A STICKY SITUATION: FUNCTIONAL RESPONSES OF MAST CELLS TO ACIDIC pH

by

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ABSTRACT

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Precise regulation of extracellular pH is critical for proper function of many cellular activities. Minor fluctuations in pH can have profound effects on ion transport, receptor function, and cellular secretions. This work examines the impact of acidic pH, universal at sites of inflammation, on the activation status of mast cells. The mast cell line HMC-1 was used to explore activation by acidic pH. Activation of HMC-1 cells was characterized in cell adhesion, secretion of cytokines, and changes in cell morphology. HMC-1 adhesion to two substrates, collagen and laminin was measured at 0, 1 hr, 2 hrs, 4 hrs, and 6 hrs. To determine the effect of acidic pH on adhesion, a comparison was made between adhesion in normal physiological pH (pH 7.4) and acidic pH (pH 6.5). The secretion of cytokines by HMC-1 was examined using a 42

cytokine array, results were quantified with ELISAs. Changes in the morphology of HMC-1 were examined with scanning electron microscopy. The involvement of a pH receptor from the TRPV family was tested by application of specific blockers. A shift to acidic pH (pH 6.5) initiated an increase in adhesion that was time, and dose dependent, and with a preference for collagen. HMC-1 secretion of the cytokine MCP-1 decreased in response to exposure to acidic pH. The receptor TRPV1 is implicated as playing a role in the response of HMC-1 to acidic pH with the finding that adhesion decreased and MCP-1 secretion increased in the presence of the TRPV1 antagonist ruthenium red. The shift to acidic pH also resulted in significant morphological changes in the morphology of HMC-1. In summary, acidic pH induces mast cells to adhere to extracellular matrix proteins, secrete cytokines as well as restructure the cytoskeleton. This study helps to define the functional responses of mast cells to acidic pH.

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CHAPTER 1

INTRODUCTION

1.1 Changes in pH, Inflammation, and Mast Cells

The regulation of extracellular pH is vital for cellular functions such as: ligand membrane-associated enzyme activities, ion transport activity and maintaining the bicarbonate buffering system of blood [34]. It is one of the most tightly regulated parameters within the human body. Changes in pH have been associated with a variety of diseases and conditions such as: allergic reactions, insect bites, migraines, tumor growth and rheumatoid arthritis. The pH is determined by the concentration of hydrogen ions, and is described by values ranging from as high as 14 to as low as 0. High concentrations have a lower pH value, and are considered to be acidic and lower concentrations of hydrogen ions have a higher pH value and are considered alkaline. Normal physiological pH, of extracellular fluids, the pH essential for the body to carry out most functions is 7.4. When the body recognizes even minor fluctuations of hydrogen ions a cascade of corrective measures at a systemic as well as a cellular level are activated. Minor fluctuations in pH have been known to cause tissue acidosis, which can result in, ischemia, tumors, toxins, inflammation, and tissue damage [26,40,42].

Cells perceive conditions in their surrounding environment by expressing a variety of membrane proteins called receptors. The stimulation of these receptors causes the activation of intracellular signaling pathways, which leads to adjustments in cell functions to the new conditions. These adjustments might include secretion of active mediators and the reformation of cytoskeletal elements. Mast cells, which are tissue resident cells positioned close to the interface between internal and external environments, possess a variety of membrane receptors allowing them to monitor the changes in the immediate neighborhood. The most well known receptor activation in mast cells is the immunolglobulin E receptor (FCεRI), which is activated by the cross linking of the receptor by immunoglobulin E [39]. This receptor is responsible for the resulting 'wheal and flare response' demonstrated in classical allergen injection reactions. Mast cells utilized Immunoglobulin G receptors and Toll-Like Receptors to monitor parasitic and microbial activities, cytokine receptors to mediate differentiation and monitor the local environment [55]. The activation of mast cell receptors under allergenic conditions has been widely studied, but the role of mast cell receptors in other conditions such as inflammation has not been described.

Inflammation has been historically characterized with five cardinal signs and symptoms: rubor (redness), calor (heat), tumor (swelling), and dolar (pain), functiolessa (loss of function) as far back as Celsus (A.D. 178) [46]). Inflammation is often associated with changes in pH toward acidity [14]. So far two well characterized families of receptors use, in the detection of hydrogen ion, concentration changes [7,67].

They are the acid sensing ion channel family (ASIC) and the transient receptor potential family (TRP). Both the ASIC family and TRPV are found in a variety of cells [38,45]

Mast cells are known to play a key role in inflammation however their exact role has not been fully characterized. It is known that mast cells respond to a variety of inflammatory mediators such as prostaglandins, bradykinin, and cytokines [10,43,68]. The response of mast cells to acidic pH has not been characterized and is important to fully understanding the role mast cells play in inflammatory diseases and conditions. Understanding this role may bring to light potential drug targets.

CHAPTER 2

BACKGROUND

Inflammatory conditions are widespread and can be debilitating. Acidic pH is often associated with inflammatory conditions, which are associated with pain. There are many receptors on Mast cells that may be used during inflammation. This work concentrates on the known ion channel receptors that can sense acidity. Further understanding of the inflammatory process with regards to pH, ion channels involved, mediators present, and the role of mast cells is important to understanding methods of inflammation and pain management.

2.1 Acid Sensing Ion Channel Family of Receptors (ASIC)

Acid sensing ion channels are voltage independent sodium channels related to amiloride-sensitive epithelial sodium channels and the degenenerin/mec (ENaCs) ion channels of *Caenorhabditis elegans* [67]. Structurally, ASIC and ENaCs are believed to have the same topology with two transmembrane domains, a cytoploasmic amino and carboxyl termini [67]. The similarities end with topology, the two ion channel families are distinct in primary sequences. There are seven isoforms within the cation channel family (Table 2.1). ASIC-1 was the first channel described; it is activated by an increase in acidity, from pH 7.4 to 6.9 or below [67]. Other members of this family respond to pH values within the physiologic range with some activation thresholds as high as pH 7.2 and ASIC2a activating as low as pH 4.1 [38].

Table 2.1. ASIC Channel Characteristics [38]**.**

Isoform	Tissue Distribution	PH 0.5
ASIC 1a	PNS, brain, spinal cord, retina, taste cells, bone	6.2 - 6.8
ASIC 1b	PNS, taste cells, cochlear hair cells	5.1 – 6.2
ASIC 1b2	?	?
ASIC 2a	PNS, brain, spinal cord, retina, cochlear spiral ganglion, bone	4.1 – 5.0
ASIC 2b	PNS, brain, spinal cord, retina, taste cells	n/a
ASIC 3	PNS, taste cells, retina, testis, lung, epithelial cells, inner ear, bone	6.2 – 6.7
ASIC 4	PNS, brain, spinal cord, retina, pituitary gland, inner ear	n/a

2.2 Transient Receptor Potential Family of Receptors (TRP)

The Transient Receptor Potential family of receptor-channels are nonselective cation channels with a strong preference for calcium [71]. The properties of this family of receptor-channels are not fully understood. Temperature is the main ligand for this receptor [49] although this family contains channels that are regulated by very diverse stimuli including: pH, capsacin [7], and mechanical stimuli such as membrane stretching [59] and osmolarity [35]. There are four known sub families within the TRP receptor family, TRP-Ankyrin (TRPA), TRP-Canonical (TRPC), TRP-Melastianin (TRPM), TRP-Mucolipin (TRPML), TRP-Polycystin (TRPP), and TRP-Vanilloid (TRPV) [6,52]. Members of this family contain six transmembrane domains, a putative pore-loop region located between the fifth and sixth transmembrane domains and similar cytoplasmic amino and carboxy termini (Figure 2.1)[44]. Several members of

the different subfamilies contain ankyrin domains that aid in protein-protein interaction [58]. For the purpose of this dissertation only TRPV will be examined.

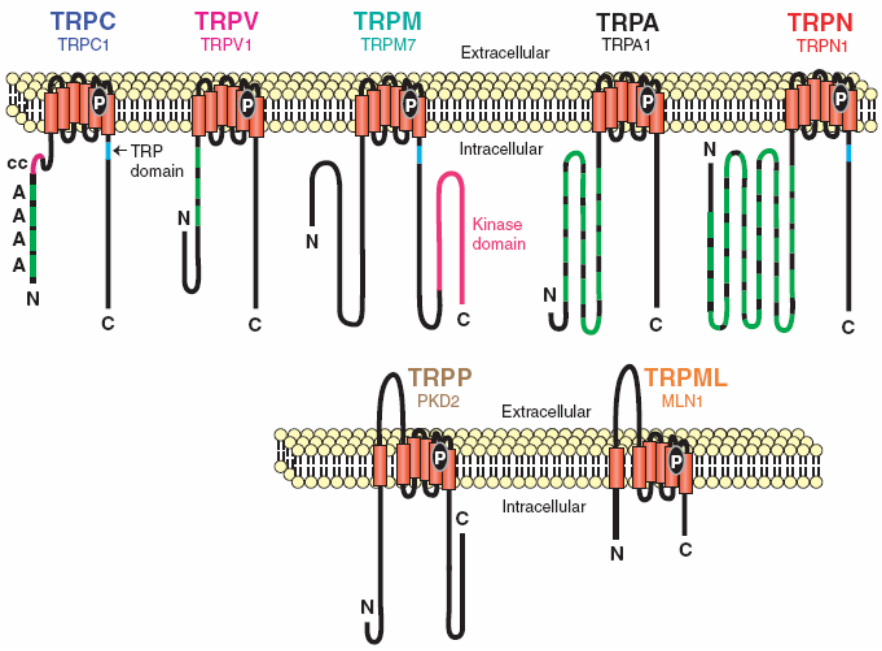


Figure 2.1. Transient Receptor Potential Family of Receptors. Proposed structural aspects of the receptor family are shown: Ankyrin repeats are shown in green (A), coiled coiled domains are shown in red (cc), protein kinase domain (TRPM only) is shown in pink, transmembrane segments are shown as red cylinders and the trp domain is shown in blue [45].

Members of the TRPV subfamily are nonselective cation channels gated by an array of stimuli. This stimuli may be physical (temperature), chemical (pH, capsaicin) [7], or mechanical (osmotic pressure) [35]. Seven members have been identified in mammals to date (Figure 2). Structurally, all members contain short sequences with open reading frames of approximately 900 amino acid residues and contain three or

more ankyrin domains within their cytoplasmic N-termini (Figure 2.1, Table 2.2)[19,21,56].

Table 2.2. TRPV Subfamily [45]**.**

Name	Activation
TRPV1	Heat (43°C), Vanilloids, Andamide, Protons, PIP_2, exocytosis
TRPV2	Heat (52°C), exocytosis, membrane stretch
TRPV3	Warm (30°-39°C)
TRPV4	Osmotic cell swelling, phorbol esters, warm (27°C), 5'6'-EET,
TRPV5	Low levels of Intracellular Ca2+, hyperpolarization, exocytosis
TRPV6	Similar to TRPV5, calcium detection store operated, exocytosis

The TRPV family of receptors is primarily temperature receptors, but one of them also functions as a pH receptor. This channel is TRPV1 (formerly known as Vanilloid Receptor 1) was the first member cloned [7]. It is expressed in small sensory neurons and believed to function as a pain receptor [7]. The mechanism for activation is unknown; however binding of more than one molecule is required to open the channel [7,19]. The pungent ingredient in hot chili peppers, capsaicin, is a known activator of the channel, resulting in an influx of calcium into the cell [11]. Capsaicin is categorized as a vanilloid molecule because it possesses vanillyl moieties. This channel

is activated by high heat, with a threshold of approximately 43°C, which is perceived as a noxious pain [7]. Acidic pH is capable of modulating TRPV1 activity by lowering the threshold to activation [7]. Stimulation with acidic pH alone does not activate the channel, however once capsaicin was added the threshold temperature was lowered [7]. Inflammatory mediators such as bradykinin produced a similar effect with the presence of acidic pH [10]. Additionally, anandimide, an endogenous cannabinoid produced in humans is an agonist of TRPV1 [74]. TRPV1 responses are antagonized by the competitive antagonist, capsazepine, and the non-competitive antagonist, ruthenium red [7]. A recent N-terminal splice variant of TRPV1 was identified. The cytoplasmic N-terminal domain and ankyrin repeats are missing and the splice variant has no identified function [56]. This seems to signify the importance of the cytoplasmic N-terminal domain and the ankyrin repeats, without them the receptor will not function [56].

The remaining six TRPV receptors are distributed widely throughout the body including the peripheral nervous system, skin, tongue, liver heart, fat, and bladder [19,23,35,71]. They are all similar in structure to TRPV1 varying only in the number of ankyrin domains [1,8]. TRPV2 and TRPV3 are activated by noxious heat, TRPV4 is activated over a range of temperatures (~27 - 42°C) [19,71]. The remaining members TRPV5 and TRPV6 are not heat activated, they are activated by low intracellular calcium levels [1]. TRPV4 is activated by osmolarity and cell volume as well as phorbol esters [35]. All of the TRPV channel receptors are blocked with ruthenium red. Only TRPV1 is activated by acidic pH and blocked by capsazepine (Figure 2.2) [49].

The TRPV family is recognized to play a role in pain reception and temperature [8]; however their function in non-neuronal tissue remains elusive

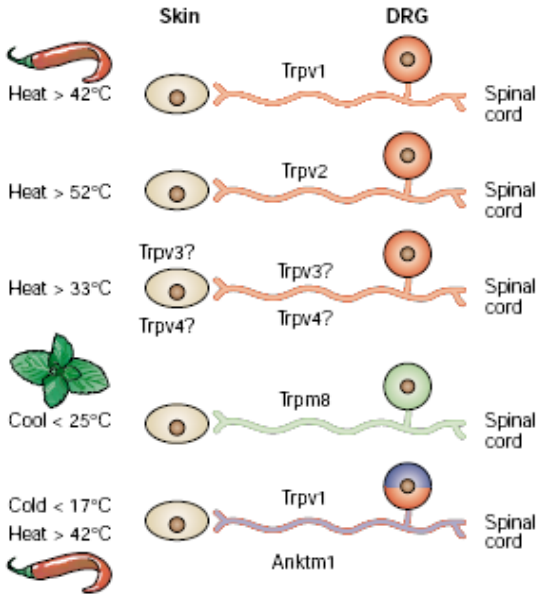


Figure 2.2. Sensitivities and Pathways of the TRPV Subfamily. The warm channels (TRPV3 and 4) have not been fully characterized. Anktm1 is included to demonstrate the multimodal activation of TRPV1 [49].

2.3 Mast Cells

Paul Ehrlich first discovered mast cells over one hundred years ago (1877). Early studies of mast cells revealed their secretory nature; however, only in the last 20 years have renewed studies using updated laboratory techniques has elucidated the more complex nature of mast cells [73]. Mast cells are key players within the immune system and there is no known disease or biological condition that exhibits a lack of mast cell influence [41]. Mast cells are secretory cells whose most important attribute is

secretion of mediators that modulate other lymphocyte behavior such as: T and B cell recruitment and response, lymphocyte growth, phagocytization and presentation of antigens to T cells [39].

Mast cells are ubiquitous throughout the body; however they do not circulate continuously within the blood stream as other immune cells. Instead, they reside in tissues all over the body, particularly near sites of contact with external environment, blood vessels and peripheral neurons [41]. Mast cells are known effectors in inflammation and chronic pain and considered to be the link between the immune and endocrine system and the brain [62,63].

Mast cells are hematopoietic in origin, developing from multipotent hematopoietic progenitors [29,73]. The development of a mast cell progenitor is influenced by the binding of stem cell factor to the Kit receptor on the progenitor cell. Newly formed from the multipotent progenitor, mast cells undergo two additional differentiations [73]. The first differentiation occurs in bone marrow. This defines which tissue the mast cell will reside in and is dependent upon localized stem cell factors and cytokines in the micro environment. Mast cells proceed to their target tissue via the blood stream. Once they reach the target tissue, mast cells undergo a second and final differentiation [73]. This differentiation is also dependent upon the localized micro environment; however this differentiation directs what mast cells manufacture and secrete as well as what mast cells will respond to [73].

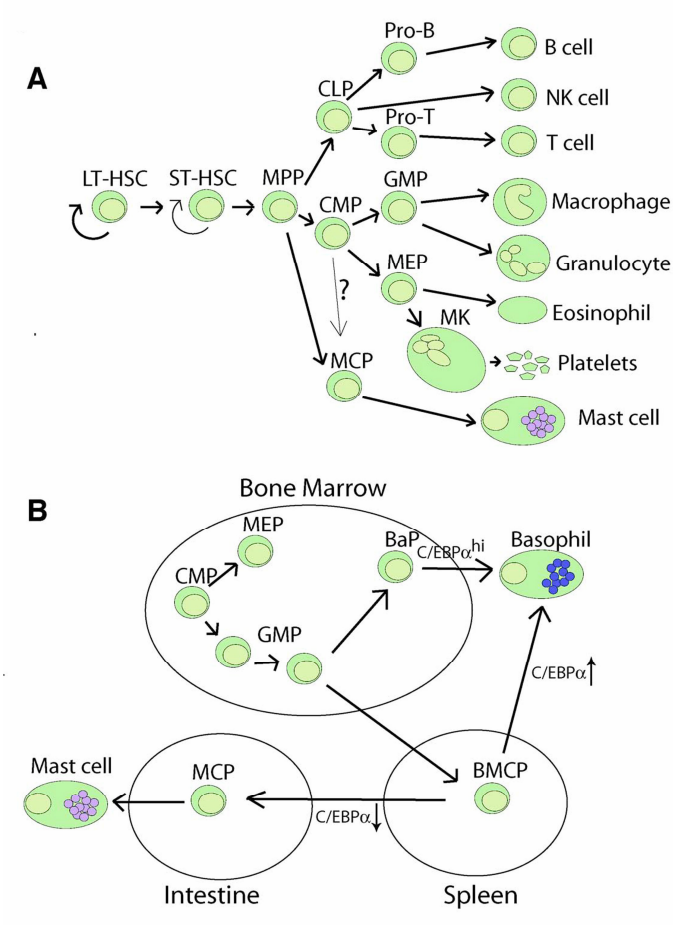


Figure 2.3. Mast Cell Differentiation. Two proposed pathways for mast cell differentiation. A). Proposes that mast cell progenitors (MCP) derive from multipotential progenitors (MPP). B). Proposes that granulocyte/ macrophage progenitors (GMP) in bone marrow are the source for basophil/mast cell progenitor (BMCP). BMCPs expressing CCAAT/enhancer binding protein α (C/EBPα) become basophil and those that do not become mast cell progenitors (MCP). This model does not show the link between Long-term hematopoietic stem cells (LT-HSC and MMPs. ST-HSC are short term heamtopoietic stem cells. [47].

Mast cells reside near sites of insult and injury and are classified according to the type of tissue they reside in. There are three main classifications of mast cells. Serosal, mast cells that reside in connective tissue, mucosal mast cells that reside in mucosal tissue, and neuronal mast cells that reside near peripheral neurons [41,62]. Mast cells that reside near peripheral neurons are of special interest as they are believed to be the neuro-immune link to the brain and may respond to some of the same stimuli that sensory neurons respond to [62].

Mast cells play an immunomodulatory or immunoregulatory role. Their role in immunoglobulin E (IgE) hypersensitivity reactions such as contact sensitivity and IgE immune responses such as bacterial and nematode infections is both complex and controversial [16]. Mast cells are well known players in immunoglobulin E (IgE) immediate hypersensitivity reactions. The cross-linking of the FcεR receptors results in mast cell activation, release of preformed inflammatory mediators such as histamine and tryptase and the secondary production and subsequent secretion of lipid mediators and cytokines [41,68]. Their role in host defense mechanisms regarding ectoparasites (ticks) has been documented, characterized and generally agreed upon however, their role regarding internal parasites is disputed. Early studies regarding internal parasitic infections, bacteria and nematodes, indicated a mast cell response, but more recent studies show the primary players are basophils and mast cells play a secondary role at best [39]. The activation of mast cells in response to an immune challenge results in a multifaceted response dependent upon the nature of the local stimuli. This response may

be within minutes or over hours, coordinated and graded to ensure an appropriate response, or in a more sinister manner resulting in a catastrophic response such as anaphylactic shock [40].

2.4 Mast Cell Morphology

Paul Ehrlich first described mast cells based on their characteristic granules which can be stained with metachromatic dyes (1870). Mast cells range in size from a mean diameter of 5 μm to 13 μm. Their shape is dependent upon their environment [68,73]. They may be spindle shaped as those found in dermal fibers, elongated or oval when near vascular tissue, or rounded when suspended in fluid as those found in vitro. Mast cell granules occupy as much as 55 % of the cytoplasm and may cause bulges and caveolae which can be seen with scanning electron microscopy [73]. The membrane of mast cells aside from the bulging appears highly ruffled with the presence of microvilli which vary in size from short enough to be lost with in the membrane ruffles to as long as twice the mean diameter [4]. The overall morphology of mast cell size, granule volume, overall shape, and ruffled membrane is dependent upon the local environment both as a limitation of space and state of activation. When a mast cell is activated, the cytoskeleton is rearranged, vesicles are moved to the periphery, the membrane is noticeably less ruffled and the volume of the cell increases all in preparation for secretion [4,12,53].

2.5 Mast Cell Adhesion

Mast cells reside in their target tissues through the attachment to extracellular matrix macromolecules such as fibronectin, laminin, collagen and protoglycans.

Adhesions between cells and the extracellular matrix may promote cell proliferation, migration, differentiation, and the production and secretion of cellular mediators [66]. This adhesion initiates cytoskeletal changes, and transport of secretory vesicles to the periphery of the cell resulting in secretion [53]. Adhesion is the first and necessary step in this signaling cascade.

Adhesion is accomplished though several cell adhesion receptors such as integrins, cadherins and the immunoglobulin superfamily. Mast cells adhere to extracellular matrix components through integrins [30]. The primary ligands of integrins receptors are laminin, fibronectin, and collagen [25]. The integrin family is formed by heterodimers of two polypedtide chains (α and β) that bind to several protein ligands providing cells with different signaling pathways. There are at least eighteen distinct α subunits and eight β subunits [25]. Integrin specificity is dependent upon the combination of the α and β subunits. Previous studies on skin mast cells have demonstrated that very late activation Ag-3 (VLA-3) integrin binds to laminin, collagen I and fibronectin. Integrin molecules VLA-4 and VLA-5 bind to fibronectin [12].

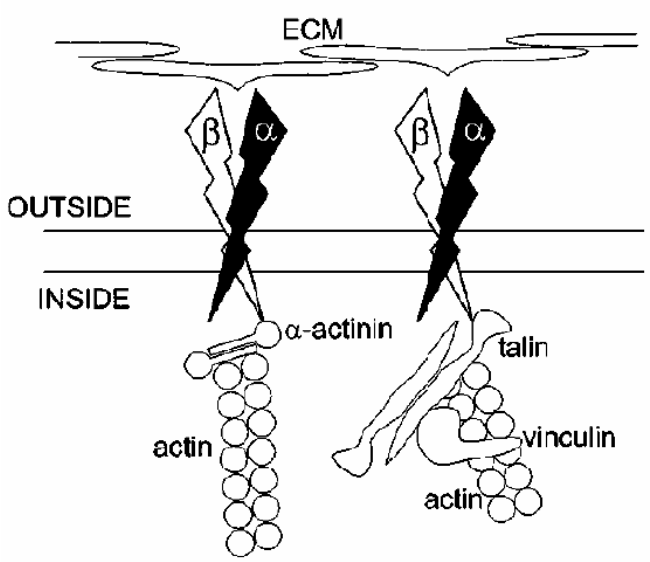


Figure 2.4. Integrin Cell Adhesion Receptor. Receptor is shown with associated cytoskeletal elements (actin, actinin, talin, vinculin) and extracellular matrix (ECM) [25].

Mast cells also express cadherins and immunoglobulin super family of cell adhesion molecules (ICAMS). The cadherin family of receptors are transmembrane proteins with a shared extracellular domain of approximately one hundred amino acids [25]. Cadherins promote cell to cell adhesion through calcium dependent associations with the actin component of the cytoskeleton or with intermediate filaments [25]. The iumunoglubulin superfamily of cell adhesion molecules (ICAMS) facilitates cell to cell adhesions primarily during inflammatory processes [65]. Structurally, ICAMS are comprised of two beta sheets formed from one hundred residues, although the number and size of the beta sheets may vary [20]. This dissertation examined mast cell

adhesion to plastic, laminin, and collagen therefore, the integrin family of receptors were the primary cell adhesion molecules involved.

2.6 Secretion of Cytokines

Mast cells have the ability of secrete anti or pro-inflammatory molecules. This is dependent upon the reception of chemical messengers within the local environment. Mast cells are a source for a variety of cytokines (Table 3) [16].

Cytokines are diverse low molecular weight proteins that regulate immune responses with a variety of effects. Rarely working alone, they are involved in a variety of biological activities including immunity, hematopoiesis, and inflammation. Their chief role is to mobilize and recruit other immune cells. Interleukins are a subfamily of cytokines that are secreted by and act on leucocytes [17]. Over twenty-five interleukins have been identified so far. Interleukins demonstrate a high degree of α-helical structure with little or no β-sheet structure [17]. They share a similar polypeptide fold containing four α-helical regions. The first and second helices run parallel and are connected by loops as do the third and fourth helices [17]. The tumor necrosis factors family of cytokines is comprised of two members, TNF-α and TNF-β. They have anti-parallel pleated β-sheets [64].

Dependent upon the stimulus, mast cell type, and individual exposure to the stimulus, mast cells may exhibit a varied activation level. Mast cells may be in a resting state in which there is no mediator release, low level activation where constitutively expressed mediators are released, to a highly activated state where large amounts and variety of mediators are released [16]. Mast cell activation and subsequent secretion of

inflammatory mediators in response to allergic conditions is well studied. Mast cell activation and subsequent secretion to inflammatory conditions has not been fully studied.

This dissertation utilizes HMC-1 to further illustrate to role of mast cells in inflammation. As stated previously, HMC-1 is an immature cell line that is not predisposed to secrete inflammatory mediators. HMC-1 can be stimulated to secrete a variety of cytokines (Table 2.3). HMC-1 has been well studied regarding secretion of cytokines in response to a number of mediators both endogenous such as substance P, and exogenous substances such as calcium ionophore and phorbol myriastate acetate (PMA). HMC-1 expression of cytokines has not been studied when stimulated with acidic pH.

Table 2.3: Mast Cells Express a Diversity of Cytokines [16,18,57]**.**

Mast Cell Expressed		
Cytokines	**Interleukins**	**Tumor Necrosis Factors**
FGF-2, IFN-α, IFN-γ, GM-CSF, LIF-2, MCP-1, MIP-1α, PDGF, TGF-α, VPF/VEGF,	IL-1β, IL-3, IL-4, IL-5, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-15, IL-16, IL-18, IL-25	TNF-α
HMC-1 Expressed		
Cytokines	**Interleukins**	**Tumor Necrosis Factors**
MIP-1α, VPF/VEGF,	L-1β, IL-3, IL-4, IL-5, IL-8, IL-10, IL-13, IL-16,	TNF-α

CHAPTER 3

MATERIALS AND METHODS

3.1 Cell Line

The human mast cell line (HMC-1) was used exclusively for this work, kindly provided by J.H. Butterfield [5]. HMC-1 was derived from a patient with mast cell leukemia [5]. This line lacks a functional IgE receptor and has mutations in the coding sequence of the C-Kit proto-oncogene [5,69]. HMC-1 is an immature mast cell line that allows for the stimulation and subsequent manipulation of this cell line for experimental purposes. This cell line is immature regarding some morphology and histamine content but still exhibit mature aspects regarding cytokine secretion. An additional advantage of this mast cell line is that it can serve as a resource for mast cell proteins including granule proteins and mast cell genetic material such as DNA and RNA [5]. In addition, HMC-1 is the only established continuously growing human mast cell line and has therefore been widely employed for *in vitro* studies of human mast cell biology.

3.2 Cell Cultures

HMC-1 cells were cultured in plastic tissue culture flasks in Cellgro COMPLETE™ with L-glutamine, (Cellgro® 40-101-cv), supplemented with 25 mM N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES)(Sigma-Aldrich, USA)

for adhesion studies and enzyme linked immuno sorbent assays (ELISA). This media is a low serum media used to minimize the presence of cytokines in the media [18]. HMC-1 cells were cultured in RPMI-1640 for the Ray Biotech cytokine assay. Regardless of media, Cells were subcultured twice a week and used the same day they were counted and plated. A hemocytometer was used to count cells.

The supernatant pH within the growing culture stocks was checked prior to all experiments with a range of pH 7.0 to 7.5 judged as being acceptable. If unacceptable the cells were either discarded or cultured and not used for 96 hours. The cells were incubated at 37°C, 95% humidity, and 5% CO_2. Experiments were performed at room temperature and incubations over 30 minutes occurred at 37°C, 95% humidity, and 5% CO_2.

3.3 Collagen Extraction

The tails from Sprague-Dawley rats (University of Texas at Arlington vivarium) were stored at -80°C until the collagen was extracted. Tails were sterilized in 70% ethanol for 20 minutes. The tails were then rinsed in sterile, distilled water. All equipment was sterilized using an autoclave prior to use. The extraction began at the tip of the tail and proceeded in approximate one-inch sections. One hemostat was used to hold the section and a second hemostat was used to stabilize the lower end of the section and crush the vertebrae. Each section is removed or cut away near the stabilizing hemostat. As each segment is detached the tendon is also pulled free and removed from the tail section. The removed tendon is then rinsed in sterilized distilled

water and placed in 0.1% v/v glacial acetic acid (Sigma-Aldrich, USA). The above steps were repeated until no more tendons could be removed from the tail. Collagen was extracted in the glacial acetic acid for 24 hours at room temperature and then an additional two to three days at 4°C with occasional swirling. The resulting solution was centrifuged at 12,000 X gravity for 1 hour at 4°C to remove insoluble material [50]. The now extracted collagen was removed, pH was checked with a pH of 7.3 to 7.5 judged as being acceptable, if judged as unacceptable the collagen was not used. Collagen within the accepted pH range was stored frozen at -20°C until use in the adhesion assay. Once thawed, collagen solutions were used within one week.

3.4 Adhesion Studies

Ninety-six well tissue culture-treated plates were used for this study. The plates were pre-coated with one of two solutions: collagen (20 ug/ml) in Saline Sodium Citrate (SSC), pH 7.0, obtained from the tail of Sprague-Dawley rats (University of Texas at Arlington vivarium) or (20 ug/ml) natural mouse laminin (Invitrogen Cat. No.23017-015) in SSC, pH of 7.0 (Fischer Biotech). Plates were coated 24 hours in advance, paraffin wrapped and kept at 4°C.

On the day of the experiment the solutions were aspirated from the 96-well plates. All plates whether coated or not were washed twice with SSC. All plates were then incubated with 3% Bovine Serum Albumin (Sigma-Aldrich, USA) in SSC for one hour at 37°C, 95% humidity, and 5% CO2. All plates were again washed twice with SSC.

The Transient Receptor Potential Vanilloid (TRPV) antagonists Ruthenium Red (RR) (Sigma-Aldrich, USA ca. # R-2751) and capsazepine (CPZ) (Sigma-Aldrich, USA ca. #C191) were used at 10uM concentrations. Cell suspensions regardless of whether RR or CPZ were added, were incubated at room temperature for 10 minutes to allow time for the blocker to bind to the Acid Sensing Ion Channel (ASIC). A concentration of 15,000 cells per 10ul was seeded in each well of the 96-well plate final volume of 10ul.

Cells were stimulated using culture media with pH adjusted to pH 7.4 and pH 6.5 using hydrochloric acid or sodium hydroxide. The stimulus was added to the wells at a volume of 40ul, and cells were incubated for 10 minutes at room temperature. Culture media (pH 7.4) was then added at a volume of 150ul. Plates were incubated at 37°C, 95% humidity, and 5% CO2. At the appropriate times (0 hours, 1 hour, 2 hours, 4 hours and 6 hours) the supernatant and any cells not adhered to the dish were aspirated. Time zero is taken after a 10 minute period of cell counting, a 10 minute incubation period with TRPV antagonists, and a 10 minute incubation period with stimulus (30 minutes total). The wells were then washed gently with culture media in order to remove any cells that had not adhered. Culture media (100 ul) was added to the sampled wells to protect the adherent cells until they could be counted. All wells were counted at the end of six hours. To control for media component effects 100ul of culture media was placed in a well at each time point.

After 6 hours, cells remaining in the wells were measured using Cyto Tox 96® Non-Radioactive Cytoxicity Assay (Promega). This took into account any cells that

had de-adhered as well as cells that were still attached. At six hours, 10ul of Lysis solution (9%(v/v) TritonÒX-100 in water) (10X concentration) was added to all wells. The plates were then incubated for 45 minutes at 37°C, 95% humidity, and 5% CO2. The supernatant was removed and placed in a round bottom 96-well plate. The plates were then centrifuged for 5 minutes at 1000 rpm. Fifty micro liters of supernatant was removed and placed in an additional flat bottom 96-well plate. Fifty micro liters of assay substrate was added to all wells, and plates were protected from light with aluminum foil and incubated at room temperature for 30 minutes. Fifty micro liters of stop solution was added and optical density was taken using an Emax precision microplate reader (Molecular Devices) and Softmax® Pro software with absorbance set at 490nm

3.5 Cytokine Assay

Final concentration of 2 million cells per ml was used for this study. HMC-1 cells were counted and re-suspended in culture media at pH 7.4 and pH 6.5, placed in separate 60 X 15 mm tissue culture treated dishes and incubated at room temperature for 10 minutes. Culture media was then added at a volume that met or exceeded 3X the stimulus volume but kept the final concentration of cells at 2 million cells per ml. HMC-1 was incubated at 37°C, 95% humidity, and 5% CO2 for 4 hours. Cells were centrifuged for 5 minutes at 1000 rpm and supernatant collected and stored at -80° C until Ray Biotech assay performed.

Two membranes were used, one membrane per pH. Each membrane was placed in a separate well of the assay tray provided. Each well received 2 mls of 1X blocking

buffer (provided with assay) and incubated at room temperature for thirty minutes under gentle rotation. Blocking buffer was decanted and 1 ml of cell supernatant was added to each well and incubated at room temperature for 2 hours. Samples were decanted from each well and each membrane was washed three times with 2 mls of 1X wash buffer #1 (provided with assay) with 5 minutes per wash. Each membrane was then washed twice with 1X wash buffer #2 (provided with assay) with 5 minutes per wash. Biotin-conjugated antibody (1 ml) was added to each membrane and incubated for 2 hours. Each membrane was then washed three times with 2 mls of 1X wash buffer #1 (provided with assay) with 5 minutes per wash. Each membrane was then washed twice with 1X wash buffer #2 (provided with assay) with 5 minutes per wash. Horseradish peroxidase conjugated with streptavidin (1,000 fold diluted) as added to each membrane at 2mls per membrane. Membranes were incubated for 2 hours at room temperature. Each membrane was then washed three times with 2 mls of 1X wash buffer #1 (provided with assay) with 5 minutes per wash. Each membrane was then washed twice with 1X wash buffer #2 (provided with assay) with 5 minutes per wash. Excess wash buffer was drained from each membrane by holding the membrane vertically.

Detection buffer C and D were mixed (500 ul from each) and 500 ul was added to each membrane drop wise. Membranes were incubated at room temperature for 2 minutes. Excess detection buffer was then removed by holding the membrane vertically. Membranes were then sandwiched between two pieces of plastic and exposed for 40 seconds to FUJIFILM Super RX (100 NIF 13 X 18 cm).

3.6 Enzyme-linked Immunosorbent Assay

Twenty-four well tissue culture treated plates were used for this study. The Transient Receptor Potential Vanilloid (TRPV) antagonist Ruthenium Red (RR) was used at 10uM concentrations. For the IL-8 ELISA, PMA / Ionophore were used as a positive control at 50ng/ml and 5 X 10-7 M concentrations respectively. Cell suspensions regardless of whether RR was added, were incubated at room temperature for 10 minutes to allow time for the blocker to bind to the Acid Sensing Ion Channel (ASIC). A concentration of 1 X 10^6 cells per 50 ul was seeded in each well of the plate.

Cells were stimulated using culture media adjusted to pH 6.5 using hydrochloric acid. The stimulus was added to the wells at a volume of 150 ul, cells were incubated for 10 minutes at room temperature, at the end of this incubation period, 300 ul of culture media was added to each well for a final cell concentration of 1 X 106 cells per ml. The plates were then incubated for the following time periods: time zero, 1 hour, 2 hours, 4 hours, 12 hours, 24 hours, 48 hours and 72 hours (dependent upon cytokine tested) at 37°C, 95% humidity, and 5% CO2. Time zero is taken after a 10 minute period of cell counting, a 10 minute incubation period with ruthenium red, and a 10 minute incubation period with stimulus.

At the appropriate times the supernatant was removed, centrifuged for 5 minutes at 1000 rpm. The supernatant was then collected in 100ul increments and placed in a 96-well plate to facilitate sample loading for the enzyme linked immuno sorbent assay (ELISA) and stored at -80°C until the ELISA was performed.

ELISA Kits were obtained from eBioscience and BioLegend. Ultra-pure dionized water, filtered at 0.2 μm (Barnstead EASYpure LF) was used for all dilutions requiring water. Corning Costar 9018 or NUNC Maxisorp 96 well ELISA plates were coated (100ul per well) with capture anitibodies (Moncyte Chemotactic Protein-1 dilution of 2 ug/ml, Tumor Necrosis Factor –alpha dilution of 4 ug/ml, Interleukin-10 dilution of 2 ug/ml, IL-8 dilution of 2ug/ml) diluted in Coating buffer from eBioscience and BioLegend (0.01 M Phosphate Buffered Saline, 0.138 M Sodium Chloride, 0.0027 M Potassium Chloride, pH 7.4, provided with ELISA), sealed with parafilm and incubated at 4°C for 24 hours. Plates were aspirated and rinsed with wash buffer consisting of 1X phosphate buffered saline (8.0 g NaCl, 1.1 g Na2HPO4, 0.2 g KH2PO4, 0.2 g KCl per 1 Liter), 0.05% Tween-20, five times. Time for soaking was allowed at approximately 1 minute per wash. Plates were blotted on absorbent paper to remove residual buffer. Assay Diluent for eBioscience and BioLegend ELISAs (5% fetal bovine serum in PBS with proclin added as a preservative, provided with ELISA) was place in each well at a volume of 200 ul. Plates were sealed with parafilm and incubated for 1 hour at room temperature. Plates were then washed (five times with for eBioscience ELISAs and 4 times for BioLegend ELISA) wash buffer, as described above. Standard (provided with the ELISA) was added to appropriate wells by performing two fold serial dilutions using assay diluent for eBioscience ELISAs and culture media for BioLegend ELISA. The resulting dilutions for Monocyte Chemotactic Protein –1 (MCP-1, recombinant human MCP-1) beginning with the top standard 1000 pg/ml, 500 pg/ml, 250 pg/ml, 125 pg/ml, 62.5 pg/ml, 31.25 pg/ml, 15.63

pg/ml, 7.81 pg/ml as the final standard concentration. Standard dilutions for Tumor Necrosis Factor –alpha (TNF-μ) beginning with the top standard 500 pg/ml, 250 pg/ml, 125 pg/ml, 62.5 pg/ml, 31.25 pg/ml, 15.63 pg/ml, 7.81 pg/ml, 3.91 pg/ml as the final standard concentration. Standard dilutions for Interluekin-10 beginning with the top standard 300 pg/ml, 150 pg/ml, 75 pg/ml, 37.5 pg/ml, 18.75 pg/ml, 9.375 pg/ml, 4.688 pg/ml, 2.34 pg/ml as the final concentration. Standard Dilutions for IL-8 beginning with the top standard 2000 pg/ml, 1000 pg/ml, 500 pg/ml, 250 pg/ml, 125 pg/ml, 62.5 pg/ml, 31.2 pg/ml, 15.60 pg/ml. One hundred micro liters per well of sample was added to the appropriate wells. Plates were sealed with parafilm and incubated at room temperature for two hours. The plate was washed a total of five washes for all eBioscience ELISAs and 4 times for the BioLegend ELISA, as described above. Detection antibody (biotin conjugated anti-human: MCP-1 1 ug/ml, TNF-μ 0.25 ug/ml, IL-10 0.125 ug/ml, IL-8 .4 ug/ml), all were provided with the ELISA) was added to all wells at a volume of 100 ul per well. The plates were sealed with parafilm and incubated at room temperature for one hour. The plates were then washed a total of five times as described above. Avidin –HRP (Avidin –Horseradish Peroxidase, provided with the ELISA) was added wells at a volume of 100 ul per well. The plates was sealed with parafilm and incubated at room temperature for thirty minutes. Plates were washed (eBioscience ELISAS , a total of seven times with the wells soaked for approximately 2 minutes per wash and BioLegend ELISA , a total of five times with the wells soaked for approximately 1 minute). Substrate solution (1X TMB, provided with the ELISA) was added to each well at a volume of 100 ul per well. The plates were sealed with parafilm and incubated

for 15 minutes at room temperature. Fifty micro liters per well of stop solution (2N H2SO4) (EM Science) was added to each well. The optical density was taken using an Emax precision microplate reader (Molecular Devices) and Softmax® Pro software with absorbance set at 450 nm .

3.7 Scanning Electron Microscopy

HMC-1 cells were centrifuged at 1000 rpm for 5 minutes, supernatent was removed and cells were resuspended in culture media (1 ml). 100 ul of cell suspension was seeded onto glass cover slips placed into 60 X 15 mm tissue culture treated dishes. Separating the cover slips into plates designated for each pH.

Cells were stimulated by culture media (300 ul) with pH adjusted to pH 7.4 and pH 6.5 using hydrochloric acid. Cells were incubated for ten minutes and 2 mls of culture media (pH 7.4) was added to each dish. The dishes were then incubated for one hour at 37°C, 95% humidity, and 5% CO2. Based on adhesion studies this is the maximal adhesion time period. Cells were then fixed in preparation for scanning electron microscopy.

Culture media was aspirated and the dishes were gently washed twice with SSC. A 2% Gluteraldehyde (Ted Pella, Inc.) solution was added to the dishes in a volume sufficient to coat the cover slips completely in order to fix HMC-1. The dishes were covered and incubated at room temperature in a fume hood for 30 minutes. The gluteraldehyde solution was aspirated and each dish was gently washed twice with SSC using a swirling motion.

A 2% Osmium solution (Ted Pella, Inc.) was added to each dish in a volume sufficient to coat the cover slips. Osmium was used as an additional fixative, cross linkage of phospholipids to achieve detailed membrane images. The dishes were incubated for 30 minutes at room temperature in a fume hood. Osmium solution was aspirated and each dish was washed twice with SSC using a swirling motion.

The dishes were then subjected to a dehydration series to replace all water in the cell with ethanol in order to critical point dry and sputter coat the cells. Subsequent ethanol solutions were then applied to each dish, beginning with 30%, 50%, 70%, 85%, 95%, 100% and ending with a final 100% dehydration step. A volume sufficient to coat the cover slips was used. Each step required an incubation of 5 minutes at room temperature. The ethanol was removed before progressing to the next dehydration step. The ethanol from the final dehydration step was removed.

Cells were critical point dried using liquid CO_2 at a maximal pressure of 1100 psi and maximal temperature of 35°C. The HMC-1 cells were then sputter coated with gold and palladium. The cells were viewed and imaged using a JOEL 35-C.

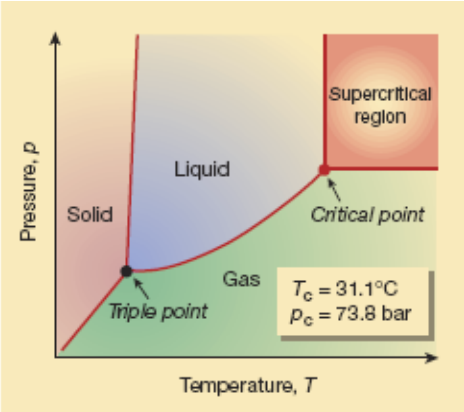


Figure 3.1. Phase Diagram for CO_2. The critical point for CO2 is reached in order to remove the liquid CO2 completely from the HMC-1 cells prior to sputter coating [36].

3.8 Statistical Analysis

An exploratory analysis was performed using a four-factor analysis of variance. This exploratory analysis showed no significant four-factor interactions. The model was constrained to three factors, which showed interactions between pH, time, and the TRPV antagonists (RR & CPZ). The four-factor analysis did show two factors of interest, pH and time. We then performed an analysis by substrate to examine the effect of pH and the effect of time. Mean comparisons were performed for cytokine secretion data and scanning electron microscopy.

3.8 Statistical Analysis

An exploratory analysis was performed using a four-factor analysis of variance. This exploratory analysis showed no significant four-factor interactions, the model was constrained to three-factor interactions and lower order effects. There was a significant three-factor interaction between substrate, pH, and time, a significant three-factor interaction between substrate, pH and the TRPV1 antagonists (RR and CPZ), and a significant three-factor interaction between substrate, TRPV1 antagonists, and time. All

three of these interactions involve substrate, meaning the joint effect of pH and time, pH and TRPV1 antagonists, as well as TRPV1 antagonists and time, depend on substrate. Based on these results, the ensuing analyses were broken up by substrate to focus on the joint effects of the other factors. The by-substrate analyses were based on an unconstrained factorial ANOVA model for the factors pH, TRPV1 antagonists, and time, which respected the blocking structure due to the plates involved in the experimental protocol. Within this model the following analyses were conducted: 1). Pairwise comparisons of pH levels at each combination of TRPV1 antagonists and time. 2). Pairwise comparisons of baseline time (time zero) verses each post-baseline time at each combination of pH and TRPV1 antagonist. Values are illustrated in Tables A6-A9. Mean comparisons were performed for cytokine secretion data and scanning electron microscopy.

CHAPTER 4

RESULTS

4.1 HMC-1 Adhesion

In order to examine the effect of substrate on mast cell adhesion, I examined the adherence of HMC-1 to two substrates, collagen and laminin under normal physiological pH (pH 7.4). BSA was used as a negative control; as mast cells are not expected to adhere to BSA more than five percent of total cells [30,33]. My results show a preference for collagen over both BSA and Laminin. HMC-1 did not spontaneously adhere to laminin. My results show an increase in number of cells adhered in Collagen over BSA, values illustrated in Figure 4.1 Table A.1.

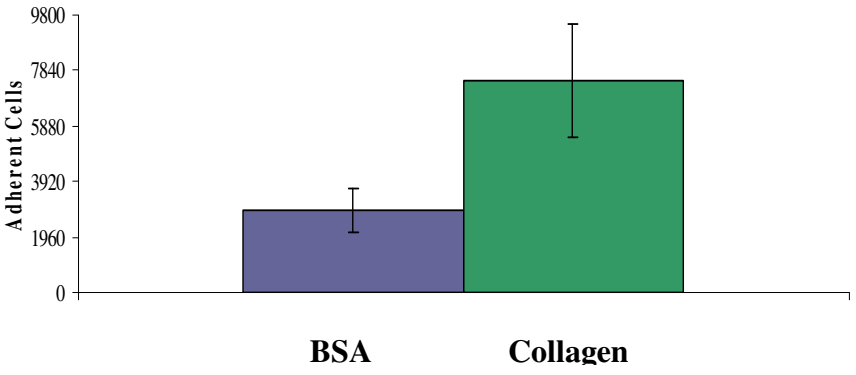


Figure 4.1. Substrate Preference of HMC-1. Spontaneous adhesion of HMC-1 was measured at time zero for three substrates.

To test whether substrate preference increased over time, I measured the number of adherent cells across five time points (0, 1hr, 2hr, 4 hr, 6 hrs) in pH 7.4, for BSA, collagen and laminin. The four-factor analyses show that adhesion is dependent upon substrate and time (p-value 0.0296). Values illustrated in Table A.6. Adhesion on BSA was significant from time zero to four hours (p-value 0.0056). Adhesion on collagen was significant from time zero to 6 hours (p-value 0.0079). Adhesion on laminin was not significant with respect to time. Values illustrated in Tables A.7-9.

HMC-1 preferred the collagen substrate over both the laminin and BSA at time 0, 1 hr, 2 hrs, and 6 hrs, values illustrated in Graph 4.2 and Table A.2. Values indicate a much weaker adhesion response overall with regards to cells cultured on laminin over time as compared with the adhesion response of cells cultured on collagen plated dishes At the four hour mark more cells did adhere to BSA, however the response does fall within the standard error of collagen (plastic 7100 ± 1897.64), collagen 14240 ± 3805.89). Values illustrated in Figure 4.2 and Table A.2.

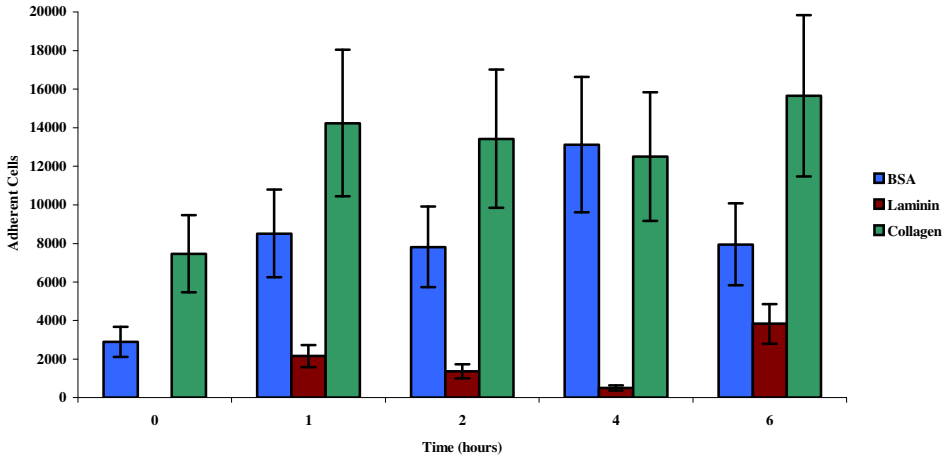


Figure 4.2. Substrate Preference Over Time. Adhesion of HMC-1 was measured for three substrates (BSA, Laminin, Collagen) across five time periods (0, 1 hr, 2 hr, 4 hr, 6 hr).

Based on the above results, I examined whether mast cell substrate preference increased in response to acidic pH. I exposed HMC-1 to pH 6.5 and measured the adhesion response at one hour. Only cells placed on laminin showed an increase in adhesion to acidic pH, although results were not significant (p-value 0.1603). Values illustrated in Figure 4.3, Tables A.3-A.5.

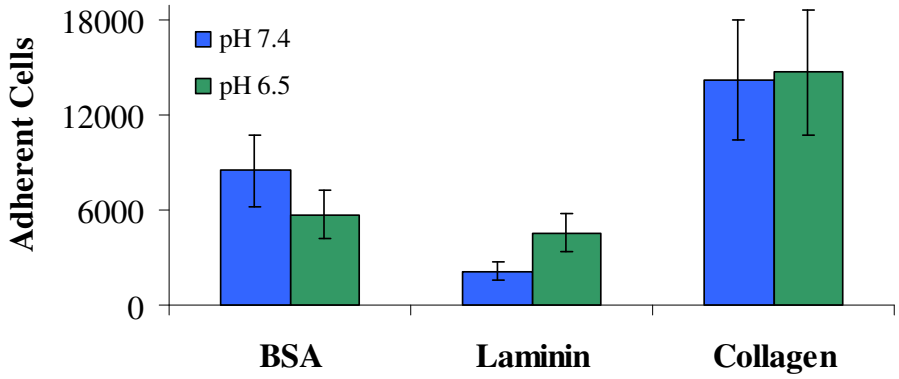


Figure 4.3. Substrate Preference of HMC-1 in Response to Acidic pH. Adhesion of HMC-1 was measured across three substrates in response to acidic pH at one hour. Values expressed are mean ± standard values.

To test whether acidic pH increases mast cell adhesion across time, HMC-1 was exposed to acidic pH (6.5) and the response compared to adhesion in normal physiological pH (pH 7.4) for five time points (0, 1 hr, 2 hr, 4 hr, and 6 hrs). Four-factor analysis indicates the effect of pH is dependent upon time (p-value 0.0125) and substrate and time (p-value < .0217). Values illustrated in Table A.6. There was a peak of adhesion at one hour, two hours, and six hours for HMC-1 cultured on collagen coated dishes, values expressed in Figure 4.4 and Table A.5. At six hours the adhesion response was maximal however, the number of adherent cells was similar regardless of pH (Figure 4.4, Table A.5). There was a decrease in adhesion at four hours. The adhesion response over time of HMC-1 cultured on laminin coated dishes shows a peak

at one hour and six hours, values are expressed in Figure 4.4, Table A.4. There is a marked decrease in adhesion at two hours and four hours (Figure 4.4, Table 4). Values indicate a much weaker adhesion response overall with regards to cells cultured on laminin over time as compared with the adhesion response of cells cultured on collagen plated dishes. The adhesion response in BSA is inconclusive; there is no clear trend in adhesion as measured across time and pH. Values illustrated in Figure 4.4 and Table A.3.

Based on the above results, analyses were performed by substrate. The adhesion response on collagen for cells in pH 7.4 shows significance between time zero and six hours (p-value 0.0079). Values are illustrated in Table A.9. The adhesion response on BSA for cells in pH 7.4 shows significance between time zero and 4 hours (p-value 0.0056). The adhesion response on BSA for cells exposed to pH 6.5 show significance between time zero and 2 hours (p-value $< .0001$), time zero and 6 hours (p-value 0.0012), 1 hour and 2 hours (p-value 0.0038), 2 hours and 4 hours (p-value 0.0092). Values are illustrated in Table A.7. The adhesion response on laminin was not significant for either pH 7.4 or pH 6.5.

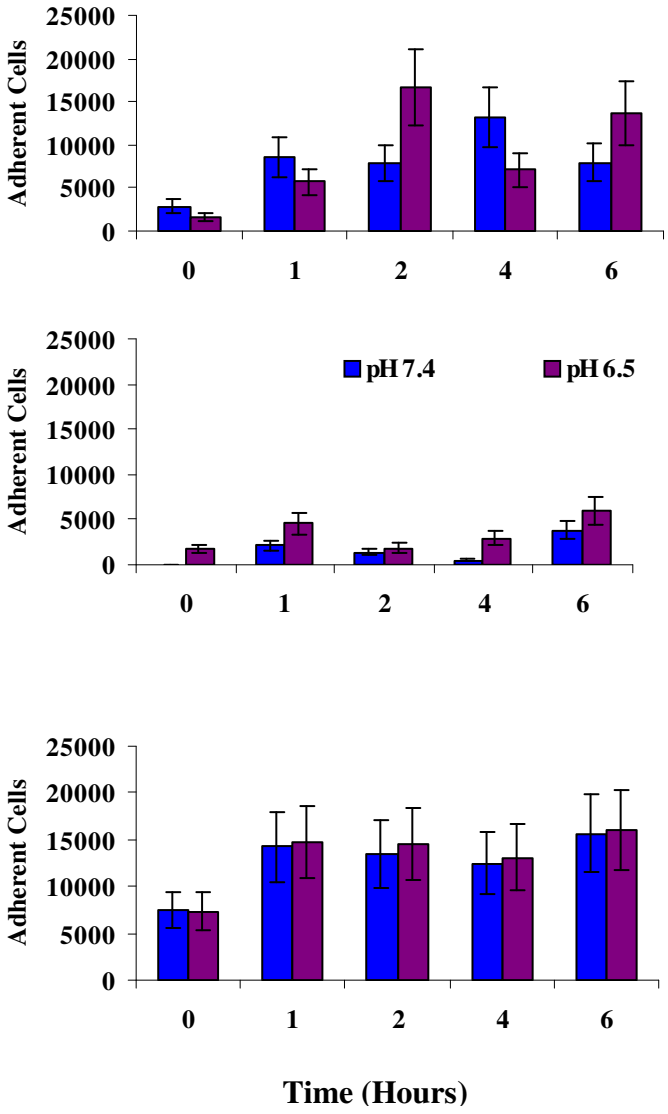


Figure 4.4. Adhesion of HMC-1 in Response to Acidic pH, Across Time by Substrate. HMC-1 adhesion was measured over 5 time points (0, 1, 2, 4, and 6 hours). Substrates tested A) BSA, B) Laminin, C) Collagen. Acidic pH (pH 6.5) was compared to normal physiological pH 7.4. Values are mean ± standard error.

To test for the activation of TRPV-1, HMC-1 was incubated with acidic pH (pH 6.5) alone and in conjunction with RR and CPZ, compared to normal physiological pH (pH 7.4) alone and in conjunction with RR and CPZ, in three substrates (BSA, laminin, collagen). Four-factor analyses showed the adhesion response by substrate was dependent upon time and the TRPV1 antagonists (RR and CPZ) (p-value 0.0055), as well as pH and the TRPV1 antagonists (RR and CPZ) (p-value 0.0288). Values illustrated in Table A.6. The TRPV1 antagonist RR decreased the adhesion response significantly on collagen from time zero to 6 hours (p-value 0.0079) for cells in pH 7.4 and for cells exposed to pH 6.5 in the presence of the TRPV1 antagonist RR, results were significant from time zero to two hours (p-value 0.0037) and time zero to six hours (p-value 0.0003). Values are illustrated in Table A.9. The TRPV1 antagonist RR decreased the adhesion response significantly in BSA for cells cultured in pH 7.4 from time zero to four hours (p-value 0.0056) and for cells exposed to pH 6.5 from time zero to 6 hours (< .0001), two hours to six hours (p-value 0.0005), four hours to six hours (p-value 0.0024). Values are illustrated in Table A.7. The TRPV1 antagonist RR decreased adhesion on laminin of cells in pH 7.4, from time 0 to 6 hours2 hours to 6 hours (p-value 0.0077) and from 2 hours to 6 hours (p-value 0.0077) for cells exposed to pH 6.5. Values illustrated in Table A.8.

The adhesion response on collagen, decreased in the presence of the TRPV antagonist CPZ for cells in pH 7.4 from time zero to 6 hours (p-value 0.0020). The adhesion response on collagen for cells exposed to acidic pH 6.5, showed a decrease **in**

adhesion from 1 hour to 6 hours (p-value 0.0046). Values are illustrated in Table **A.9.** The adhesion response on BSA for cells in pH 7.4 decreased from two hours to six hours (p-value 0.0077). Values are illustrated in Table A.7. Results for cells placed on laminin were not significant.

HMC-1 adhesion is highest when cultured with collagen regardless of pH. The number of adherent cells are highest when HMC-1 is stimulated with pH 6.5, pH 7.4 is the second highest with RR third and CPZ the lowest, values illustrated in Figure 4.5, Tables A.3-A.5. The antagonists RR and CPZ lower the adhesion response across time and regardless of substrates.

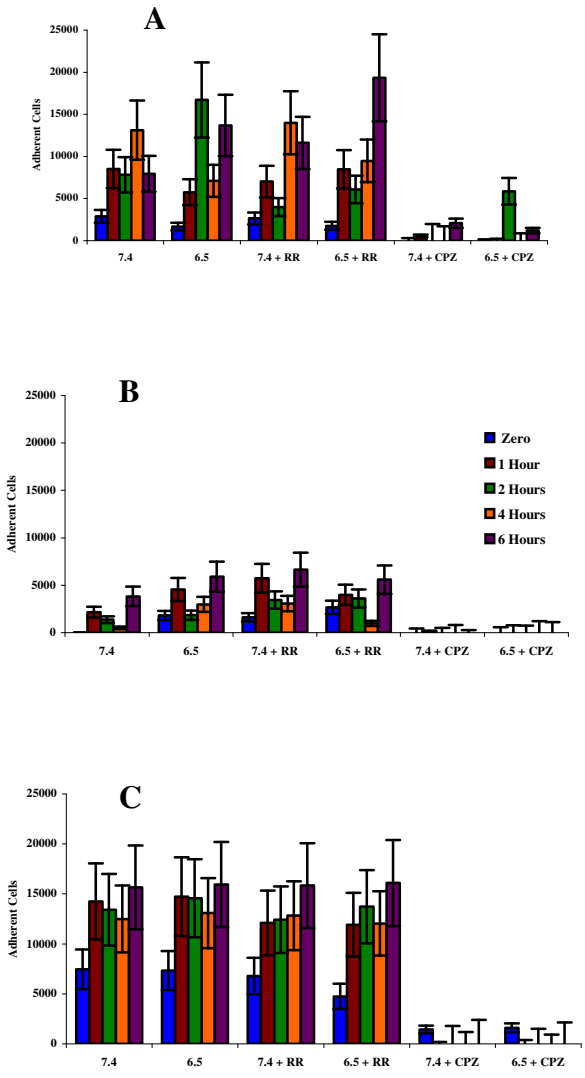


Figure 4.5. Adhesion of HMC-1 After Exposure to Acidic pH and in the Presence of TRPV Antagonists. Adhesion was measured at time 0, 1 hr, 2 hrs, 4 hrs, and 6 hrs. HMC-1 was exposed to pH 6.5 and the TRPV antangonists ruthenium red (RR) and capsazepine (CPZ). Cells were in the presence of A) BSA, B). Laminin, and C). Collagen. Values are expressed as mean ± standard error.

4.2 Cytokine Secretion of HMC-1

4.2.1 Cytokine Array

To determine whether mast cells express cytokines constitutively and upregulate cytokines in response to acidic pH, HMC-1 was stimulated with acidic pH (pH 6.5) and results compared to HMC-1 cultured in normal physiological pH (pH 7.4). I used a forty-two cytokine array from RayBiotech. Using this array I discovered that HMC-1 constitutively secreted the interleukins 10 and 8 as well as MCP-1, MCSF, MIP-1 D, RANTES, TNF-α, and Oncostatin. Values are illustrated in Table 9. An Image of the cytokine array membrane is illustrated in Figure B.1 and a map of the cytokine placement is illustrated in Table B.1.

HMC-1, when stimulated with pH 6.5, secreted those found to be constitutively expressed as well as up-regulated and secreted: MDC, SCF, TPO, and VEGF. Values are illustrated in Figure B.1 and Table B.3. HMC-1 differentially expressed the following cytokines, values expressed as fold differences: IL-10 (2.1X), IL-8 (1.9X), MCP-1 (2.8X), MCSF (2.6X), MIP-1 D (3.0X), Oncostatin (5.23X), RANTES (4.8X), TNF-α (6.0X). Values are illustrated in Figure B.1 and Table B.3.

4.2.2 Cytokine ELISAs

Based on the cytokine array results, I wanted to quantify the constitutive expression of: MCP-1, TNF-α, IL-10, and IL-8. This was done through the utilization of sandwich ELISAs. The constitutive expression was examined over several time periods dependent upon the specific cytokine examined. The constitutive expression of

MCP-1, TNF-α, and IL-10 was examined at 0, 4 hrs, 12 hrs, 24 hrs, 48 hrs, and 72 hrs. IL-8 was examined at 0, 1 hr, 2 hrs, 4 hrs, and 24 hours. HMC-1 did not express TNF-α, IL-10 constitutively. MCP-1 was secreted constitutively with cytokine levels detected at 4 hours and secretion continued to increase through 72 hours. Values illustrated in Figure 4.6 and Table B.4. Constitutive secretion of IL-8 was detected at all time points, with a peak at one hour and the lowest level at 24 hours. Values illustrated in Figure 4.7 and Table B.5

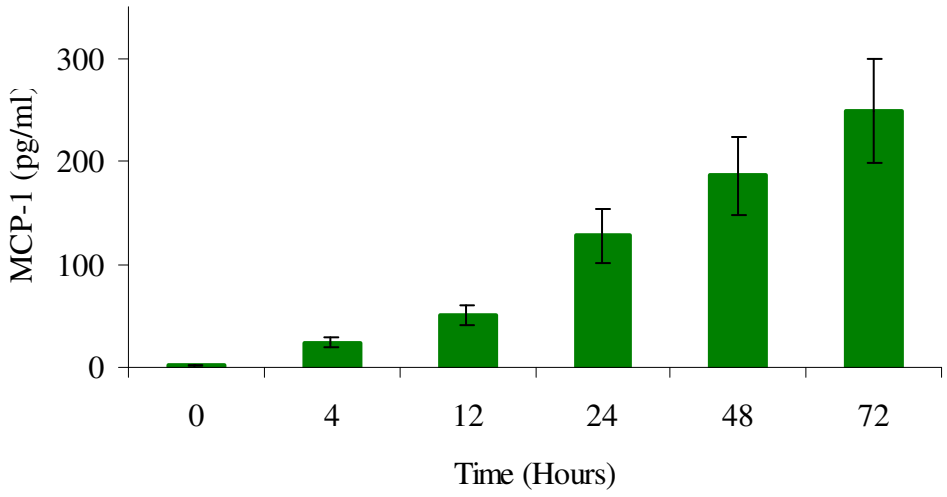


Figure 4.6 . Constitutive Secretion of MCP-1 by HMC-1. HMC-1 constitutively expressed MCP-1 over 72 hours. Values represented are mean values ± standard error.

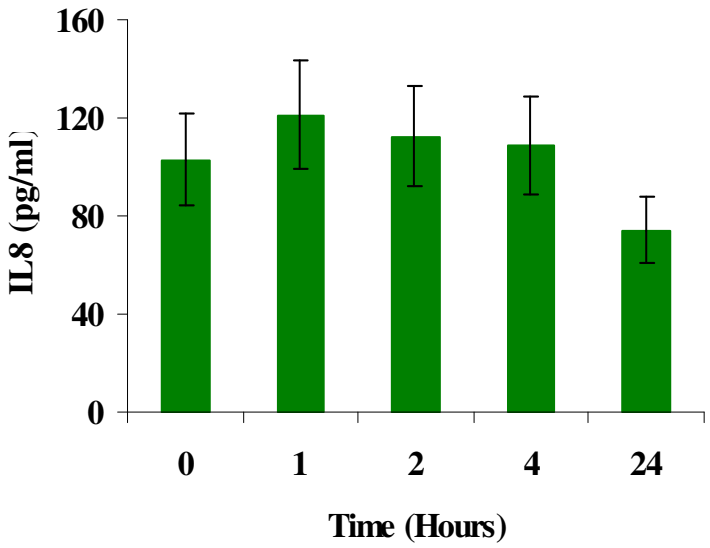


Figure 4.7. Constitutive Secretion of IL-8 by HMC-1. HMC-1 constitutively expressed IL-8 over 24 hours. Values represented are mean values ± standard error.

To test up-regulation and secretion of MCP-1, TNF-α, IL-10, and IL-8 in response to acidic pH, I stimulated HMC-1 with acidic pH (pH 6.5). The response for MCP-1, TNF-α, IL-10, was measured over a 72 hour time period at 0, 4 hrs, 12 hrs, 24 hrs, 48 hrs, and 72. The response for IL-8 was examined over a 24-hour time period at 0, 1 hr, 2 hrs, 4 hrs, and 24 hrs. HMC-1 did not express TNF-α, IL-10 upon stimulation by acidic pH (pH 6.5). The secretion of MCP-1 by HMC-1 was down-regulated in pH 6.5 beginning at 24 hours, as compared to cells exposed to pH 7.4. The amount of MCP-1 increased in cells exposed to normal physiological pH (pH 7.5) and in cells

exposed to acidic pH (pH 6.5) over time. Values illustrated in Figure 4.8, Table B.4 Acidic pH had no effect on IL-8 secretion when you consider that time point zero is baseline secretion. Values are illustrated in Figure 4.9 and Table B.5.

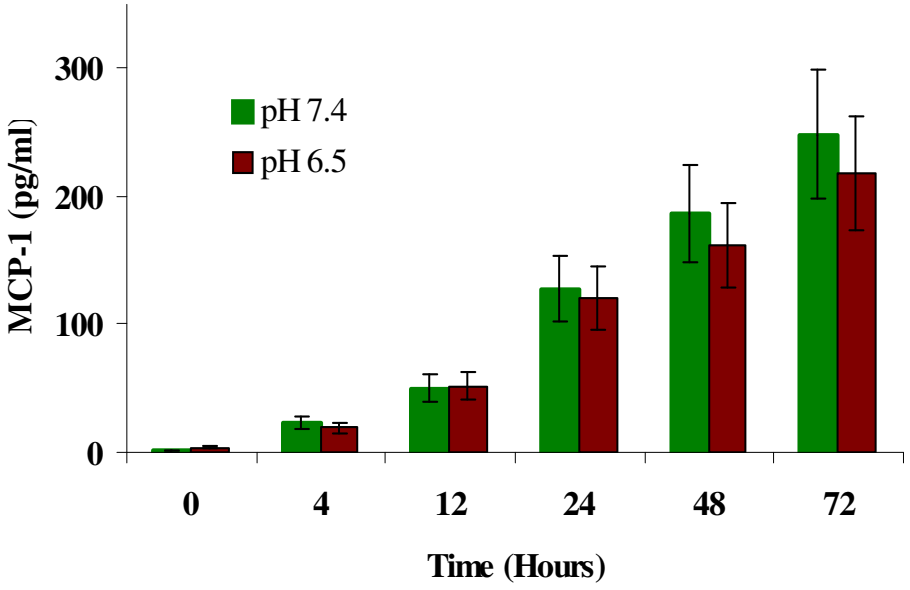


Figure 4.8. MCP-1 Secretion of HMC-1 in Response to Acidic pH. HMC-1 secretion of IL-8 after stimulation with pH 6.5 is compared to secretion of IL-8 in pH 7.4. Values expressed are mean ± standard error.

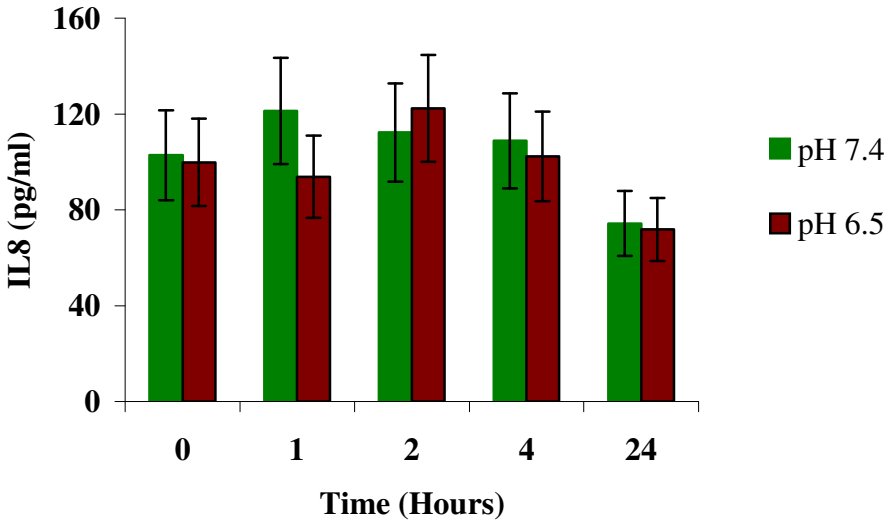


Figure 4.9. HMC-1 Secretion of IL-8 in Response to Acidic pH. HMC-1 secretion of IL-8 after stimulation with pH 6.5 is compared to secretion of IL-8 in pH 7.4. Values expressed are mean ± standard error.

To examine whether activation of TRPV1 increased the secretion of MCP-1 and IL-8, HMC-1 was exposed to acidic pH (pH 6.5) alone and in the presence of the TRPV antangonist RR and compared to HMC-1 cultured in normal physiological pH alone and in the presence of the TRPV antagonist RR. Inhibition of TRPV1 by its antagonist RR resulted in a decrease in secretion of MCP-1 by cells exposed to normal physiological pH (pH 7.4). The inhibition of TRPV1 by its antagonist RR resulted in increased secretion of MCP-1 for cells exposed to acidic pH (pH 6.5). Over time all MCP-1 secretion increased regardless of treatment. Values expressed in Figure 4.10, Table B.4.

The presence of the TRPV antagonist had no effect on IL-8 secretion. Values expressed in Figure 4.11, Table B.5.

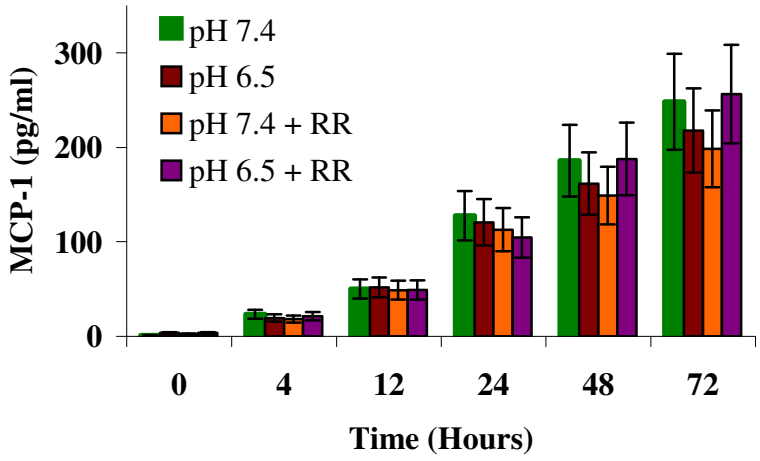


Figure 4.10. Secretion of MCP-1 in the Presence of RR.
HMC-1 secretion of MCP-1 over 72 hours. Values expressed are mean ± standard error.

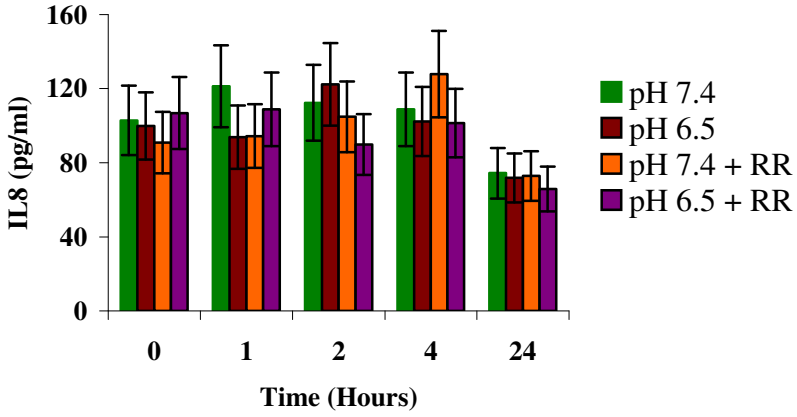


Figure 4.11. Secretion of IL-8 in the Presence of RR.
HMC-1 secretion of IL-8 over 72 hours. Values expressed are mean ± standard error.

I wanted to examine whether my HMC-1 cells were non-functioning because my results regarding cytokine expression of HMC-1 contradicted previous studies [31,18,51]. I stimulated HMC-1 with PMA, which is known to induce cytokine expression of HMC-1, and examined the expression of IL-8. HMC-1 expressed IL-8 at all time periods measured (0, 1 hr, 2 hrs, 4 hrs, 24 hrs). Values are illustrated in Figure 4.12 and Table B.5.

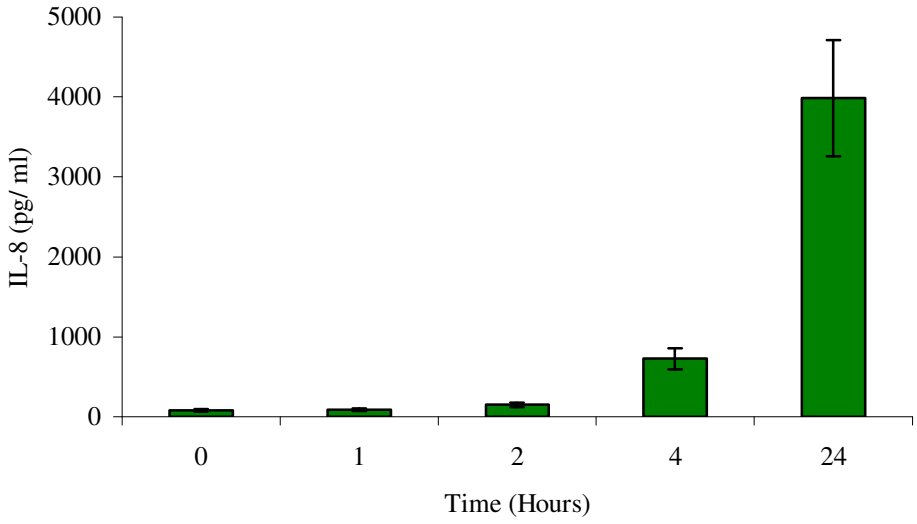


Figure 4.12. Secretion of IL-8 in the Presence of PMA.
Secretion of IL-8 from PMA stimulated HMC-1, over 72 hours. Values expressed are mean ± standard error.

4.3 HMC-1 Morphology

To further describe the response of mast cells to acidic pH, mast cell morphology was examined. HMC-1 cultured in normal physiologic pH (7.4) appear rounded with a mean diameter of 6.637 μm, the plasma membrane is ruffled in appearance with the ruffles forming rounded or shallow peaks. There is an absence of bulges, which might indicate the presence of secretory vesicles and minimal filapodia [9]. Images and values are illustrated in Figures C.1, 2, and 7, and Table C.1.

HMC-1 stimulated with pH 6.5 are rounded in shape, depict a ruffled plasma membrane as well, however the ruffles are sharper and more distinct than cells cultured in pH 7.4. The membrane exhibited bulges representative of secretory vesicles, and small. The mean diameter of these cells is 6.875; values can be seen in Table C.1. There is the presence of bulges, secreted granules, and there is minimal filipodia. Images and values are illustrated in Figures C. Figures C.3, 4, and 7, and in Table C.1.

HMC-1 stimulated with pH 5.0 are slightly irregular in shape, have sharper, longer and more pronounced ruffles and an increase in number and size of bulges as well as the appearance of lamininapodia and pronounced filipodia. The mean diameter for these cells is 7.100. Images can be seen in Figures C.5 & 6, values can be seen in Figure C.7 and Table C.1.

CHAPTER 5

DISCUSSION

This study examined several functional responses of mast cells to acidic pH: **1.** Mast cell substrate preference, **2.** Mast cell adhesion response to acidic pH, **3.** Mast cell adherence as over time, **4.** Secretion of mast cells after exposure to acidic pH, and **5.** Mast cell morphology. The mast cell line HMC-1 was used exclusively for this study. It was hypothesized that a member of the TRPV receptor channels was activated by the acidic pH so two known antagonists, RR and CPZ were used to test for this.

Two substrates, collagen, and laminin were used to examine substrate preference of HMC-1. The adhesion response to these two substrates was measured over time and after stimulation with acidic pH. HMC-1 secretion of cytokines was also examined over time and in response to acidic pH. Finally, the morphology of HMC-1 was examined across normal physiological pH (pH 7.4), slightly acidic pH (pH 6.5) and very acidic pH (pH 5.0).

5.1 HMC-1 Adhesion

HMC-1 spontaneously adhered to collagen and BSA. The spontaneous adherence to extracellular matrix components has been described previously using collagen, laminin, fibronectin and vitronectin [12,13,30,66]. HMC-1 preferred the mixture of collagen extracted from Sprague-Dawley rat tendons over the natural mouse

laminin and BSA. My study is consistent with previous studies, which examined mast cell adhesion to a variety of extracellular matrix proteins including, fibronectin, virtonectin, laminin, and collagens I-IV [31,60,66]. These studies, although they did not directly address substrate preference of HMC-1, demonstrated greater adhesion to collagen, as opposed to laminin and BSA. Previous studies examined the adhesion of HMC-1 after stimulation by the harsh stimulants, calcium ionophore and PMA [31,60,66]. My study examined the preference of HMC-1 to collagen, Laminin and BSA after stimulation by acidic pH (pH6.5), which mimics naturally occurring conditions mast cells may experience during acidosis. Previously published studies have demonstrated changes in the makeup of the extracellular matrix that may change in response to injury, parasitic infection, tumor growth, or inflammation [3,32,61]. My study demonstrates that HMC-1 prefers a mixture of collagen over laminin suggesting collagen might be the more prevalent extracellular matrix component over laminin utilized under conditions of acidosis.

HMC-1 demonstrated an adhesion response that was time dependent with peaks of adhesion at 1, 2 and 6 hours for collagen, and 1 and 6 hours for laminin. Several studies have been published which examined adhesion over time but little speculation has been made regarding the transient adhesion response [12,13,30,66]. I believe the transient adhesion response may be due in part to 'inside-out signalling' by integrins [15,28,48]. The peak of adhesion of six hours regardless of substrate may be due to the buildup of adhesion mediators in the culture media as much as recurrent 'inside-out' signaling.

Adhesion of HMC-1 was determined to be dose dependent as well as time dependent. Acidic pH resulted in increased adherence for HMC-1 exposed to collagen, but not laminin. It is possible collagen production rather than laminin production is signaled in response to acidic pH. Changes in the extracellular matrix components may change in response to acidosis, or wound repair [3,32,61].

The adhesion response of mast cells occurred preferentially when mast cells are exposed to collagen. The response occurred in a time and dose (hydrogen ion) dependent manner. I believe the time dependent response of mast cells over time maybe due to 'inside signalling' and the dose dependent response may be due to changes in the extracellular matrix components [15,48].

5.2 Cytokine Secretion by HMC-1

This study examined the effect of acidic pH (pH 6.5), on the secretion of MCP-1, TNF-α, IL-8, IL-10, through the activation of TRPV1. Acidic pH is one condition found at sites of inflammation the above cytokines were selected based on the current understanding of mast cell roles in inflammation [2,22,54,72] Initially the constitutive secretion levels were determined. A cytokine array assay showed that HMC-1 constitutively secretes the interleukins 10 and 8 as well as MCP-1, MCSF, MIP-1 D, RANTES, TNF-α, and oncostatin. Acidic pH, induces or upregulates the secretion of MCP-1, MCSF, MIP-1 D, RANTES, TNF-α, oncostatin, MDC, SCF, TPO, and VEGF. MCSF, MIP-1 D, RANTES, TNF-α, oncostatin, MDC, SCF, TPO, and VEGF were not examined further. I did examine IL-8, IL-10, TNF-α, and MCP-1 with ELISAs. I

found no detectable levels of IL-10 and TNF-α. These results differ from previously published studies that have shown that HMC-1 cell line constitutively expresses both these cytokines [18,51]. This difference may be due to the culture conditions used to maintain the HMC-1 cells prior to supernatant collection. In previous studies HMC-1 cells were grown in media supplemented with fetal bovine serum and incubated in serum-free media for 24 hours prior to collection. My cells were cultured in media supplemented with BSA rather than fetal bovine serum to eliminate the need to incubate in serum free media. Serum-free media has been suggested by several research groups, to activate many cell lines, HMC-1 included [18,5]. It may be the abrupt change from media supplemented with serum into a non-serum environment that potentially activates HMC-1. Previous studies have also utilized the unspecific mediators PMA and calcium ionophore to induce cytokine secretion in HMC-1, neither of which would mast cells normally be exposed to. To better mimic *in vivo* conditions I used acidic pH to stimulate cytokine secretion by HMC-1. Acidic pH (pH 6.5) is a condition mast cells would normally be exposed to during inflammatory conditions.

My study shows constitutive expression of MCP-1 and IL-8 by HMC-1 cells. These results agree with previous studies, HMC-1 is known to constitutively secrete both. However when I explored the effect of acidic pH on the secretion of IL-8, I found that acidic pH did not have an effect on HMC-1 secretion of IL-8. In contrast, MCP-1 secretion was down-regulated. Additionally, when RR, a known TRPV antagonist was used to block the TRPV mediated effects, MCP-1 was up-regulated in response to

acidic pH. MCP-1 has been implicated in a variety of inflammatory diseases and is known to regulate the recruitment and activation of inflammatory cells [27,37,72]. My results seem to indicate the stimulation of TRPV may inhibit MCP-1 secretion.

An additional explanation for my results might be a possible change in phenotype of cultured HMC-1 cells. It is also possible that pH is not strong enough of a stimulant to induce cytokine expression. To explore this possibility, the strong unspecific stimulators, PMA and calcium ionophore were used as positive controls for induced secretion of IL-8 by HMC-1. Secretion of IL-8 by HMC-1 upon activation by PMA and calcium ionophore has been well documented [18,31,51]. My study did show induced secretion of IL-8 by HMC-1 in the presence of both PMA or calcium ionophore. This seems to indicate my HMC-1 cells were capable of expression and secretion of these cytokines.

While the results of my study were not fully consistent with previous studies, I believe for the reasons explained above, differences in media conditions and mediators, my results are representative of HMC-1 activation by acidic pH. HMC-1 is an immature cell line with loss of function mutations that may alter its activation and subsequent cytokine secretion. I believe, HMC-1 may be unsuitable for a cytokine study as it may not respond as mast cells *in vivo*, for this part of the project animal studies may be better suited.

5.3 Morphological Changes in HMC-1

The plasma membrane of mast cells changes in preparation for secretion and subsequent recovery, this is evident in the rearrangement of membrane folds, presence

of secretory vesicles and filipodia [4,9,70]. The plasma membrane of HMC-1 was examined with scanning electron microscopy. HMC-1 demonstrated characteristic uniform folding across the whole of the membrane and an absence of secretory vesicles in normal physiological pH. When stimulated with acidic pH (pH 6.5 or 5.0) membrane folds become less uniform, with sharper peaks and greater space between folds. Secretory vesicles and filiapodia are present and in the case of filapodia, they become elongated. The filapodia are in some cases 1.5 fold longer than the body of the cell. Previous studies have described an increase in cell volume and in my study the diameter did increase although results not significant. Preliminary results using confocal microscopy showed an increase in cell volume along with cell spreading when HMC-1 was stimulated with acidic pH as compared with normal physiological pH (data not published). Previous studies have described mast cells in a recovery phase that have a non-uniform folding of the membrane that appeared as very sharp peaks, disturbances within the membrane where secretory vesicles have severed from the membrane and flattening of the cell [70]. My study did not detect cells in a 'recovery' period, which is probably due to the nature of my stimulus. Previous studies stimulated mast cells through the cross-linking of the IgE receptor, which is a strong stimulus, while my study utilized acidic ph, which lowers the threshold for activation, rather than directly stimulating mast cells.

5.4 Conclusions

The focus of this dissertation was the examination of functional responses of mast cells to acidic pH. The functional responses examined here were, adhesion,

cytokine expression and secretion, and morphology. This study utilized the human mast cell line (HMC-1). My results demonstrate that HMC-1 preferred to adhere to collagen over laminin and acidic pH (pH 6.5) increased adhesion. Activation of TRPV1 by acidic pH was shown to play a part in the adhesion response. MCP-1 secretion was down-regulated in response to acidic pH suggesting MCP-1 has a possible anti-inflammatory role. My study showed a change in the morphology of HMC-1 in response to acidic pH indicating that acidic pH is an activator of mast cells.

TRPV1receptor has been implicated in inflammatory conditions; however it is not the only receptor sensitive to acidic pH. The ASIC family is well characterized and recent literature has shown the presence of an additional acid sensing receptor, from a G-protein coupled receptor family. This receptor was first discovered in pancreatic tumors and is currently believed to be found in T-cells [24]. This receptor is not well characterized so its role in inflammation and mast cell function is still unknown. Finally, HMC-1 is the only established human mast cell line, however my results suggest it may not be the best model for all mast cell studies. Further study is needed regarding the functional responses of mast cells to acidic pH in order to fully understand the role of mast cells in inflammatory conditions. Some of these studies may need to be performed using an animal model.

APPENDIX A

HMC-1 ADHERENCE

Table A.1. HMC-1 Spontaneous Adhesion.

Substrate	Adherent Cells	Standard Error
BSA	2892.000	772.920
Laminin	0.00	-39.555
Collagen	7458.667	1993.413

Table A.2. HMC-1 Substrate Preference Over Time

Substrate	Time (Hours)	Adherent Cells	Standard Error
BSA	0	2892.000	772.920
BSA	1	8515.000	2275.729
BSA	2	7815.333	2088.736
BSA	4	13122.000	3507.002
BSA	6	7948.667	2124.371
Laminin	0	0.000	-39.555
Laminin	1	2160.333	577.373
Laminin	2	1367.000	365.346
Laminin	4	497.000	132.829
Laminin	6	3828.667	1023.254
Collagen	0	7458.667	1993.413
Collagen	1	14240.333	3805.889
Collagen	2	13423.667	3587.626
Collagen	4	12498.667	3340.409
Collagen	6	15648.667	4182.282

Table A.3. HMC-1 Adherence to BSA

Treatment	Time (Hours)	Optical Density	Standard Error
pH 7.4	0	2892	772.92
pH 7.4	1	8515	2275.73
pH 7.4	2	7815	2088.74
pH 7.4	4	13122	3507
pH 7.4	6	7949	2124.37
pH 6.5	0	1677	448.197
pH 6.5	1	5751	1537.02
pH 6.5	2	16702	4463.8
pH 6.5	4	7100	1897.64
pH 6.5	6	13664	3651.77
pH 7.4 + RR	0	2645	706.995
pH 7.4 + RR	1	7025	1877.51
pH 7.4 + RR	2	3999	1068.69
pH 7.4 + RR	4	13999	3741.3
pH 7.4 + RR	6	11617	3104.77
pH 6.5 + RR	0	1757	469.578
pH 6.5 + RR	1	8473	2264.5
pH 6.5 + RR	2	6090	1627.71
pH 6.5 + RR	4	9464	2529.27
pH 6.5 + RR	6	19337	5168.03
pH 7.4 + CPZ	0	0.00	-304.14
pH 7.4 + CPZ	1	583	155.813
pH 7.4 + CPZ	2	0.00	-1990.1
pH 7.4 + CPZ	4	0.00	-1708.6
pH 7.4 + CPZ	6	2077	555.102
pH 6.5 + CPZ	0	0.00	-143.34
pH 6.5 + CPZ	1	0.00	-231.72
pH 6.5 + CPZ	2	5865	1567.58
pH 6.5 + CPZ	4	0.00	-866.28
pH 6.5 + CPZ	6	1204	321.693

Table A.4. HMC-1 Adherence to Laminin

Treatment	Time (Hours)	Optical Density	Standard Error
pH 7.4	0	0.00	-39.555
pH 7.4	1	2160	577.373
pH 7.4	2	1367	365.346
pH 7.4	4	497	132.829
pH 7.4	6	3829	1023.25
pH 6.5	0	1814	484.723
pH 6.5	1	4564	1219.69
pH 6.5	2	1847	493.632
pH 6.5	4	2980	796.528
pH 6.5	6	5907	1578.71
pH 7.4 + RR	0	1614	431.271
pH 7.4 + RR	1	5734	1532.39
pH 7.4 + RR	2	3437	918.577
pH 7.4 + RR	4	3067	819.69
pH 7.4 + RR	6	6652	1777.82
pH 6.5 + RR	0	2654	709.222
pH 6.5 + RR	1	3997	1068.24
pH 6.5 + RR	2	3602	962.675
pH 6.5 + RR	4	987	263.787
pH 6.5 + RR	6	5592	1494.52
pH 7.4 + CPZ	0	0.00	-448.46
pH 7.4 + CPZ	1	169	45.0781
pH 7.4 + CPZ	2	0.00	-499.69
pH 7.4 + CPZ	4	0.00	-802.59
pH 7.4 + CPZ	6	0.00	-271.63
pH 6.5 + CPZ	0	0.00	-580.31
pH 6.5 + CPZ	1	0.00	-762.94
pH 6.5 + CPZ	2	0.00	-733.99
pH 6.5 + CPZ	4	0.00	-1213.7
pH 6.5 + CPZ	6	0.00	-1124.6

Table A.5. HMC-1 Adherence to Collagen.

Treatment	Time (Hours)	Adherent Cells	Standard Error
pH 7.4	0	7459	1993.41
pH 7.4	1	14240	3805.89
pH 7.4	2	13424	3587.63
pH 7.4	4	12499	3340.41
pH 7.4	6	15649	4182.28
pH 6.5	0	7337	1960.9
pH 6.5	1	14729	3936.4
pH 6.5	2	14564	3892.3
pH 6.5	4	13084	3496.76
pH 6.5	6	15942	4260.68
pH 7.4 + RR	0	6784	1813.01
pH 7.4 + RR	1	12094	3232.17
pH 7.4 + RR	2	12424	3320.36
pH 7.4 + RR	4	12820	3426.38
pH 7.4 + RR	6	15834	4231.73
pH 6.5 + RR	0	4744	1267.8
pH 6.5 + RR	1	11924	3186.73
pH 6.5 + RR	2	13717	3666.02
pH 6.5 + RR	4	12049	3220.14
pH 6.5 + RR	6	16095	4301.66
pH 7.4 + CPZ	0	1434	383.164
pH 7.4 + CPZ	1	0.00	-179.42
pH 7.4 + CPZ	2	0.00	-1784.8
pH 7.4 + CPZ	4	0.00	-1188.3
pH 7.4 + CPZ	6	0.00	-2397.7
pH 6.5 + CPZ	0	1605	429.043
pH 6.5 + CPZ	1	0.00	-373.63
pH 6.5 + CPZ	2	0.00	-1506.4
pH 6.5 + CPZ	4	0.00	-941.12
pH 6.5 + CPZ	6	0.00	-2141.1

Table A.6. Four-factor Analysis of HMC-1 Adhesion

Effect	F Value	P Value
Substrate * Time	2.16	0.0296
pH*Time	3.23	0.0125
Substrate*pH*Time	2.27	0.0217
Substrate*pH*TRPV1 Antagonists	2.73	0.0288
Substrate*TRPV1 Antagonists*Time	2.17	0.0055

*Degrees of Freedom = 454, only significant values were included in table

Table A.7. Analysis of Adhesion on BSA

Treatment	Estimate	Standard Error	t Value	P Value
pH 7.4 time 0-4 hours	1.0230	0.3636	2.81	0.0056
pH 7.4 time 0-4 hours with RR	0.9505	0.3636	2.61	0.0099
pH 7.4 time 2-4 hours with CPZ	0.9828	0.3636	2.70	0.0077
pH 6.5 time 0-2 hours	1.5025	0.3636	4.13	<.0001
pH 6.5 time 0-6 hours	1.1987	0.3636	3.30	0.0012
pH 6.5 time 1-2 hours	1.1226	0.3815	2.94	0.0038
pH 6.5 time 2-4 hours	-0.9602	0.3636	-2.64	0.0092
pH 6.5 time 0-6 hours with RR	1.7270	0.3636	4.75	<.0001
pH 6.5 time 2-6 hours with RR	1.2938	0.3636	3.56	0.0005
pH 6.5 time 4-6 hours with RR	1.1250	0.3636	3.09	0.0024

*Degrees of Freedom = 141, only significant values were included in table

Table A.8. Analysis of Adhesion on Laminin

Treatment	Estimate	Standard Error	t Value	P Value
pH 7.4 time 0-6 hours with RR	0.5038	0.1703	2.96	0.0036
pH 6.5 time 2-6 hours with RR	0.4605	0.1703	2.70	0.0077

* Degrees of Freedom = 148, only significant values were included in table

Table A.9. Analysis of Adhesion on Collagen

Treatment	Estimate	Standard Error	t Value	P Value
pH 7.4 time 0-6 hours	0.8190	0.3041	2.69	0.0079
pH 7.4 time 0-6 hours with RR	0.9050	0.3041	2.98	0.0034
pH 7.4 time 0-6 hours with CPZ	-0.9557	0.3041	-3.14	0.0020
pH 6.5 time 0-2 hours with RR	0.8973	0.3041	2.95	0.0037
pH 6.5 time 0-6 hours with RR	1.1352	0.3041	3.73	0.0003
pH 6.5 time 1-2 hours with CPZ	-0.8757	0.3041	-2.88	0.0046

* Degrees of Freedom = 147, only significant values were included in table

APPENDIX B

HMC-1 CYTOKINE SECRETION

Table B.1. RayBiotech 42-Cytokine Array. Forty-two cytokines are placed in duplicate on one a membrane.

Pos	Pos	Neg	Neg	ENA-78	GCSF	GM-CSF	GRO	GRO-a	I-309	IL-1α	IL-1β
Pos	Pos	Neg	Neg	ENA-78	GCSF	GM-CSF	GRO	GRO-a	I-309	IL-1α	IL-1β
IL-2	IL-3	IL-4	IL-5	IL-6	IL-7	IL-8	IL-10	IL-12p40p70	Il-13	Il-15	IFN-?
IL-2	IL-3	IL-4	IL-5	IL-6	IL-7	IL-8	IL-10	IL-12p40p70	Il-13	Il-15	IFN-?
MCP-1	MCP-2	MCP-3	MCSF	MDC	MIG	MIP-1d	RANTES	SCF	SDF-1	TARC	TGF-β1
MCP-1	MCP-2	MCP-3	MCSF	MDC	MIG	MIP-1d	RANTES	SCF	SDF-1	TARC	TGF-β1
TNF-α	TNF-β	EGF	IGF-I	Angiogenin	Oncostatin M	Thrombopoietin	VEGF	PDGF BB	Leptin	NEG	POS
TNF-α	TNF-β	EGF	IGF-I	Angiogenin	Oncostatin M	Thrombopoietin	VEGF	PDGF BB	Leptin	NEG	POS

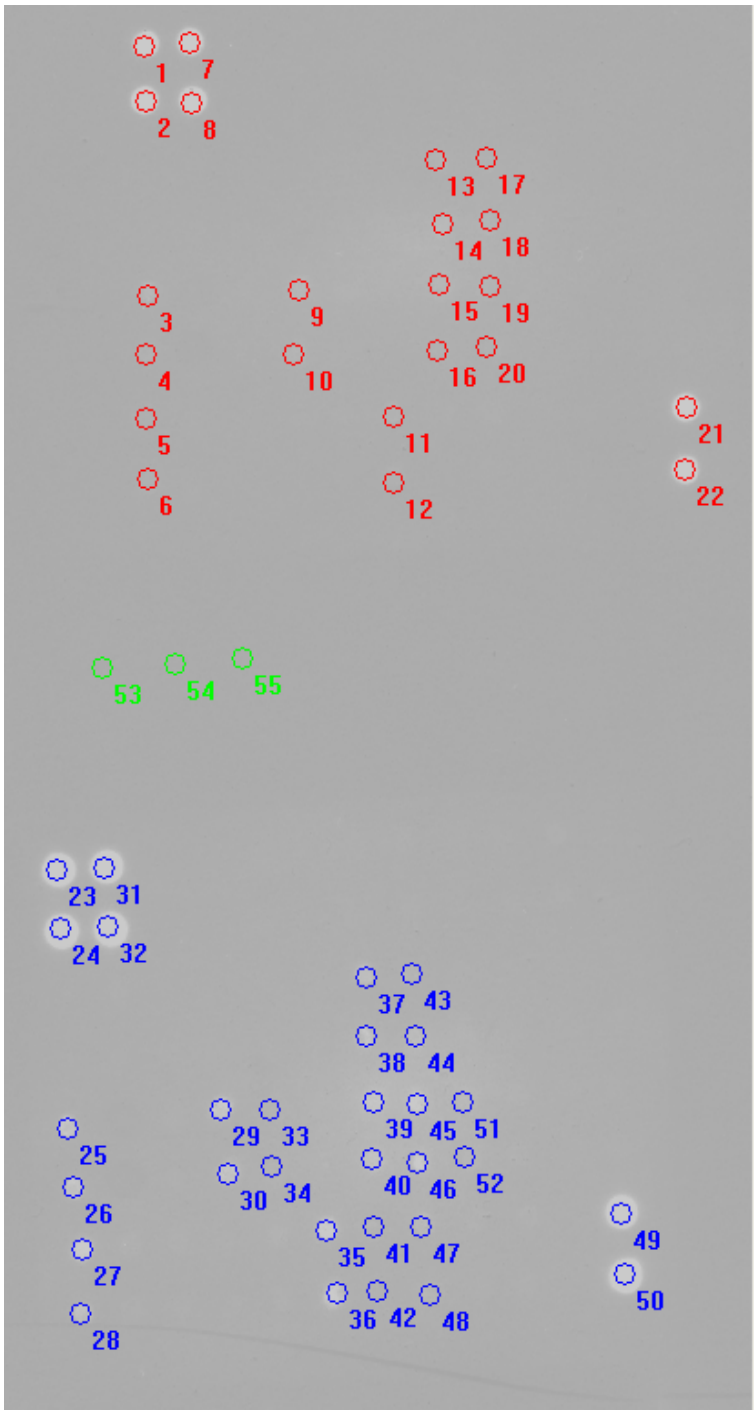


Figure B.1. Cytokine Assay Membrane. HMC-1 cells cultured in pH7.4 measurements are in red,. Cells stimulated with pH 6.5 measurements are in blue and background control values are in green.

Table B.2. Cytokine Array for Cells Cultured in pH 7.4.
Values expressed are the mean of two points.

Treatment	Cytokine	Optical Density
pH 7.4	IL-10	5.159
pH 7.4	IL-10	6.862
pH 7.4	IL-8	6.808
pH 7.4	IL-8	13.411
pH 7.4	MCP-1	10.168
pH 7.4	MCP-1	5.754
pH 7.4	MCSF	10.565
pH 7.4	MCSF	7.808
pH 7.4	MIP-1-D	10.05
pH 7.4	MIP-1-D	5.384
pH 7.4	ONCOSTATIN	6.411
pH 7.4	ONCOSTATIN	3.799
pH 7.4	RANTES	5.84
pH 7.4	TNF-α	4.01

Table B.3. Cytokine Array for Cells Stimulated with pH 6.5. Values expressed are mean values

Treatment	Dot Number	Cytokine	Optical Density
pH 6.5	43	IL-10	8.961
pH 6.5	44	IL-10	15.781
pH 6.5	37	IL-8	17.483
pH 6.5	38	IL-8	21.186
pH 6.5	25	MCP-1	22.727
pH 6.5	26	MCP-1	21.321
pH 6.5	29	MCSF	25.411
pH 6.5	30	MCSF	22.357
pH 6.5	33	MDC	7.474
pH 6.5	34	MDC	7.907
pH 6.5	39	MIP-1-D	24.961
pH 6.5	40	MIP-1-D	21.105
pH 6.5	35	ONCOSTATIN	29.547
pH 6.5	36	ONCOSTATIN	23.898
pH 6.5	45	RANTES	27.628
pH 6.5	46	RANTES	28.267
pH 6.5	51	SCF	13.285
pH 6.5	52	SCF	9.979
pH 6.5	27	TNF-α	29.610
pH 6.5	28	TNF-α	18.249
pH 6.5	41	TPO	8.105
pH 6.5	42	TPO	6.655
pH 6.5	47	VEGF	12.501
pH 6.5	48	VEGF	9.186

Table B.4. HMC-1 Secretion of MCP-1.

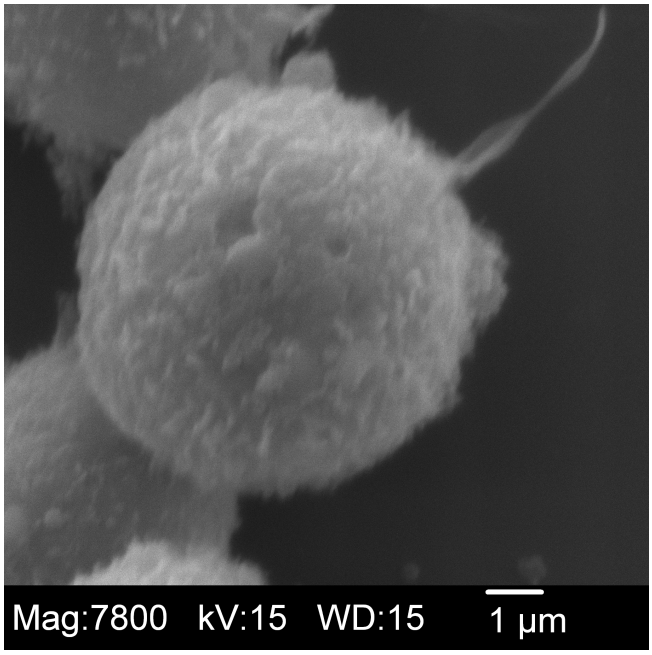
Treatment	MCP-1 (pg/ml)	Standard Error
pH 7.4	3.727	0.761
pH 7.4	19.333	3.946
pH 7.4	51.758	10.565
pH 7.4	120.697	24.637
pH 7.4	161.758	33.019
pH 7.4	217.818	44.462
pH 6.5	1.000	0.204
pH 6.5	23.424	4.781
pH 6.5	50.091	10.225
pH 6.5	127.667	26.060
pH 6.5	186.000	37.967
pH 6.5	248.273	50.678
pH 7.4 + RR	3.576	0.730
pH 7.4 + RR	21.303	4.348
pH 7.4 + RR	49.030	10.008
pH 7.4 + RR	104.636	21.359
pH 7.4 + RR	187.818	38.338
pH 7.4 + RR	256.455	52.349
pH 6.5 + RR	2.515	0.513
pH 6.5 + RR	18.273	3.730
pH 6.5 + RR	48.727	9.946
pH 6.5 + RR	112.970	23.060
pH 6.5 + RR	149.030	30.421
pH 6.5 + RR	198.424	40.503

Table B.5. HMC-1 Secretion of IL-8.

Treatment	Il-8 (pg/ml)	Standard Error
pH 7.4	102.800	18.769
pH 7.4	121.300	22.146
pH 7.4	112.300	20.503
pH 7.4	108.800	19.864
pH 7.4	74.300	13.565
pH 6.5	99.800	18.221
pH 6.5	93.800	17.125
pH 6.5	122.300	22.329
pH 6.5	102.300	18.677
pH 6.5	71.800	13.109
pH 7.4 + RR	90.800	16.578
pH 7.4 + RR	94.300	17.217
pH 7.4 + RR	104.800	19.134
pH 7.4 + RR	127.800	23.333
pH 7.4 + RR	72.800	13.291
pH 6.5 + RR	106.800	19.499
pH 6.5 + RR	108.800	19.864
pH 6.5 + RR	89.800	16.395
pH 6.5 + RR	101.300	18.495
pH 6.5 + RR	65.800	12.013
PMA	82.800	15.117
PMA	87.300	15.939
PMA	147.800	26.984
PMA	725.300	132.421
PMA	3987.800	728.069

APPENDIX C

SCANNING ELECTRON MICROSCOPY



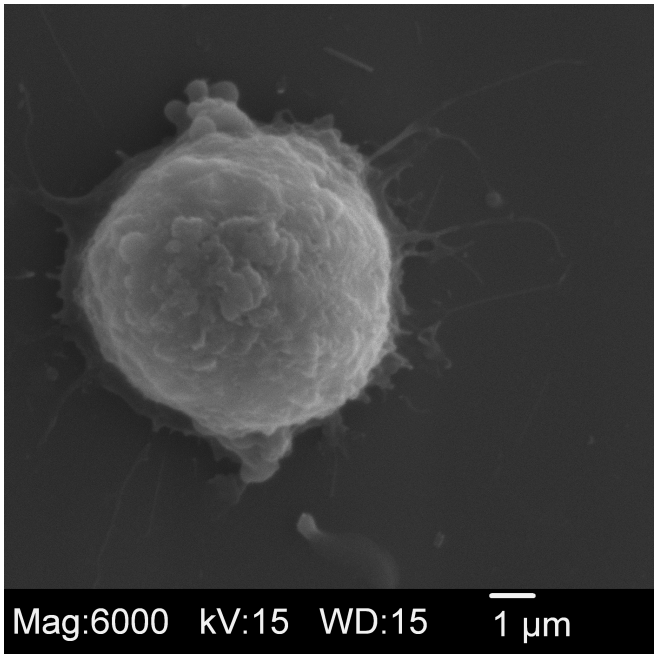
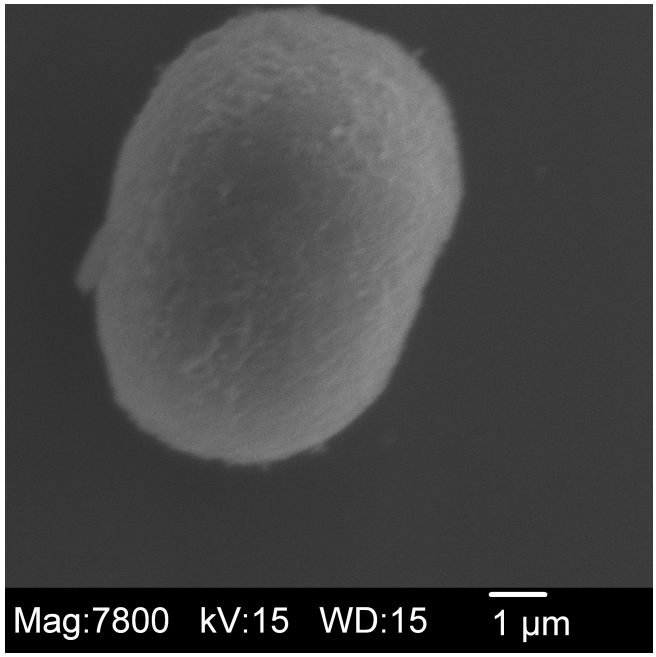


Figure C.1. Scanning Electron Micrograph of HMC-1 in pH 7.4.
Cells cultured under normal physiological pH (7.4).



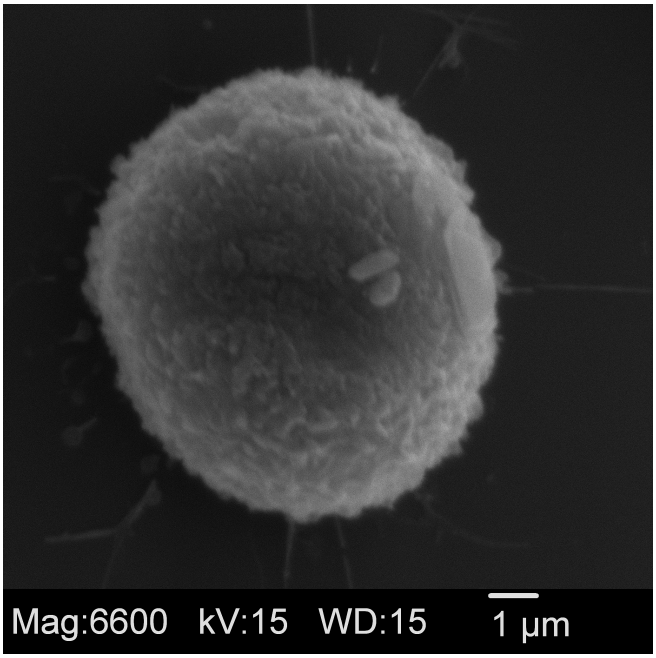
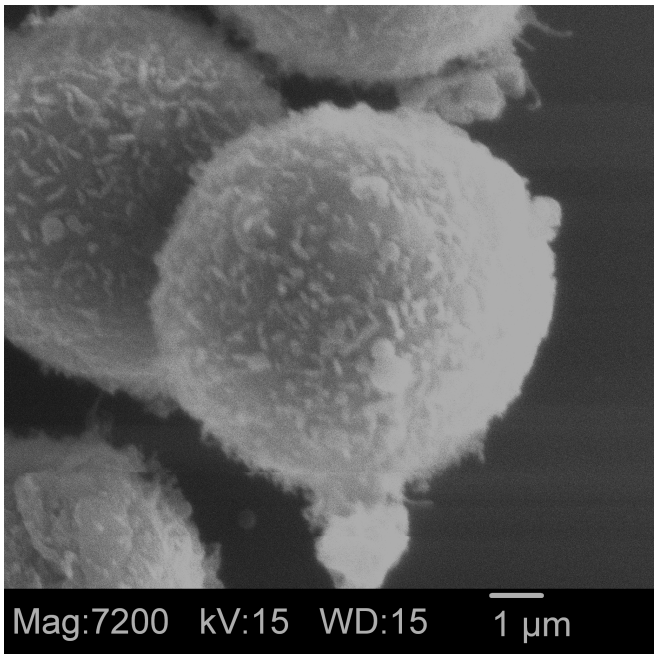


Figure C.2. Scanning Electron Micrograph of HMC-1 in pH 7.4.
Cells cultured under normal physiological pH (7.4).



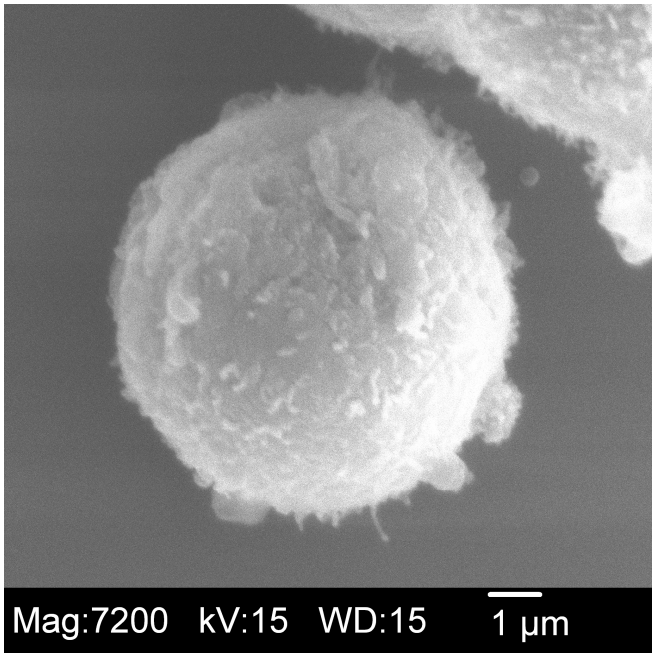


Figure C.3. Scanning Electron Micrograph of HMC-1 in pH 6.5. Cells were stimulated with acidic pH (6.5).

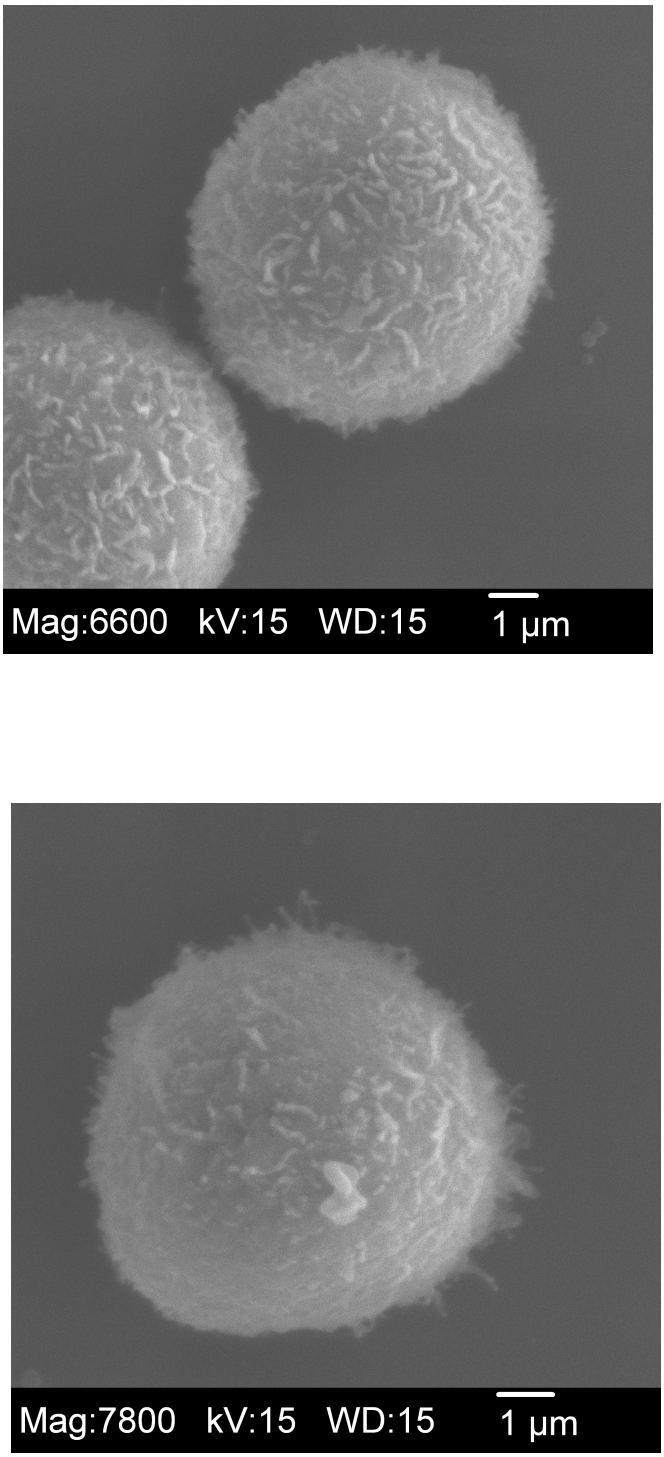
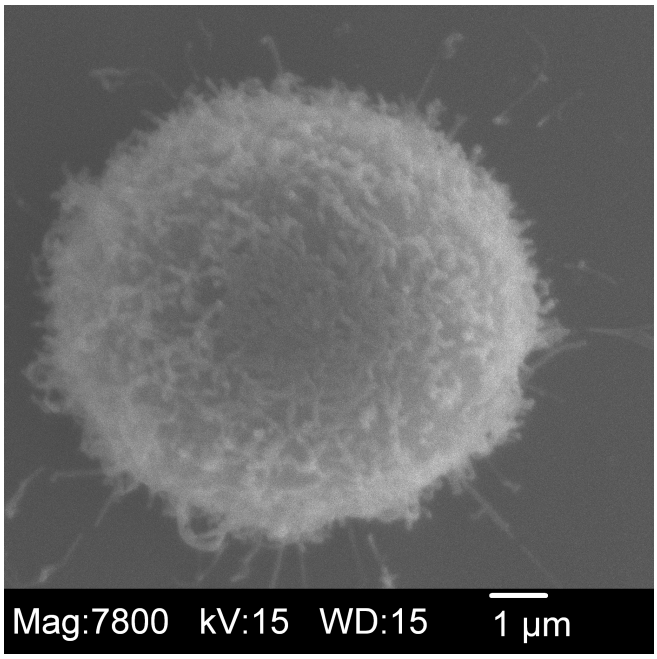


Figure C.4. Scanning Electron Micrograph of HMC-1 in pH 6.5.
Cells were stimulated with acidic pH (6.5).



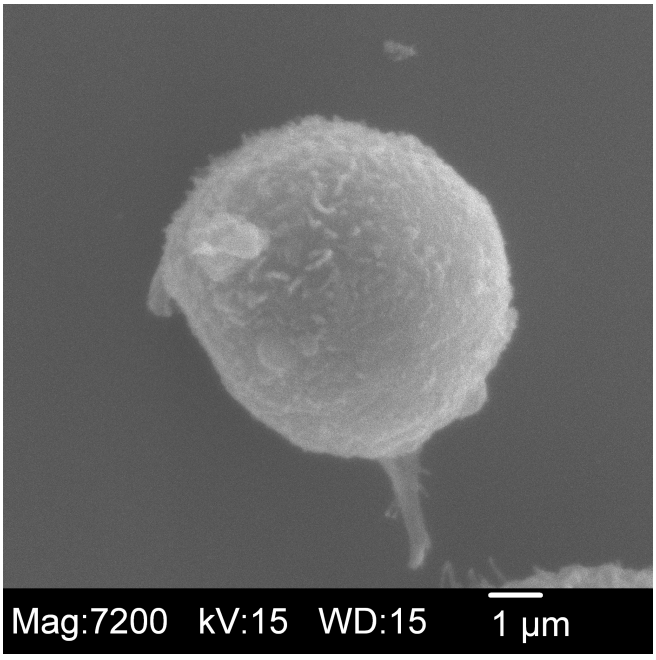
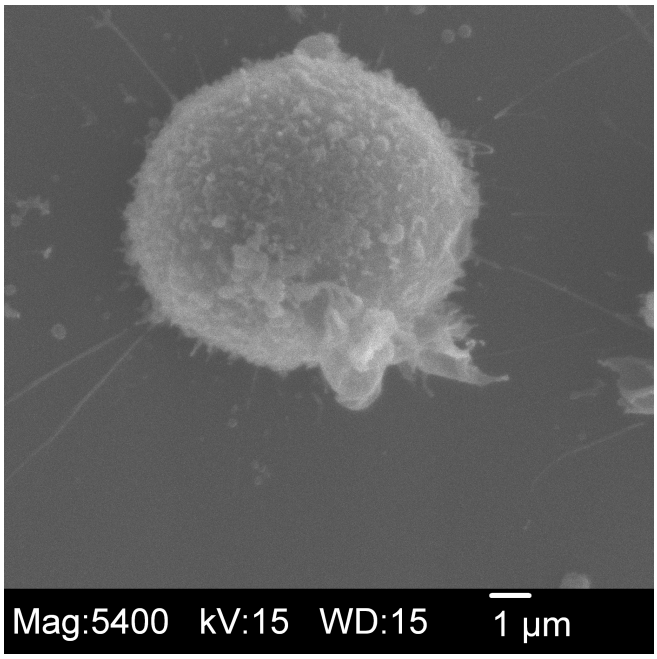


Figure C.5. Scanning Electron Micrograph of HMC-1 in pH 5.0. Cells were stimulated with acidic pH (5.0).



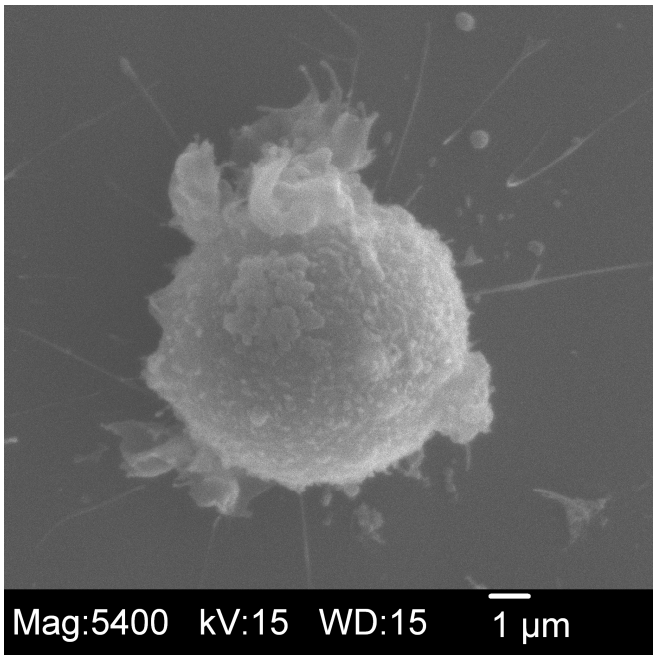


Figure C.6. Scanning Electron Micrograph of HMC-1 in pH 5.0.
Cells were stimulated with acidic pH (5.0).

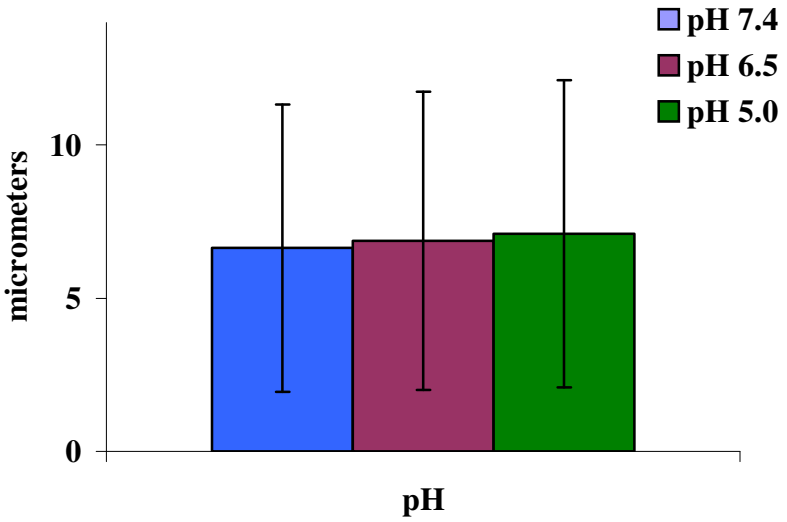


Figure C.7. Mean Diameter of HMC-1 Scanning Electron Micrographs. Mean diameter of cells under normal physiologic pH (7.4) and after stimulation with pH 6.5 and pH 5.0. Error bars represent standard error.

Table C.1. Mean Diameter of HMC-1 Scanning Electron Micrographs.

Treatment	Average Diameter	Group Mean	Standard Error
pH 7.4	7.9		
pH 7.4	6.25		
pH 7.4	5.9		
pH 7.4	6.45	6.64	4.69
pH 6.5	6.4		
pH 6.5	7.1		
pH 6.5	7.2		
pH 6.5	6.8	6.88	4.86
pH 5.0	7.0		
pH 5.0	8		
pH 5.0	5.9		
pH 5.0	7.5	7.1	5.02

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BIOGRAPHICAL INFORMATION

Nicole Conaway Grose was born in North Kansas City, Missouri. She attended the University of Missouri at Columbia before completing her Bachelor of Science degree at the University of Texas at Arlington in May 2001. She then pursued a Doctorate degree in Quantitative Biology (specializing in Physiology), which she will earn in December 2007. As a graduate student, Nicole was served as Vice President for Phi Sigma Biological Honor Society, was awarded the T.E. Kennerly award for outstanding teaching as well as the William L. and Martha Hughes scholarship. Nicole plans to continue her research of the immune system.