-10 production from monocytes. Additionally, Grissell et al. determined that IL-10 production from sputum cells was greater in virus-associated asthma exacerbations compared to either asthma or virus alone (Grissell 2005). The finding that IL-10 level returned to normal after the exacerbation resolved led to speculation that IL-10 may actually be a causative factor in virus-associated exacerbations of asthma (Busse

2005). Inhalation of IL-10 can actually enhance allergen-induced airway hypersensitivity in mice, though it also reduces eosinophilia and T cell responses (van Scott 2000). Though a definitive role for IL-10 in virus-associated asthma exacerbations has not been established, it remains possible that IL-10 could suppress both virus- and allergen-related immune responses while enhancing airway hypersensitivity.

Balancing Inflammation and Resolution: A Role for Phagocytosis

Inflammation is often necessary to effectively fight infection. Disruption of blood vessel walls during inflammation allows for accumulation of leukocytes at the site of infection and can create a physical barrier to restrict the spread of pathogens. However, inflammation must be controlled and eventually reversed. Unchecked inflammation has been shown to lead to significant pathology in murine models: mice lacking important regulatory components such as TGFβ (Shull 1992), or regulatory T cells (Rivas 2012) develop widespread autoimmunity, while mice lacking IL-10 (Kuhn 1993) develop severe autoimmune colitis. Balance between inflammatory and anti-inflammatory responses is crucial for successful elimination of pathogens and survival of the host.

Phagocytosis is an important process in both inflammation and its resolution. During inflammation, phagocytosis of invading pathogens by monocyte-derived cells is important for both removal of the pathogens (Buckley 2011) and for acquisition of antigens to present to T cells (Trombetta 2005). However, phagocytosis is also required during resolution of inflammation. During infection, allergic inflammation, or other insult, granulocytes traffic to inflamed tissues, but to prevent inflammation from continuing indefinitely these granulocytes are

eventually removed by apoptosis (Vandivier 2006). In patients with allergic asthma, an inverse correlation has been reported between eosinophil apoptosis and disease severity, suggesting that failure of granulocytes to undergo apoptosis contributes to ongoing inflammation (Duncan 2003). To restore inflamed tissue to homeostasis, apoptotic cellular debris must be removed by phagocytosis. Reed *et al*. found that severe RSV infection in humans and mice is associated with accumulation of apoptotic debris in the airways. In this murine model of infection, the authors determined that this accumulation was due to defects in alveolar macrophages (Reed 2008). This study, along with others that have observed defective phagocytosis of apoptotic cells in the context of severe asthma (Huynh 2005), suggests that defective phagocytosis of apoptotic debris may play an important role in the ongoing inflammation observed during atopic disease.

My observation that IgE cross-linking directly disrupts monocyte phagocytosis has a number of implications for control of inflammation in allergic disease. The cytokines secreted by monocytes after encountering an allergen will contribute to the inflammatory response, leading to buildup of apoptotic cells. However, monocytes may not be able to phagocytose these apoptotic cells, potentially leading to a prolonged inflammatory response. Additionally, IgE-mediated suppression of phagocytosis suggests that antigen uptake by monocytes during infection may be impaired as well. Thus, impairment of phagocytosis by IgE cross-linking suppresses a critical component of both inflammatory and homeostatic immune responses.

Future Directions

In the context of allergic disease, monocytes trafficking to areas of allergic stimulation will be exposed to allergens as they exit the bloodstream. At the same time, monocytes will

encounter the cytokine cues that direct their differentiation into macrophages and dendritic cells. My observations, along with the work of Novak *et al*. (Novak 2001a), has suggested that IgE cross-linking on monocytes induces a macrophage-like phenotype. In contrast to my experiments, which examined monocyte phenotype 2 to 4 days after IgE cross-linking, many studies of monocyte differentiation evaluate later time points (Fleetwood 2007; Novak 2001a; Popova 2011). Exposure of monocytes to αIgE over longer periods with analysis of macrophage-specific molecules such as CD68 and mannose receptor, as well as DC-specific markers such as CD1a, CD11c, and CD83, would determine the differentiation pattern after IgE cross-linking. Additionally, macrophage functions such as response to TLR ligands and phagocytosis could be compared for αIgE-differentiated macrophages and M-CSF-differentiated macrophages to identify whether the functional changes observed in my studies are acute or long lasting.

When monocytes and monocyte-derived cells are exposed to inflammatory stimuli, they secrete chemokines that recruit other immune cells to the site of the insult. Preliminary experiments from my studies (data not shown) and the work of others (Novak 2001a; Von Bubnoff 2002) have demonstrated secretion of MCP-1, MIP-1α, and MIP-1β from monocytes following IgE cross-linking. Further exploration of αIgE-induced monocyte chemokine secretion may reveal an important role for monocytes in the recruitment of granulocytes, lymphocytes, and other monocytes to sites of allergic inflammation.

Suppression of monocyte phagocytosis by IgE cross-linking may prevent monocytes from contributing to both clearance of pathogens and resolution of inflammation. Identifying the mechanism for this suppression will be important for understanding how FcεRI signals in monocytes and determining potential therapeutic targets. CD64 is known to be involved in phagocytosis (Huang 2011) and is also suppressed by IgE cross-linking; thus, CD64 down

regulation by anti-IgE could represent the mechanism of IgE-mediated impairment of monocyte phagocytosis and represents an important area for future investigation. While restoring CD64 expression in primary human cells would be technically difficult, the role of CD64 in IgE-mediated suppression of phagocytosis could be interrogated in monocyte cell lines, such as THP-1, by determining whether over-expression of CD64 rescues phagocytosis after IgE cross-linking. In chapter 3, I described another factor that could be stimulated by IgE cross-linking to regulate phagocytosis: SH2-domain-containing inositol 5' phosphatase 1. SHIP is known to suppress macrophage phagocytosis (Tamura 2009), but can promote other inflammatory functions, such as cytokine secretion (Fang 2004) and oxidative burst (Kamen 2008), making it an interesting candidate for mediating the effects of IgE cross-linking on monocyte phagocytosis. Western blots for SHIP and phospho-SHIP would determine whether SHIP expression or activation in monocytes is impacted by αIgE. If SHIP expression or activation is indeed enhanced by IgE cross-linking, commercial SHIP inhibitors are available to determine whether SHIP is necessary for IgE-mediated suppression of phagocytosis.

I have shown preliminary data revealing suppression of monocyte maturation and T cell stimulation in response to two important respiratory viruses: influenza and hRV. In addition to confirming these findings with further experiments, other aspects of viral response merit investigation. Several cytokines secreted by monocytes in response to IgE cross-linking or viral infection have also been implicated in T cell polarization. Type I interferon is secreted by monocytes following influenza and hRV exposure (Cao 2012; Schrauf 2009) and has been shown to suppress Th2 differentiation (Huber 2010). IL-6 is secreted after IgE cross-linking and is able to suppress Th1 (Diehl 2000), but augment Th2 (Rincon 1997), differentiation. IL-10 is also induced by IgE cross-linking and can prime DCs to drive Th2 commitment (Liu 1998).

Neutralization of these cytokines during co-cultures of naïve $CD4^+$ T cells with virus- and αIgE-exposed monocytes could identify a mechanism for the effects of IgE cross-linking on monocyte polarization of T cells.

In addition to influenza and hRV, other pathogens are also relevant to allergic disease. RSV has been implicated in exacerbations of asthma and is known to infect airway macrophages (Panuska 1990). Herpes simplex virus and *Staphylococcus aureus* frequently cause complications in patients with atopic dermatitis (Boguniewicz 2010), another allergic disease in which IgE plays a major role. Determination of the effect of IgE cross-linking on monocyte responses to these pathogens will provide critical insight into potential mechanisms of pathogen-associated exacerbations of atopic disease.

Monocytes and monocyte-derived cells stimulate $CD8^+$ and $CD4^+$ T cells by presenting antigens on MHC class I and class II molecules, respectively. Co-stimulation is necessary to activate naïve, but not memory T cells (Croft 1994). Interrogating the ability of monocytes to induce antigen-specific proliferation in autologous memory $CD4^+$ and $CD8^+$ T cells would provide insight into how IgE-mediated reduction in monocyte MHC molecules may affect memory T cell responses during infection. Because the co-stimulatory molecules CD80 and CD86 were differentially affected by IgE cross-linking, measuring proliferation of naïve $CD4^+$ and $CD8^+$ T cells in response to allogeneic monocytes will be useful in determining the effect of IgE cross-linking on the induction of T cell responses.

The studies presented herein were performed using an anti-IgE antibody to cross-link FcεRI and simulate allergic stimulation in monocytes. The impact of these studies would be greatly enhanced by examining monocyte responses to sensitized allergens in atopic individuals.

Identifying allergen-specific monocyte responses to a sensitized allergen, but not an irrelevant allergen, would provide valuable biological relevance to the findings presented here.

I have demonstrated in this report that IgE cross-linking induces more robust responses in monocytes from individuals with elevated serum IgE. As FcεRI expression is regulated by serum IgE concentration (Cheng 2006), it follows that increased expression of FcεRI may mediate the enhanced response to αIgE in these individuals. This could be confirmed by isolating monocytes from subjects with low serum IgE and incubating them *in vitro* with myeloma IgE to determine whether FcεRI expression and monocyte response to IgE cross-linking increase with higher concentrations of IgE. Another tool to further examine the effects of serum IgE concentration on monocyte responses to IgE cross-linking is the monoclonal antibody omalizumab. Omalizumab binds to IgE in serum, but not IgE bound to FcεRI, resulting in decreased free serum IgE concentration (Presta 1993) and down regulation of FcεRI on monocytes (Cheng 2006). Examining monocyte responses to IgE cross-linking before and after omalizumab treatment would not only determine the impact of serum IgE concentration and FcεRI expression on monocyte responses, but would also provide an opportunity to directly compare clinical disease markers – such as respiratory function and frequency of exacerbations – to the magnitude of monocyte responses to IgE cross-linking.

Clinical Implications

A significant strength of the work presented here is that it has been done entirely in primary human monocytes. Though cell lines are extremely useful for interrogating biochemical and genetic mechanisms, immortalized and cancer-derived cell lines often carry intrinsic

alterations that make extrapolation of results to primary cells difficult. Similarly, mouse models are useful for genetic manipulation and provide a platform for studying cells *in vivo*, but species differences between mice and humans frequently make comparison challenging. My research identifying the phenotypic and functional effects of IgE cross-linking on freshly isolated peripheral blood monocytes makes it highly relevant to clinical disease.

Another strength of this study is the investigation of IgE cross-linking on monocytes from atopic individuals with elevated vs. normal serum IgE concentrations. Elevated serum IgE concentration is a biomarker of atopic disease and correlates with disease severity in patients (Kovac 2007; Laske 2004). The finding that the magnitude of many responses to IgE cross-linking on monocytes is dependent on serum IgE concentration suggests that individuals with elevated serum IgE may have more robust monocyte responses to allergen exposure *in vivo*. These augmented monocyte responses may contribute to increased inflammation and more severe disease.

The monoclonal antibody omalizumab is clinically indicated for severe allergic asthma and has been shown to improve symptom control and reduce the frequency of exacerbations. Reduction of serum IgE concentration after omalizumab administration results in down regulation of FcεRI on basophils, mast cells, dendritic cells, and monocytes (Beck 2004; Cheng 2006; MacGlashan 1997; Prussin 2003). Omalizumab treatment has also been found to reduce the magnitude of allergen-mediated functions in basophils, mast cells and dendritic cells (Eckman 2010; Schroeder 2010). The results of my study suggest that reduction of serum IgE concentration with omalizumab would suppress monocyte responses to IgE cross-linking. Such a dampening of IgE-mediated monocyte responses may reduce inflammatory cytokines induced by allergen exposure. Additionally, omalizumab treatment could potentially ameliorate IgE-

mediated impairment of phagocytosis and restore monocyte-driven Th1 priming after virus infection, perhaps providing a mechanism for decreasing virus-associated exacerbations of asthma.

Antagonists of inflammatory cytokines have also been suggested as therapeutic strategies for atopic disease. Several TNFα antagonists have been examined in the context of allergic asthma. Etanercept, a TNF receptor/Fc fusion protein, reduced airway inflammation in a murine model of asthma (Hutchison 2008), but has not yielded promising results in human trials (Holgate 2011). Clinical trials investigating the effectiveness of the anti TNFα monoclonal antibodies, infliximab (Erin 2006) and golimumab (Wenzel 2009), have also demonstrated little efficacy in reducing asthma symptoms, though infliximab was found to decrease the incidence of asthma exacerbations. Though the reasons for this failure of TNFα antagonists to ameliorate allergic disease in humans remain unclear, it has been suggested that certain subgroups of patients may be more responsive than others (Holgate 2011). Antagonism of IL-1 signaling has also been examined in atopic disease. Adenovirus-mediated delivery of IL-1RA during murine OVA-induced asthma suppressed inflammation and airway hypersensitivity (Wang 2006). Recombinant human IL-1RA, marketed as anakinra, is currently undergoing investigation in a phase 1 human clinical trial for severe atopic dermatitis (ClinicalTrials.gov Identifier: NCT01122914).

A Role for Monocytes in Atopic Disease

The findings presented in this report support a critical role for monocytes in atopic disease. Monocytes express the high-affinity IgE receptor FcεRI (Maurer 1994) and are recruited

to sites of allergic inflammation (Gonzalo 1998; Jockers 2006). Activation of FcεRI on monocytes by cross-linking IgE induces secretion of cytokines that have the potential to influence allergic disease in a number of ways (**Figure 5.1**). Secretion of TNFα, IL-1β, IL-6, and IL-23 by monocytes after allergen exposure may contribute to allergic inflammation and airway hypersensitivity in allergic asthma. The potential for monocyte involvement in atopic disease is not restricted to allergic asthma: monocytes have also been proposed to play an important role in pathogenesis of atopic dermatitis (Novak 2002). Allergen-mediated cytokine secretion from monocytes may also promote skin inflammation in atopic dermatitis. Monocyte-derived cytokines also have the potential to play a role in directing adaptive immune responses to allergens. IL-10, IL-6, and IL-1β may serve to augment allergen-specific IgE production from B cells. This increased IgE production may actually feed back to up regulate FcεRI expression and further enhance allergen-induced cytokine secretion. Monocyte IL-6 and IL-23 secreted after IgE cross-linking may support Th2 differentiation from naïve $CD4^+$ T cells, which could further enhances allergic inflammation and atopic disease.

My observations also suggest an important role for IgE cross-linking on monocytes during virus-associated exacerbations of allergic disease (**Figure 5.2**). During virus infection, monocyte-derived cells present antigens to T cells to stimulate adaptive immune responses (Nakano 2009; Wu 2011b). Allergen-mediated cross-linking of IgE may impair the ability of monocytes and monocyte-derived cells to present antigens to naïve T cells and induce production of virus-specific Th1 cells, thus suppressing appropriate anti-viral responses. Monocytes exposed to allergens may instead promote Th2 differentiation, resulting in enhanced allergic inflammation. Additionally, the cytokines produced by monocytes in response to both IgE cross-linking (Pyle 2013) and virus infection (Becker 1991) could further promote inflammation.

Suppression of phagocytosis after allergen exposure may prevent monocytes from phagocytosing debris resulting from apoptosis of virus-infected cells and inflammatory granulocytes. The ensuing buildup of apoptotic debris may contribute to ongoing inflammation and exacerbation of disease (Reed 2008). In addition to virus-associated exacerbations of asthma, disruption of monocyte responses to infection could also influence other pathogen-associated exacerbations of atopic disease. In addition to virus infection, bacteria such as *Mycoplasma pneumoniae*, *Chlamydia pneumoniae*, and *Streptococcus pneumoniae* have been associated with asthma exacerbations (Freymuth 1999; Otero 2013). Patients with atopic dermatitis can acquire severe skin infections with herpes simplex virus and *Staphylococcus aureus* (Broccardo 2011). Thus, IgE-mediated secretion of inflammatory cytokines, altered T cell stimulation, and impaired phagocytosis from monocytes could potentially contribute to exacerbations of several atopic diseases associated with a variety of pathogens.

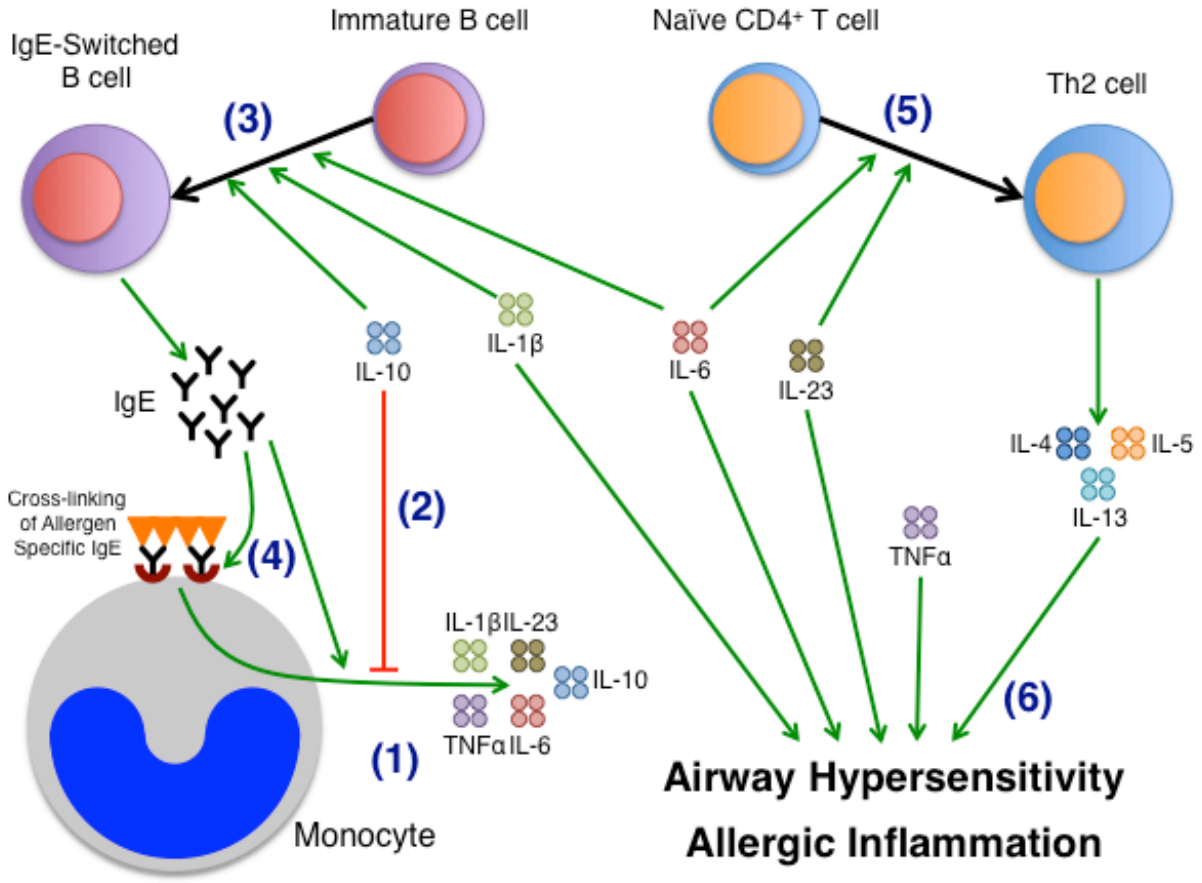


Figure 5.1 – Proposed Model of the Impact of IgE-Mediated Monocyte Cytokines on Allergic Asthma

A proposed model of the impact of IgE-mediated monocyte cytokine secretion on allergic inflammation and airway hypersensitivity. Green arrows indicate positive influence, while red blocked lines signify negative influence. Black arrows represent maturation or differentiation. (1) IgE cross-linking induces secretion of TNFα, IL-1β, IL-6, IL-10, and IL-23 from monocytes. (2) IL-10 feeds back to inhibit cytokine secretion. (3) IL-10, IL-1β, and IL-6 can support maturation and IgE class-switching from B cells. (4) IgE from B cells feeds back to up regulate FcεRI expression on monocytes and enhance allergen-mediated cytokine secretion. (5) IL-6 and IL-23 can promote Th2 responses, which include secretion of IL-4, IL-5, and IL-13. (6) TNFα, IL-1β, IL-6, IL-23, IL-4, IL-5, and IL-13 promote airway hypersensitivity and allergic inflammation.

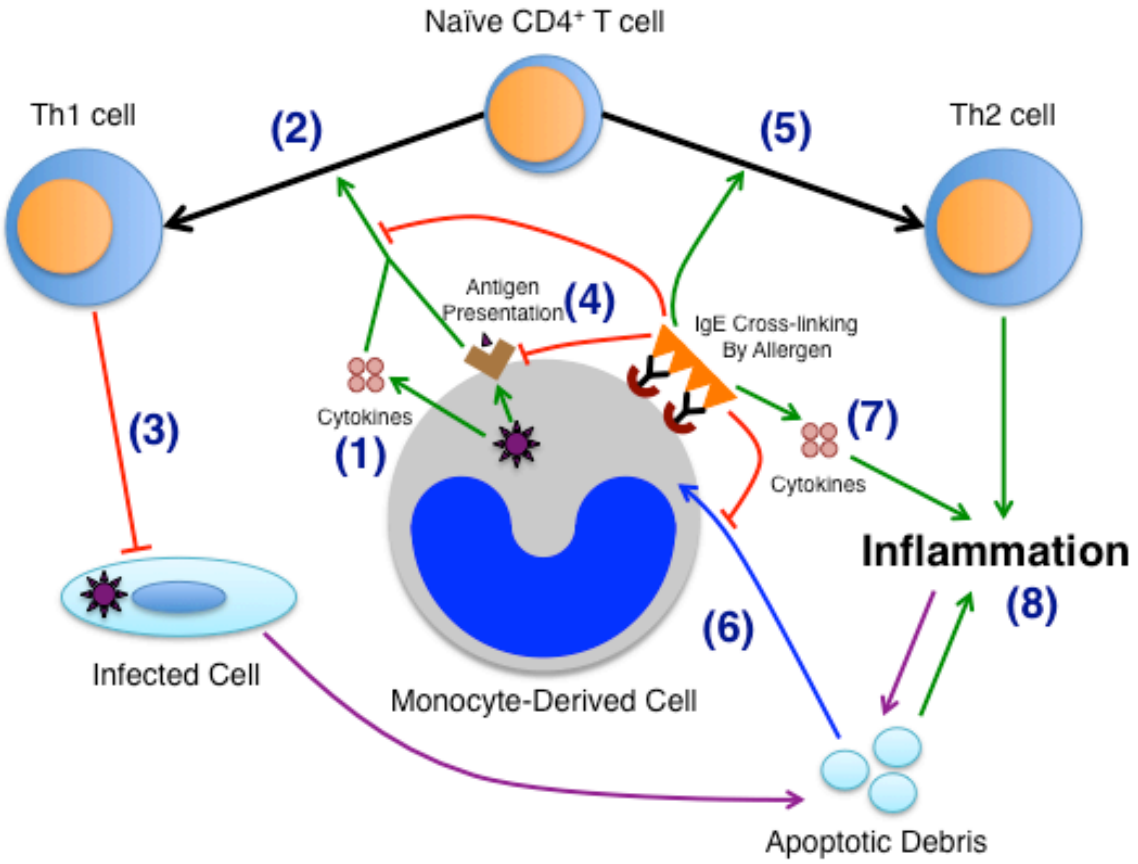


Figure 5.2 – Proposed Model of Monocyte IgE Cross-Linking During Virus Infection

A proposed model of the impact of IgE cross-linking on monocytes during viral infection and its effect on allergic inflammation. Green arrows indicate positive influence, while red blocked lines signify negative influence. Black arrows represent differentiation of naïve $CD4^+$ T cells. Purple arrows denote apoptosis of virus-infected or inflammatory cells and blue arrow indicates phagocytosis of apoptotic debris. (1) Virus infection of monocytes induces cytokines and presentation of viral antigen to naïve $CD4^+$ T cells. (2) Monocyte cytokines and stimulation of T cells induces Th1 differentiation, which (3) suppresses virus infection. (4) IgE cross-linking suppresses antigen presentation and Th1 differentiation, while (5) promoting Th2 differentiation. (6) IgE cross-linking also suppresses phagocytosis of apoptotic debris and (7) induces inflammatory cytokines. (8) Inflammatory cytokines, Th2 response, and residual apoptotic debris result in enhanced inflammation.

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