DELINEATING SIGNALING MECHANISMS INVOLVED IN LYMPHOCYTE CHEMOTAXIS, IMMUNE HOMEOSTASIS AND ALLERGIC ASTHMA

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ABSTRACT

The vasoactive intestinal peptide (VIP) signaling axis constitutes VIP and its two G protein coupled receptors (GPCR) termed vasoactive intestinal/pituitary adenylate cyclase activating polypeptide (VPAC) 1 and 2. This signaling axis regulates numerous biological actions within the endocrine system, the nervous system and the immune system. Working as a gut hormone, VIP can increase cAMP signaling within beta-islet cells of the pancreas to impact insulin production. As a neurotransmitter, it acts as a master circadian regulator controlling light and dark cycling. Lastly, VIP regulates immune processes such as activation, chemotaxis, development and cytokine secretion. The focus of my doctoral research was to delineate VIP signaling mechanisms controlling immunity. We aimed at understanding: 1.) the molecular mechanism of VIP-induced T cell trafficking 2.) ability for VPAC2 signaling to regulate immune homeostasis and 3.) a phenotype of a B cell subset during asthma, an immune pathology devoid of VIP protein due to excessive protease activity. Methods employed utilized isolated primary mouse immune cells to measure a VIP-induced signaling pathway centered on the epidermal growth factor receptor (EGFR), a tyrosine kinase receptor, by qPCR and chemotaxis assays. Flow cytometry to enumerate immune cell numbers in VPAC2 deficient mice was done to accomplish aim 2. Lastly, using a published in vivo allergic asthma mouse model, we used qPCR, immunoblotting and flow cytometry analyses to measure expression of Hyaluronic acid binding proteins on B cells. Results from these studies revealed that VIP signaling in T cells is regulated by EGFR as inhibitors against its enzymatic activity abolished T cell movement towards VIP. Immune cell numbers were lowered as a consequence of VPAC2 deficiency, suggesting its involvement in homeostasis. Lastly, a unique B cell population homing to asthmatic lung secretes an anti-inflammatory mediator, TGF-beta, through the HA binding
protein called RHAMM. Collectively, these data emphasize the importance of VIP signaling in the immune system controlling cell migration and homeostasis.
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DEDICATION

I dedicate this dissertation to my very supportive, understanding and loving family. My Wife, Shannon, has been a shoulder I could always lean on and a source of constant encouragement and counsellor. To my sons, Caleb, Elijah and Kyson, I couldn’t have pushed this hard to achieve my goals without your constant reminder of what is important in life. To my father and mother, Joseph and Naomey Wanjara, and to my brothers Ben, Evans, Joshua, Moses, James, David and my sisters Emma, Lilian and Brenda, your prayers and encouragement has always kept me focused on achieving my dreams ever since I was a young boy. To my father-, mother-, sister-, and brother-in-law, you truly have embodied what it means to be a second family to me. Thank you Jim, Melody, Pam and Nick for taking me in as one of your own. To you Grace, my dear cousin, let this be a testimony that it can be done if you set your eyes on the prize. More importantly, to my Lord and Savior, I am very thankful for your guidance and control of everything in my life.
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LIST OF ABBREVIATIONS

$^{51}$Cr .......................... Radiolabeled chromium 51
AC ............................ Adenylate cyclase
ACLB .......................... Ammonium chloride lysis buffer
ADAM ......................... A disintegrin and metalloprotease
ADAM15 ...................... A disintegrin and metalloprotease 15
AIDS .......................... Acquired immunodeficiency syndrome
ANOVA ....................... Analysis of variance
APC ............................ Allophycocyanin
APC ............................ Antigen presenting cell
APP ............................ Amyloid beta (A4) precursor protein
BCR ............................ B cell receptor
BM .............................. Bone marrow
Ca$^{2+}$ .......................... Calcium ion
cAMP .......................... Cyclic adenosine monophosphate
CCL ........................... CC chemokine ligand
CCR ........................... CC chemokine receptor
CD ............................. Cluster of differentiation
cDNA ......................... Complementary deoxyribonucleic acid
CFSE .......................... Carboxyfluorescein succinimidyl ester
COPD .......................... Chronic obstructive pulmonary disease
CTL ............................ Cytotoxic T lymphocyte
DAG .......................... Diacyl-glycerol
DAMPS..........................Damage-associated molecular pattern
DC..................................Dendritic cell
DMEM..........................Dulbecco’s Modified Eagle Medium
DMSO..........................Dimethyl sulfoxide
DNA............................Deoxyribonucleic acid
EDTA............................Ethylenediaminetetraacetic acid
EGF..............................Epidermal growth factor
EGFR............................Epidermal growth factor receptor
EOS..............................Eosinophils
EPAC............................Exchange protein directly activated by cAMP
ER..............................Endoplasmic reticulum
FACS............................Fluorescence-activated cell sorting
FASL............................Fas ligand or CD95L
FITC.............................Fluorescein isothiocyanate
GAG.............................Glycosaminoglycan
GPCR............................G protein coupled receptor
GRB7............................Growth factor receptor-bound protein 7
HA...............................Hyaluronic acid or hyaluronan
HEPES..........................4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HER2............................Human epidermal growth factor Receptor 2
HEV............................High endothelial venules
HIV..............................Human immunodeficiency virus
HMM HA........................High molecular mass hyaluronic acid
IBD.............................Inflammatory bowel disease
IFN.............................Interferon
Ig..............................Immunoglobulin
IL...............................Interleukin-
ILC2............................Type 2 innate lymphoid cells
IP₃..............................Inositol triphosphate
LMM HA.......................Low molecular mass hyaluronic Acid
LPS.............................Lipopolysaccharide
LYVE-1........................Lymphatic vessel endothelial hyaluronan receptor
MALT...........................Mucosa associated lymphoid tissue
ME..............................Mercaptoethanol
mRNA..........................Messenger ribonucleic acid
PAMPs.........................Pathogen-associated molecular patterns
PBS.............................Phosphate buffered saline
PI..............................Propidium iodide
RBCs............................Red blood cells
TGF-β1..........................Transforming growth factor-beta 1
Th...............................T helper
TNF-γ...........................Tumor necrosis factor-gamma
CHAPTER ONE: GENERAL INTRODUCTION

Overview of the immune system

Two important goals of the immune system are eradication and limitation of infections to the host. These goals are illustrated in immune-compromised people who are pre-disposed to opportunistic pathogenic infections, as seen in those with genetic immune disorders like X-linked severe combined immunodeficiency (SCID)\(^1\)-\(^3\), and immune-compromised infections like acquired immunodeficiency syndrome (AIDS)\(^4\),\(^5\). The importance of the immune system can also be demonstrated in expecting mothers, who are also more susceptible to microbes that normally do not infect healthy individuals\(^6\)-\(^8\). For the eradication and limitation of infections, normal, healthy cells and unhealthy cells are distinguished by the immune system by recognition of “danger” signals called damage-associated molecular patterns (DAMPs) that are expressed on unhealthy cells\(^9\). Infections of the cells by pathogens or cellular damage caused by non-infectious agents like sunburn or cancer, trigger the infected and damaged cells to express DAMPs on their surfaces that mark them for destruction by immune cells\(^10\). Cells that are infected by microbes such as viruses and bacteria trigger a different “danger” signal called pathogen-associated molecular patterns (PAMPs)\(^11\), also marking them for destruction. Hence, failure by the immune system to respond accordingly to DAMPs and PAMPs when there is sufficient need, can lead to problems like infections or cancer. Conversely, over-reaction by the immune system without any real threat or the inability to be turned off after clearance of a threat is detrimental as seen in allergic reactions, chronic inflammation and autoimmune disease, when the immune system attacks the host\(^12\)-\(^14\). Therefore, the responses of the immune system are tightly regulated.
Organs and tissues of the immune system

The immune system is made up of primary lymphoid organs (e.g., bone marrow and thymus) and secondary/peripheral lymphoid organs (e.g., lymph nodes and spleen) (Figure 1-1). In the bone marrow, precursor cells called hematopoietic stem cells (HSCs) can differentiate into three major lineages of blood cells. The first is called the lymphoid lineage, which constitutes T and B lymphocytes. These cells make up the adaptive immune system. B lymphocytes mature in the bone marrow, while thymocyte precursors are generated in the bone marrow and migrate to the thymus to develop into mature T lymphocytes. The second is called the myeloid lineage that represents eosinophils, neutrophils, basophils, mast cells, dendritic cells, monocytes and macrophages. These cells make up the innate immune system. The last lineage represents erythroid progenitor cells, which generate red blood cells and platelets. Innate immune cells are important as first-line responders to infection. The innate immune cells lack the diversity to recognize microbes as compared to the adaptive immune system. Whereas, our innate immune cells have only evolved the ability to sense and recognize approximately one thousand different PAMPs, the adaptive immune system can recognize antigens in the order of billions.

Thymus, which is a small organ located in the thoracic cavity near the heart, and functions to produce mature naïve T cells. Lymphoid progenitors egress from the bone marrow and migrate into the thymus. Through a thymic education process called negative and positive selection, mature naïve T cells are produced. During the final stages of T cell development, those cells that fail to recognize self-tissue did by neglect (negative selection), while those T cells that might attack “self” are eliminated. Selected mature T cells that bind tissue with moderate affinity only are then released into the bloodstream to migrate to peripheral lymphoid organs such as spleen and lymph nodes.
Lymph nodes are immune compartments that can be thought of as immunological conference centers or communication hubs where antigen presenting cells (APCs) concentrate information from the body to be sampled by T and B lymphocytes. If the T or B lymphocytes in the lymph nodes recognize pieces of a microbe (antigen) brought in from an area of infection and presented by APCs, they respond by clonally expanding (proliferating) and migrating to the infected area in an attempt to clear the invading microbe. Thus, an active immune response can be assessed by both visual and physical examinations for swollen lymph nodes in the neck, armpit or inner thighs.

Another peripheral immune organ is the spleen which is located behind the stomach and acts as an immunological filter of the blood. The Spleens contain APCs that interrogate blood plasma for PAMPs from invading pathogens that enter the blood. Such infection route could be due to blood-feeding insects. It can also be thought of as an immunological conference center like the lymph nodes even though it is not directly connected to the lymphatic system. It consists of circulating B cells, macrophages, dendritic cells, natural killer cells and red blood cells. These immune cells are enriched in specific regions of the spleen, and upon recognizing blood-borne pathogens, they activate and respond accordingly. In addition, damaged or senescent red blood cells get removed by the spleen.

The mucosal tissue is made up of mucosal surfaces known as mucosal associated lymphoid tissues (MALT). Since mucosal surfaces, like the respiratory tract and the gut are vulnerable entry points for pathogens, specialized immune hubs or “rest areas” are strategically located in the MALT. For instance, in the small intestines, specialized lymphoid organs called Peyer's patches and mesenteric lymph nodes are responsible for organizing immune cells access to sampling from the MALT of the gastrointestinal tissue (GALT) and pulmonary tissue.
Due to the sheer numbers of potentially life-threatening bacteria in the vertebrate gut, more than 70% of immune cells line this tissue.  

The vascular is a plasma “highway” that enables constant circulation of immune cells that assists in their surveillance of protein antigens originating from pathogenic microbes. Immune cells or white blood cells are designated as leukocytes. Overabundance or scarcity of (an) immune cell type(s) in the blood may reflect a dysregulation with respect to homeostasis and is a major goal of my doctoral research (chapter 3).  

The last component of the immune system is the lymphatic system. The lymphatic system consists of a network of vessels and tissues containing an extracellular fluid known as lymph and organs, such as lymph nodes. It is an elaborate system for immune cells to traffic between tissues and the bloodstream. Immune cells traffic through the lymphatic system and converge in lymph nodes found throughout the body.  

Figure 1-1. Organs and tissues of the immune system. The immune system is made up of primary lymphoid organs (bone marrow and thymus) and secondary lymphatic tissues (spleen, tonsils, lymph vessels, lymph nodes, adenoids, and skin). The right panel shows organs and tissues of the human immune system, whereas the left panel shows those of the mouse. Experiments used in our research utilized mouse immune cells. https://www.aids.gov/hiv-aids-basics/just-diagnosed-with-hiv-aids/hiv-in-your-body/immune-system-101/
http://ctrgenpath.net/static/atlas/mousehistology/Windows/lymphatic/pictures/lymphdiagram.gif
Immune cells

Studies presented in this dissertation characterized different types of immune cells and therefore, general background of the cells are provided. The immune system is made up of numerous types of cells that either reside in particular tissues or traffic throughout the body. Each cell type plays a unique function due to distinct abilities to differentially recognize host microbial infection and/or host damaged cells (e.g. dying or cancerous cells) through PAMPs and DAMPs (innate immune cells) or through the lymphocyte receptors (T and B cell receptor complexes). Such communication requires cell/cell contact called juxtacrine signaling and via cytokine and chemokine soluble mediators. Immune cells are divided into two major categories called innate and adaptive immunity.

Innate immune cells

Innate immune cells are made up of cells that provide the first-line of defense against infections. These include granulocytes (basophils, eosinophils, and neutrophils), mast cells, monocytes, macrophages, and dendritic cells.

Granulocytes are also known as polymorphonuclear (PMN) leukocytes. Neutrophils are the most numerous of the granulocytes in the blood and patrol for pathogens by constantly circulating the bloodstream. They protect against infections by ingesting (phagocytosis) pathogens such as bacteria, and degrading them inside special vesicles that contain a battery of degradative enzymes and toxic substances that degrade the engulfed pathogen\textsuperscript{35, 36}. Neutrophils have a greater range of particulate that they engulf as compared to macrophages, which also clear infection by phagocytosis\textsuperscript{35}. Basophils and eosinophils are key to host defense against parasites through the extracellular release of toxic molecules contained in their granules\textsuperscript{37}. Eosinophils also play a key role in allergic inflammation such as during asthma\textsuperscript{38}. Since mature granulocytes
cannot replenish their granule contents, they undergo apoptosis when these organelles are depleted\textsuperscript{39, 40}.

Mast cells are resident in all connective tissues\textsuperscript{41}. They have granules like the basophils and play an important role in allergic reactions by secreting pro-inflammatory chemicals like histamine, which causes blood capillaries to become more permeable to leukocytes and other proteins leading to inflammation to affected areas\textsuperscript{42, 43}. They are also important for defense against parasites through their release of toxic granular contents such as mast cell protease-1\textsuperscript{41, 44, 45}.

Monocytes are the mobile progenitors of macrophages, which are sedentary tissue cells, and can also differentiate into dendritic cells\textsuperscript{46}. Monocytes travel from blood to tissues where they mature into macrophages and take up residence\textsuperscript{47, 48}. Macrophages ingest and degrade bacteria\textsuperscript{49}. When activated, monocytes and macrophages educate other immune cells against the foreign pathogens. Macrophages also have non-immune functions that are “housekeeping” roles where they phagocytose dead and dying cells in the tissues such as bone marrow, the spleen and the thymus\textsuperscript{50}. This is important since the dead cells can become necrotic, promoting inflammation and autoimmunity\textsuperscript{51}.

Dendritic cells (DCs) are professional antigen presenting cells that develop from the bone marrow and monocytes\textsuperscript{46, 52}. They are considered as both innate and adaptive immune cells that process large molecules into recognizable fragments (antigens) since unprocessed antigens alone cannot be recognized by B and T cells\textsuperscript{53}. DCs present antigens to T cells with the appropriate major histocompatibility complex (MHC) expressed on them providing checkpoints to help immune cells distinguish between host and foreign cells\textsuperscript{52, 53}.
Adaptive immune cells

B and T cells make up the adaptive arm of the immune system. B and T cells are commonly known as lymphocytes. B cells produce antibodies to neutralize infectious microbes and present antigens to T cells. Antibodies are the secreted forms of activated B cell receptors (BCR). Membrane bound and secreted BCRs are collectively called immunoglobulins (Ig). This dual expression is important since surface bound BCR is used for recognition of pathogen antigens leading to activation of B cells. Upon activation, the B cell responds by secreting antibodies. This ensures that the response is specific against a particular antigen. Antibodies are produced by plasma cells that differentiate from B cells. Every antibody is unique and encoded by heavy and light chain immunoglobulin (Ig) genes. Even though every antibody is unique, they fall under five general categories differentiated by the heavy chain constant regions possessed: IgM, IgD, IgG, IgA, and IgE. Antibodies serve three major roles: neutralization, opsonization, and complement activation. Pathogens are neutralized when antibodies cover pathogens making them unable to bind and infect host cells. Opsonization serves as “red flags” on antibody-bound pathogens to alert phagocytes (neutrophils and macrophages) to engulf and digest the pathogen. Complement is a series of reactions resulting in lysing of extracellular pathogens like bacteria. While they have overlapping roles, IgM generally is important for complement activation, IgD is involved in activating basophils, IgG is important for neutralization, opsonization, and complement activation, IgA is essential for neutralization in the gastrointestinal tract and IgE is necessary for activating mast cells in parasitic and allergic responses.

B cells play a central role in adaptive immunity through antigen presentation to CD4 T cells, production of cytokines, provision of co stimulatory signals, and activating naïve CD4
T cell differentiation into T helper 1 (Th1) or Th2 subsets. More recently, a subset called regulatory B cells was identified. This subset functions in the suppression of immune response by secreting suppressive cytokines, IL-10 and TGF-β1 once an infection has been cleared. Deficiency of B cells or the inefficient regulatory B cell function result in autoimmunity and chronic inflammatory diseases.

T cells are divided into two major subsets based on the T cell receptor (TCR) co-receptor expressed on their surfaces. These subsets are CD4 and CD8 (CD, cluster of differentiation). TCRs bind to unique non-polymorphic regions presented on major histocompatibility complexes (MHCs) by APCs. Antigen processing and presentation is the process by which APCs express antigen on their cell surface in a form recognizable by lymphocytes. It consists of protein fragmentation through proteolysis, association of fragments with MHC and expression of peptide-MHC complex at the cell surface where they can be recognized by T cell receptors (TCRs). The co-receptors, CD4 or CD8, on the surface of T cells aid in stabilizing the interaction between the MHC and TCR. CD8 T cells only respond to antigen peptides presented by class I MHC (MHCI), while CD4 T cells only recognize peptide presented by class II MHC (MHCII). The antigen peptide processing differs for presentation by MHCI and MHCII. For antigen presentation by MHCI, cytosolic antigen proteins are degraded through the proteasome mechanism and resulting peptides are transported to the endoplasmic reticulum (ER) by TAP (transporter associated with antigen processing). The MHCI molecules are secreted to the ER, associate with the peptide, and are transported to the plasma membrane in vesicles.

MHCII antigen presentation process entails exogenous proteins being endocytosed and degraded by acidic proteases within endosomes and lysosomes. By enzymatic cleavage of disulfide bonds, gamma-interferon-inducible lysosomal thiol reductase, IP-30, then facilitates...
unfolding of the endocytosed antigens in MHCII-containing compartments. The MHCII is then secreted in the ER and transported in a vesicle to the peptide phagolysosome, where the MHCII binds to a peptide and gets transported to the plasma membrane for presentation.

Upon MHCII interaction with the TCR, CD4 T cells differentiate into T helper (Th) cells that are subdivided into four main types - Th1, Th2, Th17, and regulatory T cells (Tregs) - based on their distinct cytokine-secretion phenotype and functional characteristics. There are several factors that are involved in directing this differentiation, which include the nature and affinity of the antigen, the type of TCR and co-receptor signaling, and, most importantly, the cytokine environment. Cytokines are signaling proteins and glycoproteins that can act like hormones or neurotransmitters secreted by innate, adaptive, and nonimmune cells. They are a means of cellular communication, and they function to provide signals for survival, proliferation, and differentiation.

Th1 cells participate in both cell-mediated immunity and antibody-mediated immunity. They provide protection against intracellular bacteria, protozoa and viruses. They are triggered by presence and increase in IL-12, IL-2 in the serum. These cells then secrete IFN-γ as their effector cytokine to activate macrophages, CD8 T cells, IgG B cells and other CD4 effector cells. The secreted IFN-γ directly inhibits differentiation to the Th2 pathway and polarizes other Th cells to develop into Th1 cells.

In contrast, Th2 helper cells provide host immunity against extracellular parasites such as helminths. They are triggered by increase in IL-4. In response, Th2 cells secrete cytokines IL-4, IL-5, IL-9, IL-10 and IL-13 which activate basophils, mast cells and eosinophils as well as B cells. Th2 secreted IL-4 stimulates B-cells to produce IgE antibodies, which stimulates mast cells to release histamine, serotonin, and leukotriene. The secreted histamine, serotonin and
leukotriene induces broncho-constriction, intestinal peristalsis and gastric fluid acidification that help expel helminths\textsuperscript{83}. IL-5 from CD4 T cells activate eosinophils to attack helminths\textsuperscript{84}. Th2 secreted IL-10 suppresses Th1 cells differentiation and dendritic cells function\textsuperscript{80}. Th2 over-activation against autoantigens such as \textit{aspergillus fumigatus} results in IgE-mediated allergy and hypersensitivity. Allergic rhinitis, atopic dermatitis, and asthma belong to this category of autoimmunity\textsuperscript{79,80,85}.

The third Th subset, Th17, was discovered just over the last 5-10 years. Cytokines IL-6, IL-23, and TGF-\beta are essential for the development of Th17 cells\textsuperscript{86}. Th17 cells are developmentally distinct Th1/2 and secrete IL-17\textsuperscript{86}. In addition, they also secrete IL-6, IL-22, and TNF-\alpha and function in inflammation of tissues, neutrophil activation to combat extracellular bacteria, and in autoimmune diseases\textsuperscript{87-89}.

The fourth Th subset, Treg, has also recently been discovered and function in suppressing T cell responses through secretion of IL-10 and TGF-\beta. These cells are important in regulating self-tolerance and may also function in interfering with immunity to tumor\textsuperscript{90,91}.

Upon stimulation from MHCI, CD8 T cells differentiate into cytotoxic T lymphocytes (CTLs) that respond to infections against viruses, intracellular bacteria, and protozoan pathogens. IL-2 is important for the induction and elimination of CTLs at the start and end of an infection, respectively. Also, the IL-15 cytokine has been shown to be needed for the maintenance of CD8 memory T cells. Once fully activated, CTLs enter circulation and home to the site of infection and directly destroy pathogens and infected cells. The methods by which CTLs kill targeted infected cells and pathogens are through the use of perforins, granzymes and the secretion of the apoptotic protein, Fas ligand (FasL). Perforins form pores in the plasma membrane of the targeted pathogen or infected cell causing disruption of ion balance. Granzymes act as cytotoxic
granules to mediate cell death. The secreted FasL will bind to the Fas receptor on the targeted pathogen or on Th cells that are no longer needed for the immune response so that the cells will be initiated to undergo apoptosis.

**Vasoactive Intestinal Peptide (VIP) and the immune system**

VIP is a 28 amino acid neuropeptide that was first isolated by Said and Mutt in swine small intestines. It belongs to the glucagon/secretin super family and exhibits structural similarities with other gastrointestinal hormones such as secretin, glucagon, gastric inhibitory peptide, and growth hormone releasing hormone. In fact, VIP is the most abundant hormone in the gut. It evokes its biological activities by binding two structurally similar receptors called vasoactive intestinal peptide receptor 1 (VPAC1) and vasoactive intestinal peptide receptor 2 (VPAC2). A second super family peptide, pituitary adenylate cyclase activating peptide (PACAP), also binds VPAC1 and VPAC2. VIP and PACAP share a remarkable sequence homology. Their amino acid sequences have remained nearly unchanged for over 700 million years of evolution. Through exon duplication, VIP is thought to have evolved from PACAP, which is believed to have been associated with evolution of the adaptive immune system. It is synthesized from a 170-amino acid preproVIP precursor molecule into a series of intermediate products to eventually yield a final 28-amino acid VIP product.

VIP is one of the most abundant cytokines of the immune system and is found in spleen, thymus, mesenteric lymph nodes, and mucosa-associated lymphoid tissues (MALT) of the pulmonary and gastrointestinal tissues. It gets delivered to the body by VIPergic nerve fibers innervating both primary and secondary immune organs. Some immune cells such as mast cells and CD4 Th2 cells also act as sources of VIP.
VIP and its receptors are believed to have co-evolved with establishment of the adaptive immune system\textsuperscript{103}, which may explain why VIP modulates numerous functions important to the immune response, such as proliferation\textsuperscript{104}, cytokine expression, inhibition of apoptosis\textsuperscript{105}, chemotaxis\textsuperscript{106} and differentiation\textsuperscript{107}. Many studies have since verified that VIP’s biological effects or functions have been generally associated with increased adenylate cyclase activity and cAMP concentrations through binding to the VPAC receptors\textsuperscript{108}.

The VIP/PACAP receptors, VPAC1 and 2, belong to class B of the seven-transmembrane (7TM) G-protein coupled receptor (GPCR) superfamily\textsuperscript{109}. They are encoded by separate genes, and share 50\% amino acid sequence identity\textsuperscript{109}. VIP binds at the receptor’s N-terminal extracellular domain through a Venus flytrap mechanism\textsuperscript{110}. The N-terminus of VIP then docks to the transmembrane regions and intervening loops, causing a conformational change in the receptor\textsuperscript{111}. This enables the intracellular loops to trigger at least three major signaling pathways, including $G_{\alpha_s}$/cAMP/PKA, and $G_{\alpha_q}$/PLC/Ca$^{2+}$ and $G_{\alpha_i}$/PLC/Ca$^{2+}$ activation\textsuperscript{125-128, 123-126}.

The VPAC receptors are expressed by numerous types of immune cells, which is consistent with the immunomodulatory activities of VIP/PACAP. VIP/PACAP receptors are present on T lymphocytes, macrophages, dendritic cells, eosinophils, with expression on B cells still being controversial\textsuperscript{92}. Mouse B cells are thought not to express any binding sites whereas human B cells have been shown to express the receptors. VPAC1 is most highly expressed in macrophages and T lymphocytes\textsuperscript{112}, while VPAC2 is expressed lowly in resting lymphocytes and macrophages until after TCR or lipopolysaccharide (LPS) stimulation when VPAC2 expressions are seen to increase\textsuperscript{113}. VPAC2 has therefore been termed the inducible VIP receptor.
Figure 1-2. Mechanisms of VIP signaling. VPAC receptors are preferentially coupled to G\textsubscript{as} leading to activation of adenylate cyclase (AC) and subsequent cAMP production and activation of protein kinase A (PKA). The VPAC receptors can activate phospholipase C (PLC) pathway after coupling to G\textsubscript{i}, G\textsubscript{aq}, or G\textsubscript{a\textsubscript{16}} and stimulate calcium levels and protein kinase C (PKC). G\textsubscript{i}-dependent activation of PLC by VPAC\textsubscript{2} relies on the availability of free G\textsubscript{\beta\gamma} and on Ca\textsuperscript{2+} entry through receptor-operated Ca\textsuperscript{2+} channels while G\textsubscript{i}-dependent activation of PLC by VPAC\textsubscript{1} is not affected by chelating of extracellular calcium.

Thymocytes (immature T cells) and epithelial cells in the thymus predominantly express VPAC\textsubscript{2} with a more restricted expression of VPAC\textsubscript{1}\textsuperscript{114}. VIP affects numerous aspects of thymus biology such as cytokine production, apoptosis, differentiation, and mobility through the direct effects on T cells\textsuperscript{115}. Therefore functions therefore predispose VIP and its receptors as candidates for molecules that can regulate immune cells homeostasis.

Currently, molecular mechanisms regulating VIP-induced immune cell migration are not well understood. In this dissertation, we used VIP as a chemoattractant to naïve mouse CD4 T cells in order to investigate the molecular mechanisms regulating this process. In addition, little data on the role of VIP/PACAP signaling in regulation of immune cell homeostasis are available. To investigate the role of VIP/PACAP signaling through VPAC\textsubscript{2} receptor utilized a VPAC\textsubscript{2}
knockout mouse strain to collect data investigating the homeostatic regulation of immune cells by VPAC2 activity.

Asthma

Asthma is a lung disease characterized by a reversible airway obstruction, airway inflammation and increased airway responsiveness to a different stimuli\textsuperscript{12,116}. Currently, it is one of the most common chronic diseases, affecting over 300 million people worldwide and nearly 20 million in the US\textsuperscript{117}. Even though the death rate due to asthma has been decreasing as a result of improved health care in the US, annual costs associated with it have continued to increase over the years to around 30 million per year. Coupled to missed school and work days, financial losses from asthma attacks are staggering\textsuperscript{117,118}. In most cases, genetic predispositions towards development of hypersensitivity reactions against environmental allergens lead to a dysregulated cellular and humoral responses\textsuperscript{119,120}. Environmental factors such as lifestyle, infections and pollution can also cause asthma\textsuperscript{118,120}.

Asthma can either be intrinsic or extrinsic\textsuperscript{121}. However, symptoms associated with both cases are similar\textsuperscript{122}. These may include wheezing, coughing, shortness of breath and chest pains amongst others\textsuperscript{117,123}. Intrinsic asthma, also known as non-allergic asthma, may be initiated by factors such as viruses, stress, anxiety, cold air, dry air, exercise, hyperventilation or other irritants\textsuperscript{124}. Extrinsic asthma, also known as allergic asthma, occurs in response to allergen inhalations. It is the most common form of asthma. It can be caused by inhalation of house dust mites, molds, cockroach antigens and animal dander\textsuperscript{123,125}.

Inflammation of several different immune cells in the lungs characterize asthma\textsuperscript{126}. These cells play key roles in pathogenesis and persistence of asthma. Immune cells that have been shown to play key roles in asthma include mast cells, basophils, eosinophils, macrophages,
neutrophils, dendritic cells, T and B lymphocytes\textsuperscript{127}. Eosinophil inflammation is considered a hallmark of allergic asthma\textsuperscript{38,128}. In addition, secreted cytokines also help orchestrate the chronic inflammation and structural changes of the respiratory tract in allergic asthma by recruiting, activating and promoting survival of various inflammatory cells\textsuperscript{129}. For example, IL-5 involvement in differentiation and recruitment of eosinophils to allergic lungs from the bone marrow is well documented\textsuperscript{130-132}. On the other hand, the role of B lymphocytes in asthma is not completely understood. While some studies shown that B lymphocytes exacerbate asthma through antibody secretion\textsuperscript{133,134}, others have also shown that mice lacking B lymphocytes and antibodies exhibit a more severe form of asthma\textsuperscript{135}. In this dissertation, data are collected to characterize the phenotype of B lymphocytes in a murine model of fungus-induced allergic asthma. This is critical in better understanding functions of B cells during asthma.

**Purpose of my doctoral research**

The vertebrate immune system entails a physiological mechanism to defend against invasion by foreign microbes or pathogens. Normally, when confronted with foreign antigens, specific and appropriate responses are initiated to clear the threat without harming “self.” This is followed by restoration to the pre-infection state of the immune system, also known as the homeostatic state, which is necessary for a balanced and effective immune system. However, under certain circumstances, restoration to homeostasis is not achieved, resulting in an imbalance to the immune system\textsuperscript{136-139}. Primary immune deficiency diseases (PIDD)\textsuperscript{140,141} and autoimmunity are examples of pathologies associated with either unresponsive or over-reactive immune reactions. Often, such conditions can be due to inherited genetic defects\textsuperscript{142}. Some causes of inefficient immune responses are decreased numbers and/or absence of particular type(s) of immune cell population(s) as in the case of T cell deficiency\textsuperscript{143-145}. Conversely, when the
immune response over-reacts, it can result in conditions that harm “self” such as in autoimmunity, or as seen in allergic conditions like asthma. Thus, identifying molecular signaling cues governing immune balance or homeostasis, including recruitment of immune cells and immune homeostasis is vital for understanding how the immune system works.

The recruitment and migration of immune cells are critical for immune responses. For effective clearance of infections, immune cells developed in germinal centers of the body (e.g., bone marrow and thymus) must be rapidly mobilized and expanded to eliminate pathogenic microbes while limiting host cell damage. To accomplish such an important task, vertebrates (e.g., mammals) have evolved a lymphatic system that assists in immune cell movement throughout the body. The adaptive arm of the immune system is comprised of T and B lymphocytes. T lymphocyte populations are made of two major subsets called CD4 T cells and CD8 T cells. Lymphocytes migrate through a lymphatic system “highway” in search of foreign antigens. The “rest areas” that reside along this “highway”, are called lymph nodes and are where T and B lymphocytes can interrogate foreign antigens presented by antigen presenting cells (APCs). APCs concentrate foreign antigens into nearby lymph nodes in an attempt to accelerate their recognition by lymphocytes.

Chemokines, the chemical substances secreted by target cells, enable immune cells to navigate the lymphatic system and arrive at predetermined lymphoid compartments. Vasoactive intestinal peptide (VIP) is one such chemokine secreted by neurons and some immune cells. VIP binds two major receptors known as vasoactive intestinal/pituitary adenylate cyclase activating polypeptide receptor 1 and 2 (VPAC1/2) with equal affinity. VIP signaling is required for maximal recruitment of naïve CD4 T lymphocytes to the gut, specifically to Peyer’s patches and mesenteric lymph nodes. This was first recognized when
 naïve CD4 T lymphocytes with reduced VIP binding sites failed to traffic through the gut as compared to other lymphoid compartments after reintroduction into mice\textsuperscript{164, 165}. Several other studies since have also shown that VIP is a potent chemoattractant to different immune cells such as macrophages, eosinophils and B lymphocytes\textsuperscript{106, 166-169}. Despite knowing that VIP is a potent chemoattractant to immune cells, molecular mechanisms controlling VIP-induced chemotaxis are not well defined almost 30 years after the earliest report in 1984\textsuperscript{164}.

The overall goal of this research was to understand mechanisms involved in immune cell migration and homeostasis. We investigated how VIP affects immune cell biology in non-disease conditions and characterized an allergic asthma induced recruited B cell population to the lung microenvironment. The purpose of the first project was to investigate the molecular mechanisms governing VIP-induced migration of immune cells. The second project investigated the role of VIP/VPAC2 receptor signaling in immune cell homeostasis. Lastly, the third project characterized effects of pulmonary hyaluronic acid (HA) on B lymphocytes during asthma. From these three projects, we present findings that are statistically significant to understanding the following: 1) molecular mechanisms guiding lymphocyte trafficking to the gut, 2) regulation of immune cell homeostasis, and 3) the response of B cells to HA during asthma.

\textit{Egfr signaling is required for VIP-induced naïve mouse CD4 T cell migration.}

We previously discovered that VIP upregulated mRNA levels genes in an epidermal growth factor receptor (EGFR) chemotactic pathway in mouse primary resting CD4 T cells (subsequently referred to as resting CD4 T cells). The working hypothesis was that EGFR-signaling is required for VIP-induced chemotaxis of resting CD4 T cells. Using resting CD4 T cells, time and concentration curves were carried out to determine optimal parameters (time and ligand concentration) for VIP-induced EGFR-pathway mRNA upregulation over a 24 hour
period. Once established, we tested our hypothesis whether the putative chemotactic EGFR-pathway mediated VIP’s chemotactic activities by utilizing sensitive EGFR inhibitors and measured gene expression and cell migration by RT-qPCR and Boyden Chamber assays.

VPAC2 deficiency results in impaired immune cell homeostasis

Currently, only a few molecules have been identified that control steady-state (homeostatic) conditions of the immune system. For this reason, identification of additional molecules controlling homeostatic steady-state levels of the immune system is an area of intense research. Improper homeostatic conditions of the immune system can lead to decreased numbers in some or all immune cell populations resulting in attenuated immune responses leading to immunodeficiency or autoimmunity\textsuperscript{145, 170, 171}. While utilizing a VPAC2 knockout mouse we discovered that their spleens were smaller compared to age-and sex-matched wild type counterparts. The difference in spleen sizes could not be explained by weight of the mice. This startling discovery led us to hypothesize that VPAC2 signaling plays a crucial role in immune cell homeostasis. The evidence presented in this dissertation shows for the first time the importance of VIP/VPAC2 signaling in immune cell homeostasis. Using flow cytometry, we compared immune cell population numbers and cellular percentages in different immune organs. Rate of survival and proliferation of immune cells were also investigated to understand potential reasons for differences in total cell numbers. Therefore, from these discoveries, we expect that our findings will firmly establish the role of VIP signaling in regulating immune homeostasis.

Phenotypical characterization of B lymphocytes in response to hyaluronan in a murine fungal allergic asthma model

The last part of this dissertation was performed to characterize B cells in asthma. Asthma is a reversible obstructive lung disease that affects more than 300 million people worldwide.
Asthma is caused by increased hypersensitivity of the airways to various stimuli. It is a chronic inflammatory condition with acute exacerbations and can be life-threatening if not properly managed. B cells get expanded and recruited to the lungs of chronically asthmatic mice. This recruitment of B cells to the lungs was hypothesized to be due to components of the lung extracellular matrix called low molecular mass hyaluronic acid (LMM HA). Upon binding LMM HA, B cells secret immunosuppressive chemicals (IL-10 and TGF-β1). These cytokines are needed to resolve inflammation and remodel asthmatic lungs. CD44 was shown to be important for B cell LMM HA recruitment and IL-10 production. However, the identity of the HA binding receptor needed for production of TGF-β1 and whether LMM HA induces differentiation and/or expansion of a suppressive subset of B cells during allergic asthma are unknown. We hypothesized that an HA binding receptor, other than CD44, was required for HA-induced TGF-β1 production by B cells. We also hypothesized that B cells from allergic asthmatic mice differentiate into an inflammatory suppressive subset of B lymphocytes known as regulatory B cells (CD19+CD5+CD1dhi B cells) upon binding LMM HA. Using a mouse model that closely mimics human asthma developed by the Schuh research group here at NDSU, we characterized the expression of HA binding receptors on B cells during allergic asthma and induction of regulatory B cells in response to HA treatment. Evidence collected will help in understanding the role of B cells in the microenvironment of asthmatic lung in response to increase LMM HA, with the goal of developing new therapeutic targets against asthma in the future.

**Organization of the dissertation**

This dissertation has been organized to provide the reader with background information to understand the rationale motivating the collection of the presented research. It starts by providing literature review intended to provide a sufficient overview of the immune system,
including the immune cells, and their responses. The reader is also introduced to a focused description of the immunoregulatory role of VIP and its receptors. The subsequent chapters after that provide an account of the research successfully accomplished in each individual research project. In conclusion, a general discussion will follow each result section complete with an integration of how our reported discoveries match the literature as well as and future directions intended to expand on these results.

References


84. Martin, C., Al-Qaoud, K. M., Ungeheuer, M. N., Paehle, K., Vuong, P. N., Bain, O., Fleischer, B., and Hoerauf, A. (2000) IL-5 is essential for vaccine-induced protection and


122. Barnes, P. J. (2009) Intrinsic asthma: not so different from allergic asthma but driven by superantigens?, *Clin Exp Allergy* 39, 1145-1151.


CHAPTER TWO: EGFR SIGNALING IS REQUIRED FOR VIP-INDUCED NAÏVE MOUSE CD4 T CELL MIGRATION

Introduction

Over half a century ago Gowans and colleagues \(^1-5\) made pioneering observations regarding the central contributions of immune cell migration to the regulation of mammalian immunity. Gowans and colleagues showed that T lymphocytes recirculated from blood to lymph by migrating across specialized high endothelial venules of lymph organs. Hence, by showing that lymphocytes recirculated from blood to lymph, they solved the mystery of the “disappearing lymphocytes”\(^2\). These authors showed that lymphocytes did not disappear at all, but rather trafficked into secondary lymphatic organs only to re-enter the blood. Contrary to Gowan’s studies on immune cell movement, there was little evidence in early 1960s implicating lymphocytes as possessing immunological function\(^1,6\). Distinct migratory paths for naïve T cells, and other immune cells, such as B cells and antigen-presenting cells (APCs) to secondary lymphoid organs and non-immune tissues such as mucosal tissues and lungs have been elucidated\(^7-14\). Naïve T cells are quiescent and non-dividing since they have never encountered foreign antigens. The movement of lymphocytes between various lymphoid organs and the blood is called lymphocyte recirculation, whereas the process by which particular subsets of lymphocytes selectively enter some tissues but not others is called lymphocyte homing\(^15\).

Immune cell migration from their sites of development through the blood stream into the appropriate secondary lymphoid organs (SLOs) such as lymph nodes and Peyer’s patches require specialized endothelial interfaces known as high endothelial venules (HEVs). HEVs are found at the entrance of all secondary lymph organs except the spleen. The primary step in leukocyte migration is the establishment of weak and transient adhesive interactions between leukocytes
(white blood cells) and the endothelial cells of post capillary venular walls in close proximity to secondary lymphoid organs or inflamed tissues. Leukocytes are very sensitive and can sense as little as a 1% difference in chemoattractants or chemorepellants concentration across their diameter, resulting in a steady movement towards or away from their sources.

Establishment of weak and transient adhesive interactions mediated by selectins, is facilitated by stimulation of leukocytes when chemoattractants (e.g., VIP) are displayed on the luminal side of blood vessels and bind to its receptors on the leukocyte (e.g., T cell). This alters integrin from low- binding affinity to high-binding affinity resulting in firm adhesion to endothelium at highest chemoattractant concentration and subsequent transmigration of cells through the high endothelial venules out of the blood vascular as depicted in Figure 2-1.

**Figure 2-1. Depiction of steps of leukocyte transmigration in response to chemoattractant.** Leukocytes migration towards a chemoattractant involves 5 major steps (1) cells come to a slow stop and (2-3) start rolling upon binding of cell adhesion molecules expressed by endothelial cells of the high endothelial venules. (3) At this point, the chemoattractant released activate the integrins on the rolling leukocytes to switch convert from a low-affinity to a high-affinity state. (4) This causes firm adhesion to the high endothelial venules followed by (5) transmigration out of the blood.
Migration of immune cells into SLOs is needed for the formation and maintenance of an organized secondary lymphoid tissue compartment\textsuperscript{19-21}. Once there, lymphocytes receive soluble signals such as cytokines for long-term survival and homeostatic proliferation\textsuperscript{22, 23}. The net flux of lymphocytes through lymph nodes is very high, and it has been estimated that approximately $2.5 \times 10^{10}$ cells pass through lymph nodes each day (i.e. each lymphocyte goes through a node once a day on average)\textsuperscript{24, 25}. Antigens are concentrated in the lymph nodes and spleen, where they are presented by mature dendritic cells, the APCs that are best able to initiate responses to naïve T cells. Thus, movement of naïve T cells through the lymph nodes and spleen ensures that maximum stochastic probabilities of productive recognition of an antigen which would result in an efficient peripheral immune response is achieved\textsuperscript{26}. Furthermore, during tissue injury and infection, the circulating blood leukocytes are required to migrate to immune foci with the aim of eliminating the primary pathogen and to help in tissue repair\textsuperscript{27}. Therefore, the encounter of antigen-loaded APC by naïve T cells in lymph tissue is arguably the central event leading to a successful adaptive immune response. Failure of T cell/APC encounter would jeopardize the ability for a host to clear infecting microbes. Hence, determination and characterization of molecular mechanisms that guide APCs and T cells to the appropriate microenvironments to interact is critical for understanding how the immune system initiates activation.

In the past few years, experimental evidence has accumulated demonstrating that chemokines and their receptors are key elements that direct lymphocytes and APCs to distinct anatomical areas in the secondary lymphoid organs\textsuperscript{16}. These ligands, for example CCL21 (also known as secondary lymphoid-tissue chemokine or SLC) act upon their GPCRs such as the C-C chemokine receptor 7 (CCR7) to achieve the regulated homing and retention of lymphocytes in their respective anatomical areas of lymph nodes \textsuperscript{28}. By doing so, chemokines and their cognate
GPCRs have emerged as major molecular regulators of naïve T cell entry into lymph nodes from the blood stream. Chemokines acting as chemo-attractants or chemo-repellants through their GPCRs also act as key mediators of leukocyte chemotaxis to inflammatory sites during infectious diseases\textsuperscript{29}, asthma\textsuperscript{30, 31}, rheumatoid arthritis\textsuperscript{32, 33}, and inflammatory bowel disease\textsuperscript{34}. In fact, GPCRs comprise the largest (~50%) group of cell-surface receptors in the human/mouse genome\textsuperscript{35}.

GPCRs are made up of seven transmembrane domains with an extracellular amino terminus and an intracellular carboxy terminus\textsuperscript{36}. They lack intrinsic enzymatic activity therefore differ from tyrosine kinase receptors like epidermal growth factor receptor. Instead, they are coupled to heterotrimeric G proteins that propagate ligand functions. Upon binding their ligands at their N-terminus, GPCRs undergo conformational changes in their transmembrane alpha helices which results in dissociation of GTP-bound α subunit (G\(_{\alpha}\)) from the βγ complex (G\(_{\beta\gamma}\))\textsuperscript{37}. Subsequently, the active form, GTP-bound α subunit (G\(_{\alpha}\)) and the βγ complex (G\(_{\beta\gamma}\)) initiate intracellular signaling responses by modulating the activity of specific effectors including adenylate cyclase (AC), phospholipase C\(_{\beta}\) and a number of other chemotactic pathway kinases resulting in generation of intracellular second messengers that enable cross-talk between different signaling pathways that control cellular functions such as motility\textsuperscript{37}.

Cross-talk between different signaling systems plays a key role to coordinate the plethora of extracellular stimuli to which a cell is subjected to under physiological conditions. For example, GPCRs can utilize receptor tyrosine kinases (RTKs) to mediate important cellular responses such as proliferation, differentiation, survival and motility\textsuperscript{38}. RTKs are primary mediators of physiological cell responses such as differentiation, survival, proliferation and motility\textsuperscript{39-41}. A classic example of the RTK family is epidermal growth factor receptor (EGFR).
EGFR is one of the most studied tyrosine kinase receptors involved in the regulation of cell proliferation, differentiation and migration both in normal and cancer cells\textsuperscript{42-44}. When EGFR ligands bind to its extracellular domain they induce dimerization of the receptor. This leads to autophosphorylation of tyrosine residues within the cytosolic domain of EGFR resulting in the formation of phosphor-tyrosine binding (PTB) domains\textsuperscript{45,46} for docking of adaptor proteins that contain SH2 domains capable of recognizing PTB domains. This then initiates downstream signaling responses; for example, cell migration\textsuperscript{47,48} and angiogenesis\textsuperscript{49}. Autophosphorylation of tyrosine residues within the cytosolic domains of EGFR is called transactivation\textsuperscript{50}. EGFR activation was determined to be required for the influx of a subset of T cells (CD4 T cells) to the lungs of asthmatic mice, illustrating a role of EGFR signaling in T cell migration during asthma\textsuperscript{51} and in cancerous cell metastasis to the colon\textsuperscript{40}. Whether GPCR-induced EGFR signaling is required for T cell recruitment in non-inflammatory conditions or during inflammatory conditions to the gut is still not known.

Transactivation of EGFR by GPCR signaling can occur through two mechanisms. In ligand-dependent triple-membrane-passing-signal (TMPS) mechanism, GPCR-mediated EGFR transactivation depends on activation of membrane-bound metalloproteases (MMPs), like \textbf{A Disintegrin And Metalloprotease} (ADAM) family members\textsuperscript{50,52,53}. The activated MMPs then cleave off the inactive membrane-bound pro-EGFR ligand(s) which then binds and transactivate EGFR (\textbf{Figure 2-2})\textsuperscript{50}.

The second mechanism is a ligand-independent mechanism. This mechanism requires GPCR signaling, including the src kinase pathway, phosphorylates tyrosines within the cytosolic region of the EGFR initiating its activation and downstream signaling responses\textsuperscript{54,55}.
Figure 2-2. Ligand-dependent EGFR transactivation. Agonist (A) activation of GPCR triggers different intracellular signaling mediators via activation of Gα and/or Gβγ subunits. Intracellular signaling mediators then activate metalloprotease-mediated proteolytic cleavage of a pro-EGFR ligand to generate an EGFR ligand which binds and transactivate EGFR.

VIP is a 28-amino acid peptide with structural similarities to other gastrointestinal hormones such as; secretin, gastric inhibitory peptide, glucagon, glucagon-like peptide 1, calcitonin peptide histidine methionine, growth hormone releasing hormone, helodermin and PACAP56-59. The biological functions of VIP are mediated mainly by two structurally similar receptors called VPAC-1 and VPAC-260,61. The VIP receptors belong to the class II of GPCRs known as the secretin receptor super family. VPAC1 and VPAC2 bind VIP with equal affinity and are encoded by separate genes containing 13 and 16 exons respectively60. VIP binding to its receptors evokes three major signaling pathways: Gαs/cyclic AMP/Protein Kinase A, Gq/Phospholipase C/Ca^{2+} and Phospholipase D activation, which can lead to RTK transactivation62-64.
The mammalian gut harbors almost 100 trillion microorganisms composed of more than 1000 different bacterial species as determined by high-throughput microbial 16S ribosomal RNA gene characterization. It is therefore very important that immune cells traffic to the gut to establish an immune balance with commensal gut bacteria that are essential for nutrient absorption by the host while keeping any potentially harmful pathogens at bay. During intestinal inflammation as seen in inflammatory bowel disease (IBD) or in experimental colitis, this selective tolerance is lost. Currently, an area of intense research is to understand the molecular mechanisms that control the homing of immune cells such as CD4 T cells to the gut in hope of developing drugs to fight gastrointestinal inflammatory diseases, immunodeficiency disorders and to understand gut immunity better.

In the adaptive immunity, T cells are a major target for VIP regulation. T cells predominantly express VPAC1 with low levels of VPAC2 expression (100-1000X less at mRNA level). Hence, VPAC1 is probably the main mediator of VIP’s biological effects on naïve CD4 T cells. VIP inhibits CD4 T cell activation and proliferation, enhances naïve CD4 T cell differentiation to Th2 effectors, favors Th2 cytokines secretion and is a potent chemoattractant to resting CD4 T cells.

In the gastrointestinal tract VIP is secreted by a dense network of VIPergic nerves in a subset of innervating myenteric neurons with nerve endings in close proximity to smooth muscles. VIPergic nerve fibers are also present in both central (thymus/bone marrow) and peripheral lymphoid organs. VIP is an important chemokine that is involved in recruitment of resting CD4 T cells to the gut. Nearly 30 years ago, Cliff Ottaway showed that VIP has a striking capacity to recruit T cells to Peyer’s patches and mesenteric lymph nodes (MLN) within the gastrointestinal tissue, but not to the spleen and other lymph tissues. Preincubation of T cells
in the presence of VIP resulted in a decrease in available VIP binding sites without altering binding affinity for VIP\textsuperscript{70}. VIP pretreated resting CD4 T cells were radio-labelled with $^{51}$Cr and injected into recipient mice. Results from this study clearly showed a failure of resting T cells to migrate to the PP and MLN, but did not affect their trafficking to the spleen and other lymphoid tissues\textsuperscript{70}. This was attributed to lack of VIP receptors presented on their plasma membranes, which hindered their ability to bind to HEV and subsequent homing to PP and MLN. Since then, several additional studies implicate VIP as a potent chemoattractant for many immune cells\textsuperscript{69, 74-78}. Despite the numerous studies in support of VIP as a potent chemoattractant to immune cells including T cells, the molecular mechanism of VIP-induced T cell migration remains elusive. This is critical in identifying the potential molecular mechanism that regulates VIP-induced recruitment of resting T cells to the PP and MLN in the gut where they can be presented with foreign antigens by APCs. This would also further our understanding of how the immune system works.

The VIP-induced chemotaxis of immune cells is sensitive to tyrosine kinase inhibition\textsuperscript{78} and through VPAC1, VIP rapidly autophosphorylates EGFR and human epidermal growth factor 2 (HER2) and slowly upregulates EGFR protein expression in human breast cancer cells\textsuperscript{79} to provide precedence for a connection between VIP and EGFR signaling. Interestingly, both VIP receptors and the epidermal growth factor receptor are known to enhance cell proliferation, survival, migration, adhesion and differentiation in cancer cell lines\textsuperscript{40, 57}.

Our group has published a mouse VIP-induced transcriptome in resting CD4 T cells which revealed that VIP treatment of resting CD4+ T cells results in differential mRNA upregulation of genes whose translational products are involved in biological pathways such as lipid metabolism, molecular transport, cell cycle regulation and cellular movement\textsuperscript{68}. This latter
cellular affect supports the fact that VIP is a potent chemoattractant to T cells. To further expand this study, we set to understand the role of EGFR in VIP-induced chemotaxis of resting murine CD4 T cells. Since both VIP and EGFR signaling can induce cell movement, we hypothesized that VIP signaling through its G protein coupled receptor, VPAC1, requires EGFR signaling to induce resting CD4 T cell migration. To test this hypothesis, our first goal was to determine the effect of EGFR kinase activity inhibition on VIP-induced chemoattraction of resting CD4 T cells by pharmacological inhibitor studies. Using a modified Boyden-chamber / transwell chemotaxis assay, we investigated the effect of a potent and highly specific EGFR kinase activity inhibitor (AG-1478) on VIP chemoattraction of resting CD4 T cells. Secondly, since we had previously shown that VIP upregulated EGFR mRNA, we set to characterize the VIP-induced mRNA upregulation of putative chemotactic EGFR pathway genes comprised of genes encoding EGFR, App, Adam15, Grb7, Pak1 and Snail. We next asked whether EGFR kinase activity was also needed for gene regulation of the putative chemotactic EGFR pathway genes. To answer these questions, murine CD4 T cells were isolated and used to investigate whether the putative chemotactic EGFR pathway genes were expressed prior to encountering VIP. In addition, a 24 hour kinetic study of VIP-induced EGFR chemotactic pathway genes was completed using SYBR green qRT-PCR assay to determine the speed, co-ordination and longevity of mRNA regulation by VIP. Finally, the necessity of EGFR signaling for VIP-induced transcriptionally upregulation of the putative chemotactic EGFR genes was also done by way of SYBR green qRT-PCR assay.

This analysis revealed that inhibition of EGFR kinase activity abolished VIP chemoattraction of CD4 T cells suggesting the necessity for EGFR signaling in VIP-induced CD4 T cell chemotaxis. CD4 T cells express mRNA of genes encoding proteins in the putative
chemotactic EGFR pathway prior to VIP treatment. We showed that VIP rapidly, coordinately and transiently upregulated six genes encoding a putative chemotactic EGFR pathway in resting CD4 T cells. This upregulation of the putative chemotactic EGFR pathway genes was also sensitive to a potent and specific EGFR kinase inhibitor (AG-1478). Thus, these findings offer a potential molecular mechanism for VIP-induced resting CD4 T cell chemoattraction in vivo.

Materials and methods

Mice

Wild-type C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME) and bred in a mouse facility (ANPC) at North Dakota State University. Mice were housed in ventilated cages. Mouse cages, water bottles and lids were purchased from Jackson labs. Mice food was purchased from animal supply and the mice were fed the Purina mouse chow (ckrs-5015) (Animal Supply Company; MN). Mice had food and water ad lib. They were bred at no more than 4 males or 5 females per cage. All mouse protocols were approved by the NDSU IACUC board and met all federal guidelines.

Splenocytes isolation

For all studies we used male or female mice between 8- to 15 weeks old. Mice were euthanized by CO₂ narcotization followed by rapid cervical dislocation. The following steps were performed under a clean UV irradiated PCR quality hood (VWR; Radnor, PA): Scissors and forceps were sterilized with 70% ethanol. The mouse in a supine position was rinsed thoroughly with 70% ethanol on a dissecting tray as a first line of disinfection. An incision of the abdomen was made by a transversal cut of the skin while avoiding opening of the peritoneal cavity. The peritoneal sac was completely exposed by pulling the skin in opposite directions using gloved hands or forceps. At this point, the spleen became visible on the left side of the
mouse. A second cut was made to open the peritoneal sac and the spleen was collected with sterile forceps avoiding contact with the skin to preserve sterility. Any visible residual debris or fat tissue was removed from the spleens which were then placed in 30 ml of RPMI supplemented with 10% characterized fetal bovine serum. Spleens were transferred into a 10 cm Petri dish containing 20 ml of RPMI growth media. The spleens were minced on a metal mesh and passed through a 40 µm sieve to disperse splenocytes into single cells using 50 ml centrifuge tube for collection. A homogenous cell suspension was obtained by inverting the tube 5 times. Cells were centrifuged at 300xg for 10 min at 4 °C in order to pellet them. Red blood cells (RBCs) were lysed by resuspending cell pellets in 5 ml of RBC lysis buffer at RT for 3 minutes. Immediately afterwards, cells were diluted with 47 ml of 1X PBS to neutralize the RBC lysis buffer. Cells were centrifuged at 400xg for 10 min at 4 °C to and resuspended in 10 ml of RPMI growth media. The successful lysis of red cells was inspected by the appearance of a whitish cell pellet. Splenocytes were counted by a hemocytometer by pipetting 10 µl of 0.4% trypan blue stained cell suspension between the chamber and the cover glass slide. All the cells contained in the four outer quadrants of the counting grid printed on the hemocytometer surface were counted using light microscopy. The number obtained was divided by 4 to determine the average number of cells per quadrant. Total cells were then calculated by following the formula below.

\[
\text{Total cells/ml} = \frac{\text{Total cells per quadrant} \times \text{dilution factor} \times 10,000 \text{ cells/ml}}{}
\]

*Primary mouse splenic CD4 T cell isolation and enrichment*

To remove adherent splenocytes such as macrophages and dendritic cells, single cell suspensions of splenocytes were cultured in complete RPMI growth media and incubated for 45 minutes at 37°C, 5% CO₂/95% air in a humidified incubator. Non-adherent splenocytes were collected and passed through a 40 µm sieve, washed with 1 X PBS, centrifuged as above and
resuspended in 90 µl of PBS/0.5% BSA (bovine serum albumin) with 10 µl of anti-mouse CD4 magnetic beads / 10^7 nonadherent cells and refrigerated at 4°C for 20 min. Mouse splenic CD4 T cells were positively purified to a minimum purity of ≥95% by passing cells through a metallic micro bead column bearing anti-CD4 monoclonal antibodies using an AutoMacs cell isolation instrument. For some studies, mouse CD4 T cells were negatively isolated using EasySep™ Mouse Naïve CD4+ T Cell Isolation Kit. Briefly, total splenocytes including RBCs were prepared at a concentration of 1 x 10^8 cells/mL in phosphate-buffered saline (PBS) or Hank’s balanced salt solution (HBSS) plus 2% fetal bovine serum (FBS). Cells were placed in a 5 mL (12 x 75 mm) polystyrene tube to properly fit into the EasySep™ Magnet. Normal Rat Serum and EasySep™ Mouse CD4+ T Cell Isolation Cocktail were added at 50 µl /mL of cell suspension, mixed well then incubated at room temperature (15 - 25 °C) for 15 minutes. EasySep™ Streptavidin RapidSpheres™ 50001 were vortexed for 30 seconds and added at 75 µL/mL of cell suspension. Thoroughly mixed cells were incubated at room temperature (15 - 25°C) for 3 min. Cells were diluted up to 2.5 mL total volume with suspension medium and gently mixed by pipetting up and down 2 - 3 times. Tubes were placed into the EasySep™ magnet, incubated at room temperature (15 - 25°C) for 3 minutes and inverted to pour off supanatant (CD4 T cells) into a polystyrene tube. The magnetically labeled, unwanted cells remained bound inside the original tube held by the magnetic field of the EasySep™ Magnet. CD4 T cell yield was determined as described previously using a hemocytometer.

**Determination of percent CD4 T cell enrichment by flow cytometry**

To determine percent purity, 1.0 x 10^6 post-enrichment CD4 T cells were suspended in 200 µL 1 X PBS/0.5% BSA and incubated with 1:200 (0.25 µg) FITC conjugated rat anti-mouse CD4 antibody for 30 min at 4°C in the dark. Cells were washed twice with 4 ml PBS/ 0.5%
BSA, centrifuged for 10 min at 300xg, 4°C and resuspended in 200 µl PBS/0.5% BSA. Flow cytometry analysis was performed by analyzing for percentage of CD4 positive cells on an Accuri 6 flow cytometer. Both positive (Miltenyi) and negative (EasySep) CD4 T cell purification techniques consistently yielded ≥95% splenic mouse CD4 T cells.

*Primary mouse splenic CD4 T cell in vitro culture with VIP*

Highly purified resting CD4 T cells were cultured at 2 X 10⁶ cells/mL of RPMI complete growth media for 1 hour under 5% CO₂, 85% humidity air-controlled atmosphere at 37°C to allow them to “equilibrate” to the media conditions. After that, the following studies were performed; a) Kinetics: In order to understand the kinetics and peak time of VIP induced EGFR pathway genes CD4 T cells were treated with either water or 10⁻⁷M VIP for indicated times in 6 well plates. b) Optimal VIP concentration determination: To determine the optimal VIP concentration that would induce highest steady state mRNA upregulation, cells were exposed to various VIP concentrations ranging from 10⁻¹¹ to 10⁻⁶M or water control for 1.5 hours. c) Effects of AG1478: To determine effects of AG 1478 on VIP-induced steady state mRNA upregulation, VIP [10⁻⁷] was exogenously added with or without AG-1478 EGFR kinase inhibitor [30 nM]. After appropriate incubation times, cells were harvested, washed twice with ice cold 1X PBS, pH 7.0 and centrifuged at 300xg at 4°C for 5 minutes. Cell pellets were immediately lysed and applied to Qiashredder (Qiagen) to shear genomic DNA. Total RNA was either immediately isolated (see below) or lysates stored immediately at -80°C processed when needed.

*Total RNA isolation, first strand cDNA synthesis and quantitative RT-PCR (qRT-PCR) analysis*

Cells were washed twice with PBS, centrifuged at 300xg at 4°C for 5 minutes and total RNA isolated using SurePrep™ TrueTotal™ RNA Purification Kit purification columns as
described by the manufacturer but with a few modifications. RNA was eluted in 150 µl elution buffer at 42°C instead of 50 µl at room temperature. On-column DNase I treatment was done using DNase I protocol (Qiagen, Valencia, CA) followed by a second off-column DNase I treatment at 37 °C for 45 minutes. Briefly, to each total RNA elution, 1 µl of DNase I, 6 µl of 10X DNase I buffer (Promega, Madison, WI) and 3 µl of nuclease free water was added and incubated for 45 minutes at 37 °C. RNA samples were concentrated by ethanol precipitation by the addition of 2.5 volumes of 100% ethanol and a 1/10 dilution of 3 M sodium acetate solution, pH 5.2. Samples were stored at -80°C for at least 2 hours, centrifuged at 16,000xg at 4°C for 15 min. Samples were washed with 1 ml nuclease free 70% ethanol, centrifuged as before and air dried for 10 minutes at room temperature and reconstituted in 20 µl nuclease-free water at 70°C. Total RNA concentration was determined by ultraviolet spectroscopy and using 260 nm as the absorbing wavelength for RNA and 280 nm for protein contamination. RNA purity was determined spectrophotometrically by calculating absorbance ratio (A_{260/280}) where a value of 1.8-2.0 was deemed suitable for cDNA synthesis.

First-strand cDNA was synthesized by reverse transcription of 1 µg total RNA with either superscript II reverse transcriptase (Life technologies, Grand Island, NY) or M-MLV Reverse Transcriptase RNase H- (New England Biosciences, Ipswich, MA) with a mixture of random hexamers and oligodT primer mixes according to the manufacturer’s protocol. Amplifications by qRT-PCR contained 2.5 µl of cDNA template with 5 µl of master mix containing 5X HOT FIREPol EvaGreen qPCR supermix (Solis Biodyne, Tartu, Estonia), 250 nM of respective sense and antisense primer sets (table 1) and nuclease free water to bring to 20 µl total volume. Reactions for all primer sets were conducted with nuclease-free water alone, and in the absence of reverse transcriptase (RT-) to ensure ≥ 6 cycle thresholds between RT+ and RT- reactions.
This would verify ≤1.6% genomic DNA contamination in reactions as described by Applied Biosystems (ABI, Carlsbad, CA). The qRT-PCR reaction was conducted using a 7500 ABI instrument with the following parameters: 15 min at 95°C to activate the Taq polymerase, followed by 40 cycles of 15 s at 95°C and 30 s at 60°C (data collected at this stage) followed by a dissociation curve option to monitor the melting curve of each amplicon. Cycle thresholds (ΔC<sub>T</sub>) were used for relative steady-state quantification of gene transcription normalized to β-actin. Fold changes of mRNA levels in VIP or VIP and AG-1478 treated cells relative to respective water/ DMSO controls were calculated using the 2<sup>ΔΔCt</sup> method. Data are averages from at least three independent experiments fold changes ± SEM.
Table 2-1: Oligonucleotide primers used in SYBR Green qRT-PCR analysis

<table>
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<th>mRNA</th>
<th>PRIMER</th>
<th>Ref Seq. #</th>
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</thead>
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<td>NM_001198825</td>
</tr>
<tr>
<td></td>
<td>antisense CTT CCT GTT CCA GAG ATT CCA C</td>
<td>NM_001198825</td>
</tr>
<tr>
<td>adam15</td>
<td>Sense GCC GCT GCC AAA TAT AGG A</td>
<td>NM_001037722</td>
</tr>
<tr>
<td></td>
<td>antisense CCT CAG GTA AAC CAG TCT GAA G</td>
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</tr>
<tr>
<td>egfr</td>
<td>Sense CCT TCA CAT CCT GCC AGT G</td>
<td>NM_207655</td>
</tr>
<tr>
<td></td>
<td>antisense CAG TCC AGT TAT CAG GCC AAG</td>
<td>NM_207655</td>
</tr>
<tr>
<td>grb7</td>
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<td>NM_010346</td>
</tr>
<tr>
<td></td>
<td>antisense GCA ACC TTC ATC TTC GCT TG</td>
<td>NM_010346</td>
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</tr>
<tr>
<td>snail</td>
<td>Sense GTCAGCAAAAGCAGGGTTG</td>
<td>NM_011427</td>
</tr>
<tr>
<td></td>
<td>antisense CTTGCTCGACGACCT</td>
<td>NM_011427</td>
</tr>
<tr>
<td>β-actin</td>
<td>Sense TGTCACCTCCAGCAGATGT</td>
<td>NM_007393</td>
</tr>
<tr>
<td></td>
<td>antisense AGCTCAAGTAGTCGCCCTAGA</td>
<td>NM_007393</td>
</tr>
</tbody>
</table>

Chemotaxis assay

A Transwell chemotaxis system (Corning, NY, USA) was used to evaluate cell migration. The upper and lower chambers were separated by a polycarbonate membrane with pores of 5 μm diameter and were coated with collagen IV (Tocris, Minneapolis, MN, USA). Approximately $5 \times 10^4$ purified CD4 T cells suspended in 100 μl serum-free medium (RPMI-1640) were seeded onto the upper chamber, and 600 μL of serum free RPMI-1640 medium with
either water, water and DMSO, VIP \([10^8M]\), VIP \([10^8M]\) plus 30 nM AG1478 or 30 nM AG1478 only was added to the lower chamber. After 4 hours of incubation at 37°C with 5% CO₂, the medium was aspirated from the upper chamber. The non-migrated cells on the upper side of the chamber were gently scraped off with a cotton swab and washed twice with 1 X PBS. Cells on the underside of the membrane were fixed with glutaraldehyde, stained with crystal violet and counted by light microscopy. The migration activity of CD4 T cells was determined by counting number of cells per high powered field from five random fields at x 200 magnification. Fold increase of migrating cells was calculated by dividing average total number of cells from each unknown by total number of cells migrating towards water control. Each assay was repeated two times with each experiment having 3 replicates per treatment. Results plotted are average fold changes ± SEM.

Statistical analysis

Data are presented as the average plus or minus standard error of the mean (SEM). Differences between two groups were compared by student t test and considered significant at a P value of less than 0.05 or as stated. Differences between multiple groups were evaluated by ANOVA.

Results

**VIP chemoattractant activity on resting murine splenic CD4 T cells is sensitive to EGFR kinase inhibitors**

Several reports have demonstrated that VIP is a potent chemoattractant to numerous immune cells\(^{57, 69, 70}\) including resting CD4 T cells homing into PP and MLN\(^{70}\). However, a critical gap in the knowledge base exists regarding the molecular mechanism controlling VIP-induced CD4 T cell migration. We have presented evidence indicating that VIP upregulates
mRNA levels of genes encoding proteins that make up a putative chemotactic EGFR pathway in resting CD4 T cells. In addition, VIP has the capability of impinging on EGFR signaling by rapidly transactivating EGFR, but slowly increasing its protein expression in human breast cancer cells. Here, we set to determine the molecular mechanism governing the VIP-induced T cell migration. We hypothesized that EGFR signaling is required for resting CD4 T cell migration towards VIP.

As this study aimed to determine the significance of EGFR signaling in primary resting CD4 T cells, isolated these cells from harvested spleens from 8-15 weeks old mice and performed magnetic bead chromatography to obtain highly purified (≥ 95%) T cells as determined by flow cytometry (Figure 2-3A). Because strong adherence of naïve T cells to a surface such as high endothelial venules is a required first step during T cell migration, we postulated that it was reasonable to assume that the optimal VIP concentration that induced the highest percentage of adherence would theoretically induce the highest rate of CD4 T cell migration towards VIP. Using a collagen IV coated transwell assay, different VIP concentrations (10^{-11} through 10^{-6} M) or water control were used to treat highly purified CD4 T cells. Percent adhesion to collagen IV was measured in comparison to water control which was arbitrarily set to 1 % (Figures. 2-3 A and B). All concentrations tested in this experiment induced significant induction of CD4 T cell adhesion to collagen IV with VIP 10^{-8} M resulting in the highest amount of CD4 T cell adhesion.

To evaluate the importance of EGFR signaling in VIP-induced T cell migration, optimal VIP concentration that induced the maximum adhesion to collagen IV (10^{-8} M) was used in a modified Boyden Chamber / transwell assay. We tested whether an EGFR kinase inhibitor, AG-1478, affected the extent of chemotaxis of CD4 T cells to VIP. Inhibition of EGFR kinase
activity led to a significant decrease in VIP-induced CD4 T cell migration (Figure 2-3B). These data strongly support that EGFR kinase activity was necessary for VIP-induced migration of CD4 T cells. From this, we further concluded that VIP’s chemotactic activity is upstream from EGFR signaling, which for the first time sheds important light on this molecular mechanism.

Resting mouse splenic CD4 T cells endogenously express EGFR pathway mRNA at varying levels prior to VIP treatment.

As VIP appears to require EGFR kinase activity to enable resting CD4 T cells to migrate towards the source of VIP, we tested whether VIP treatment of resting CD4 T cells upregulates the mRNA of genes involved in a known putative chemotactic pathway initiated by EGFR signaling. To bolster the role of EGFR signaling in VIP-induced CD4 T cells migration, we first investigated whether or not resting CD4 T cells prior to VIP treatment at the mRNA level endogenously expressed genes involved in the EGFR pathway known to affect survival, proliferation and chemotaxis. The mRNA expression of a subset of 6 genes including EGFR highlighted as a putative chemotactic pathway hypothesized to explain the molecular mechanism of the chemo attractive activity of VIP on T cells was determined. Using magnetic bead technology highly pure (≥95%) resting mouse CD4 T cells were enriched. Basal expression levels of the subset of 6 genes were then measured by qRT-PCR assay normalized to β-Actin (materials and methods). Our comparison showed that resting mouse CD4 T cells endogenously express all the predicted genes involved in the putative EGFR-centered chemotactic pathway at different levels. The relative expression range was over 96-fold with \( \text{app} > \text{pak1} > \text{snail1} > \text{grb7} > \text{egfr} \geq \text{adam15} \) (Figure 2-4). These results demonstrated that resting primary mouse splenic CD4 T cells differentially express all measured EGFR pathway genes known to code for proteins involved in chemotaxis at varying levels prior to VIP.
treatment suggestive of a pathway already genetically imprinted in these cells ready to be turned on given the right signaling cues.

Figure 2-3. VIP induced CD4 T cell chemotaxis is dependent on Epidermal growth factor receptor signaling. A) Highly purified mouse splenic CD4 T cells were purified by magnetic bead technology; used for (B) adhesion assay and (C) chemotaxis analyses. Purified CD4 T cells were incubated on collagen IV coated plates and exposed to various VIP concentrations as indicated for 2 hours at 37°C. The extent of adhesion was assessed as the mean fluorescence emitted by Calcein AM stained cells and percentages compared to water control. (C) CD4 T cells were placed on transwell with either VIP [100nM] ± AG1478 [30nM] or relevant controls as indicated. Number of migrating cells was determined microscopically by counting 10 random fields (x20) of stained cells and fold-change over controls determined by dividing average number of cells per high powered field by that of water control. Results show average fold changes ± SEM from 2 independent experiments (p-value< 0.01).
Figure 2-4. Chemotactically relevant genes within an EGFR pathway are detectable, but differentially expressed in resting primary CD4 T cells. (A) Non-adherent splenocytes were stained with rat anti-mouse CD4 FITC antibody (materials and methods) prior to and post magnetic anti-CD4 bead chromatography technology. Side/forward scatter was initially used to gate splenocyte population for CD4 purification determination. (B) qRT-PCR analysis of EGFR, Adam15, App, Grb7, Pak1 and Snail1 normalized to β-Actin mRNA was carried out with total RNA isolated from freshly purified CD4 T cells. (C) Proposed model of EGFR pathway. Data is graphed by means ± SEM from 5 independent experiments.
VIP rapidly, coordinately, but transiently upregulates a putative chemotactic EGFR pathway in resting primary murine CD4 T cells

Coordinate upregulation by a soluble factor or environmental stimuli of a signaling pathway bolsters its biological relevance as illustrated in the coordinate upregulation of glycolytic enzymes and glucose transporters by hypoxia-induced factor-1 (HIF-1) transcription in response to hypoxia in rat brain cancer cells. In addition, it is crucial that the mRNA of most or all of the genes follow similar kinetics as evidenced in the carotenoid biosynthesis pathway. Based on these biological precedents, we measured the expression levels of all six EGFR pathway genes at various times throughout 24 hours to test whether VIP coordinately upregulated them. Also, in order to substantiate this gene expression change by VIP in chemotaxis of T cells, we need to show that they are upregulated prior to when chemotaxis occurs. To collect this data, resting CD4 T cells were culture plus or minus VIP as indicated, cells lysed and total RNA isolated followed by first strand cDNA synthesis. Measurement of relative expression normalized to β-actin was performed by SYBR-green qRT-PCR. Analysis by quantitative RT-PCR (qRT-PCR) showed a rapid and coordinate upregulation of all genes as early as after 1.5 hours post-VIP additions. These changes were transient as their levels uniformly were back down to basal levels by 24 hours post VIP treatment (Figure 2-5).
VIP rapidly, coordinately but transiently upregulates steady state mRNA levels of all putative chemotactic EGFR pathway genes. Purified splenic CD4 T cells (>95%) were treated with or without VIP [10^{-7} M] over a 24 hour period. Total RNA was extracted and gene expression measured by qRT-PCR SYBR green analysis. Data is presented as mean ± SEM normalized to β-actin from 3 independent experiments (*p-value ≤0.05).

Grouping of the VIP-induced putative chemotactic EGFR pathway genes and determination of optimal VIP concentration.

Upon further analysis of the kinetics of VIP transcriptional regulation of the EGFR pathway genes, we grouped the putative chemotactic EGFR genes into three groups according to their kinetic fold-changes over a 24 hour VIP exposure. Group 1 was made up of genes that all were rapidly and coordinately upregulated after 1.5 hours with a quick return to basal levels immediately thereafter. These genes were EGFR, App and Grb7. The second group only had the snail1 gene. In this group, snail was also coordinately upregulated as the other genes after 1.5 hours upon VIP treatment of the CD4 T cells, but its levels persisted longer than group 1 genes.
before returning to basal levels. The third group, made up of Adam15 and Pak1, involved genes that had the highest fold-induction after VIP treatment. They were also coordinately upregulated with all the other genes in the EGFR pathway after 1.5 hours, declined after 5 hours, but peaked after 10 hours post VIP treatment. All the genes in the groups returned to basal levels after 24 hours post VIP treatment (Figure 2-6).

The effect of different VIP concentrations was tested to determine one that would illicit the highest fold increase in mRNA of the EGFR pathway genes at their earliest peak time of 1.5 hours. Primary resting CD4 T cells were subjected to varying VIP concentrations and fold changes over water control of two representative EGFR pathway genes (pak1 and grb7) was conducted similarly to above. The fold changes by $10^{-7}$ M VIP resulted in the highest steady state mRNA levels of the representative genes assessed (Figure 2-6B). Importantly, this response showed a unimodal response pattern typical of GPCRs where higher concentrations of ligands result in homologous desensitization of ligand receptors, hence decrease in ligand signaling responses. From this, we concluded that resting primary murine CD4 T cells coordinately upregulate EGFR pathway genes upon VIP treatment. Furthermore, the profile of the mRNA levels mirrored each other over the different VIP concentration curve study, supporting a coordinate regulatory control with a maximum effect with $10^{-7}$ M VIP (100x its Kd).
Figure 2-6. Kinetic VIP response grouping of the EGFR pathway genes and determination of optimal VIP concentration. Purified mouse spleen CD4 T cells (>95%) were treated with or without VIP [10^{-7} M] for indicated time intervals or (B) for 1.5 hours with indicated VIP concentrations. Total RNA was extracted and gene expression measured by qRT-PCR analysis. (A) The EGFR pathway genes were grouped according to expression patterns over a 24 hour period. Group 1 peaked after 1.5 hours and returned to basal levels afterward, group 2 went up after 1.5 hours and persisted until after 5 hours before returning to basal levels and group 3 did not return to basal levels until after 10 hours.

EGFR kinase inhibitor abolishes VIP-induced coordinate upregulation of a putative chemotactic EGFR pathway.

Activation of receptor tyrosine kinases such as EGFR require the enhancement of intrinsic catalytic activity that provide new binding surfaces for the recruitment of downstream signaling proteins. Both of these processes are accomplished by auto-phosphorylation/transactivation on tyrosine residues by ligand binding or transactivation\(^{52}\). VIP signaling through VPAC1 can transactivate EGFR proteins in human breast cancer cells within minutes\(^ {79}\). To gain support for the putative EGFR pathway as the molecular mechanism by which VIP is chemotactic in resting CD4 T cells, we hypothesized that EGFR kinase activity was required for the VIP-induced EGFR pathway gene suite upregulation. Gene expression studies were repeated (materials and methods) with a highly specific and potent EGFR kinase inhibitor, tyrphostin
AG-1478 inhibition of EGFR kinase activity by tyrphostin AG-1478 resulted in complete abolishment of VIP mRNA upregulation using four representative EGFR pathway genes. For reasons we currently do not understand, tyrphostin AG-1478 inhibition of VIP-induced Snail mRNA upregulation resulted in a drastic reduction of Snail1 mRNA compared to basal levels (Figure 2-7). Overall, these results suggest that EGFR kinase activity is vital and upstream of VIP-induced coordinate gene regulation of the EGFR pathway gene-suite; and potentially other gene targets.

![Graph](image)

**Figure 2-7. Coordinate transcriptional upregulation of an EGFR-pathway mRNA is sensitive to EGFR kinase inhibitors in resting CD4 T cells.** Highly purified resting splenic mouse CD4 T cells were isolated and cultured with either vehicle control or 10^{-7} M VIP +/- 30nM AG1478 for 1.5 hours (Materials and Methods). Relative steady-state mRNA levels for four of six EGFR-pathway genes were measured by qRT-PCR analysis and normalized to β-actin. Values are shown as means ± SEM from 3 independent experiments with statistical differences determined by student t-test (* p< 0.05).

**Discussion**

Constant migration of naïve immune cells throughout the lymphatic system to patrol concentrated foreign antigens is crucial for an effective immunity. Previous studies have demonstrated that VIP can alter murine T cell homing to the gut and induces directional
migration of resting human T cells towards it in a study that was done in vitro. In this research study, we present the first potential molecular mechanism involved in VIP-induced resting murine CD4 T cell migration. The chemotaxis assay analysis used in this study allowed us to determine that inhibition of EGFR kinase activity completely abolished VIP-induced CD4 T cell migration. Therefore, for the first time, EGFR signaling has been implicated in VIP’s chemoattractant activity. More importantly, we discovered that a putative chemotactic EGFR-pathway made of egfr, app, adam15, grb7, pak1 and snail1 genes was rapidly, coordinately, but transiently upregulated at the mRNA level upon VIP treatment. The upregulation of representative EGFR pathway genes by VIP was also abolished upon pretreatment of the cells with a potent EGFR kinase inhibitor CD4 T cells. We found that resting CD4 T cells endogenously express all the six EGFR pathway genes prior to VIP treatment.

The fact that VIP is a potent chemoattractant to resting T cells has been known for nearly 30 years, but how it achieves this at the molecular level has been elusive. Currently, there is no known molecular mechanism that has been proposed to explain how VIP induces chemotaxis in T cells. Our results agree with previous studies of VIP induced T cell adhesion and migration. Here, we have expanded the previous studies by showing that EGFR signaling is required for CD4 T cells to migrate towards a source of VIP. These results are in agreement with a chemotactic study done in human cells that found that VIP chemoattraction of these cells was sensitive to tyrosine kinase inhibition. Using non-specific tyrosine inhibitors, it is possible that these authors inhibited EGFR signaling, which blocked cell migration towards VIP. Our results also demonstrated that CD4 T cell adhesion to collagen IV can be induced by VIP at a wide range of concentrations, and this was most likely homologously desensitized at higher concentrations, reminiscent of GPCR signaling. Coming to a stop before starting to migrate.
towards a chemoattractant is a critical step in T cell migration, further illustrating how potent VIP is in inducing T cell migration.

There are no previous quantitative analyses of the putative chemotactic EGFR pathway gene expression in CD4 T cells of any species. Our quantitative gene expression analysis results are the first to show that resting murine CD4 T cells endogenously and differentially express all the six genes involved in an EGFR pathway important for chemotaxis in epithelial cells. However, the importance of EGFR signaling in migration of CD4 T cells to the lungs in an asthma model has been shown previously. Here we are extending this observation illustrating the importance of EGFR signaling in resting T cell homing by demonstrating that all the key players in a putative EGFR chemotactic pathway are present at the mRNA level in resting CD4 T cells prior to activation of the pathway.

For coordinate upregulation of a signaling pathway that results in sustained biological response to occur, it is crucial that the mRNA of most or all of the genes in that pathway follow similar kinetics. In this work, the mRNA of all the molecules involved in a putative chemotactic EGFR pathway that could explain the molecular mechanism of VIP induced T cell chemotaxis were rapidly, coordinately, but transiently upregulated. It was enticing to propose that the upregulated mRNA was being translated to their respective proteins involved in CD4 T cell chemotaxis. However, further analysis indicated that the kinetics of the mRNA formation was slower than the time needed for VIP induced CD4 T cell adhesion to collagen IV. Because of this, we can conclude that VIP induced CD4 T cell chemotaxis involves two phases. The first is the rapid transactivation of the already present EGFR protein to initiate the chemotactic machinery. The second phase involves the replenishment of the proteins in the pathway through de novo synthesis as a result of the mRNA upregulation. This second phase is slower and may be
important to sustain the ongoing chemotactic machinery by increasing the protein expression of all molecules involved in the pathway. Interestingly, VIP can also significantly increase protein expression of EGFR in human breast cancer cells which gives biological precedent for this proposal.

Activation of EGFR by metalloproteases after their activation by GPCRs has been shown. The activated metalloproteases can cleave off and activate the inactive membrane-bound pro-EGFR ligand(s) which would bind and transactivate EGFR. Present results demonstrate that VIP upregulates Adam15, a member of the ADAM family. The upregulated Adam15 can further enhance EGFR transactivation by cleaving and releasing any CD4 T cell membrane bound ligands such as epidermal growth factor (EGF) or amphiregulin. Interestingly, during DSS-induced colitis in mice, VIP downregulates the activity of MMP-9, a different kind of metalloprotease. This differences might be due to the immune state in that during inactivation conditions, VIP activates metalloproteases which would facilitate T cell migration, but during inflammatory conditions, the opposite occurs to limit inflammation. Our future goal is to measure the levels of EGFR ligands in VIP treated CD4 T cell media as compared to their controls. It is also our aim to measure the ligands serum ligand levels in VIP knockout mice that we recently secured. We hypothesize that VIP knockout mice will have lower levels of EGFR ligands in the serum as compared to their wild type controls. We also plan to determine whether homing of CD4 T cells isolated from wild type mice to PP and MLNs is affected when adoptively transferred into VIP knockout and VPAC2 knockout mice.

In conclusion, we propose a potential EGFR-mediated mechanism that could contribute to the VIP-induced resting CD4 T cell homing to the gut. Although VIP is well established as a potent chemoattractant to immune cells for many years, the molecular mechanism mediating its
chemotactic effects were still largely unknown. We described the importance of EGFR signaling in mediating VIP-induced CD4 T cell migration and propose a model by which this pathway is required for T cell chemotaxis towards VIP. In our proposed hypothetical model (Figure 2-8) there is a quick EGFR transactivation by VIP/VPAC1 signaling that primes and initiates CD4 T cell chemotaxis towards a VIP source. At the same time, a slower (hours) and coordinated induction of mRNA expression of all the putative chemotactic EGFR pathway genes represents a cellular response to VIP resulting in elevation of EGFR signaling. It is therefore reasonable to envision that when the VIPergic nerves in the gut secrete this chemokine near HEV lining the vasculature, it could attract circulating resting CD4 T cells to the gut lymph tissue by activating EGFR signaling.

![Figure 2-8. Hypothetical model of a putative chemotactic EGFR pathway mediating VIP-induced CD4 T cell movement.](image)

Binding of VIP to its receptor (VPAC1) on resting CD4 T cell leads to rapid transactivation of EGFR within minutes to initiate cell migration towards a gradient source of VIP. Transactivation of EGFR induces a putative chemotactic EGFR pathway resulting in CD4 T cell movement towards source of VIP such as the gut. At the same time a rapid increase in mRNA expression of EGFR pathway genes is induced. This increased mRNA expression leads to replenishment of proteins involved in the EGFR pathway that facilitates sustainment of the chemotactic response towards source of VIP.
References


CHAPTER THREE: VPAC2 DEFICIENCY RESULTS IN IMPAIRED IMMUNE CELL HOMEOSTASIS

Introduction

Mammals possess control mechanisms capable of maintaining an internal equilibrium of biochemical, phenotypical and physiological features. Well characterized examples of internal equilibriums, in spite of changes in their environments, include near constant body temperature, blood pH, size of organs, levels of nutrients and waste products. Deviation from this internal equilibrium can have adverse effects as illustrated in the cases of metabolic acidosis which may result in cardiac arrest and/or mental disorders\textsuperscript{220, 221}. This property of a system in which variables are regulated so that internal conditions remain stable and relatively constant is known as homeostasis\textsuperscript{222}.

The immune system is no exception to this control. When a foreign antigen is recognized, the immune system becomes activated resulting in immune cell activation, proliferation, differentiation into effector cells and cytokine secretion; all of which in turn lead to increased immune cell numbers and antigen clearance. Once the foreign antigen is removed, the immune system returns to its preactivation state, to maintain a constant number of immune cells and to prevent uncontrolled inflammatory responses despite frequent stimulations by antigens. This homeostatic regulation of maintaining a constant number and ration of more than ten different immune cell phenotypes ensures that once antigens are cleared, elevated levels of activated immune cells are killed off to restore pre-infection cell numbers and to limit inflammatory diseases such as autoimmunity or immunodeficiency\textsuperscript{223}.

Immune homeostasis plays a role in shaping immune cell repertoires. For instant, as the number of T and B lymphocytes is kept constant, any newly produced cell can only survive if
another resident cell dies. Because each lymphocyte does have a different antigen binding receptor, homeostatic mechanisms cellular survival and death are crucial; it is crucial for a balanced immune system. Thus, homeostatic control of immune cell numbers provides a fundamental mechanism that shapes the immune repertoire and bolsters its capacity to respond to foreign. Immune homeostasis also enables the reestablishment of the immune system following its disruption due to irradiation and/or chemotherapy. During the process of restoration, the immune system can be reset at a new equilibrium overcoming any of its previous malfunctions. This capacity of homeostatic regulation may therefore be used in potential therapeutic strategies to radically modify lymphocyte repertoires, immune responses, autoimmune diseases, allergy and cancer treatments.

In adult mouse, despite continuous production of new cells in the bone marrow, thymus, and peripheral cell division, the number and diversity of B and T lymphocytes remains relatively constant. Homeostasis of the immune system is well established although molecules controlling it are not fully understood. Factors that affect immune cell homeostasis are those involved in their production, differentiation, activation or death (Figure 3-1).

Figure 3-1. Basic mechanisms regulating immune cell homeostasis. As immune cells are continuously generated, the stationary distribution of immune cells is a steady-state of generation, differentiation, survival, and death.
VIP and its receptors may be candidates for immune homeostatic regulators based on over two decades of research. Anatomical connections and factors released and recognized by the neuroendocrine and the immune systems enable bi-directional interactions between them. There are extensive peptidergic innervations in both primary and secondary lymphoid organs that delivery neuroendocrines to immune cells in these microenvironments. Vasoactive intestinal peptide (VIP) and the structurally related pituitary adenylate cyclase activating polypeptide (PACAP), are secreted by the peripheral nervous system and are soluble neuropeptides present in the immune microenvironment. VIP/PACAP elicit a broad spectrum of biological functions, including actions on innate and acquired immunity such as differentiation and development. VIP and PACAP bind to their GPCRs, VPAC1 and VPAC2, expressed on several immune cells with equal affinity. VIP and PACAP signaling through these receptors inhibit the production of proinflammatory cytokines such as IL-2, TNFα, IL-12, and IL-6 in activated T cells and macrophages. They can also inhibit activation induced apoptosis in T cells through reduction in FasL expression, resulting in increased survival rates. In addition, VIP inhibits the proliferation of bone marrow progenitors implicating it in immune cell homeostasis. The fact that VIP promotes a positive Th2/Th1 balance, and is capable of stimulating regulatory T-cell production through VPAC2 signaling axis suggests that endogenous VIP might play a role in immune cells homeostasis. Curiously, in a recent study investigating the role of VIP in experimental autoimmune encephalomyelitis, spleens of control animals appeared smaller than their age and sex matched littermates. However, the role of VIP in immune cell homeostasis was not investigated in this study.

VPAC2 is prominently expressed in immune cells such as lymphocytes, macrophages, thymocytes and immune microenvironments such as in the thymus, gut, and bone
Upon ligand binding, VPAC2 can engage at least three G proteins, including $G_{\alpha S}$, $G_{\alpha i}$ and $G_{\alpha q}$ that regulate signaling molecules as diverse as adenylate cyclase, PKA, PKC, PLC, PLD and EPAC, and elevate the intracellular secondary messengers, cAMP, IP$_3$, DAG and Ca$^{2+}$, (Figure 3-2)$^{24}$. 

Figure 3-2: VPAC2 signaling upon ligand binding$^{24}$

Previous studies, using VPAC2 transgenic mice showed increased serum IgE antibody levels, with heightened cutaneous allergic reactions and depressed delayed-type hypersensitivity$^{242}$. On the other hand, mice deficient in VPAC2 expression presented an increased basal metabolic$^{243}$ rate and decreased serum interleukin (IL) - 5$^{242}$. VPAC2 signaling stimulates IL-5 secretions from type 2 innate lymphoid cells$^{143}$. VPAC2 stimulation by VIP increases IL-4 secretion by T lymphocytes$^{244}$. During the first 4 months, mice deficient in VPAC2 show no difference in growth and weight gain$^{243}$. However, the role and mechanism of signal transduction of VPAC2 in immune cell homeostatic regulation has not been reported in detail. In the present studies, we characterized the immune cell homeostatic regulation by VPAC2 by utilizing knockout mice. We characterized these mice as having a global decrease in
immune cell numbers and diminished T cell activation responses. In contrast, VPAC2-deficient cells showed less potential for apoptosis.

**Materials and methods**

**Mice**

C57BL/6 were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). VPAC2 knockout mice were a kind gift from Dr. Jane Schuh at NDSU. Male or female mice were weighed, age- and sex-matched for all experiments. The mice used in these studies were between ages 8 to 12 weeks old. All animal procedures were performed according to National Institutes of Health guidelines and approved by the North Dakota State University Institutional Animal Care and Use Committee (Fargo, ND, USA). Experimental groups consisted of at least three mice, and each experiment was repeated a minimum of two times.

**VPAC2 knockout mouse strain genotyping**

A 2-4 mm tail clip was collected per mouse and placed in a microfuge tube on ice. Tweezers and scissors were sterilized in between mice tail clip collection with an alcohol swab to prevent contamination between samples. For DNA extraction, the SYBR® Green Extract-N-Amp™ Tissue PCR Kit (Sigma Aldrich, Catalog # XNATG-1KT) was used. Briefly, we added 100 µl extraction solution to each tail sample followed by 25 µl of tissue prep solution. The tubes were then incubated at room temperature for 10 minutes followed by 95 ºC for 3 minutes. Neutralization solution b (100 µl) was added and samples vortexed for 30 seconds. Samples were now ready for PCR reaction. PCR was performed with Expand Long Template PCR system (Cat. #. 11681834001, Roche diagnostics, Mannheim, Germany). Two sets primer pairs were utilized; one to test for WT gene and one for the mutant gene.
Primers P1 (5'-TTCAGAGGGAAGTAGGCTGGACTAGAGGACG-3') from the 5' region of exon 1 and P2 (5'-TACCTCTCTGATTCTCCGCTGC TTAGC-3'), spanning the junction of exon 2 and intron 2, to giving rise to a 2.5 kb product from the unmodified Vipr2 allele and a 7.2 kb product from the disrupted allele. Primers P3 (5'-GCTTCTCGTACGTTCACAGTGTCGCTCC-3') from the 3' end of the Neor gene and P4 (5'-TCCCCACTGTCACAAGGCTACATTAGTTTTGC-3') in intron 2 giving rise to a 2.5 kb product from the targeted allele but no product from the wild type allele or from a randomly integrated event. PCR was performed under the following conditions: 94°C (2 min) for one cycle; 94°C (10 s), 65°C (30 s), and 68°C (12 min) for 10 cycles; 94°C (10 s), 65°C (30 s), and 68°C (12 min) with 20 s extension/cycle for 20 cycles, and finally 68°C for 7 min. Products were then resolved in a 1% agarose gel electrophoreses and imaged by Gene Genius Bio imaging system (Syngene, Frederick, MD) after ethidium gel staining-distaining using 254 wavelength UV transmission.

![Diagram](https://example.com/diagram.png)

**Figure 3-3. Schematic of VPAC2 knockout generation and genotyping procedure.** Schematic diagram of (A) wild-type allele, (B) targeting vector, and (C) targeted allele showing restriction sites for EcoRI (E), EheI (Eh) Scal (Sc), SacII (Sa), and XbaI (X) and positions of probes A and B and primers P1, P2, P3, and P4. EcoRI digests of genomic DNA from ES cells hybridized with probe A (D) and probe B (E).
Euthanasia of mouse: carbon dioxide asphyxiation

Mice were placed in a CO\textsubscript{2} chamber that was filled with CO\textsubscript{2} over a 4 minute 24 seconds timed program as mandated by the NDSU IACUC. This was followed by cervical dislocation to ensure humane death.

Removal of mouse lymphoid organs

After sacrificing the animal in a humane manner, they were placed on their backs on clean, dry, dissection board. The fur was wetted with 70\% ethanol to sterilize the area and reduce the possibility of contamination. A midline incision was made with sterilized iris scissors. The skin was then retracted above the head and below the thighs.

To harvest the spleen, a 1-inch incision was made at the left of the peritoneal wall with surgical scissors and using sterile forceps the connective tissue was grasped around the spleen to expose it. Gently the spleen was cut off from the connective tissue into a clean 50 ml tube with complete RPMI growth medium (recipe listed in previous chapter). Single cell suspensions were prepared and counted as described in previous chapter.

For thymus removal, an incision in the chest was made, beginning at the xiphoid and extended to the neck with surgical scissors. Ribs were retracted with curved forceps. At this point, the thymus was located as a yellowish-white bi-lobed organ found just under the ribs, attached above the heart in the midline. Thymus was gently grasped with curved forceps for removal from the connective tissues. Harvested thymus was placed in 15 ml of complete culture media. Single cell suspensions were prepared by collagenase digestion by incubating the thymus tissue at 37\textdegree C for 45 minutes with 50 mg/ml Collagenase D (Roche, Indianapolis, IN), 50 \( \mu \)g/ml DNase I (Qiagen, Germantown, MD). After that, 5 mM EDTA was added to each dish and incubated for an additional 5 minutes. Each dish was pipetted vigorously to break up tissue aggregates and
strained through a 70 µm strainer. Remaining tissue on the strainer was mashed further with the back of syringe, followed by a wash with 5 mM EDTA in PBS until a volume of 30 ml was reached. Cells were centrifuged at 300xg for 10 minutes at 4°C. Red blood cells were lysed, cells washed with ice cold PBS and centrifuged as above. Cell suspensions were the prepared in complete culture media.

**Bone marrow cells isolation**

Mice were doused with 75% ethanol to reduce the possibility of contamination and placed onto a dissecting table. The abdominal skin was cut open to expose thigh muscles. To remove the femur and tibia, all the muscles and tendons around them were scrapped off using a sterile pair of scissors and a scalpel. Once all bones were scrapped clean, they were placed in a petri dish with isolation media (PBS, 2% FBS and 1mM EDTA. The epiphysis region of the bone was cut off each end of bone and marrow flashed with 3-4 ml of fresh isolation media using a 26 gauge needle. The cell suspension recovered was passed through a 70 µm cell strainer into a 50 ml centrifuge tube and centrifuged at 300xg at 4°C for 10 minutes. Media was aspirated and red blood cells lysed with 3 ml of eBiosciences 1X RBC lysis buffer solution (Cat# 00-4333-57; 200 ml) with 10 seconds of vortexing and incubated at room temperature for 3 minutes. After 3 minutes, RBC lysis buffer was diluted to 50 ml by addition of 1X PBS, centrifuged as above and passed through a 70 µm cell sieve. Cells were resuspended in 2.5 ml of complete media (RPMI, 20 FBS (Atlanta Biological), 1% P/S, 1% Glutamine, 25 mM Hepes, 1X NEAA, 1 mM sodium pyruvate, 50 µM beta-ME) for every two mouse equivalents of bone marrow. Triturated ten times or until cell suspension was homogeneous and serial dilutions (1/10, 1/20, 1/40, 1/80 and 1/160) prepared for cell counting. The high and low cell counts were removed and counts calculated using the mean of the middle two cell counts.
Ex vivo culture of mouse bone marrow-derived eosinophils (bm-Eos)

Bone marrow cells were collected from the femurs and tibiae of mice by flushing the opened bones with Iscove's Modified Dulbecco's Medium (IMDM; Invitrogen). Red blood cells were lysed in RBC lysis buffer, followed by the addition of 1 X PBS. After centrifugation, the cells were washed once in PBS containing 0.1% BSA and cultured at 5 X 10^6/mL in media containing RPMI 1640 (Invitrogen) with 20% FBS (Atlanta biologicals), 100 IU/mL penicillin and 10 μg/mL streptomycin (Cellgro), 2 mM glutamine (Invitrogen), 25 mM HEPES and 1x non-essential amino acids and 1 mM sodium pyruvate (Gibco) and 50 μM β-mercaptoethanol (Sigma) and supplemented with 100 ng/mL stem-cell factor (SCF; PeproTech) and 100 ng/mL FLT3-Ligand (FLT3-L; PeproTech) from day 0 to day 4. On day 4, the media containing SCF and FLT3-L was replaced with media containing 10 ng/mL recombinant mouse interleukin-5 (rmIL-5; R&D Systems) only. Cells were cultured for another 4 days. On day 8, the cells were moved to new flasks and maintained in fresh media supplemented with rmIL-5. Every other day from this point forward (days 10 and 12), one-half of the media was replaced with fresh media containing rmIL-5. Cells were enumerated at day 0 and on days indicated thereafter in a hemocytometer.

Isolation of peritoneal cavity cells

Mice were euthanized as described above, sprayed with 70% ethanol and mounted on a Styrofoam block on its back. Using a scissors and forceps outer skin of the peritoneum was cut and gently pulled back to expose the inner skin lining the peritoneal cavity. Ice cold 1X PBS (with 3% characterized fetal calf serum, 5 ml) was injected into the peritoneal cavity using a 27g needle by pushing the needle slowly in the peritoneum being careful not to puncture any organs. After injection, the peritoneum was gently massaged to dislodge any attached cells into the PBS
solution. A 25 g needle was inserted into the peritoneum and the fluid collected being careful with the needle to avoid clogging with fat tissue. About 3 ml of fluid was collected and cell suspensions kept on ice. An incision in the inner skin of the peritoneum was made and while holding up the skin with forceps, the remaining fluid from the cavity was collected using a plastic Pasteur pipette. At this point, if visible blood contamination was detected then the contaminated sample was discarded. Samples were centrifuged at 600xg at 4°C for 8 minutes. Cell pellets were then resuspended in complete RPMI culture media. Cell concentrations were determined by counting serially diluted samples with trypan blue on a hemocytometer.

Flow cytometry analysis

Single-cell suspensions from spleens, thymus, blood, lymph nodes and peritoneal cavity were prepared as described above. For cell surface markers, cells were stained with 0.25 μg antibodies per 1 x 10^6 cells (all antibodies used were of rat anti-mouse, clone RM4-4, Biolegend) in PBS with 2% BSA for at least 30 minutes at 4°C in the dark. After washing twice with 3 ml ice cold 1X PBS, immunolabeled cells were analyzed on an Accuri 6 flow cytometer (BD Biosciences). Data analysis was performed using the Accuri 6 software.

5-(and 6)-Carboxyfluorescein diacetate succinimidy l ester (CFSE) CD4 T cell proliferation assay

Single cell splenocyte suspension from wild type and VPAC2KO mice were washed twice with 1 X PBS to remove serum and resuspended at 10 X 10^6 cells/mL using room temperature 1X PBS. CFSE [5 μM] was added to the cell suspension, mixed immediately and incubated for 10 minutes at 37°C in the dark. After which, CFSE labeling was stopped by adding 4-5 volumes of cold complete media and incubated on ice for 5 minutes. Cells were washed three times with complete media and activated using 5 μg/ml anti-mouse CD3e functional grade
antibody coated plates plus 2 µg/ml anti-mouse CD28 functional grade antibody (both from eBiosciences) in complete RPMI culture media at 37°C, 5% CO₂. Control samples (inactivated) were plated without either antibodies. Cells were cultured for 72 hours before analysis of CFSE degradation by flow cytometry.

Cell apoptosis analysis

Freshly isolated splenocytes and thymocytes were incubated with 0.25 µg per 1x10⁶ cells anti-annexin V-APC (eBiosciences) for 30 minutes at 4°C. Cells were washed twice with ice-cold 1X PBS and resuspended in 200 µl of 1XPBS plus 2% BSA buffer. Immediately before flow cytometry analysis, 1 µg/ml propidium iodine dye was added to cell suspension for 5 minutes. Percent apoptotic cells were calculated by determining percent PI⁺ and annexin V⁺ cells.

Statistical analysis

Data are graphed with means ± SEM from independent experiments. Unpaired two-tailed Student t-tests were used for statistical analysis to compare VPAC2 knockouts and wild type samples. A value of p≤ 0.05 was considered statistically significant.

Results

VPAC2 deficiency causes decrease in total splenocytes

Maintenance of lymphocyte homeostasis is crucial to allow rapid protective response against foreign pathogens while simultaneously preventing diseases of the immune system, such as autoimmunity or immunodeficiency. One mechanism to maintain immune homeostasis is the maintenance of an adequate number of immune cells. When the number of immune cells decreases, a state of immunodeficiency can result with inability to clear pathogens as quickly as needed. To elucidate the roles of VPAC2 in immune cell homeostasis, we used a VPAC2
deficient mouse strain. Mice homozygous for VPAC2 gene mutation developed normally and were born with the expected Mendelian ratio. However, we observed that these mice consistently had smaller spleens as compared to their age-and-sex matched wild type counterparts. To elucidate the importance of homeostatic regulation of immune cells by VPAC2 in vivo, we characterized the immunophenotype of the mutants as compared to wild type. In order to confirm the above observation was not due to weight differences, we measured weights of mutants and compared them to those wild types mice. There was no significant difference in the average weights between VPAC2 homozygous mutants and wild type mice used in all the experiments (Figure 3-4A). Some of the VPAC2 knockout mice were housed in Dr. Schuh research groups mouse room here at NDSU. Our analysis also revealed that VPAC2-deficient mice consistently had smaller spleens, which was associated with a marked decrease in the number of splenocytes by homozygous mutants as compared to age-and sex-matched wild types (Figures 3-4B and C). VPAC2-deficiency resulted in approximately 50% fewer total splenocytes after red blood cells lysis. These results demonstrate that VPAC2 plays a critical role in maintaining immune homeostasis since lack of VPAC2 resulted in fewer splenocytes as compared to wild types.
Figure 3-4. VPAC2 deficiency leads to smaller spleens and fewer total splenocytes. (A) Average weights of 15 age matched WT and VPAC2 deficient mice between 8-12 weeks old were determined. Mice were weighed prior to sacrificing (B) Photograph of representative WT and VPAC2¿/¿ spleens. Each measurement is 0.5 cm apart (C) Single cell suspensions were prepared from spleens and WT vs. VPAC2¿/¿ total splenocytes numbers compared after RBC lysis (materials and methods n=8, Mean± SEM, **p<0.05 was considered significant).

VPAC2 deficiency causes a defect in immune cells homeostasis

Given the reduction in total splenocyte numbers in VPAC2¿/¿, we next investigated the homeostatic role of VPAC2 signaling. Using flow cytometry analysis, we calculated percent composition and numbers of lymphocytes (B and T cells) in different immune tissues of WT and VPAC2 deficient mice. Flow cytometry analysis was done to determine percentages of B cells (CD19+) and T cells (CD3+ or CD4+ or CD8+) in spleen, thymus and blood from WT or VPAC2 deficient mice. Average total cell numbers were calculated by multiplying total cell number by the percentage of each cell population. Our analysis revealed that VPAC2 ablation did not have a significant effect on the percent composition of lymphocytes in the immune compartments investigated. However, there was a reduction in absolute cell counts in all compartments investigated (Figure 3-5A). And since there was a reduction in peripheral T cells, we
investigated if this could have arisen from defects in thymic differentiation. Analysis of proportions of double-negative (CD4-, CD8-), double-positive (CD4+, CD8+) and single-positive (CD4+ or CD8+) thymic subsets did not reveal any differences between wild type and VPAC2 deficient mice (Figure 11A). But, analysis of naïve and effector/memory revealed a significant reduction in naïve CD4 T cells (CD4+/CD44\text{low}/CD62L\text{hi}) balanced by a slight increase in central memory CD4 T cells (CD4+/CD44\text{hi}/CD62L\text{low}) in VPAC2 deficient mice as compared to wild type age and sex matched counterparts. There was no significant difference in effector memory cells percentages in the spleen (Figure 3-5B). From this, we concluded that VPAC2 plays a significant role in maintaining immune cell homeostasis and that the reduction in number of CD4 T cells in peripheral lymphoid tissues is caused in part by a specific reduction in the number of naïve CD4 T cells in VPAC2 deficient mutants.
Figure 3-5. VPAC2 deficiency causes loss of lymphocytes. (A-E) Single cell suspensions from spleen, thymus, and blood were immunostained with anti-CD19, anti-CD3, anti-CD4 and anti-CD8 antibodies, and analyzed by FACS. Percent composition and numbers are graphed as means ± SEM. (B) Splenocytes were stained for naïve, effector and central memory CD4 T cells with a cocktail of anti-CD4, anti-CD44 and ant-CD62L antibodies. Numbers of naïve, effector, and central memory CD4 T cells are shown. Data are graphed as means ± SEM, *p value≤ 0.05 for 3 independent experiments.

Deletion of VPAC2 results in heightened apoptosis resistance by T cells

Survival regulation is crucial for immune cell homeostasis. Heighted rate of survival can result in increased number of immune cells if their output is maintained. Given that VPAC2 deficient mice have a lower number of peripheral T cells, we hypothesized that VPAC2 deletion would result in defective survival of immune cells leading to decreased total cells. To test this hypothesis, single cell suspensions of thymocytes were prepared, and subjected to Annexin-V
and propidium iodide staining analysis. Surprisingly, we found that VPAC2 deficient T cells were more resistant to apoptosis than their WT counterparts (Figure 3-6A). We then cultured total non-adherent splenocytes for 24 hours in complete growth media (RPMI-1640, 10% characterized FBS, and 1% Penn-strep) and tested for percent apoptosis. Consistent with thymocytes assayed immediately after isolation, cultured splenocytes lacking VPAC2 were significantly (3 fold) more resistant to cell death after 24 hours in culture as compared to WT (Figure 3-6B).

Interleukin-7 (IL-7) is a nonredundant cytokine required for the survival of both naïve and memory T cells. IL-7 receptor alpha (IL7Rα/CD127), which regulates IL-7 signaling is expressed on T cells at almost all stages of development, from early CD4-CD8- progenitors in the thymus to mature T cells in the peripheral lymphoid tissues. In an attempt to explain the difference in survival of T cells between VPAC2 deficient T cells and their wild type counterparts, we used flow cytometry analysis to measure IL7Rα protein and qRT-PCR to measure relative mRNA expression. We found that compared to wild type, VPAC2 deficient T cells had higher relative mRNA expression levels of IL7Rα (Figure 3-6C). This was consistent with a greater percentage of cells expressing comparable amounts of IL-7Rα protein. These data revealed that VPAC2 deficiency is associated with increased resistance to apoptosis. This could be due to increased IL7Rα signaling which have prosurvival influences on T cells.
Figure 3-6. VPAC2 deficient T cells display increased resistance to apoptosis and higher IL7R alpha expression. Single cells suspensions of non-adherent WT or VPAC2 deficient splenocytes were used immediately (A) or cultured for 24 hours (B) and late stage apoptotic analysis conducted using annexin V and PI staining method. Representative flow cytometry result of 3 independent experiments are shown (left panel) with means ± SEM bar graph combined data (right panels). (C) CD4 T cells were isolated and IL-7Rα expression determined by flow cytometry and qRT-PCR analysis. Far left is a representative flow analysis of IL-7Rα expression on total splenocytes, middle right panel bar graph shows means ± SEM of percent IL7Rα expression on CD4 and CD8 T cells and far right shows relative mRNA levels of total splenocytes (n=3, p-value ≤ 0.05).

VPAC2 deficiency leads to impaired activation induced T cell proliferation

Since lack of VPAC2 endowed thymocytes and T cells with increased survival capacity, and previous studies have demonstrated that VPAC2 expression is increased during T cell activation and lymphopoiesis, we next examined TCR signaling in VPAC2 deficient T cells as compared to their wild type counter parts. Highly purified splenic CD4 T cells were
labeled with proliferative dye CFSE and stimulated with plate bound functional grade anti-CD3e and anti-CD28 antibodies in complete growth media. After 72 hours cells were analyzed for CFSE dilution. The more a cell divides, the lower the fluorescent intensity of CFSE from which percent division rate can be calculated (materials and methods). Compared with WT controls, VPAC2-deficient CD4 T cells displayed diminished TCR-induced proliferation activity showing only 25% of cells undergoing proliferation verses 80% for wild type cells (Figure 3-7) suggesting that VPAC2 deletion impairs T cell activation and proliferation.

**Figure 3-7.** VPAC2 deficiency leads to impaired activation induced T cell proliferation. Purified splenic CD4 T cells were labeled with CFSE and cultured with or without 10 µg/mL plate bound anti-CD3 antibody together with 2 µg/mL anti-CD28 antibody for 72 hours and assessed for percent proliferation by CFSE fluorescent intensity assay (materials and methods). Bar graphs show means ± SEM of 2 independent experiments with 3 replicates per experiment, p-value ≤ 0.05.
VPAC2 deficiency is associated with delayed production of bone marrow eosinophils and decreased peripheral eosinophil counts

During an infection, innate immunity provides a first line of defense which allows for a nonspecific, but rapid response before adaptive immunity takes over if needed. Eosinophils are a type of granulocyte that plays a key role in innate immunity to defend against parasitic infections\(^{245}\). IL-5 is a homeostatic cytokine required for production and proliferation of eosinophils\(^{143}\). VPAC2 signaling induced secretion of IL-5 by type 2 innate lymphoid cells (ILC2) maintains homeostatic levels of eosinophils\(^{143}\). To study the potential role of VPAC2 in innate immune homeostasis, we compared the homeostatic levels of eosinophils in VPAC2 deficient mice to that of WT counterparts. To do so, we isolated single cell suspensions of splenocytes and identified eosinophils as high side scatter, siglec F\(^+\), and CD11c\(^{-}\) cells by flow cytometry analysis. VPAC2 deficiency resulted in drastically fewer splenic eosinophils (Figure 3-8A). Based on these results, we hypothesized that production of eosinophils was diminished in the bone marrow of VPAC2 deficient mice\(^{246}\). We tested this hypothesis by conducting a bone marrow (BM) eosinophil differentiation. Total BM cells were isolated and eosinophil differentiation performed as described in materials and methods. Lack of VPAC2 resulted in delayed production of differentiated eosinophils with no effect on the ability for differentiation towards eosinophils lineage (Figure 3-8B); as we were able to consistently get \(\geq 92\%\) high side scatter and siglec F\(^+\) cells (eosinophils) after 14 days. Therefore, lack of VPAC2 results in decreased eosinophil production in the bone marrow which could in part explain the decreased peripheral eosinophil numbers under homeostatic conditions.
Figure 3-8. Lack of VPAC2 results in fewer peripheral eosinophils and delayed ex vivo bone marrow eosinophil differentiation. (A) Total splenocytes were isolated, stained and analyzed for percent eosinophils (right) and absolute numbers (left) calculated based on total splenocytes numbers (materials and methods). Bar graphs are for 2 independent experiments with means ± SEM. (B) Eosinophils were differentiated from total bone marrow cells for 14 days (materials and methods). On days 0 and 14 of differentiation, high side scatter and siglecF+ cell percentages were determined to identify eosinophils in cell population. Right: Graph of average total nonadherent cell counts for WT and VPAC2−/− through day 14. Left: Percent high side scatter and siglecF+ cells on days 0 and 14 for WT and VPAC2−/− cells. Data is for means ± SD for 2 independent experiments for spleen analysis and 1 independent experiment for ex vivo bone marrow eosinophils analysis.

Discussion

The results presented in this study reveal that VPAC2 deficiency results in impaired immune cells homeostasis. VPAC2 deficient mice are born with the expected Mendelian ratio
and grow normally as compared to WT mice with relatively equal gain in weight within the first fifteen weeks which was consistent with literature\textsuperscript{243}. However, lack of VPAC2 results in decreased numbers of both innate and adaptive immune cells. In addition, we show that VPAC2 knockout is associated with increased splenocyte and thymocyte resistance to apoptosis as compared to their wild type counterparts. In agreement with other studies, we also found that ablation of VPAC2 led to diminished peripheral eosinophils\textsuperscript{143, 246} which could be due to slower production in the bone marrow. Interestingly, VPAC2 deficiency did not affect thymopoiesis or induce T cell activation as was the case for cdc42 deficient mice\textsuperscript{247}, but resulted in approximately 50\% decrease in naïve CD4 T lymphocytes in the spleen which was similar to the cdc42 deficient mutants. When stimulated with a mitogenic signal in culture, VPAC2-deficient CD4 T cells had diminished proliferative response as shown with proliferation studies.

Maintenance of an adequate number of immune cells is crucial to having an efficient protection against foreign antigens without adverse effects against self. To date, no study has identified the role of VPAC2 in immune cell homeostasis. In this study, we examined the role of VPAC2 in immune cell homeostasis by using a VPAC2 knock out mouse strain. We provide evidence showing that VPAC2 signaling activity is likely required in order to maintain normal homeostatic conditions in the immune system. Mice lacking VPAC2 have fewer total immune cells representing both the innate and adaptive arms of the immune system. Total splenocytes, thymocytes and blood T cell numbers were reduced as compared to wild type counterparts. This reduction in total immune cells could not be explained by the size of mice as VPAC2-deficient mice used in these experiments grew normally to wild type within the first 15 weeks. Analysis of percent composition of lymphocytes in the spleen indicated that there are no defects in development and lineage commitment. This lack of development could explain why previous
studies done on these mice concluded that there was no homeostatic difference due to lack of VPAC2 as they only looked at the percentages of lymphocytes instead of total cell numbers as well\textsuperscript{246}. To support our results, VIP knock out mouse strain have smaller spleens as compared to their age and sex matched counterparts\textsuperscript{239}, illustrating a potential signaling axis involving VIP\textrightarrow VPAC2 to maintain normal homeostasis of immune cells.

Thymus is the primary site of T lymphopoiesis. VPAC2 is predominantly the VIP receptor expressed in thymocytes\textsuperscript{248} and supports the generation of CD4+CD8- thymocytes\textsuperscript{249}. Once differentiated to CD4+CD8- thymocytes, these cells migrate to the peripheral immune organs. The findings that VPAC2 supports production of naïve CD4 T cells in the thymus which would migrate to the periphery is in agreement with our results demonstrating that VPAC2 deficient mice have decreased number of peripheral naïve CD4+ T cells as lack of VPAC2 would lead to diminished output of naïve CD4 T cells from the thymus. However, in contrast to an \textit{in vitro} mouse thymocyte cell line study\textsuperscript{248}, we did not find a decrease in percentage of CD4+CD8- thymocytes in our \textit{in vivo} studies of VPAC2 deficient mice as compared to the wild type.

A surprising result of these studies was the finding that VPAC2-deficient splenocytes and thymocytes are more resistant to apoptosis. Survival/apoptosis regulation in T and B lymphocytes is a major element controlling immune homeostasis. Increased survival can result in increased numbers of immune cells if production is maintained. We previously reported a microarray study where we found that VIP treatment of naïve CD4 T cells resulted in differential regulation of 68 cell death genes as compared to control\textsuperscript{205}. These results provided evidence showing that VIP signaling through either VPAC1 or VPAC2 can affect the rate of T cell death. VPAC2 signaling protects Th\textsubscript{2} cells from apoptosis by inhibiting granzyme B and therefore is a
survival factor and promoter of Th2 memory cells\textsuperscript{250,251}. Here, we found that CD4 T cells lacking VPAC2 expression survive longer than wild type controls. Our analysis of IL7Rα expression revealed that VPAC2-deficient CD4 T cells also have higher IL7Rα expression as compared to wild type. IL7 signaling through IL7Rα is central to mediating T cell survival and maintaining peripheral T cells\textsuperscript{229}. Since VPAC2-deficient CD4 T cells express higher levels of IL7Rα one would expect them to have higher resistance to apoptosis as we found. Our future goal is to investigate the potential differences in IL-7 induced survival between VPAC2-deficient CD4 T cells as compared to wild types given the higher expression of IL7Rα in VPAC2-deficient mice. These results were surprising because we expected to find that VPAC2 deficiency would have resulted in increased apoptosis to explain the lower total immune cell numbers.

VPAC2 deficiency led to a hypo-responsiveness of T cells to TCR ligation illustrated by diminished TCR-induced proliferation. We attributed this to the lack of VPAC2 signaling. During T cell activation, VPAC2 expression consistently increases while that of VPAC1 decreases\textsuperscript{242} suggestive of a requirement of VPAC2 in T cell activation. Whereas VPAC2 deficient mice have been shown to have an immune system that is skewed towards a Th\textsubscript{1} response, the rate of proliferation of T cells following TCR activation has not yet been investigated. Also, whether down regulation of VPAC1 in VPAC2 deficient CD4 T cells occurs during T cell activation is yet to be determined. We have collected evidence to indicate that the upregulation of VPAC2 during CD4 T cell activation might actually be more important than previously appreciated. VPAC2 deficient T cells do not proliferate as much as wild type counterparts which might suggest that induction of VPAC2 during TCR activation is be needed for CD4 T cell activation-induced proliferation. To further this study, our future goal is to use highly selective VPAC2 antagonist to inhibit VPAC2 signaling to shed light into importance of
VPAC2 in CD4 T cell activation responses. It would also be informative to confirm this observation of diminished T cell hyporesponsiveness to TCR activation in vivo.

Consistent with previous observations, VPAC2 deficient mice have decreased basal eosinophil numbers. These mice have been reported to be skewed towards a Th1/Th2 cytokine phenotype which also supports our observations as Th2 cytokines, such as IL-5 play a significant role in maintaining eosinophil homeostatic levels. Here, we further establish that the decreased levels of eosinophils could be explained by a defective production of eosinophils in the bone marrow. Our bone marrow cytokine-stimulated eosinophil differentiation studies revealed that VPAC2-deficient bone marrow progenitor cells lagged in total cells production as compared to wild type. However, there was no defect in the ability of the VPAC2-deficient bone marrow progenitor cells to differentiate into mature eosinophils as analyzed by flow cytometry.

Collectively, here we demonstrated a homeostatic role of VPAC2 in immune cell production that was revealed through utilization of a VPAC2-deficient mouse strain. Mice lacking VPAC2 have fewer total immune cell numbers as compared to wild type counterparts. We identified VPAC2 as important for CD4 T cell TCR activation proliferative responses and maintenance of peripheral naïve T cell pool. Loss of VPAC2 results in decreased bone marrow production of eosinophils. Finally, it is important to point out that the results observed might have been contributed to by heightened VPAC1 signaling. We intend to repeat these experiments using VIP knockout and VPAC1 knockout mouse strains as well to determine the signaling axis involved.

References


CHAPTER FOUR: CHARACTERIZATION OF B LYMPHOCYTES IN RESPONSE TO HYALURONIC ACID IN A MURINE FUNGAL ALLERGIC ASTHMA MODEL

Introduction

According to world health organization, asthma is a prevalent disease of the lung that affects over 300 million people in the world\(^1\). It is characterized by a complex interplay of environmental factors, airway obstruction, bronchial hyperresponsiveness, inflammation, cytokines and chemokines, which results in narrowing of the airways\(^2-4\). Airway inflammation in asthma is often triggered by exposure to environmental allergens such as molds and arthropods\(^5\)-\(^6\). Repeated exposures to allergens can result in asthma attacks which can be fatal\(^5\). Classical hallmarks of chronic airway inflammation of patients with severe or persistent asthma; include accumulation of activated eosinophils, neutrophils, lymphocytes, and extracellular matrix (ECM) components in the airways\(^7-9\).

Hyaluronan (HA) is a major component of ECM\(^10\). It is a negatively charged non-sulfated glycosaminoglycan (GAG) polymer consisting of repeating disaccharide subunits of N-acetyl glucosamine and glucuronic acid\(^10, 11\). Fibroblasts produce the vast majority of HA with smooth muscles producing HA to a lesser extent\(^12\). Under normal physiological conditions HA exists as a high-molecular-weight polymer (HMM HA), but can undergo a dynamic breakdown into several lower-molecular-weight (LMM HA) forms which are prevalent during inflammation\(^10, 13-15\). HMM HA has a molecular mass > 1X10\(^6\) Da and plays homeostatic roles in normal healthy tissues\(^14\), whereas LMM HA masses range from 0.8 to < 500 Da and accumulate during inflammatory responses\(^12, 16\). HMM HA can be broken down to LMM HA fragments by the activity of hyaluronidases and reactive oxygen species (ROS)\(^10, 15, 17, 18\). Differences between HMM HA and LMM HA can be detected by immune cells through multiple receptors\(^19-22\). LMM
HA fragments possess the ability to activate inflammatory gene expression in epithelial cells, endothelial cells, fibroblasts, dendritic cells and macrophages and therefore considered to be proinflammatory. LMM HA is associated with active inflammation and lack of its clearance leads to enhanced inflammation-induced pathology further highlighting the importance of HA in regulating inflammatory responses.

Conditions that result in tissue damage such as lung ozone damage, and chronic obstructive pulmonary diseases like chronic obstructive pulmonary disease (COPD), idiopathic arterial pulmonary hypertension, acute respiratory distress syndrome (ARDS) and allergic asthma are accompanied by increase in HA production. In the lungs, HA can exist in a soluble form which can covalently bind to a variety of protein receptors to influence their functions. HA-binding proteins are known as Hyaladherins. Hyaladherins include receptors for hyaluronan mediated motility (RHAMM), CD44, tumor necrosis factor-α-stimulated glycoprotein-6 (TSG-6), lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1), and toll-like receptors (TLR)-2 and -4. Binding of HA to its receptors can result in proinflammatory or suppressive/pro-remodeling responses depending on the size of HA.

The role of T lymphocytes in asthma has been intensively investigated. T helper 2 (Th2) lymphocytes and Th2 cytokines are thought to drive the formation of allergic reactions such as activation of inflammatory cells including eosinophilia, mast cell accumulation/activation and airway remodeling in asthma. Through co-stimulatory molecules, Th2 lymphocytes have been shown to engage allergen-specific B cells. Activated Th2 secretion of IL-4 and IL-13 promotes antibody class switching B cells to synthesize IgE antibodies, a classical hallmark of asthma. However, less is known about the biology of B cells in allergic asthma, including the role, phenotype and mechanisms that govern this pathology. The functional roles of B
lymphocytes in allergic asthma are still controversial, with some studies showing that B lymphocytes play a minimum role\textsuperscript{41,42}, if any, in the development of asthma; while others have shown that IgE production by B lymphocytes is needed for the development of asthma\textsuperscript{43-45}. These controversial results were recently attributed to differences in types of allergens used in murine asthma models, which resulted in non-identical inflammatory responses\textsuperscript{40,46}. By using a cocktail of commonly used allergens to induce asthma, mice lacking B lymphocytes had attenuated eosinophilic airway inflammation, decreased levels of Th2 cytokines and chemokines, which were accompanied by decreased airway hyperresponsiveness. Lack of B cells also led to decreased allergen-induced CD4\textsuperscript{+} T lymphocytes expansion in asthmatic lungs\textsuperscript{40}, suggesting that B lymphocytes could be playing a key role in resolving eosinophilic airway inflammation and expansion of CD4\textsuperscript{+} T lymphocytes during asthma.

TGF-β1 and IL 10 are important immunosuppressive cytokines that help resolve inflammation and promote tissue remodeling\textsuperscript{47}. These immunosuppressive cytokines were shown to be elevated in \textit{aspergillus fumigatus} induced asthma\textsuperscript{31}. Likewise, low molecular mass hyaluronic acid (LMM HA) levels are increased during \textit{aspergillus fumigatus} induced asthma\textsuperscript{31}. B lymphocytes express at least four HA-binding proteins; CD44, TLR-2, TLR-4 and RHAMM. During \textit{aspergillus fumigatus} induced asthma, B lymphocytes from asthmatic lungs undergo a CD44-dependent chemotaxis and interleukin-10 (IL-10) secretion\textsuperscript{48}. LMM HA elicited a CD44-independent production of TGF-β1 by B lymphocytes from asthmatic lungs and spleens\textsuperscript{48}. However, the authors of that study did not identify which HA receptor was required for HA-induced B lymphocytes’ TGF-β1 production. Therefore, an HA receptor other than CD44, expressed on the surface of B lymphocytes must regulate TGF-β1 production.
The first aim of this study was to characterize which functional B lymphocyte LMM HA receptor mediated TGF-β1 production during asthma. We isolated lung and spleen B lymphocytes from non-asthmatic and asthmatic mice and compared the expression of TLR-2,-4 and RHAMM on them. We found that B cells upregulate RHAMM during asthma, while both non-asthmatic and asthmatic spleen and lung B lymphocytes had no detectable expression of TLR2 and 4. These results revealed a previously unknown characteristic of B lymphocytes in aspergillus fumigatus induced allergic asthma. From these results we concluded that RHAMM is most likely the required receptor for production of TGF-β1 in response to LMM HA binding by B lymphocytes during asthmatic.

The second aim of this study was to determine if LMM HA treatment of resting or activated B cells can induce a regulatory B lymphocyte phenotype. We treated resting or LPS treated splenic B cells with LMM HA and compared the percentage of CD19+, CD5+ and CD1dhi cells as compared to relevant controls. Our results indicated that LMM HA expands the percentage of regulatory B cell phenotype when cells were activated, but had no effect without activation. Together with RHAMM protein upregulation, it is enticing to speculate that activated B lymphocytes homing to the allergic lung via LMM HA is an anti-inflammatory suppressive response. These results provide a critical step towards characterizing the phenotype of B cells in allergic asthma.

Materials and methods

Ethics statement

All experiments were performed in accordance with the Office of Laboratory Animal Welfare guidelines and were approved by the North Dakota State University Institutional Animal Care and Use Committee, Fargo, ND, USA.
**Experimental animals**

C57BL/6 male and female mice (6–9 weeks of age) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) and bred in house. Animals were housed on Alpha-dri™ paper bedding (Shepherd Specialty Papers, Watertown, TN, USA) in micro filter-topped cages (Ancare, Bell-more, NY, USA) in a specific pathogen-free facility with ad libitum access to food and water.

**Antigen preparation and conidia culture**

Soluble *A. fumigatus* extract was purchased from Greer Laboratories (Lenoir, NC, USA) and fungal culture stock (strain NIH 5233) was purchased from American Type Culture Collection (Manassas, VA, USA). The *A. fumigatus* was reconstituted in 5 ml PBS, and 60 µl aliquots were stored at 4 °C until use. A single aliquot of *A. fumigatus* was grown on sabouraud dextrose agar (SDA) in a 25 cm² cell culture flask for 8 days at 37 °C. All experiments that utilized *A. fumigatus* were conducted with prior approval of the institutional biological safety committee of North Dakota State University.

**Allergen sensitization and challenge by nose only inhalational model**

Animals were sensitized per Hogaboam’s published protocol\(^*\), with the exception that alum was used as the adjuvant. To elicit allergen sensitization, mice were sensitized globally with 10 µg of *A. fumigatus* antigen (Greer laboratories) in 0.1 ml normal saline (NS) mixed with 0.1 ml of Imject Alum (Pierce, Rockford, IL, USA) and injected subcutaneously (0.1 ml) and intraperitoneally (0.1 ml). After two weeks, mice were given a series of three, weekly 20 µg doses of *A. fumigatus* antigen in 20 µl of normal saline intranasally (IN). One week after the final IN inoculation, mice were exposed to *A. fumigatus* by inhalation of mature, airborne conidia. To do this, mice were anesthetized using a cocktail of ketamine (75 mg/kg) and xylazine.
(25 mg/kg), and their noses were placed in the inoculation chamber where they inhaled mature *A. fumigatus* conidia for 10 minutes. The sensitization and challenge model is illustrated in Figure 4-1. After 5 days of allergen challenge, animals were anesthetized with pentobarbital (150 mg/kg) and tracheostomized for sample collection.

Figure 4-1. Sensitization, challenge, and analysis schedule for the *A. fumigatus* murine model of allergic asthma. Naïve mice (N) groups received neither sensitization nor conidia challenges. Mice were sensitized to *A. fumigatus* extract via a series of injections and intranasal inoculations, after which they were exposed to 2, nose-only inhalation doses of live conidia 2 weeks apart. Groups of animals were assessed 5 days after allergen challenge (2A3K)D5.

*Preparation of lung and spleen cell suspensions*

Mice were anesthetized using sodium pentobarbital (Butler, Columbus OH; 100 mg/kg of mouse body weight). Each experiment consisted of 5 mice per group. Lungs and spleens were removed from five animals to from which single cell suspensions were derived. For lung preparations, lungs were minced and subjected to collagenase IV (Sigma–Aldrich) digestion in DMEM at 37 °C for 1 hour with gentle agitation. Cells were then dispersed through a 10 ml syringe and passed through a 40 µm cell strainer (BD Biosciences, San Jose, CA, USA). The cells were washed with sterile PBS twice before they were subjected to ammonium chloride cell lysis buffer (ACLB) to remove red blood cells (RBCs). To prepare a single cell suspension of
spleen perfusate, spleens were perfused with DMEM. Spleen cells were washed with sterile PBS and treated with ACLB to lyse red blood cells. Lung and spleen cell preparations were counted and resuspended in PBS with 1% BSA (Sigma Aldrich) to a final concentration of $1 \times 10^7$ cells/ml.

**Negative isolation of highly purified B lymphocytes**

For the purification of B lymphocytes, spleen and pooled total lung cells were negatively isolated using EasySep™ Mouse B Cell Isolation Kit (Stem Cell technologies, Vancouver, CA). Total splenocytes including RBCs were prepared at a concentration of $1 \times 10^8$ cells/mL in phosphate-buffered saline (PBS) or Hank’s balanced salt solution (HBSS) plus 2% fetal bovine serum (FBS). Cells were placed in a 5 mL (12 x 75 mm) polystyrene tube to properly fit into the EasySep™ Magnet. Normal Rat Serum and EasySep™ Mouse B lymphocytes Isolation Cocktail were added at 50 μl/mL of cell suspension, mixed well then incubated at room temperature (15 - 25 °C) for 15 minutes. Vortexed EasySep™ Streptavidin RapidSpheres™ 50001 were added at 75 μL/mL of cell suspension. Thoroughly mixed cells were then incubated at room temperature (15 - 25°C) for 3 minutes and diluted up to 2.5 mL total volume with more suspension medium. Cells were gently pipetted up and down 2 - 3 times and placed (without cap) into the magnet. Samples were incubated at room temperature (15 - 25°C) for 3 minutes and inverted to pour off supanatant (B lymphocytes) into a polystyrene tube. The magnetically labeled, unwanted cells remained bound inside the original tube held by the magnetic field of the EasySep™ Magnet. To determine percent purity, $1.0 \times 10^6$ post-enrichment B lymphocytes cells were suspended in 200 μL 1XPBS/0.5% BSA and incubated with 1:200 (0.25 μg) FITC conjugated rabbit anti-mouse CD19 (clone MB19-1) or APC conjugated rabbit anti-mouse B220 (clone RA3-6B2) antibodies (eBioscience, San Diego, CA) for 30 min at 4°C in the dark. Cells were washed twice with 4 ml PBS/ 0.5% BSA, centrifuged for 10 min at 300xg at 4 °C and resuspended in 200 μl PBS/0.5%
BSA. Flow Cytometry was performed on an Accuri 6 flow cytometer (Ann Arbor, MI) and percent purity determined. CD19 lymphocyte purification consistently yielded ≥95% mouse CD19+ cells. B lymphocytes yield was determined as described previously using a hemocytometer.

**Determination of Toll-Like Receptor (TLR)-2 and TLR-4 receptor expression on B lymphocytes**

Individual mouse total spleen and lung single cell suspensions from either naïve or challenged groups' surface Fc receptors were blocked with 1 µg of anti-mouse CD16/CD32 antibodies per 10^6 cells (eBioscience, San Diego, CA, USA) for 10 min on ice. The following Abs were used for phenotypic characterization of B lymphocytes using flow cytometry: FITC-anti-TLR-2 (clone: 6C2), PE-anti-TLR-4 (clone: UT41), and APC conjugated anti-CD19 monoclonal antibodies (eBioscience). Samples were stained with labeled Abs (0.5 µg/million cells) for 30 minutes in the dark at 4°C and then washed twice with 1X PBS 1% BSA before analyzing using an Accuri C6 flow cytometer (Accuri Cytometers, Ann Arbor, MI, USA). TLR2/4 expression was determined by first gating on CD19+ cells, followed by TLR2/4 cell surface expression compared between naïve and challenged groups. The data was analyzed using the Accuri 6 software.

**Determination of RHAMM expression level**

RHAMM expression between naïve and challenged splenic and lung B lymphocytes was determined at the mRNA and protein levels. The steady-state mRNA levels of RHAMM was compared between highly purified B lymphocytes from naïve and challenged mice lungs or spleens by SYBR-green qRT-PCR. Briefly, total RNA was isolated, first strand cDNA synthesized and relative steady-state mRNA levels for Rhamm determined using sense: 5’-GCG
TCA GAA TGT CCT TTC CTA-3’ and antisense: 5-GAC ACT GGT CCT TTA GTT GCT-3’
primers normalized to β-actin. For RHAMM protein expression determination, highly purified (≥95%) splenic or lung CD19+ cells (B lymphocytes) were isolated and 1.0 X 10^7 cells were lysed in 250 µl of radio-immuno precipitation assay (RIPA: 20 mM Tris pH 7.5, 150 mM NaCl, 1% nonidet P-40, 0.5% sodium deoxycholate, 1 mM EDTA, 0.1% SDS) containing 1:100 diluted Halt protease and phosphatase inhibitor cocktail (100X), catalog #78440 (life technologies, Grand Island, NY, USA). Protein concentration was determined by Bradford assay kit catalog #500-0001 (BIO-RAD, Hercules, CA, USA) following the manufacturer’s instructions. After which, 10 µg of protein was resolved over a 12% SDS-PAGE gel followed by protein transfer to a nitrocellulose membrane. Nitrocellulose membranes were incubated at room temperature for at least 4 hours in 5% skim milk/Tris-buffered saline/0.1% NP-40 to block nonspecific protein binding sites. Membranes were then incubated overnight at 4°C with Tris-buffered saline/1% skim milk/0.1% NP-40 containing 1:2000 diluted anti-RHAMM goat polyclonal antibodies (E-19) (Santa Cruz, Dallas, Texas, USA). Membranes were washed four times with Tris-buffered saline/0.1% NP-40 for 15 minutes each at room temperature followed by a 2 hour incubation with 1:5000 diluted horseradish peroxidase–coupled anti-rabbit secondary Ab. Next, membranes were washed six times with Tris-buffered saline/0.1% NP-40 at room temperature for 15 minutes each followed by protein detection with ECL Plus kit (Amersham/GE Healthcare) according to the manufacturer’s instructions. Membranes were developed using an X-ray developer and signal intensity determined using chemigenius2 imager (Syngene, Frederick, MD, USA).
**CD19^+CD5^+CD1D^{hi} induction by low molecular mass hyaluronic acid (LMM HA) on resting or LPS activated mouse splenic B lymphocytes.**

To assess the capacity of LMM HA to induce a regulatory B cell phenotype, total splenocytes were either treated with or without 10 µg/ml lipopolysaccharide (LPS) plus or minus LMM HA as indicated for 48 hours. They were then cultured for 48 hours before being analyzed by flow cytometry for regulatory B cell markers. Briefly, surface Fc receptors were blocked with 1 µg of anti-mouse CD16/CD32 antibodies per 10^6 cells (eBioscience, San Diego, CA, USA) for 10 min on ice. Cells were washed twice with ice cold 1X PBS and then incubated with FITC-anti-CD1d, PE-anti-CD5 and APC-anti-CD19 (clone MB19-1) monoclonal antibodies or with their respective isotype controls (all from eBioscience). Samples were stained with labeled Abs (0.5 µg/million cells) for 30 minutes in the dark at 4°C and then washed twice with 1X PBS 1% BSA before analyzing using an Accuri C6 flow cytometer (Accuri Cytometers, Ann Arbor, MI, USA). For analysis, cells were first gated on lymphocyte gates based on forward and side scatter. CD19+ cells were gated for CD5+CD1D^{hi} identification.

**Statistical analysis**

Allergic C57BL/6 wild type animals were compared to their respective naïve controls after 5 days post challenge. B cells cultured in the presence of LPS and LMM HA were compared to control B cells. Results were expressed as mean ± SEM. Data were evaluated using an unpaired, two-tailed student t test to determine significance differences. P-value of < 0.05 was considered significant when treatments were compared to their respective controls.
Results

_Determination of TLR-2 and TLR-4 expression on naïve and allergic mice spleen and lung B lymphocytes_

We previously discovered that during _A. fumigatus_ conidia induced allergy, IL-10 and TGF-β1 serum levels are increased. _In vitro_ studies also showed that B lymphocytes isolated from the spleens and lungs of allergic mice secreted IL-10 and TGF-β1 in response to LMM HA. IL-10 production was CD44-dependent whereas the hyaluronic acid binding receptor responsible for TGF-β1 secretion in the _A. fumigatus_ conidia-induced allergic mouse B lymphocytes was unknown. To identify HA binding receptor(s) needed for TGF-β1 secretion by B lymphocytes in response to LMM HA binding, we compared the expression of TLR-2 and TLR-4 on B lymphocytes on naïve and allergic mice spleens and lungs. To determine the changes in the TLR-2/4 expression in the lung and spleen B lymphocytes population after _A. fumigatus_ conidia challenge, we prepared single cell suspensions of lung and spleen cells from naïve and allergic mice. We found that CD19+ cells increased at day 5 after two conidia challenges as compared to non-asthmatic naïve controls in both the lungs and spleens (Figure 4-2). We then analyzed the B lymphocyte population (CD19+) for TLR2 and TLR4 expression by flow cytometry. We did not detect any TLR-2 or TLR-4 expressing CD19+ spleen and lung B cells from naïve or allergic mice as determined by flow cytometry (Figure 4-2). This data suggests that TLR-2 or TLR-4 receptors are not expressed on B lymphocytes during non-asthmatic or _A. fumigatus_-induced mouse allergic asthma.
RHAMM expression is upregulated in splenic and lung B lymphocytes isolated from allergic mice.

Due to lack of TLR 2/4 expression and upregulation in asthmatic B lymphocytes, we next focused on RHAMM expression to identify the HA receptor needed for HA-induced B lymphocyte TGF-β1 secretion. Expression of RHAMM mRNA and protein in highly purified allergic mice spleen and lung B lymphocytes was compared to non-asthmatic counterparts. Highly pure spleen and lung B lymphocytes were purified (materials and methods) and total
RNA isolated followed by first strand cDNA synthesis. Relative steady-state mRNA levels were then determined by SYBR green qRT-PCR assay normalized to β-Actin (materials and methods). Comparison between naïve and allergic B cells showed that during A. fumigatus-induced allergic asthma, there was approximately 8- and 6-fold increase of RHAMM relative steady-state mRNA in lung and splenic B cells as compared to controls respectively (Figure 4-3A and B). RHAMM protein level was also increased in splenic B cells from allergic compared to control mice (Figure 4-3C). From these results, we concluded that B lymphocytes express RHAMM endogenously, and RHAMM expression is elevated at both the mRNA and protein levels during asthma as compared to non-asthmatic conditions.

**Figure 4-3. Upregulation of RHAMM mRNA and protein in A. fumigatus conidia-induced allergic mouse B lymphocytes.** (A-C) Purified lung and spleen B lymphocytes from naïve and allergic animals were analyzed for (A-B) RHAMM mRNA and (C) protein expression levels (M&M). Representative analysis for RHAMM protein expression is shown in (C). Data is presented as means ± SEM for mRNA and protein normalized to β-actin from 3 independent experiments comprising of 4-5 mice per group. **P≤0.05
**LMM HA induces a regulatory B lymphocyte phenotype in activated, but not naive splenic B lymphocytes**

IL-10 and TGF-β1 are immunosuppressive cytokines. A subset of B cells known as regulatory B cells can produce IL-10. In our *A. fumigatus*-induced allergic asthma murine model, both IL-10 and TGF-β1 levels were elevated, which coincided with peak recruitment of B cells in the lungs and spleens of allergic mice. In parallel *in vitro* studies, we further discovered that LMM HA treatment of splenic B cells from allergic mice and not their naïve control counterparts resulted in secretion of both IL-10 and TGF-β1 suggesting an induction of a regulatory B cell phenotype. A phenotypically unique CD19+CD5+CD1d hi subset of regulatory B cells are capable of producing both IL-10 and TGF-β1 cytokines in response to inflammatory conditions. In this study, we investigated whether LMM HA can induce the expansion of a phenotypically unique CD19+CD5+CD1d hi subset of regulatory B cells in resting and LPS activated splenic B cells. Highly purified splenic B cells were stimulated with or without 10ng/ml LPS subsequently with different concentrations of LMM HA. As shown in Figure 4, LMM HA stimulated the expansion of CD19+CD5+CD1d hi B cells in LPS activated (Figure 4B), but not resting (Figure 4A) splenic B cells. This data suggests that LMM HA and B lymphocyte interactions may be involved in induction of IL-10 and TGF-β1 production by a subset of regulatory B lymphocytes during chronic allergic asthma.
Figure 4-4. Effects of LMM HA treatment on resting and activated splenic regulatory B cell phenotype. Highly purified B lymphocytes were analysed by flow cytometry after treatments (A) Percentage of CD5+Cd1d^{hi} cells within resting CD19+ B cells after treatment with or without LMM HA. Highly purified splenic CD19+ B cells (resting) were cultured with or without LMM HA for 24 hours. (B) Effects of LMM HA on expansion of CD19+CD5+CD1d^{hi} LPS activated total splenocytes. Total splenocytes were cultured with or without LPS, plus or minus LMM HA for 24 hours and percent CD5+Cd1d^{hi} cells within CD19+ cells analyzed by flow cytometry. Figures for representative of 2 independent experiments with 3 replicates each. Means ± SEM values are graphed for each study. *p-value ≤0.05 was considered significant when compared to the respective controls. NS means not statistically significant.
Discussion

In the current study, we aimed to determine which HA binding receptor mediated TGF-β1 upregulation and secretion that might be contributing to a pro-fibrotic remodeling of lung environment following a chronic allergic asthma. CD44 receptor is expressed on B lymphocytes and bind LMM HA to induce chemotaxis and IL-10 secretion by these cells. However, the LMM HA receptor required for TGF-β1 upregulation and secretion by B lymphocytes in the *A. fumigatus*-induced allergic asthma lungs was unknown. We analyzed RHAMM, TLR2 and TLR4 expression on B lymphocytes during *A. fumigatus*-induced chronic murine allergic asthma. We showed that RHAMM mRNA expression was increased in both splenic and lung B lymphocytes from chronically *A. fumigatus*-induced allergic mice as compared to their naïve controls. RHAMM protein was elevated in splenic B cells as well supporting the mRNA changes observed. There was no detectable toll-like receptors 2 and 4 expression on splenic and lung B lymphocytes from either allergic or non-allergic mice, but lung RHAMM protein was not measured due to low cell yields. In addition, we showed that treatment of activated, but not resting splenic B lymphocytes with LMM HA results in an expansion of a subset of B lymphocytes (CD19⁺CD5⁺CD1dhi) known to be immunosuppressive. From these data, we concluded that RHAMM is the most likely HA binding receptor mediating LMM HA induced TGF-β1 elevation and secretion in B lymphocytes during allergic asthma.

B lymphocytes are important inflammatory cells associated with asthma that exacerbate granulocytic inflammation during asthma. In agreement with other studies, we found that B lymphocyte production and recruitment to allergic lung increases during asthma. Allergic lung recruited B cells are important for IgE secretion and airway hyperreactivity during asthma. However, local inflammatory environment and the extracellular matrix in particular are
underappreciated partners of the adaptive immune response. During allergic asthma, the production of Hyaluronic acid increases. HMM HA gets broken down into its pro-inflammatory smaller size fragments, LMM HA. LMM HA recruits and activates macrophages at sites of tissue damage. LMM HA, and not HMM HA is chemotactic to B lymphocytes from allergic asthma mice in a CD44-dependent mechanism. The increase in B lymphocytes to the lungs has been correlated to serum LMM HA increase during asthma. In addition, these cells respond to LMM HA by producing TGF-β1 through an unknown receptor that is not CD44. Because RHAMM, TLR2 and 4 are the remaining major HA-binding receptors currently known to be expressed on B cells, we compared their expression on naïve and allergic mice B lymphocytes. Analysis of TLR2 and 4 indicated that they were not modulated on B lymphocytes at the conditions we investigated. Our study was the first to show that lung and spleen B cells do not express TLR2 and 4 in normal or asthmatic mice. In contrast to B lymphocytes, alveolar macrophages express both TLR 2 and 4 in allergen-induced asthma.

Analysis of RHAMM expressed on naïve and allergic mice B lymphocytes indicated an increase in a 90-95-KDa protein species comparable to that described on malignant B cells during allergic asthma. Since this is the only other HA binding protein other than CD44 expressed on B lymphocytes during A. fumigatus-induced allergic asthma, these results suggest that RHAMM is the required HA binding receptor for production of TGF-β1 by B lymphocytes upon binding LMM HA in our allergic asthma model. Hence, our results suggest a “division of labor” for the HA-binding receptors during asthma. CD44 is needed for B lymphocyte recruitment and IL-10 production, whereas RHAMM is important in HA-induced TGF-β1 secretion. Interestingly, in fibroblasts, TGF-β stimulates RHAMM and hyaluronic acid synthesis. However, whether this is true for B lymphocytes also was not studied here. More
importantly, RHAMM can compensate for the loss of CD44 in binding hyaluronic acid, increasing leukocyte migration, up-regulating genes involved with inflammation and exacerbating collagen-induced arthritis, a condition similar to asthma in terms of immune cell inflammation. Understanding whether this compensation is possible in B lymphocytes in asthmatic patients will advance our understanding of the role of B cells during asthma.

Once stimulated, immune responses need to be regulated to prevent the responding effector cells from causing detrimental effects. IL-10 and TGF-β1 are key immunoregulatory cytokines produced during allergic asthma. The secreted IL-10 and TGF-β1 during allergic asthma can regulate Th2 mediated inflammatory responses and promote tissue repair. A very rare subset of splenic B lymphocytes characterized by CD19+CD5+CD1dhi (B10) surface markers make up only about 1-2% of splenic B cells and have been shown to produce large amounts of IL-10 and TGF-β1. Toll-like receptor ligation by LPS can induce regulatory B cells to induce tolerance and downregulate inflammatory reactions in conditions such as asthma and arthritis. In agreement with this study, we showed that TLR ligation by LPS induced a regulatory B cell phenotype. Importantly, a major contribution of this research study was the discovery that LMM HA treatment of LPS activated B lymphocytes increased the expansion of these rare B10 cells. This expansion required that the B lymphocytes be in an activated state such as that seen during allergic asthma conditions and was LMM HA concentration independent. This data suggested that LMM HA and B lymphocytes interactions may be involved in secretion of IL-10 and TGF-β1 secretion by regulatory B lymphocytes during chronic asthma. Regulatory B lymphocytes are important in resolving inflammation as mice lacking B lymphocytes have exacerbated inflammation and tissue damage in autoimmune conditions. Identifying regulatory B lymphocytes in our murine allergic asthma model could explain the cell source of IL-10 and
TGF-β1. Whether RHAMM signaling is required for regulatory B lymphocytes induction and expansion is still unknown.

In conclusion, the roles of B lymphocytes in allergic asthma other than antibodies production are starting to be appreciation. Mice lacking B lymphocytes have exacerbated inflammatory responses\textsuperscript{50}. Therefore, our identification of RHAMM as the only non-CD44 receptor expressed by B lymphocytes during allergic asthma provides an opportunity for development of drug therapies that target B lymphocytes during asthma. TGF-β1 production is important for immunosuppression and tissue remodeling; and is secreted by regulatory B cells. Even though LMM HA is generally regarded as a pro-inflammatory ECM, our results suggest that it could also be playing a key role in resolving inflammation by inducing the expansion and activity of a regulatory B cell subsets. Increase in soluble LMM HA in asthmatic lungs and serum could provide a “danger signal” hence expanding and promoting anti-inflammatory phenotypes to resolve inflammation and promote tissue repair.

References


35. Barnes, P. J. (2009) Intrinsic asthma: not so different from allergic asthma but driven by superantigens?, *Clin Exp Allergy* 39, 1145-1151.


CHAPTER FIVE: OVERALL DISCUSSION, FUTURE STUDIES AND CONCLUSIONS

Effective immune surveillance by lymphocytes requires that they be able to continuously circulate from lymphoid organs back into the blood stream. Peripheral lymphoid organs provide regions where close interactions between antigen presenting cells and lymphocytes are maximized. Multiple ligand-receptor interactions are employed by the immune system to regulate migration into lymphoid tissues under homeostatic conditions or in response to infections and inflammation. VIP is a potent chemoattractant to naïve CD4 T cells to the gut. Ottaway discovered that VIP signaling was required for efficient migration of these cells into the GALT. This research expanded the knowledgebase regarding molecular mechanisms required for VIP-induced chemotaxis of CD4 T cells. There were three main findings from the first study presented here: 1) VIP upregulated the mRNA levels of a known chemotactic pathway, centering on EGFR signaling. This upregulation was found to be rapid, transient and coordinated. 2) We found that VIP-induced upregulation of all the six putative-chemotactic EGFR pathway genes was sensitive to a potent EGFR kinase inhibitor, Tyrphostin AG-1478. 3) VIP-induced CD4 T cell chemotaxis was sensitive to Tyrphostin AG-1478 as well.

The determination of the molecular mechanism of VIP-induced chemotactic activity is significant because VIP is the highest secreted neuropeptide in the gut. 70% of immune cells reside in the mucosal tissues of the GALT and continued understanding of factors orchestrating their migration there is required to understand their biology. These studies have implications in potential therapeutic developments for the treatment of intestinal diseases such as intestinal bowel disease and colitis. Other elements that will help elucidating the molecular basis of VIP-induced CD4 T cell movement is the investigation of EGFR signaling requirement in expression
of adhesion molecules after VIP treatments. However, many unanswered questions still remain regarding the signaling mechanism orchestrating VIP-induced CD4 T cell recruitment. The studies done here were in vitro, which have a chance of not being replicated in vivo. To investigate this, our lab has secured, a VIP knockout mouse strain to begin investigating the levels of recruited CD4 T cells to the GALT. It would also be interesting to investigate the same studies in vitro and in vivo using EGFR knockout mice. If our findings are accurate, we would expect to find that VIP and EGFR knockouts have fewer CD4 T cells in the GALT and prone to intestinal infections. Understanding which VIP receptor is responsible for VIP-induced T cell migration is also one of our lab’s future goals.

The immune system is under homeostatic control. It responds to changes in the environment to maintain homeostasis. In doing so, the immune system tends to maintain a steady near constant number of immune cells throughout the adult life of an individual. The concept of homeostasis implies that the production and elimination of cells through death remains relatively equal. In case of a perturbation, the number of lymphocytes may change, but once the perturbation is eliminated, cell numbers return to re-establish immune homeostasis. However, it is noteworthy to mention that comparing two similar animals with different number of a subset of immune cells doesn’t necessarily mean that one of them has a perturbed homeostasis. The molecular mechanisms regulating immune system homeostasis are not well known. VIP receptor, VPAC2 is widely expressed by immune cells and has been reported to be involved in a wide array of immunological functions including, immune and inflammatory responses, neuromuscular transmission, metabolic rhythmic activities as well as cognition and behavior. However, VPAC2 signaling functions in immune homeostasis has never been reported. Using a VPAC2 knockout mouse strain, we demonstrated that VPAC2 is required to maintain immune
homeostasis. Mice deficient in VPAC2 had fewer total splenocytes, thymocytes and leukocytes as compared to their wild type age, sex and weighed matched counterparts. However, the expected ratios of B and T cell splenocytes were not affected. This indicates that VPAC2 signaling is required to maintain normal production of immune cells. But, because the ratios were not affected, lineage commitment immune cell generation in the bone marrow is not affected by lack of VPAC2.

Since immune homeostasis is a product of production minus cell death, we determined the rate of survival. We showed that VPAC2 cells survived longer than wild type cells. This observation disproved our hypothesis that VPAC2 deficient immune cells would die faster resulting in fewer peripheral cell numbers, thus explaining the lower numbers seen in mutants as compared to wild types.

The VPAC2 knockouts exhibited lower levels of eosinophils, which could possibly be due to dysregulated eosinophil generation in the bone marrow. We would like to investigate this further by comparing IL-5 levels in VPAC2 knockouts. IL-5 is key to inducing eosinophil generation. Furthermore, VPAC2 agonists have been shown to stimulate an potent IL-5 secreting cell type called type 2 innate-like lymphoid cells (ILC2), which in turn regulated eosinophil homeostasis\(^5\). By comparing percent ILC2 cells and IL-5 levels in VPAC2 knockouts to wild types, we will be able to better understand why the mutants have lower levels of eosinophils. We hypothesize that VPAC2 knockouts have lower levels of ILC2 cells resulting in lower IL-5 levels. Further research is necessary to investigate homeostatic regulation by VIP and VPAC1 as well. We will be repeating these studies and expanding on them to determine the VIP signaling axis controlling immune cell homeostasis.
We expanded our investigation of VPAC2 immune homeostatic role into hematopoiesis. We found that differentiation of VPAC2 deficient bone marrow cells into eosinophil lineage lagged the pace of wild type age, weight and sex-matched counterparts. VIP is secreted in the bone marrow where it’s been shown to inhibit VPAC2 is also expressed. We hypothesize that the late proliferative burst seen at the end of eosinophil differentiation in the knockouts is related to late down regulation of VPAC1 expression in VPAC2 knockout mice as compared to wild types. This would be reminiscent of the down regulation of VPAC1 in the thymus where a switch is seen in VPAC1:VPAC2 ratio expression in DN thymocytes. Therefore, our data suggests that VPAC2 signaling is needed for normal bone marrow cell differentiation. One weakness to this research is the drawbacks associated with using knockout strains. Knockout strains generally do not have all the components of normal development. VPAC2 knockout mice have abnormal reproduction. Male VPAC2 knockouts have been shown to have fewer numbers of sperms as they get older and in our hands produced very few litters. These mice also do not gain as much weight as the wild types after around 15 weeks. We utilized mice below the age of 15 weeks for our studies. We also limited any variables by age and sex matching all the mice we used. All mice were also weighed prior to studies carried out and therefore difference in results presented could not be explained by either age, sex or weights of the mice. Our future studies will be to compare basal cytokine levels between VPAC2 knockouts and wild type mice. Cytokines such as IL-7, IL-2 and IL-15 have been shown to control immune cell homeostasis. We predict that VPAC2 knockout mice will have lower levels of one or all of these homeostatic cytokines.

Using experimental animal systems disease models offers the benefit of the ability to recreate similar human disease environment like during asthma. Using a fungus-induced asthma
model developed by the Schuh research group, we were able to study the expression profile of hyaluronic acid receptors on B cells from allergic asthma as compared to those from naïve mice. We found that B cells from allergic mice increased their expression of RHAMM as compared to non-asthmatic mice B cells. RHAMM is a HA-binding protein whose role in B cells is not well understood. We hypothesize that RHAMM activity is required for LMM HA induced TGF-β1 secretion.

Another important finding from our research was the discovery that LMM HA induced a regulatory B (B10) cell phenotype when B cells were in activated state. Discovery of existence of regulatory B cells is very recent and factors that are responsible for their expansion and induction are not well known. In sum, our data support the conclusion that B lymphocytes express CD44 and RHAMM as the only major HA-binding proteins. RHAMM expression is increased in B lymphocytes during chronic allergic asthma. RHAMM is potentially the receptor required for TGF-B1 secretion by B lymphocytes in asthmatic patients. Cross-linking of HA-receptors by LMM HA and potentially other ECM components promote regulatory B lymphocyte expansion and persistence. We propose a two-step model, in which the initial activation of regulatory B (B10) cells through the LPS-TLR signaling is sufficient to induce a B10 phenotype, followed by a requirement for a costimulatory signal to support the regulatory B lymphocyte phenotype expansion and persistence. This latter step can be achieved by presence of LMM HA, such that in the absence of LMM HA there is no expansion of regulatory B lymphocytes. This pathway can contribute to the support of peripheral immune tolerance as mice lacking regulatory B cells develop autoimmune disorders. This mechanism may be one way in which viable regulatory B10 populations expand and persist in injured tissues like asthmatic lungs to resolve inflammatory responses and maintain peripheral tissue tolerance. Even though
LMM HA is generally regarded as a pro-inflammatory ECM, our results suggest that it could also be playing a key role in resolving inflammation by inducing the expansion and activity of a regulatory B cell subset. We propose that the presence of soluble LMM HA in asthmatic lungs conveys an inflammatory and tissue damage signal which functions as a danger signal to expand and promote the responses of regulatory B cells promoting immune homeostasis and tissue repair. Therefore, showing that LMM HA expands this subset of B cells is very exciting to the asthma community as a potential advancement towards development of immunotherapeutic strategies against asthma and other autoimmune diseases. LMM HA expression is increased in allergic mice lungs. Our next goal is to identify the correlation between LMM HA levels and expansion of regulatory B cells during asthma. Therefore, our findings further illustrate the role of B cells in allergic asthma.

In sum, our main aim for the studies carried out here were to further our understanding of immune cell biology and responses in normal and asthmatic conditions. We revealed that VIP beckons T cells by igniting a known chemotactic pathway centered on EGFR signaling. We have also identified a previously unknown role VPAC2 in immune cells homeostasis. We showed that VPAC2 signaling is necessary to have a normal immune homeostasis. Mice lacking VPAC2 appear to be lymphopenic and have less eosinophils. Even though most in the VIP/VPAC2 field have generally regarded its biological functions as being generally limited to modulating activated immune cells, our data suggest otherwise. We show that play important biological roles in quiescent immune cells by controlling chemotaxis and homeostasis. In conclusion, by characterizing B cell RHAMM expression during asthma and collecting data indicating that LMM HA expands/induces regulatory B cells, we have provided a platform that can be expanded
on to better understand the roles of B cells during asthma with the goal of developing of therapeutic interventions against asthma in the future.

References