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Inflammatory Regulation of Cysteine Cathepsins

Blaine Creasy
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A Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

by

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This work is dedicated in loving memory of my mother Delores Martin Creasy. Her emphasis on the importance of education was a main influence on my decision to continue my education for so many years. She always encouraged me to thrive in school, in life, to climb mountains and achieve my goals no matter what. She is forever in my heart and constantly reminding me to be the person she always knew I could be.
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Abbreviations

Ab.................................................. Antibody
AMC.............................................. 7-amino-4-methylcoumarin
AP-1........................................... Activator protein-1
APC............................................... Antigen presenting cell
C. parvum........................................ Corynebacterium parvum
cAMP........................................... Cyclic adenosine monophosphate
Cat .................................................. Cathepsin
CB1.............................................. Cannabinoid receptor subtype 1
CB2.............................................. Cannabinoid receptor subtype 2
CLIP............................................. Class II-associated invariant peptide
DC................................................ Dendritic cell
ELISA ............................................. Enzyme-linked immunoabsorbant assay
ERK1............................................ Extracellular signal-regulated kinase 1
ERK2............................................ Extracellular signal-regulated kinase 2
FCM............................................. Flow cytometry
FITC............................................ Fluorescein isothiocyanate
h.................................................. hour
IFN............................................... Interferon
Ig.........................................................Immunoglobulin
Ii................................................................Invariant chain
IL........................................................................Interleukin
iNOS..........................................................Inducible nitric oxide synthase
IP_{3}...........................................................Inositol-1,4,5-triphosphate
IRAK.......................................................Interleukin-1 receptor associate kinase
IRF................................................................Interferon response factor
IRSE.......................................................Interferon-stimulated response elements
JNK1.........................................................Jun N-terminal kinase 1
JNK2.........................................................Jun N-terminal kinase 2
LPS.........................................................Lipopolysaccharide
mAb.........................................................Monoclonal antibody
Mal or TIRAP..........................................MyD88 adaptor-like
MAPK.....................................................Mitogen-activated protein kinase
M-CSF......................................................Macrophage-colony stimulating factor
MFI..........................................................Mean fluorescence intensity
MHC.........................................................Major histocompatibility complex
min.............................................................Minute
MKK.......................................................Map kinase kinase
MR.........................................................Magic Red®
mRNA .................................................................Messenger RNA
MyD88 .................................................................Myeloid differentiation primary response gene 88
NFκB .................................................................Nuclear factor-kappa B
NK ..............................................................Natural killer
NS .................................................................Not Significant
PAMP .................................................................Pathogen associated molecular patterns
PEC .................................................................Peritoneal exudate cells
PGN .................................................................Peptidoglycan
PI3K .................................................................Phosphoinositide-3 kinase
PIP2 .................................................................Phosphatidylinositol bisphosphate
PKA .................................................................Protein kinase A
PKB or Akt ..........................................................Protein kinase B
PKC .................................................................Protein kinase C
PLC .................................................................Phospholipase C
Poly I:C ..............................................................Polyinosinic-polycytidylic acid sodium
PRR .................................................................Pattern recognition receptors
RA .................................................................Rheumatoid arthritis
ROS .................................................................Reactive oxygen species
RT-PCR ............................................................Reverse-transcriptase polymerase chain reaction
Th1 .................................................................T helper type 1 cell
Th2.................................................................T helper type 2 cell
THC.................................................................delta-9-tetrahydrocannabinol
TIR.................................................................Toll/IL-1 receptor
TLR.................................................................Toll-like receptors
TNF...............................................................Tumor necrosis factor
TRAF............................................................TNF receptor associated factor
Tram............................................................Trif-related adaptor molecule
Trif..............................................................Tir-related adaptor protein inducing interferon
Abstract

Inflammatory Regulation of Cysteine Cathepsins

By Blaine Madison Creasy, Ph.D

A Dissertation submitted in partial fulfillment of the requirements for the degree of Doctorate of Philosophy at Virginia Commonwealth University.

Virginia Commonwealth University, 2008

Major Director: Kathleen L. McCoy
Department of Microbiology and Immunology

Cysteine cathepsins B, L and S are endosomal/lysosomal proteases that participate in numerous physiological systems. Cathepsin expression and activity are altered during various inflammatory diseases, including rheumatoid arthritis, atherosclerosis, neurodegenerative diseases and cancers. Early immune responses to microbial pathogens are mediated by pattern-recognition receptors, including Toll-like receptors (TLR). Signaling through TLR causes cell activation and release of inflammatory mediators, which can contribute to the severity of chronic inflammatory diseases. The impact of TLR cell activation on cathepsins B, L and S activities was investigated using live-cell
enzymatic assays. Individual ligands of TLR4, TLR2 and TLR3 increased intracellular activities of the three cathepsins indicating the involvement of both MyD88-dependent and -independent pathways. To investigate the role of inflammatory cytokines in regulating these proteases, a lipopolysaccharide (LPS) non-responsive cell line was utilized. LPS non-responsive cells co-cultured with LPS responsive macrophages upregulated cathepsin activities. Furthermore, culture supernatants from LPS-stimulated macrophages increased cathepsin activities in LPS non-responsive cells, which could be reduced by neutralizing antibodies to TNF-α or IL-1β. These findings indicate cytokines regulate cathepsin activities during macrophage responses to TLR stimulation. Using LPS as a model for inflammation, the ability of the cannabinoids, delta⁹-tetrahydrocannabinol (THC), and CP55940 to suppress cysteine cathepsins during an inflammatory response was investigated. Cannabinoids, including the major psychoactive component of marijuana THC, modulate a variety of immune responses and have been proposed as possible therapeutics to control chronic inflammation. Cannabinoids may mediate their effects through receptor-dependent or independent mechanisms. Cannabinoid receptor subtype 1 (CB1) and receptor subtype 2 (CB2) have differential expression in leukocytes. Dose response studies showed that 1 nM THC was sufficient to inhibit cathepsin enhancement in LPS-stimulated cells. P388D1 macrophages expressed CB2 mRNA, but had no detectable CB1 mRNA indicating a role for the CB2 receptor. Utilizing a CB2⁺/macrophage cell line, the role of CB2 receptor participation in THC inhibition of cysteine cathepsin upregulation was explored. THC did not affect cathepsin activity in LPS-stimulated cells lacking CB2 expression. These findings support the possibility of receptor selective...
agonists as therapeutic treatment during inflammatory diseases to prevent cathepsin involvement in pathological tissue destruction.
Chapter 1: Introduction

The immune system is an intricate organization of multiple cell types required for effective host response to invading pathogens. The two arms of immunity are innate immunity, which recognizes invading pathogens in a non-specific manner, and adaptive or specific immunity. Innate immunity is considered the first line of host defense, and a number of cells are involved in the initial response to pathogens, including neutrophils, macrophages, dendritic cells (DC) and natural killer (NK) cells. The adaptive immune response involves antigen-specific B and T lymphocytes, which lead to antibody formation and the development of immunological memory. The two types of adaptive immune responses are cell-mediated immunity and humoral immunity. Cell-mediated immunity involves T cell-mediated cytotoxicity and further activation of macrophages for enhanced pathogen killing. Humoral immunity is the antibody-specific arm of immunity and requires T cell-B cell interaction. The adaptive immune response is dependent on cells activated during the innate response. The innate response begins within moments of pathogen invasion and last for a few days, whereas the adaptive response is activated within 24 hours and continues for weeks.

Immune cells involved in both innate and adaptive immunity express surface receptors known as pattern recognition receptors (PRR) that recognize invading pathogens via pathogen-associated molecular patterns (PAMP). Toll-like receptors (TLRs) are one of
the most evolutionarily conserved members of PRR. These receptors are especially important in innate immunity, and genetic mutations in these receptors leave hosts unable to eliminate some infections effectively. For instance, people with a mutated TLR-4 gene are more susceptible to Gram-negative bacterial sepsis (Janeway, 2001). TLR recognition of commensal microflora in the intestine is required for intestinal homeostasis (Rakoff, 2004). Loss of homeostatic balance in the intestine contributes to diseases, such as chronic inflammatory bowel disease and Crohn’s disease (Rakoff, 2004). Upon pathogen recognition through PRR, immune cells are activated to produce chemical mediators, such as cytokines and chemokines. Subsequent events, such as recruitment of neutrophils and macrophage activation, lead to direct killing of microbes and inflammation. The five classic signs of inflammation are heat, swelling with cell infiltration, pain, redness, and altered function. Inflammation induced by TLRs plays a role in a number of inflammatory diseases, including rheumatoid arthritis (RA), atherosclerosis and Crohn’s disease (Karin, 2006; Huang, 2007; 2008). Therefore, understanding the effects of TLR activation and regulation has important implications for not only host responses but also chronic diseases.

**Antigen-Presenting Cells and Antigen Processing**

Antigen-presenting cells (APC), such as macrophages and DC, are important players in both arms of immunity. APC play a critical role in initial pathogen recognition and inflammation leading to acquired immunity. A very important function of macrophages is phagocytosis. Phagocytosis is defined as the internalization of extracellular particulate matter by cells; in many cases, this matter is bacterium. The
internalized pathogen is contained within a vesicle called the phagosome, which then fuses with more acidic vesicles including endosomes and lysosomes (Janeway, 2001). The endosomal/lysosomal enzymes are important for pathogen destruction and antigen presentation. Intracellular proteases within the vesicles help destroy the pathogen and process proteins that will serve as cell surface antigens to alert other cells of infection. Cell surface molecules expressed on APC, such as major histocompatibility complex (MHC) and co-stimulatory molecules, interact with receptors on T cells. T cells, in turn, proliferate and differentiate into effector cells, and cell-mediated and humoral immunity are activated.

The MHC class II antigen presentation pathway is initiated in the endoplasmic reticulum (ER) where MHC class II αβ heterodimers are assembled with the assistance of invariant chain (Ii), which serves as a molecular chaperone (Honey, 2003). Ii occupies the peptide-binding groove, thereby preventing premature loading of peptides in the ER and during trafficking to the endosomal compartments. Once the MHC class II complex reaches endosomal/lysosomal compartments, Ii is cleaved by lysosomal proteases called cathepsins, leaving only the class II associated invariant chain peptide (CLIP) bound. Pathogen destruction and peptide formation occurs simultaneously within the endosomal/lysosomal compartments. Lysosomal proteases degrade pathogen proteins and form peptides, which are then loaded into the MHC II peptide-binding groove. The MHC class II-like molecule HLA-DM in humans or H-2M in mice mediates the exchange of CLIP for antigenic peptide (Chapman, 2006). The MHC class II-peptide complex is then transported to the cell surface where interaction with CD4⁺ T cells occurs.
Whereas MHC class II molecule is only expressed on professional APC under normal conditions, MHC Class I molecule is found on almost all nucleated cells. MHC class I molecule is constructed of a 45kDa α chain that associates with β2 microglobulin. MHC class I molecules have binding grooves with closed ends limiting the peptide size to 8-10 amino acids. MHC class I antigen processing pathway does not involve Ii, and peptides are derived from endogenous cytoplasmic proteins, such as viral proteins.

Antigenic peptides are generated by the proteasome, and unlike the MHC class II pathway does not involve endosomal/lysosomal proteases. The proteasome is a multi-subunit molecular complex present in the cytoplasm and nucleus. While the proteasome’s primary role is to degrade misrouted or improperly assembled proteins, specific subunits are substituted to form the immunoproteasome. Unlike MHC class II molecules, MHC class I molecule is not exported from the ER until peptide loading has occurred. Peptides generated by the proteasome are transported to the ER lumen through the transporter associated with antigen processing (TAP). Once the peptide is within the ER, it is loaded into the MHC class I binding groove. This binding, in turn, stabilizes the MHC class I complex allowing for transport from the ER to the cell surface. Cells expressing MHC class I-antigen complexes then interact with CD8⁰ T cells.

The T-cell receptor (TCR) interacts with peptide-loaded MHC molecules on APC. APC also express co-stimulatory molecules, such as B7-1 (CD80) and B7-2 (CD86), which interact with co-receptors on T cells. Proper T cell activation requires both TCR and co-stimulation signals. T cells are divided into two subpopulations based on cell surface expression of co-receptor CD4 or CD8 proteins. Cytotoxic CD8⁰ T lymphocytes
(CTL) are MHC class I restricted and aid in response to intracellular pathogens by killing infected cells that are expressing foreign antigen in the context of MHC class I molecules. CTL contain intracellular proteins, such as perforin and granzymes, which are secreted from activated CTL. These proteins aid in the destruction of infected cells by punching holes in their cell membrane, which causes cell death. CD8\(^+\) T cells can also induce target cells to undergo apoptosis via Fas-Fas ligand interaction. This function is particularly important for killing tumor cells. Tumor cells can evade killing by immune cells by down regulating Fas expression, shutting off the internal Fas pathway or even expressing Fas ligand thereby inducing apoptosis of immune cells expressing Fas (Abrams, 2005).

CD4\(^+\) T cells exert their effects primarily by cytokine secretion. CD4\(^+\) T cells can further be divided into two subclasses called T helper 1 (Th1) and T helper 2 (Th2) cells. Another subclass of T cells called T regulator cells also play an important role in immune regulation. Cytokines produced by Th1 cells act as a positive feedback for APC, increasing MHC class II expression and inducing cytokine production by APC. Th2 cells are the humoral immunity helper cell. These cells secrete cytokines, such as interleukin (IL)-4, and express CD40 ligand that induce antibody class switching and differentiation of B cells into antibody-producing plasma cells or memory cells. Efficient antigen processing and B-cell activation are required for the memory and longevity of adaptive immunity.

**Macrophages**

All cells of the immune system arise from pluripotent hematopoietic stem cells in the bone marrow. These pluripotent cells differentiate into either lymphoid or myeloid
progenitor cells. Circulating monocytes give rise to macrophages and are derived from myeloid progenitors (Janeway, 2001). Peripheral blood monocytes circulate throughout the body before entering tissues and becoming macrophages. Inflammatory stimuli increase cell recruitment to peripheral sites where macrophage maturation occurs (Gordon, 2005). As described in preceding sections, macrophages play multiple roles in host defense, including pathogen recognition via PRRs, induction of inflammation, phagocytosis, antigen presentation and cytotoxic killing. Tissue macrophages also have a crucial role in tissue homeostasis by clearing senescent cells and debris, and participating in tissue remodeling (Gordon, 2005). A great amount of heterogeneity exists within macrophage populations, in part, as a result of specialization due to the local anatomical microenvironment. Differences among these cell types can often be detected by distinct expression of cell surface molecules. For instances, Mac-1 molecule is highly expressed on splenic and peritoneal macrophages, but virtually undetectable on alveolar macrophages (Gordon, 1992).

Alveolar macrophages are the guardian of the respiratory tract. These phagocytes are responsible for clearing inhaled debris. These cells are in a quiescent state producing minimal amounts of inflammatory cytokines and displaying poor phagocytic activity. In several lung injury models, activated pulmonary macrophages release cytokines tumor necrosis factor-α (TNF-α) and IL-1β as well as chemokines monocyte chemoattractant protein-1 and macrophage inflammatory protein-1β, which are associated with the acute phase response (Fels, 1986). Alveolar macrophages secrete proteases with elastinolytic activity, which contributes to their role in pulmonary diseases. For example, cigarette
smoke induces distinct changes in gene expression leading to an altered activation state of
alveolar macrophages contributing to chronic obstructive pulmonary disease and
emphysema (Heguy 2006; Muley, 1994; Woodruff, 2005).

Microglial cells are within the central nervous system (CNS) making them unique,
since the CNS is considered to be “immune privileged”. Microglia function as
surveillance cells, constantly moving and analyzing the CNS for damaged neurons,
plaques, and infectious agents (Gehrmann, 1995). Similar to macrophages in the rest of
the body, microglia primarily use phagocytic and cytotoxic mechanisms to destroy foreign
materials (Aloisi, 2001). Over-activation or loss of microglial cell regulation contributes
to the severity of several neurological diseases, such as Alzheimer’s disease, Parkinson’s
disease, multiple sclerosis, human immunodeficiency disease, dementia and traumatic
brain injury.

Osteoclasts, found in bones, regulate bone remodeling and are responsible for bone
resorption. They are characterized by high expression of tartrate-resistant acid phosphatase
(TRAP) and cathepsin K as well as other proteases. While not always considered as
macrophages, their high expression of proteases, production and response to pro-
inflammatory cytokines, and contribution to disease make them macrophage-like. Loss of
osteoclast regulation contributes to the bone disease often suffered by aging women,
omeoporosis. Osteoporosis is caused by an imbalance between bone resorption and bone
formation, and over-activation of osteoclasts promotes bone destruction (Holtrop, 1977;
Vaananen, 2000).
Kupffer cells and splenic macrophages have similar functions. Kupffer cells are specialized macrophages found within the liver. The primary function of Kupffer cells is to recycle old red blood cells that no longer are functional. The red blood cell is broken down by phagocytic action, and the hemoglobin molecule is split. Both globin chains and the heme iron-containing portion are reutilized (Haubrich, 2004). During liver injury and inflammation, Kupffer cells secrete cytokines, reactive oxygen species (ROS) and enzymes, which contribute to hepatocyte destruction. Splenic macrophages within the white pulp make up approximately 5% of the splenocyte population. Splenic macrophages are heterogeneous due to the various microenvironments in the spleen and resemble Kupffer cell function. Via phagocytosis, they remove parasites, bacteria, damaged or old red blood cells, apoptotic leukocytes, platelets, and other particles circulating in the blood (Bennett, 1981; Klonizakis, 1981; Nursat, 1988). Functional failure of marginal zone macrophages impairs the induction of tolerance to cell-associated antigens leading to autoimmune disease (Miyake, 2007).

Peritoneal macrophages are resident cells in the peritoneal cavity and make up approximately 85% of the cells present in peritoneal fluid of humans. Due to their location, they can affect multiple aspects of immunity and have been linked to diseases, such as endometriosis. Peritoneal macrophages are also potent cytokines producers, which can be distributed throughout the body, thereby affecting systemic immunity. Thioglycollate-elicited peritoneal macrophages are commonly used in scientific animal studies. Thioglycollate recruits bone marrow monocytes that differentiate into macrophages, which are not quiescent.
Monocytes can differentiate into macrophages or DC depending upon the environmental signals received. Some stimuli can promote macrophages to differentiate into DC-like cells as well (Conit, 2008; Saxena, 2003; Shen, 2008). Macrophages can exist in different activation states depending upon the stimulus. Macrophages respond to a TLR ligand, such as bacterial lipopolysaccharide (LPS), or interferon-γ (IFN-γ) and become primed. IFN-γ upregulates MHC class II expression (Boss, 1997) and lysosomal cysteine cathepsins B and L (Lah, 1995). In this state, they are capable of phagocytosis, antigen processing and presentation, however their cytotoxic killing is still inefficient (Adams, 1992). Stronger stimuli, such as IL-1β and TNF-α fully activate macrophages to promote cell killing of tumor cells or pathogens through increased production of nitric oxide and ROS. These inflammatory mediators can also be produced by primed macrophage and act in an autocrine manor to activate macrophages fully. Furthermore, macrophages can be fully activated by a two-step process involving LPS stimulation followed by IFN-γ. Primed and activated macrophages serve as APC but also have another important immune function, antibody-dependent cell-mediated cytotoxicity (ADCC). (Janeway, 2001) Macrophages have high cell surface expression of Fc receptors for IgG, which bind the Fc portion of IgG. During acquired immune responses, B cells are activated to produce antigen-specific IgG, which can then bind bacterial cell surface or virally infected cells, a process termed opsonization. Opsonized infected cells may then be killed by ADCC. Alternatively, opsonized infected cells or bacteria undergo Fc receptor-mediated phagocytosis by macrophages. Complement receptors on macrophages can also mediate phagocytosis of pathogens bound by complement proteins. Macrophages can clear
immune complexes formed by immunoglobulin binding to soluble antigen or binding of complement components to immune complexes. These latter functions are why macrophages are called scavenger cells.

Macrophages are potent producers of pro-inflammatory cytokines and also promote other cell types to synthesize cytokines. While under normal conditions the macrophage serves to protect and maintain tissue homeostasis, during chronic inflammatory diseases they are a major contributor to pathological tissue destruction. Macrophage activation through TLRs induces distinct changes in gene expression and promotes upregulation of proteins involved in tissue destruction. For example, macrophages also secrete a number of proteases, such as matrix metalloproteases (MMP) and cathepsins. These enzymes degrade extracellular matrix components causing tissue damage or facilitating tumor metastasis.

**Cathepsins**

Cathepsins are lysosomal proteases that are divided into three groups: cysteine, serine, and aspartyl based on active site amino acid residues. Lysosomal cysteine proteases belonging to the papain gene family were first discovered in the 1940s when cathepsin C was identified. Forty years elapsed before other members were identified and the amino acid sequences were determined for some mammalian cathepsins (Turk, 2001). Currently 11 cysteine cathepsins have been identified in humans: cathepsins B, L, H, S, K, F, V, X, W, O, and C, of which 9 have catalytic activity (Turk, 2001). These enzymes are synthesized as inactive precursors, which then undergo proteolytic cleavage by another protease or autocatalytic cleavage to achieve their active form. The pro-enzyme contains
sugar moieties of mannose-6-phosphate, which targets the enzyme to lysosomal compartments, where it is transferred through interaction with the mannose-6-phosphate receptor (Ishidoh, 2002). Once cathepsins reach the proper intracellular compartment, their activation is regulated by pH, endogenous inhibitors, such as cystatins, and secretion.

Cysteine cathepsins participate in antigen processing that is required for T cell immune responses (Berdowska, 2004; Hsing, 2005; Reinheckl, 2001; Rudensky, 2006). Active site-labeling demonstrates active cathepsins B, L, and S in phagosomal compartments of APC (Lennon-Duménil, 2002). One of the first indicators of the importance of these proteases in antigen processing was the treatment of macrophages with ammonium chloride or chloroquine, which increases lysosomal pH, inhibits the MHC class II antigen processing pathway (Ziegler, 1982). Disruption of lysosomal acidification by lysosomotropic reagents or treatment of cells with the protease inhibitor leupeptin leads to accumulation of Ii chain fragments associated with MHC class II molecules (Honey, 2003). Similar to most cysteine cathepsins, aspartyl cathepsins D and E require an acidic pH for activity; these enzymes belong to the pepsin family. Pepstatin A, an aspartyl protease inhibitor, interferes with Ii cleavage and processing of intact chicken ovalbumin antigen, indicating a role for cathepsins D and E in antigen processing as well (Zhang, 2000). While there is some redundancy among these proteases, studies using genetically deficient mice indicate specific roles for cathepsins. Not only do cathepsins appear to have specific roles, but also in many cases various cell types have differential expression or specific activity. **Table 1** gives an overview of selected cathepsin expression, and the phenotype of genetically deficient mice.
Table 1

<table>
<thead>
<tr>
<th>Protease</th>
<th>Expression</th>
<th>Knockout Phenotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cathepsin B</td>
<td>B cells, DC, macrophages</td>
<td>Decreased susceptibility to TNF-α induced hepatocyte apoptosis</td>
<td>Honey, 2003</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Guicciardi, 2001</td>
</tr>
<tr>
<td>Cathepsin L</td>
<td>Cortical thymic epithelial cells, macrophages, B cells and DC</td>
<td>Decreased CD4^+ T cells, decreased NKT, epidermal hyperplasia, hair follicle deficiencies and dilated cardiomyopathy</td>
<td>Honey, 2003</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Hsieh, 2002</td>
</tr>
<tr>
<td>Cathepsin S</td>
<td>B cells, DC, macrophages, epithelial cells, smooth muscle cells</td>
<td>Decrease MHC II presentation, NKT, cells, deficient germinal center formation and impaired class switching to IgG2a and IgG3</td>
<td>Honey, 2003</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Shi, 1999, 2003</td>
</tr>
<tr>
<td>Cathepsin K</td>
<td>Macrophages and osteoclasts</td>
<td>No immune phenotype but osteopetrotic phenotype</td>
<td>Saftig, 2000</td>
</tr>
<tr>
<td>Cathepsin D</td>
<td>B cells, DC, macrophages</td>
<td>Dies at day 21 due to atrophy of ileal mucosa, lysosomal storage disorder in the CNS neurons</td>
<td>Honey, 2003</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Koike, 2000</td>
</tr>
</tbody>
</table>
Cysteine cathepsin regulation is not well elucidated. Cytokines, such as IFN-γ, TNF-α, IL-1β, IL-6 and IL-10, can regulate cathepsins, however differential regulation appears to occur among different cell types (Chae, 2007; Fiebiger, 2001, Lah, 1995; Beers, 2003; Watari, 2000), which is discussed later in this chapter. These enzymes can also be secreted into the extracellular matrix to prevent their over-accumulation within lysosomal compartments. Endogenous inhibitors, particularly cystatins, regulate cathepsin activity by binding the active site. Cystatins A, B, C and F are expressed in a number of immune cells (Kopitar-Jerala, 2006). Cystatins are inversely affected during inflammatory disease, whereby expression is often decreased, in contrast to cathepsin expression. Cystatins A and B, also referred to as stefins, are primarily intracellular proteins, whereas cystatin C contains a peptide signal sequence for extracellular targeting. Cystatin A is expressed in epidermal cells, neutrophils, hepatocytes, follicular dendritic cells and splenocytes (Kopitar-Jerala 2006). Gene expression studies show LPS-stimulated monocytes decrease cystatin A synthesis, possibly allowing increased cathepsin activity during monocyte differentiation into APC. Opposite effects occur with cystatin B in monocytes differentiating into macrophages, whereby expression increases (Hashimoto, 1999). Mutations in this gene are responsible for Unverricht Lundborg disease, a form of epilepsy. The phenotype of cystatin B deficient mice mimics human disease with progressive ataxia, myoclonic seizures and apoptotic cerebellar granular cells (Pennachio, 1998). Furthermore, lack of cathepsin B in cystatin B−/− mice abolish neuronal cell death implicating cystatin B directly controls cathepsin B involvement in neuronal apoptosis (Houseweart, 2003). Cystatin C is a very potent inhibitor of cysteine cathepsins with an
inhibition constant below the nanomolar range (Lindahl, 1992). Decreased cystatin C expression is associated with a number of diseases, including atherosclerosis and RA. During DC maturation intracellular cystatin C levels decrease, and incubation of DC with TNF-α increases cystatin C secretion, thereby increasing intracellular cathepsin activities (Kopitar-Jerala, 2006). Due to the inverse correlation of cystatins with cathepsins during disease, both are often investigated.

Recent research on cathepsins focuses on their importance in various types of pathophysiological processes. Increased cathepsin activity is associated with multiple tumor types, including breast, lung, brain, thyroid, pancreatic and melanomas. They are also associated with RA, osteoporosis, arteriolsclerosis, and other chronic inflammatory diseases (Berdowska, 2004). Cathepsin K, highly expressed in osteoclasts, plays a crucial role in progression of osteoporosis and has been suggested as a target for therapy. Hereditary disorders, such as pycnodysostosis and Papillon-Lefevre, and Haim-Munk syndromes, are linked to mutations in cathepsin genes (Berdowska, 2004). Cathepsins B, L and S have been extensively studied over the past decade and have a wide range of physiological roles. These proteases will be discussed in detail. Infiltrating immune cells are thought to be the major cause of tissue destruction in numerous inflammatory diseases. Cell types separated by flow cytometry indicate cathepsin expression is highest within the GR-1⁺/Mac-1⁺ myeloid cell population (Joyce, 2004). Therefore, understanding the regulation of cathepsins and cystatins in macrophage populations has important implications for controlling tissue damage mediated by theses proteases.

**Cathepsin B**
Cathepsin B (Cat B) is ubiquitously expressed in various tissues and is the most abundant cysteine cathepsin. Protein and mRNA expression is highest in the liver, thyroid gland, kidney, and spleen (Berdowska, 2004; Honey, 2003). Enhanced expression of Cat B is prevalent in colon, thyroid, liver, breast, melanoma, and prostate cancers (Berdowska, 2004). Mice deficient in Cat B are not defective at processing ovalbumin and hen egg lysozyme indicating it is not required for processing these antigens (Duessing, 1998). However inhibition of Cat B by the selective inhibitor CA074, produces a Th1 immune response in BALB/c mice infected with *Leishmania major* allowing them to overcome disease (Maekawa, 1997). These mice typically promote a Th2 response to *L. major* and lack resistance to this infection. The inhibitor alters processing of soluble *L. major* antigens suggesting Cat B could be destroying the necessary epitopes required to drive a Th1 response (Zhang, 2000).

Cat B has a critical role in initiating pancreatitis and TNF-α-induced apoptosis of hepatocytes based on genetically deficient mice (Guicciardi, 2001). Cat B is released from lysosomal compartments and interacts with mitochondria triggering release of cytochrome c. The death ligand TRAIL (tumor necrosis factor-related apoptosis inducing ligand) triggers apoptosis in a variety of cancer cells. Inhibition of Cat B blocks TRAIL-induced cell death in oral squamous cell carcinoma implicating its possible role in caspase activation as well (Nagaraj, 2006). Cat B is capable of degrading extracellular matrix components such as collagen and elastin, thereby playing a role in angiogenesis (Joyce, 2004). In pancreatic tumors, Cat B expression is highest within angiogenic islets, and Cat B deficient mice have fewer angiogenic islets overall (Gocheva, 2006). Furthermore,
Gocheva et al. examined upregulation of these cathepsins through various stages of tumor angiogenesis and detected highest enzyme activity at the invasive tumor fronts (Gocheva, 2006). Cat B is detected in fibroblast- and macrophage-like cells at sites of cartilage and bone destruction, particularly in patients with osteoarthritis and RA (Hashimoto, 2001). In addition, Cat B activity is increased in centrum semiovale specimens from the brains of deceased multiple sclerosis patients and is localized to fibrillary tangles and degenerated neurites of Alzheimer’s disease and dementia patients (Bever, 1994; Bever, 1995; Berdowska, 2004).

Cat B proteolytic activity plays a role in multiple aspects of immunity, however little about its regulation is understood. Doxorubicin, a commonly used cancer treatment drug that induces cell death, increases Cat B expression and activity in tumor cells. This regulation is mediated by transcription factor NFκB identifying a NFκB binding site in the Cat B promoter region (Bien, 2003). IFN-γ decreases mRNA expression of Cat B in microglia, primary alveolar macrophages and macrophage cell lines, however an interferon response element has not been identified in the Cat B promoter (Liuzzo, 1999). The murine Cat B gene does contain several SP-1 sites in the promoter region (Qian, 1991). IL-6 indirectly increases Cat B activity in osteoblasts through mechanisms involving mitogen activated protein kinase (MAPK) signaling and cyclic adenosine monophosphate (cAMP) (Chae, 2007). The effects of inflammatory stimuli, such as LPS or other TLR ligands, on Cat B have not been well studied. TLR activation can contribute to chronic inflammatory diseases and may lead to overexpression of this protease. Furthermore, the mechanisms involved in cathepsin regulation by infectious ligands are unknown. Therefore studies
investigating the regulation of Cat B by immune modulating substances would be progression to understanding and controlling activation during disease states.

**Cathepsin L**

Cathepsin L (Cat L) is also implicated to be important for antigen processing and Ii degradation in APCs (Honey, 2003; Reinheckl, 2001). Cat L deficient mice have decreased CD4+ T-cells and NK T-cells due to impaired Ii chain degradation (Honey, 2003). Cortical thymic epithelial cells from Cat L deficient mice cannot properly cleave Ii to CLIP, causing a decrease in MHC class II surface expression and ultimately affecting positive selection (Nakagawa, 1998). Cat L also plays an important role in antigen processing. Unlike Cat B, which possibly destroys the antigenic epitope required for a Th1 response during infection with *L. major*, Cat L is required for a Th1 response. Inhibition of Cat L with CLIK148, a Cat L selective inhibitor, exacerbates the disease and increases Th2 cytokines (Onishi, 2004).

Cat L−/− mice have epidermal hyperplasia and hair follicle deficiencies as well. A Cat L isoform localizes in the nucleus and processes CDP/Cux transcription factors involved in cell cycle progression (Goulet, 2004). The CDP/Cux transcription factors are also involved in hair follicle morphogenesis possibly explaining the hair follicle deficiency in Cat L−/− mice. This finding has led to the idea that other cathepsins may also be players in cell cycle regulation. Similar to Cat B, Cat L can hydrolyze extracellular matrix components, such as collagen and laminin. Low-density lipoprotein receptor deficient mice on a high fat diet are used as a model for atherosclerosis. Cat L deficiency in this model reduces the number of atherosclerotic plaques and leukocyte migration due to its role in
remodeling of the arterial extracellular matrix (Kitamoto, 2007). Cat L is also increased in synovial fluid from patients with RA but is not increased in plasma indicating the change in activity is localized to cells in the synovial joint region (Keyszer, 1998).

Pro-inflammatory cytokines, such as TNF-α and IL-1β, are increased in the synovial joints of RA patients and are current targets for RA therapy. Both cytokines stimulate secretion of Cat L from synovial fibroblasts indicating their role in the regulation of Cat L during inflammatory disease (Huet, 1993). Interestingly a NFκB site has not been identified in the Cat L promoter region, suggesting these cytokines regulate Cat L through post-translational mechanisms. Cat L is upregulated in macrophages treated with IFN-γ correlating with increased antigen processing (Lah, 1995). However, an IFN-γ response element has not been identified in the Cat L promoter indicating IFN-γ indirectly regulates Cat L. The promoter region for Cat L contains two AP-2, a SP-1 cluster and a cAMP response element (CRE) (Ishidoh, 1989). Unique to Cat L is regulation by li p41 splice variant. Studies show p41 binds the Cat L active site inhibiting activity, possibly protecting important epitopes from degradation (Fineschi, 1997; Bevec, 1997). Other studies suggest p41 binding stabilizes Cat L allowing it to retain its activity in the extracellular environment when secreted due to inflammatory stimuli (Fiebiger, 2002). LPS increases Cat L expression in DC, however these studies did not investigate changes in enzymatic activity or the effect on macrophages (Lautwein, 2002). It is unclear whether the effects of LPS are directly due to TLR signaling or to cytokines produced during the inflammatory response.

Cathepsin S
Cathepsin S (Cat S) is highly expressed in the spleen, lymph nodes and heart, and has a role in antigen processing and Ii degradation in APC (Berdowska, 2004; Honey, 2003). Cat S is unique compared to other cathepsins, because it is not tightly regulated by pH and can retain approximately 70% of its activity at a neutral pH (Turk, 2001). Cat S deficient mice have decreased MHC class II antigen presentation, deficient germinal center formation, and impaired immunoglobulin class switching (Honey, 2003). These mice also have diminished susceptibility to collagen-induced arthritis and autoimmune myasthenia gravis indicating a possible role in production of autoantigens (Nakagawa, 1999; Yang, 2005).

Similar to Cat B and L, increased Cat S levels are associated with atherosclerosis, inflammatory myopathies, and prostate, lung, and kidney cancers. Studies indicate Cat S may be involved in the activation of caspases 3,8, and 9 therefore regulating caspase-mediated apoptosis (Zheng, 2005). Endothelial cells from cathepsin S\(^{-/-}\) mice poorly degrade elastin and collagen and display decreased migration across Matrigel or type I collagen membranes indicating its importance in angiogenesis (Berdowska, 2004). Cat S produces pro-angiogenic factors derived from laminin IV and degrades anti-angiogenic factors, thereby increasing angiogenesis (Wang, 2006). Cat S involvement in atherosclerosis has been reported in numerous studies. Whereas Cat S expression is low in normal arteries, in patients with atherosclerosis it is highly expressed (Sukhova, 1998). Deficiency of Cat S in the atherosclerosis mouse model led to decreased plaque size, number of macrophages, T cells, and increased plaque stability (Sukhova, 2003).
While Cat S clearly participates in multiple autoimmune and chronic inflammatory diseases, its regulation is poorly understood. IFN-γ increases Cat S expression in smooth muscle cells, epithelial cells, and lung tissue (Storm, 2002; Zheng 2005). Studies indicate IFN regulatory factor-1 (IRF-1) regulates Cat S expression by IFN-γ suggesting an IFN response (ISRE) element in the Cat S promoter (Storm, 2002). IFN-γ induces expression of Cat S in alveolar remodeling, and pulmonary emphysema is linked to epithelial cell apoptosis. However, the role of apoptosis in pulmonary disease is not fully understood (Zheng, 2005). The 5′ untranslated region of the human Cat S gene contains two SP-1 sites and at least 18 AP1 sites (Shi, 1994). LPS induces Cat S expression in cervical smooth muscle cells and DC (Lautwein 2002; Watari 2000). LPS leads to the activation of transcription factors binding AP1, which may contribute to this regulation. LPS also induces expression of pro-inflammatory cytokines TNF-α, IL-β, and IL-6. Monoclonal anti-TNF-α antibody partially inhibits LPS upregulation of Cat S mRNA expression in cervical smooth muscle cells demonstrating cytokine regulation of the enzyme as well. IL-6 controls Cat S activity in DC in an indirect way by decreasing cystatin C expression (Kitamura, 2005). This is particularly important, because over-activation of Cat S decreases MHC class II expression and suppresses CD4+ T-cell responses.

Regulation of cysteine proteases by TLR ligands other than LPS has not been studied. Studies using LPS indicate there may be both direct and indirect regulation of these proteases. Studies investigating TLR regulation and direct signaling effects of cathepsins would increase our understanding of their regulation during infection and chronic disease. Furthermore, understanding the role of cytokines in TLR-mediated effects...
on cathepsins could promote finding of new therapeutic treatments.

**Toll-Like Receptors**

As mentioned previously, initial immune responses to microbial pathogens are mediated by a family of PRR, called TLRs. TLRs were first identified in *Drosophila* as an essential receptor for proper dorso-ventral pattern development in embryos (Lemaitre, 1996). TLR mutations cause flies to be highly susceptible to fungal infections (Hoffmann, 1996). This was an important discovery making researchers aware of the importance of TLR in immunity and led to the finding of TLR homologues in mammals. Pathogen recognition via PAMPs play an important role in innate immunity. This initiates acute inflammatory responses promoting production of cytokines and chemokines. Subsequent events include recruitment of neutrophils and activation of macrophages leading to the direct killing of microbes. During this process adaptive immunity may also be activated to further control infection.

There are 10 TLR identified in humans, named TLR 1-10, and each of these receptors has different specificity (Pasare, 2004). **Table 2** is a summary of TLR ligand specificity and cellular expression location. TLR-1, 2 and 6 bind lipoproteins from the cell wall of Gram-positive bacteria, whereas TLR-4 recognizes the Gram-negative bacterial cell wall component, LPS. TLR-3 plays an important role in viral immunity by recognizing double stranded RNA. TLR-7 and TLR-8 recognize RNA from single stranded RNA viruses. Unmethylated CpG DNA found in prokaryotic genomes and DNA viruses is recognized by TLR-9 (Pasare, 2004). There is also evidence that endogenous ligands such as oxidized lipids, heat shock proteins and DNA from apoptotic cells can activate these
## Table 2
TLR pathogen associated molecular patterns.

<table>
<thead>
<tr>
<th>TLR</th>
<th>Pathogen</th>
<th>PAMP</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/2</td>
<td>Gram Positive Bacteria</td>
<td>Triacylated Lipoprotein</td>
<td>Cell Surface</td>
</tr>
<tr>
<td>2/6</td>
<td>Gram Positive Bacteria</td>
<td>Peptidoglycan (PGN)</td>
<td>Cell Surface</td>
</tr>
<tr>
<td>3</td>
<td>Double Stranded RNA</td>
<td>dsRNA, Poly I:C</td>
<td>Cell Surface and</td>
</tr>
<tr>
<td></td>
<td>Viruses</td>
<td></td>
<td>Cytoplasm</td>
</tr>
<tr>
<td>4</td>
<td>Gram Negative Bacteria</td>
<td>Lipopolysaccharide (LPS)</td>
<td>Cell Surface</td>
</tr>
<tr>
<td>5</td>
<td>Flagellated Bacteria</td>
<td>Bacterial Flaggelin</td>
<td>Cell Surface</td>
</tr>
<tr>
<td>7</td>
<td>Single Stranded RNA</td>
<td>ssRNA, Imidazoquinolines</td>
<td>Cytoplasm</td>
</tr>
<tr>
<td></td>
<td>Viruses</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Single Stranded RNA</td>
<td>ssRNA</td>
<td>Cytoplasm</td>
</tr>
<tr>
<td></td>
<td>Viruses</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Bacteria and DNA</td>
<td>CpG</td>
<td>Cell Surface and</td>
</tr>
<tr>
<td></td>
<td>Viruses</td>
<td></td>
<td>Cytoplasm</td>
</tr>
<tr>
<td>10</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Surface</td>
</tr>
</tbody>
</table>

Table derived from: Pasare, 2004.
receptors (Karin, 2006; Pasare, 2004). TLR signaling pathways have been extensively studied. Cell activation by TLR requires receptor dimerization. In some cases such as TLR-4, homodimerization occurs; for others such as TLR-1,2, and 6, heterodimerization plays a role in specificity. TLR-1/2 dimerization leads to specific recognition of triacylated lipoproteins, whereas TLR-2/6 dimers bind diacylated lipoproteins (Takeda, 2004; Trinchieri, 2007). TLR-4 is unique in that it requires the co-receptor CD14 and a stabilizing protein MD-2 for efficient signaling. Upon ligand binding these receptors dimerize, and a series of signaling events occurs that results in cell activation and an inflammatory response. TLR signaling cascades lead to activation of MAPK pathways and novel transcription factors, including NFκB and AP-1 (Figure 1) (O’Neil, 2006). The cytoplasmic portion of TLR receptors has high similarity to that of the IL-1 receptor family now referred to as Toll/IL-1 receptor domain (TIR) (Yamamoto, 2004). The importance of the TIR domain was first recognized in the C3H/HeJ mouse strain, which has a point mutation in the TLR-4 gene that results in the change of a cytoplasmic proline at position 712 to histidine. Loss of this proline residue resulted in a dominant negative effect on TLR-4 mediated signaling, and these mice are hyporesponsive to LPS-induced endotoxic shock (Qureshi, 1999; Poltorak, 1998). Among the first studies of TLR signaling was the identification of the common adaptor protein MyD88 (myeloid differentiation primary-response gene 88) (Takeuchi, 2000). MyD88 is a common adaptor protein for all TLRs except for TLR-3, which signals independently of MyD88 (Figure 1). TLR-4 is unique because it signals through both MyD88-dependent
Figure 1. Schematic representation of TLR signaling pathways.

Binding of LPS induces TLR-4 receptor dimerization and allows for MyD88 or TIRAP binding to the TLR cytoplasmic domain. Upon activation of the MyD88-dependent pathway, IRAK-1, IRAK4 and TRAF6 are recruited to the receptor complex and activated. TRAF6 then activates TAK-1 and/or ECSIT. Activated TAK-1 phosphorylates IKKβ, and NFκB translocates into the nucleus. TLR-4 MyD88-independent pathway involves adaptor molecules TRAM and TRIF, which activate TBK-1. TBK-1 can crosstalk with MyD88-associated signaling components leading to MAPK and NFκB activation or activate interferon response factors (IRFs) inducing the production of Type I IFN. TLR-4 signaling can also activate multiple MAPK pathways independent of MyD88 leading to activation of SRE and AP-1 transcription factors. Binding of dsRNA to TLR-3 activates only the MyD88-independent pathway involving TRIF and leads to the activation of NFκB and IRFs.
MyD88 possesses a TIR domain in the C-terminal portion and a death domain in the N-terminal portion. TIR-TIR interaction is hypothesized to occur between TLR receptor and MyD88 when receptor dimerization occurs. MyD88 deficient mice have diminished responses to all TLR ligands that activate the MyD88 pathway. MyD88 deficiency yields similar results as individual TLR deficiencies except for in the case of TLR-3 and TLR-4. Loss of MyD88 delays NFκB activation and TNF-α production in response to LPS, however responses are not completely abolished. The fact that MyD88−/− mice still induce responses to TLR-3 and TLR-4 ligands suggesting a MyD88-independent pathway exists. The TLR-3 ligand dsRNA induces NFκB and IRF3 activation in MyD88−/− cells, and IRF3 interacts with tank binding kinase-1 (TBK-1). This leads to robust IFN-β production, which is completely independent of MyD88 (O’Neil, 2002; O’Neil 2006; Takeda, 2004).

The TIR-TIR platform leads to recruitment of interleukin-1 receptor associated kinase-4 (IRAK-4), which interacts with MyD88 through the death domain (Figure 1). IRAK-4 then phosphorylates IRAK-1. Interestingly, deficiency of IRAK-4 causes severe impairment in response to TLR-2, -3, -4, and -9 ligands, whereas IRAK-1 deficiency only partially impairs LPS response (Suzuki, 2002; Swantek, 2000). IRAK-1 then activates TNF-receptor associated factor-6 (TRAF-6). A series ubiquitinylation reactions occurs on TRAF-6 and TGF-β activating kinase-1 (TAK-1). TAK-1 then activates the IKK complex leading to NFκB activation (O’Neil, 2006). The IKK complex is composed of IKKα and IKKβ, which phosphorylate IκB leading to its degradation. As a result the NFκB subunits are released and translocate into the nucleus to regulate gene transcription. TRAF-6
activation can also lead to activation of evolutionary conserved signaling intermediate in
toll pathways (ECSIT) and activation of MAP kinase pathways (O’Neil, 2006). Activation
of MAP kinases leads to activation of NFκB, SRE and AP-1 transcription factors. MAP
kinases are activated by other means as well, although this signaling pathway is not fully
understood (Figure 1). Signaling through TLRs can also lead to activation of
phosphoinositol kinase-3 (PI3K) resulting in Akt activation, which causes phosphorylation
of IκB and activation of NFκB (Jones, 2001). These activated transcription factors induce
transcription of immune modulating proteins, in particular TNF-α, IL-1β, IL-6, IFN-γ,
Type I IFN, IL-12 and IL-10.

Other adaptor proteins have been identified including MyD88 adaptor-like (Mal),
also known as TIRAP, TIR-related adaptor protein inducing interferon (Trif) and Trif-
related adaptor molecule (Tram). Mal plays a role in TLR-4 and TLR-2 signal
transduction, and mice deficient for Mal have a similar phenotype to MyD88-deficient
mice. Rapid induction of TNF-a production is abolished and activation of MAP kinases
and NFκB are delayed (O’Neil, 2002). Trif is recruited to TLR-3 and TLR-4 and is
responsible for interferon response factor-3/7 (IRF3/7) activation via IKK-like kinase,
TBK-1. Trif is thought to be responsible for the IFN-β production in response to TLR-3
and TLR-4 ligands that is absent with other TLRs stimulation.

Differential effects of TLR responses have been extensively studied in
macrophages. For instance, in RAW 264.7 murine macrophage cell line LPS induces
strong IL-1β production that is not seen in response to TLR-2 ligands (Jones, 2001).
Similar to that result is induction of inducible nitric oxide synthase (iNOS) gene
expression. However, TNF-α production occurs in response to both TLR-2 and TLR-4 ligands (Vogel, 2001). Similar findings are also seen with thioglycollate-elicited peritoneal macrophages (Jones, 2005). These differences may be attributed to differential activation of MAPK pathways (Jones, 2001). Studies performed using MyD88−/− mice reveal the induction of TNF-α, IL-1β, and IL-12 secretion is MyD88-dependent. The time course of cytokine production shows that TNF-α, IL-6 and IL-12 production peaks at four to six hours. In the case of TLR-4 signal transduction, cytokine production still occurs but is delayed without the presence of MyD88. Other genes, such as chemokine CXCL 10 (IP-10), IRF-1, and macrophage colony stimulating factor 1 (M-CSF), are regulated independent of MyD88 (Bjorkbacka, 2004). TLR-3 ligands induce robust IFN-b secretion, and the rapid TNF-α production seen with other TLRs does not occur (Takeda, 2004; Trincheiri, 2007).

TLRs are implicated as contributors to severity of numerous diseases that also involve increased cathepsin activity and expression. Recent human genetic studies indicate TLR polymorphisms are associated with susceptibility to several diseases (Misch, 2008). Activation of TLR on tumor cells enhances proliferation and metastasis by increasing expression of MMPs and integrins (Huang, 2007). Possibly, cathepsins are affected as well, and infiltrating immune cells appear to have the highest cathepsin expression. In atherosclerosis, TLR-2 activation of multiple cell types with either endogenous or exogenous ligands promotes atherogenesis (Tobias, 2007). TLR-2 and TLR-4 expression is increased on macrophages isolated from the joints of RA patients. Furthermore, inflammatory cytokine production in response to peptidoglycan (PGN) and LPS is
increased in cells from diseased patients (Huang, 2007). TLR regulation of cathepsins is not well elucidated. Hence, my research project investigated the impact of macrophage stimulation via different TLRs on cysteine cathepsin activities and expression.

**Cannabinoids**

The plant *Cannabis sativa*, better known as marijuana, has been used in traditional medicine for millennia to treat various ailments, including chronic inflammatory and autoimmune diseases. Delta⁹-tetrahydrocannabinol (THC), the major psychoactive component of marijuana, has immune modulating abilities in both humans and experimental animal models. THC has been used to treat ailments, including but not limited to pain, asthma, glaucoma, multiple sclerosis, AIDS and cancer (Benamar, 2006; Klein, 2005). THC along with the endogenous cannabinoids 2-arachidonylethanolamide and 2-arachidonoylglycerol (2-AG), as well as synthetic derivatives of THC are currently being investigated as possible therapeutic candidates.

Cannabinoids, including THC, alter cellular responses by immune cells, in particular macrophages. THC and its associated derivatives suppress mitogen-induced B and T lymphocyte proliferation. In addition, THC also suppresses cell-mediated immunity by decreasing cytotoxic T cell activity. Studies using sheep red blood cell plaque-forming cell assays suggest THC decreases B cell antibody responses indicating cannabinoids can affect both arms of adaptive immunity. In other model systems, cannabinoids interfere with Th1, but not Th2, cytokine production (Cabral, 2005; Klein, 2003). These latter findings have lead to the hypothesis that cannabinoids skew helper T cell responses toward humoral immunity while impairing cell-mediated immunity and inflammation. However,
this hypothesis cannot easily explain diminished IgE response caused by cannabinoid exposure in an ovalbumin-asthma mouse model (Schatz, 1997). Furthermore, cannabinoids affect multiple aspects of innate immunity. Cannabinoids impair multiple macrophage functions, including phagocytosis, antigen processing, and co-stimulatory activity; all of which are required for CD4⁺ helper T cell responses (Klein, 2006; Lopez-Cepero, 1986; McCoy, 1995; McCoy, 1999; Spector, 1991). For example, alveolar macrophages isolated from chronic marijuana users are compromised in their ability to produce pro-inflammatory cytokines, such as TNF-α and IL-6, in response to LPS stimulation (Baldwin, 1997). Cannabinoids decrease IFN-γ, IL-1β and TNF-α production by synovial monocytes in murine models of collagen-induced arthritis (Klein, 2005). Pro-inflammatory cytokines are key players in the pathogenesis of various diseases including RA. In addition, macrophage cell line RAW 264.7 treated with THC has attenuated LPS-stimulated iNOS gene expression, which is mediated by inhibited NF-κB activation (Jeon, 1996). ROS and nitric oxide are key players in macrophage-mediated pathogen killing.

THC exposure of PEC also decreases APC co-stimulatory activity, ultimately leading to a defective T-cell response and decreased IL-2 production (Chuchawankul, 2004). THC interferes with the processing of intact protein antigens by macrophages and increases aspartyl cathepsin D activity (Matvayeva, 2000; McCoy, 1995; McCoy, 1999). These findings indicate a possible affect of cannabinoids on the enzymes involved in antigen processing, which may extend to cysteine cathepsins. Cannabinoids, including THC, may mediate immune modulation through cannabinoid receptors called CB1 and CB2 (Schatz, 1997). Peripheral leukocytes have been shown to express the CB2 receptor
or both receptors. In contrast, microglial cells express either the CB1 receptor, or both receptors (Klein, 2003). Studies utilizing receptor selective agonists, antagonists and receptor deficient mice indicate the immune modulating effects of cannabinoids are primarily mediated through the CB2 receptor (Buckley, 2008; Chuchawankul, 2004; McCoy, 1999). However, other reports support the involvement of the CB1 receptor in immune suppression, particularly in models of endotoxic shock (Cabral, 2005). Recently, a homozygous CB2 receptor gene polymorphism was found to be associated with RA, multiple sclerosis and myasthenia gravis, suggesting this may be a genetic risk factor for autoimmune disease development (Sipe, 2005). The best evidence that cannabinoid receptors are important for immune regulation is derived from mice lacking both CB1 and CB2 receptors. These mice are extremely sensitive to developing delayed-type hypersensitivity and chronic inflammation (Karsak, 2007).

Cannabinoid receptors are seven-transmembrane-spanning G protein-coupled receptors. CB1 receptor, originally identified in rat cerebral cortex, is primarily expressed in the CNS (Matsuda, 1990; Thomas, 1992). CB2 receptor was originally identified in the promyelocytic leukemic cell line HL60 and is prevalent in lymphoid organs (Munro, 1993; Bouaboula, 1993). Cannabinoid receptors were identified as $G_{i/o}$ protein-coupled receptors linked to inhibition of adenylate cyclase, when studies demonstrated submicromolar THC concentrations decrease cAMP (Howlett, 1984). This finding was confirmed by studies utilizing pertussis toxin, which inhibits $G_{i/o}$ protein signaling (Howlett, 1986). The high-affinity radiolabeled ligand $[^3H]CP55940$ led to identification of a binding site in brain membranes confirming receptor expression (Howlett, 2005). However, 3 µM to 10 µM
THC can have non-receptor-mediated effects by increasing membrane fluidity, thereby influencing receptor dimerization and synapse formation (Hilliard, 1985). Non-psychoactive cannabidiol has a very low affinity for CB1 and CB2 receptors, yet has anti-inflammatory activity *in vitro* and *in vivo*, suggesting a non-receptor-mediated mechanism (Pertwee, 2005). Additional complexity to this situation may be the involvement of other receptors besides CB1 and CB2. Endogenous cannabinoids can activate the vanilloid TRPV1 receptor, and several studies also suggest there may be other cannabinoid receptors that remain unidentified. In mouse models of inflammatory or neuropathic pain palmitoylethanolamide produces antinociception by acting on a CB2-like receptor. Palmitoylethanolamide lacks affinity of the CB2 receptor however the CB2 selective antagonist SR144528 opposed the antinociceptive effects (Pertwee, 2005). Other studies using anadamide, WIN55,212-2 and cannabidiol have demonstrated their actions can be mediated through unidentified non-CB1/non-CB2 receptors particularly in endothelial cells and the CNS (Begg, 2004). While multiple studies indicate the existence of non-CB1/non-CB2 receptors their specific identities and functions remain unclear.

Since the identification of cannabinoid receptors, studies have been underway to gain insight into the cellular signaling mechanisms of cannabinoids. While CB1 and CB2 receptors share some similar signaling cascades, distinct differences in the activity of theses receptors have been identified. G proteins are heterotrimers, and upon activation the \(\alpha\) subunit dissociates from the \(\beta\gamma\) dimer. The \(G_{i/o}\) a subunit inhibits adenylate cyclase. Cannabinoid receptor-mediated decreases in cAMP cause decreased protein kinase A (PKA) activity, which can have multiple biological effects. In contrast, increased PKA
activity has also been reported via CB1 receptor and may contribute to activation of Raf and MAPK pathways (Figure 2) (Howlett, 2005). CB1 receptor-mediated increases in cAMP and PKA activity are due to CB1 association with $G_s$ proteins. Furthermore, CB1 receptor may associate with $G_q$ proteins leading to phospholipase D activation (Howlett, 2005). CB1 receptor signaling can cause increased intracellular $Ca^{++}$, which may be a result from the activation of phospholipase C (PLC) (Howlett, 2005). PLC cleaves membrane-bound PIP$_2$ to IP$_3$, and IP$_3$, in turn, opens ER $Ca^{++}$ channels, thereby increasing intracellular $Ca^{++}$. CB1 signaling affects other ion channels in neural cells as well. CB1 receptor activation of protein kinase C (PKC) and regulation of MAPK have also been reported (Rubovitch, 2004). Activation of extracellular signal-regulated kinase1 and 2 (ERK1 and ERK2) and jun N-terminal kinases 1 and 2 (JNK1 and JNK2) via p42/44 and p38 MAPK pathways has been reported as well (Figure 2). One proposed mechanism is that the G protein $\beta\gamma$ dimer provides a scaffold for proteins involved in the MAPK pathway like PI3K. PI3K activation leads to Akt activation, Akt activates downstream molecules leading to MAPK activation. This hypothesis was supported when it was demonstrated that PI3K inhibitors could attenuate cannabinoid activation of MAPK (Bouaboula, 1995).

CB2 receptor signaling is less understood. Whereas CB1 receptor may couple to $G_s$ proteins leading to increased cAMP and PKA activity, CB2 receptor does not (Demuth 2006). Likewise, CB2 receptor has not been found to associate with $G_q$ proteins. Stimulation of HL60 cells with cannabinoid agonists does not lead to Akt activation (Howlett, 2005). However, stimulation of rat microglial cells with 2-AG leads to MAPK activation, and PKC-dependent activation of MAPK has been reported (Carrier,
Figure 2. Generalized cannabinoid receptor signaling cascades.

Upon agonists binding to the receptor the G protein subunits disassociate. The α subunit interacts with adenyl cyclase inhibiting its activity leading to a decrease in cAMP levels and PKA activity. The βγ subunit can activate PI3K and MKKs. This, in turn leads to the activation of Akt, p38 MAPK, p42/p44 MAPK and ultimately the activation of JNK1/2 and ERK1/2. PI3K can also activate phospholipase in the plasma membrane, which leads to the formation of IP₃ thereby increasing intracellular Ca²⁺ levels.
THC activates the PI3K/Akt pathway in epithelial cells, which leads to Raf-1 mediated activation of p42/p44 MAPK (Demuth, 2006). However, opposite effects with high WIN 55,212-2 concentrations have been reported, whereby inhibition of p42/p44 MAPK activation occurs in murine splenocytes (Faubert, 2003). The discrepancies in the literature may due to differences in cannabinoid concentrations or cell types with differential cannabinoid receptor expression. MAPK regulation by cannabinoid receptors is not well elucidated but is thought to be involved in the immune modulating effects seen with cannabinoids.

Multiple synthetic derivatives have been developed as possible therapeutics. THC is a partial receptor agonist, whereas CP55940 is a full agonist and exerts potent biological effects. There are also receptor selective agonists, such as JWH-015, which is selective for CB2 (Klein, 2005). CB2 receptor selective agonists are appealing for therapeutic use, because they avoid the psychoactive effects accompanied by CB1 activation. Respective CB1 and CB2 selective antagonists SR141716A and SR144528, inhibit agonist biological effects. Currently SR141716A, also known as Rimonabant, is used in Canada to treat obesity and is in clinical trial in the U.S. Other THC-derived drugs such as nabilone (Cesamet®) and dronabinol (Marinol®) are currently used in Canada and the U.S. as antiemetic to treat nausea and vomiting associated with chemotherapy (Benamar 2006). They are also used as appetite stimulants for treatment of anorexia and HIV-associated weight loss. In Canada Marinol® and Cesamet® are used to treat chronic inflammatory
diseases such as multiple sclerosis and are currently in clinical trials in the U.S. and parts of Europe (Benamar, 2006).

The immune modulating capabilities of cannabinoids make them a potentially attractive anti-inflammatory drugs to treat a multitude of diseases. Studies utilizing animal models of multiple sclerosis, experimental autoimmune encephalomyelitis, and collagen-induced arthritis suggest cannabinoids are an effective treatment for a variety of illnesses (Ramirez, 2005; Sumariwalla, 2004; Wirguin, 1994). Cannabinoids affect a broad spectrum of immune responses, thereby attacking multiple mechanisms involved in chronic inflammatory diseases. Studies utilizing LPS as a model for inflammation show cannabinoids decrease pro-inflammatory cytokine responses and may enhance anti-inflammatory cytokine production (Cabral, 2005; Benamar, 2007). Cytokines suppressed by cannabinoids include those known to regulate cysteine proteases. Cannabinoids inhibit tumor angiogenesis by decreasing MMP activity (Blazquez, 2003; 2008). MMP and cathepsins are both upregulated in a multitude of cancers and chronic inflammatory diseases (Keyszer, 1998). As mentioned before, macrophages are often the primary producers of cytokines and proteases during inflammatory responses. These studies combined support the need for investigation on cannabinoid modulation of cysteine cathepsins in macrophage populations during inflammation, which nothing was known when this research project began.

**Rationale and Objectives**

Increased cathepsin expression and activity are associated with rheumatoid arthritis, multiple sclerosis, atherosclerosis, a multitude of cancers, and other diseases. The
mechanisms resulting in increased cathepsin activities during these disease processes remain poorly understood. Infiltrating immune cells producing inflammatory mediators and cathepsins are thought to be the major cause of tissue destruction in numerous inflammatory diseases. Among the infiltrating immune cell types, cathepsin expression is highest within GR-1⁺/Mac-1⁺ macrophages. Therefore, this study focuses on understanding the regulation of cysteine cathepsins in macrophages.

The objective of this study is to elucidate the regulation of cathepsins by TLR and to investigate the use of cannabinoids as pharmacological therapeutics to alter cathepsins during an inflammatory response. Signaling through TLR causes cell activation and release of inflammatory mediators as important defenses against pathogens. Very few studies have examined the impact of TLR activation on cysteine cathepsins. TLR signaling could increase cathepsin gene expression, enzymatic activity or decrease gene expression of endogenous inhibitors. TLR4 signal transduction involves pathways that are dependent and independent of the MyD88 adaptor molecule. Differential cellular responses are obtained depending on the signaling pathway activated. Therefore, this study examines TLR ligands, which are MyD88-dependent and or independent. This study also investigates the role of inflammatory cytokines produced in response to TLR ligands in cathepsin regulation.

LPS is the most extensively studied TLR ligand and is a classic model for inflammation. Cannabinoids modulate a variety of immune responses, including the LPS response. Little is known about cannabinoids’ impact on macrophage responses to other TLR ligands. The drugs suppress production of inflammatory mediators, including
chemokines and cytokines. Cannabinoids may mediate their effects on the immune system through receptor-dependent or -independent mechanisms. Cannabinoid receptor subtype 1 (CB1) and receptor subtype 2 (CB2) have differential expression in immune cells with the CB2 receptor being more prevalent. This study investigates the use of cannabinoids to alter cathepsin activity during the inflammatory response and the involvement of cannabinoid receptors.

These studies combined will address multiple questions pertaining to the regulation of cysteine cathepsins. Does macrophage activation by MyD88-dependent and -independent TLR ligands affect cysteine cathepsins? Are the changes in cathepsins at the level of gene expression or enzymatic activity? Are the endogenous inhibitors, cystatins, affected by cell stimulation with TLR ligands? Are the changes in cathepsins a direct effect due to TLR signaling cascades and cell activation or indirect effect due to pro-inflammatory cytokines? Can cannabinoids alter cathepsins during the inflammatory response? Is this action cannabinoid receptor mediated? The answers to these questions will provide insight to the regulation of cathepsins during infection and characterize the mechanisms involved. Furthermore, the studies focusing on cannabinoids may lead to the use of cannabinoid receptor selective agonists as therapeutic agents for chronic inflammatory diseases to prevent cathepsin involvement in pathological tissue destruction.
Chapter 2: Materials and Methods

Mice

Female C3D2F1/J (The Jackson Laboratory, Bar Harbor, ME) were used from 9 to 21 weeks of age. Mice were housed under specific pathogen-free conditions. Research complied with all relevant laws, guidelines, and policies. Protocols were approved by IACUC at VCU.

Cell Lines

Culture medium for murine macrophage J774 cell line (American Type Culture Collection (ATCC), Manassas, VA) was previously described (Harrison, 2003). Murine macrophage cell line P388D1 (ATTCC) was grown in RPMI 1640 containing 10% heat-inactivated fetal bovine serum (FBS) (Biofluids, Rockville, MD) and antibiotics. A macrophage cell line was generated from bone marrow cells of cannabinoid receptor type 2 deficient (CB2−/−) mice by J2 retroviral infection (Blasi, 1989). CB2−/− cells were cultured in DMEM containing 10% FBS and antibiotics. Macrophage/microglial cell line EOC 20 derived from C3H/HeJ mice (ATCC) were grown in DMEM containing 10% FBS, antibiotics and 10% LADMAC (ATCC) conditioned medium as a source of CSF-1.

Monoclonal and Polyclonal Antibodies
Cell-free culture supernatants from monoclonal antibody-producing rat-mouse fusion B cell hybridomas (ATCC) produced anti-B220 (clone RA3-3A1), and anti-Thy1.2 (clone J1j.10), anti-CD8 (clone 53-6.72) and anti-FcgII/III receptor (clone 2.4G2). Monoclonal anti-CD11b (Mac-1; clone M1/70), anti-HSA (clone J11d), anti-B7-1 (clone 1G10), anti-B7-2 (clone GL1), anti-CD11c (clone HL3), anti-ICAM-1 (3E2), fluoresceinated anti-I-E^k^ (clone 17-3-3S), anti-CD14 (clone rmC5-3) and fluoresceinated anti-rat Ig kappa light chain (clone Mrk-1) antibodies were purchased from PharMingen (San Diego, CA). Monoclonal anti-ICAM-1 (clone KAT-1), anti-CD48 (clone MRC OX-78) and fluoresceinated anti-I-A^b^ (clone MRC OX-3) were purchased from Serotec USA (Washington, DC). Monoclonal anti-TLR4/MD2 (clone MTS510) and anti-TNF-α (clone TN3-19.12) were purchased from e-Biosciences (San Diego, CA). Monoclonal rat anti-mouse IL-1β (clone 30311.11) was purchased from R&D Systems (Minneapolis, MN). FITC anti-mouse MHC Class I H-2K^k^ (clone 36-7-5) was purchased from Biolegend (San Diego, CA). Mouse IgG technical grade was purchased from Sigma Aldrich Chemical Co. (St. Louis, MO). Rabbit complement and monoclonal anti-Dec 205 (clone NLDC-145) antibody were purchased from Cedarlane (Ontario, CA). Polyclonal fluoresceinated anti-hamster IgG antibodies and MOPC-104E, were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA) and Cappel Organon Teknika (Durham, NC), respectively.

**Splenocyte Preparation**

Spleens were harvested from C3D2F1/J mice and homogenized by hand into a single-cell suspension. Erythrocytes were removed by hypotonic lysis in Tris-buffered
ammonium chloride. Nucleated cells were prepared for cathepsin assays and immunofluorescence staining. For some experiments, splenic macrophages and B cells were enriched by Ab- and complement-mediated cytolysis as described (Gondre-Lewis, 2003; Hartmann, 2005). Briefly, macrophages were enriched by 30 min incubation of cells on ice with saturating amounts of anti-B220 and anti-Thy1.2 antibodies followed by 45 min incubation at 37°C with low toxicity rabbit complement. The enriched cells were cultured for 2 h at 37°C in complete medium, non-adherent cells were removed and adherent cells were considered the splenic macrophage population. For B cell enrichment splenocytes were incubated on ice with anti-Thy1.2 for 30 min followed by addition of rabbit complement as above. The enriched cells were cultured for 2 h 37°C in complete medium, and non-adherent cells were collected as the B cell population.

**Cathepsin Substrates and Inhibitors**

Respective Magic Red™ (MR) substrates (Immunochemistry Technologies, LLC, Bloomington, MN) for Cat B, L and S were Z-Arg-Arg-MR-Arg-Arg, Z-Phe-Arg-MR-Arg-Phe, and Z-Val-Val-Arg-MR-Arg, respectively. General cysteine cathepsin inhibitor was E-64d (Peptide International, Inc., Louisville, KY), and selective inhibitors for Cat L and S were Z-Phe-Phe-CHN₂ and Z-Val-Val-Nle-CHN₂, respectively (Bachem Bioscience, Inc, King of Prussia, PA). Respective 7-aminomethyl-coumarin (AMC) substrates (Bachem Bioscience) for Cat B, L and S were Z-Arg-Arg-AMC, Z-Phe-Arg-AMC, and Z-Val-Val-Arg-AMC. Stock solutions of substrates and inhibitors in DMSO were stored at -80°C.

**Confocal Microscopy**
Macrophages at 5 x 10⁵ were plated in Lab-Tek chambered coverglass plates (Nunc Inc. Naperville, IL) in phenol red-free complete DMEM. Cells were incubated with 500 nM LysoTracker DND-26, an acidic vesicle marker, (Molecular Probes, Eugene, OR) for 1 h at 37°C followed by addition of 1 µM Cat L MR-conjugated substrate for 15 min. Intracellular red fluorescent hydrolyzed product from Cat L substrate and green LysoTracker DND-26 were detected with a Zeiss LSM 510 Meta confocal microscope (VCU Imaging and Flow Cytometry Core Facility) using dual excitation wavelengths of 488 and 543 nm.

**Cathepsin Enzymatic Activity in Live Cells Using Magic Red Substrates**

Spleen cell suspensions with erythrocyte hypotonic lysis were prepared or J774 macrophage cell line was utilized. For initial assays, cells at 4 x 10⁶ cells/ml in PBS, or RPMI 1640 containing 5% heat-inactivated FBS, L-glutamine, 2-ME and antibiotics were incubated with or without various substrate concentrations for 1 h at 37°C in 5% CO₂-humidified atmosphere. Substrate concentrations for subsequent experiments were 6 µM for Cat B and Cat L and 24 µM for Cat S. For time course experiments, cells were incubated with substrate from 15 min to 3 h, and a 45-min incubation was selected for all subsequent assays. Cells were washed twice with PBS containing 0.02% sodium azide to remove excess substrate, and in some experiments, cells were fixed with 0.5% paraformaldehyde in PBS before analysis. Red fluorescence intensity of cells was measured with logarithmic amplification using a Becton Dickinson FACScan equipped with a 15 mW 488 nm argon laser and appropriate excitation filters (BD Biosciences, San
Jose, CA). Data on 20,000 splenocytes and macrophage cell lines were collected, and forward-angle side scatter gates were set to exclude dead cells and cell clumps. Compensations were set to remove green emission due to uncleaved substrate.

**Substrate in Stimulated Cells Not Limiting**

Macrophages at 2 x 10^6 cells/well were cultured in complete medium in 6-well culture dishes with or without 10 µg/ml LPS for 48 h. Cells were harvested, washed twice in PBS and cathepsin activities were assessed using MR-conjugated cathepsin substrates as described above or cell lysate assays were performed as described below.

**Protease Activity in Cell Lysates**

Cathepsin activities in cell lysates were measured as described with modifications (9,10). Lysates were prepared in 0.75% Triton X-100 lysis buffer at 1 x 10^8 cells/ml. Protein concentration in the lysates was measured by bicinchoninic acid assay. Cat B and L were activated by incubation at 37°C for 15 min before substrate addition. Cat S activation time was 1 h at 37°C. Reactions contained 6.25 µg protein for Cat B and L, and 25 µg protein for Cat S assay. Activation buffer for Cat B was 87.7 mM KH₂PO₄/12.3 mM NaHPO₄ containing 4 mM EDTA, pH 6 and 2.6 mM dithiothreitol (DTT). Cat B activity was measured by cleavage of 5 µM AMC selective Cat B substrate for 2 h. Activation buffer for Cat L was 340 mM Na acetate/60 mM acetic acid containing 4 mM EDTA, pH 5.5 and 1.3 mM DTT. Cat L activity was assessed by cleavage of 6.6 µM AMC Cat L selective substrate for 1 h. Activation buffer for Cat S was 0.1 mM KH₂PO₄ containing 5mM EDTA, pH 7.5 and 5 mM DTT. Cat S activity was measured by cleavage of 10 µM AMC selective Cat S substrate for 3 h. Reactions were stopped by 1mM
iodoacetamide (Sigma). Background controls were reactions lacking cell lysate.

Fluorescence was measured by a Shimadzu spectrofluorophotometer RF5000 (Columbia, MD) with an excitation wavelength of 370 nm and emission wavelength of 460 nm. One activity unit is 1.0 fluorescence unit per cell.

**MR Substrate Specificity**

For specificity experiments, spleen cells at 4 x 10^6/ml were pre-incubated with 0.45 mM E-64d for 3 h, 0.14 mM Z-Phe-Phe-CHN_2 or 0.95 mM Z-Val-Val-Nle-CHN_2 for 30 min before addition of substrate. Control cells were incubated with the corresponding DMSO concentration, which was 2.3% for E-64d and 3.5% for selective inhibitor assays. To detect viable cells, 10 nM carboxyfluorescein diacetate, acetoxymethyl ester (5-CFDA, AM), a cell viability marker, (Molecular Probes) in DMSO/PBS was added at the time of substrate addition. Substrate concentrations were increased to 8.3 µM for Cat B and 10 µM for Cat L to enhance detection sensitivity, when substrates were used in conjunction with 5-CFDA,AM. Green and red fluorescence intensities of cells were measured with logarithmic amplification using a Becton Dickinson FACScan as described above. For assays with inhibitors, two-color flow cytometry (FCM) was performed with compensations to eliminate emission spectral overlap of 5-CFDA, AM and cresyl violet. Compensation settings on the FACScan were determined by analyzing single-stained cells. Histograms represent red fluorescence intensity of green 5-CFDA, AM^+ viable cells.

**Cathepsin Activity in Live Cells Using AMC Substrates**

For initial experiments, macrophages at 4 x 10^6 cells/ml in PBS were incubated with or without a cathepsin inhibitor under conditions described above. Cells were washed
twice in PBS to remove excess inhibitor and resuspended in PBS and incubated with 10 
µM of AMC selective Cat B, L or S substrate for 1 h at 37°C. Fluorescence was measured 
by a spectrofluorophotometer as described above. One activity unit is 1.0 fluorescence unit 
per cell. Background controls were cells incubated with the corresponding DMSO 
concentration but without a cathepsin substrate. Percent inhibition was calculated as [1- 
(fluorescence units without inhibitor – fluorescence units with inhibitor) / fluorescence 
units with inhibitor] x 100%. For subsequent experiments utilizing AMC-conjugated 
substrates with live cells at 1 x 10^6 cells/ml were incubated in PBS with 10 µM of AMC 
selective Cat B, L or S substrate for 1 h at 37°C. As above fluorescence was measured by a 
spectrofluorophotometer. Fold increase values were calculated by dividing fluorescent 
values of treated cells by values for medium control cells.

**Cathepsin Assays Combined with Immunofluorescence Staining**

Single cell suspensions of splenocytes were prepared as described above. In some 
cases, macrophages and B cells were enriched using Ab- and complement-mediated 
cytolysis as described above. Spleen cells were incubated with substrate and washed. Cells 
were then incubated with 1 µg of monoclonal anti-B220, anti-Thy-1.2, or 3 µg of anti-
Mac-1 Ab along with 25 µg of mouse of IgG to block Fc receptors for 30 min at 4°C, and 
washed in PBS containing 0.1% BSA and 0.02% sodium azide. Cells were then incubated 
with 0.5 µg of fluoresceinated monoclonal anti-rat kappa Ab for 30 min at 4°C, washed 
twice in PBS containing 0.02% sodium azide and fixed in 0.5% paraformaldehyde. 
Fluorescence intensity with logarithmic amplification was measured by a Becton 
Dickinson FACScan as described above. Fluorescence intensity of cells for two-color
analysis was measured by FCM, and compensations were set to eliminate emission spectral
overlap of fluorescein and cresyl violet. Analysis of cathepsin activity in splenocyte
subpopulations was performed with gating on Mac1⁺, B220⁺, and Thy1.2⁺ cells.

**TLR Ligands**

Bacterial lipopolysaccharide (LPS) from *E. coli* 055:B5, peptidoglycan (PGN) from
*S. aureus*, and polynosinic-polycytidylic acid sodium salt (Poly I:C) were purchased from
Sigma-Aldrich (St. Louis, MO). Formalin-killed *Corynebacterium parvum* (*C. parvum*)
was purchased from Biotechnology Limited (Beckenham, England).

**TLR Stimulation**

For initial experiments, macrophage cell lines at 2 x 10⁶ cells/well were cultured in
complete medium with various doses of LPS, PGN, Poly I:C or *C. parvum* at 37°C for time
points ranging from 12 to 48 h. Cells were harvested, washed in PBS and protease activity
of cathepsins was assessed using FCM or spectrofluorophotometry as described above.
For subsequent experiments, 2 x 10⁶ P388D1, EOC 20 or CB2⁻/⁻ cells were incubated
without or with 1 µg/ml LPS, 4 µg/ml PGN, 10 µg/ml Poly I:C, *C. parvum* or complete
medium for 6, 24 or 48 h as indicated. Cells were harvested and washed twice in PBS, and
cell viability was assessed by trypan blue exclusion. Live cell cathepsin assays using MR-
or AMC-conjugated substrates were performed as described above. Cell-free culture
supernatants were collected or RNA was isolated as described below.

**ImageStream® Cell Analysis**

Cells were incubated without or with 1 µg/ml LPS for 24 h and harvested as above.
Cells at 1 x 10⁶ cells/ml in PBS were incubated with 6 µM Cat L or B substrate or 15µM
Cat S substrate for 45 min. Cells were then incubated with 100 nM LysoTracker DND-26 for 15 min and analyzed using ImageStream® Cell Analysis System (Amnis Corporation, Seattle, WA) courtesy of Amnis Corporation and University of Virginia, School of Medicine FCM Core Facility. Analysis and similarity bright detail scores were performed in collaboration with Dr. Philip Morrissey at Amnis Corporation, as previously described (Beuma, 2006). Similarity bright detail is based on identification of the small puntate staining in a pair of images. The value is based on how well fluorescence due to LysoTracker DND-26 in channel 3 correlates with MR fluorescence in channel 5.

**Cathepsin Secretion**

CB2−/− macrophages at 4 x 10^6 cells per well were cultured with and without LPS or 10 mM NH₄Cl/mannose-6-phosphate in phenol red-free DMEM containing 10% FBS and supplements at 37°C for 24 h. Cell-free culture supernatants were collected and centrifuged through Centricon-30 microconcentrators (Amicon, Beverly, MA) at 2000g for 30 min. Cell number and viability for each treatment was assessed using trypan blue exclusion. Protein concentration in concentrated supernatants was measured by bicinchoninic acid assay. Cathepsin activity in culture supernatants was measured according to protocols for cellular lysates as described above. Cathepsin activities were measured by cleavage of 5 μM selective Cat B AMC-substrate for 2 h, 6.6 μM Cat L selective AMC-substrate for 1 h, and 10 μM selective Cat S AMC-substrate for 3 h. Background controls were reactions containing phenol red-free DMEM containing 10% FBS. Fluorescence was measured by a spectrofluorophotometer RF5000 as above. Data
values are expressed as secreted activity per cell, which was calculated as fluorescence units/total cell number per culture.

**Semiquantitative Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)**

RNA was isolated using Trizol reagent (Invitrogen). Cat B primers (forward primer: 5′-TGC TTA CTT GCT GTG GCA TC-3′; reverse primer: 5′-GGG AGT AGC CAG CTT CAC AG-3′), Cat L primers (forward primer: 5′- CCC CAA GTC TGT GGA CTG GAG AGA-3′; reverse primer: 5′-TTT ACA AGA TCC GTC CTT TGC TTC-3′), Cat S primers (forward primer: 5′-TAA TCG GAC ATT GCC TGA CA-3′; reverse primer: 5′-CTG GAA AGC TTC GGT CAT GT-3′), cystatin B primers (forward primer: 5′-GTC CCA GCT TGA ATC GAA AG-3′; reverse primer: 5′-GGG TCA AA GCT TGT TTT CA-3′), cystatin C primers (forward primer: 5′-TCG CTG TGA GCG AGT ACA AC-3′; reverse primer: 5′-ATC TGG AAG GAG CAG AGT GC-3′), CB1 receptor primers (forward primer: 5′-GCT TGC GAT CAT GGT GTA TG-3′; reverse primer: 5′-CAT GCT GGC TGT GTT ATT GG-3′), CB2 receptor primers (forward primer: 5′- ATG TAC CCA CCT TGG CTG AG-3′; reverse primer: 5′- ACC TTG GGC CTT CTT CTTC-3′), β-actin primers (forward primer: 5′-ATC TAG AGG GCT ATG CTC TCC-3′; reverse primers: 5′-TCT GCA TCC TGT CAG CAA TGC C-3′) were synthesized and purified by the Virginia Commonwealth University DNA core laboratory (Richmond, VA). Cat B, L and S primers produce a 237-bp product from bases 447-683, a 294-bp product from bases 410-704, and a 241-bp product from bases 357-597 respectively. Cystatin B primers produce a 174-bp product from bases 74-244 and cystatin C primers produce a 217-bp
product from bases 146-362. CB1 receptor, CB2 receptor and β-actin primers produce a 250-bp product from bases 1079-1328, a 355-bp product from bases 655-1009, and a 451-bp product from bases 415-865 respectively. RNA at 1µg was treated with amplification grade DNase I (Invitrogen, Carlsbad, CA) for 20 min at room temperature to remove genomic DNA. Yield was determined by absorbance at 260 nm, and purity was verified by 260:280 and 260:230 nm absorbance ratios. RNA was reversed transcribed with Superscript™ first strand synthesis kit (Invitrogen) using oligo-dT primers according to manufacturer’s directions. Controls omitted reverse transcriptase to verify that genomic DNA was not amplified by PCR. Products were amplified by PCR using Platinum Taq DNA polymerase (Invitrogen). Following initial denaturizing at 94°C for 2 min, samples were denatured at 94°C for 30 s, annealed at 55°C for 30 s and extended at 72°C for 30 s. Each cDNA was amplified separately up to 44 cycles. PCR products were separated by agarose gel electrophoresis. Gels were stained with ethidium bromide, and bands were visualized by a UV transilluminator with a CCD camera. Band intensities were determined by AlphaEase FluorChem 8900 (Alpha Innotech Corporation, San Leandro, CA). Relative expression for each sample was calculated by dividing the intensity of the target band by the β-actin band intensity.

Co-culture Experiments

Co-cultures were 1 x 10^6 P388D1 and 1 x 10^6 EOC 20 cells in complete RPMI with or without 1 µg/ml LPS at 37°C for 24 h. Single cell type control cultures had 2 x 10^6 cells per well in medium with or without 1 µg/ml LPS. Cells were harvested and washed in PBS, and flow cytometric cathepsin assays and immunofluorescence staining were
performed as described above. Cells were incubated with 25 µg of mouse IgG to block Fc receptors and 0.25 µg of FITC-conjugated anti-H-2K<sup>k</sup> antibody to detect EOC 20 cells. Fluorescence intensity was measured by a Becton Dickinson FACScan as described above. Red fluorescence intensities of EOC 20 cells in medium control and LPS-stimulated co-cultures were compared with gating on H-2K<sup>k+</sup> cells. Cells negative for H-2K<sup>k</sup> were analyzed for red fluorescence to measure cathepsin activity in the P388D1 macrophage population.

**Activation of Non-responsive Bystander Cells with Culture Supernatants**

P388D1 cells at 2 x 10<sup>6</sup> cells per well were cultured in complete RPMI without or with 1µg/ml LPS, and cell-free culture supernatants from medium control and LPS-stimulated cultures were collected at 6 or 24 h. Cell-free culture supernatants were concentrated with Centricon-10 microconcentrators (Amicon) and filter sterilized using 0.2 µm low protein binding syringe filters. EOC 20 cells at 2 x 10<sup>6</sup> cells per well were cultured in 2.5 ml of conditioned DMEM and 0.5 ml of concentrated culture supernatants at 37°C for 24 h. Cells were harvested and washed, and cathepsin activities in EOC 20 cells were assessed using AMC-conjugated substrates as described above. Fold increase was calculated by dividing fluorescence values for LPS-stimulated culture supernatants by the values for medium control supernatants.

**Cytokine Neutralization Studies**

As above, P388D1 cells were stimulated with 1µg/ml LPS, and cell-free culture supernatants from medium control and LPS-stimulated cultures were collected at 6 h. Cell-free culture supernatants were concentrated and filter sterilized as described above.
Concentrated culture supernatants were then incubated on ice for 1 h with 0.75 to 3 µg/ml anti-TNF-α, 5 to 20 µg/ml anti-IL-1β mAb, or 3 µg/ml or 20 µg/ml of mouse IgG as corresponding controls. EOC 20 cells at 2 x 10⁶ cells per well were cultured in 2 ml of conditioned DMEM and 1 ml of antibody-treated culture supernatants at 37°C for 24 h. EOC 20 cells were harvested, washed and assayed for cathepsin activity in live cells using AMC-conjugated substrates as described above.

**Cannabinoid Receptor Expression**

LPS stimulation of P388D1 or CB2⁻/⁻ macrophages was performed as described above. Cells were harvested and washed, and RNA was isolated using Trizol reagent (Invitrogen). Semiquantitative RT-PCR was performed as described above.

**Cannabinoid Treatment of Cells**

THC and CP-55,940 (VCU Center for Drug Abuse Research) were prepared in ethanol as described (McCoy, 1995). Vehicle was 0.1% ethanol as in final drug preparations. P388D1 or CB2⁻/⁻ cells at 2 x 10⁶ cells per well were pre-incubated with THC, CP-55,940 or vehicle for 4-h and then stimulated with 1 µg/ml LPS for another 24 or 48 h. Cells were harvested and washed, and viability was determined by trypan exclusion. Cathepsin activities were assessed using AMC-conjugated substrates as described before.

**Characterization of CB2⁺/⁻ Macrophages**

Immunofluorescence staining of CB2⁻/⁻ BM cells was performed by Constance Hartmann and Mika Shima (VCU). CB2⁻/⁻ BM cells were stained as described (Clements et al., 1996). Briefly, cells were co-incubated with 25 mg of mouse IgG to block Fcγ receptors. Cells were incubated with a saturating amount of monoclonal antibody followed
by the appropriate fluoresceinated secondary antibody. Controls for nonspecific fluorescence were cells incubated with irrelevant species- and isotype-matched antibody (MOPC 104E, 53-6.72 or hamster IgG), followed by the appropriate fluoresceinated secondary antibody. For MHC class II expression, cells were incubated with fluoresceinated MRC OX-3 or irrelevant 17-3-3S. Fluorescence intensity was measured as described above.

**Apoptosis Studies**

P388D1 macrophages at 2 x 10⁶ cells per well in complete medium were incubated with vehicle or THC at 37°C for 18 h. Apoptotic cells were detected by annexin V binding to phosphatidylserine using an annexin V-FITC apoptosis detection kit (BD Pharmingen, San Diego, CA). Harvested cells were washed, and 5 x 10⁵ cells were resuspended in 200 µl of binding buffer followed by addition of 0.25 µg of FITC annexin-V. Cells were incubated at room temperature for 15 min. Excess FITC annexin-V was removed by centrifugation, and cells were resuspended in 800 µl of binding buffer and analyzed by FACSCAN as described above. Propidium iodide was added to cell samples immediately before flow cytometric analysis. These studies were performed by Constance Hartmann.

**TLR Cell Surface Expression**

P388D1 macrophages at 2 x 10⁶ cells per well were cultured in complete medium with vehicle or THC for 4 h. Cells were harvested, washed and incubated on ice for 30 min with 3 µg FITC-conjugated anti-TLR-4/MD2, 2µg FITC-conjugated anti-CD14, or controls were incubated with 0.5 µg of fluoresceinated monoclonal anti-rat kappa Ab. Cells were washed twice in PBS containing 0.02% sodium azide and fixed in 0.5%
paraformaldehyde. Fluorescence intensity with logarithmic amplification was measured by a Becton Dickinson FACScan as described above.

**Statistical Analyses**

Parametric analysis of variance was performed by two-tailed Student’s *t* test. TLR ligand-treated or inhibitor-treated groups were compared with medium or DMSO controls, and *P* values < 0.05 were considered significant. Mean fluorescence intensities (MFI) of cell subpopulations gated by cell surface marker expression were compared, and *P* values < 0.05 were considered significant. Comparisons between THC experimental samples and controls were made by Dunnett’s *t* test.
Chapter 3: New Assay Staining to Measure Cysteine Cathepsin Activity in Live Cells

Introduction

During the past few years, cysteine cathepsins are being proposed as candidate disease markers. Increased levels of Cat B, L and S are associated with various inflammatory myopathies and cancers therefore this study focused on these three proteases (Berdowska, 2004). This correlation has led to the possibility that cathepsins could serve as useful diagnostic and prognostic markers of disease. In previous studies, reverse transcriptase-PCR (RT-PCR) or Western blot analysis are utilized to assess mRNA and protein expression of cathepsins. RT-PCR determines a change in gene expression but does not indicate the amount of translated cathepsin. Western blot analysis performed with cell lysates requires a high number of cells and is a time consuming technique. Although these methods are good indicators of expression, they do not render any information about enzymatic activity. Enzyme-linked immunosorbent assay (ELISA) technology measures the amount of cathepsins in serum or extracellular fluid. Antibodies typically used for this technique and western blot analysis recognize both precursor and mature forms of cathepsins. Procathepsins lack enzymatic activity (Berdowska, 2004; Turk, 2001), and their presence in serum or extracellular fluid may be irrelevant. Secreted mature cathepsins may be bound by
endogenous inhibitors, which also cannot be distinguished using this method. Lysate enzymatic assays are typically employed to measure cathepsin activity through the use of selective substrates attached to a fluorogenic molecule. These assays require an activation step (Barrett, 1981), which could drive procathepsin to mature cathepsin (Berdowska, 2004; Turk, 2001), and, therefore, may not be accurate of what is present in live cells. A recent approach uses activity-based probes for determination of active intracellular cathepsins in intact cells (Falgueyret, 2004; Lennon-Deménil, 2002). The fluorescent or radiolabeled probes bind active sites of the enzymes, and their binding affinity is then measured in cell lysates, which requires a high number of cells. Each of these techniques has advantages and disadvantages. All these methods measure a parameter as an average of a cell population and require cell purification to evaluate individual cell types within a heterogeneous cell population.

To be a useful disease marker, clinical diagnostic and prognostic tests necessitate sensitive, accurate and reliable assays with high-throughput capacity. This study investigated a novel method for measuring cathepsin activity on a per cell basis inside cells that have been immunofluorescence stained for cell surface molecules. Enzymatic activity of cathepsins in intact cells was assessed through FCM using selective fluorogenic peptide substrates. The fluorophore cresyl violet is bi-substituted with peptide sequence selective for a particular cathepsin (Boonacker, 2001). When enzymatic cleavage occurs at one or both sites, a peptide is released, and red fluorescence can be measured (Boonacker, 2001). These substrates have been mainly used for confocal microscopy with live cells due to the fluorophore’s emission spectral
properties (Figure 3) (Boonacker, 2001; Du, 1998). This new approach utilizes these substrates for single argon laser flow cytometers. Cathepsin activities were measured on a per cell basis in murine macrophage J774 cell line and spleen cells. Selectivity of substrate cleavage was confirmed with cysteine cathepsin inhibitors. The activity assay was combined with immunofluorescence staining for cell surface molecules to assess cathepsin activities in a heterogeneous leukocyte population. Thus, this novel assay would be an important advance for assessing cathepsin activities in cells from patients with related diseases and disorders.

Results

Titration of Cathepsin Substrates

Cresyl violet-conjugated peptide substrates are cell permeable and very quickly enter live cells (Falgueyret, 2004). Cat B selectively cleaves the Z-Arg-Arg substrate under acidic conditions (Barrett, 1981), whereas Z-Phe-Arg and Z-Val-Val-Arg substrates are preferentially cleaved by Cat L and S, respectively (Barrett, 1981; Kinschke, 1994). First, the substrates were utilized for confocal microscopy (Figure 3). Red fluorescent product generated from the Cat L substrate localized within vesicular structures as indicated by punctate cytoplasmic fluorescence. These cytoplasmic vesicles were acidic based on co-localization with a green fluorescent acidotropic probe. Similar results were observed with Cat B and S substrates (data not shown).

I examined the usefulness of these substrates for measuring proteolytic activity inside cells by FCM. When excited at 488 nm, cresyl violet-conjugated substrates emit fluorescence with maximal emission in the green FL-1 channel with minimal emission
**Figure 3.** Localization of Cat L activity inside a macrophage cell line.

Top left panel shows differential interference contrast (DIC) of labeled cells. Intracellular red fluorescent hydrolyzed product from Cat L substrate and green LysoTracker DND-26 were detected with a Zeiss LSM 510 Meta confocal microscope using dual excitation wavelengths of 488 and 543 nm. Co-localization is indicated by yellow fluorescence.
in the red FL-3 channel (Falgueyret, 2004). Once the peptide is removed, fluorescence shifts to longer wavelengths, and the highly fluorescent leaving group has maximal emission in the red FL-3 channel (Falgueyret, 2004). Compensations were set to remove signals in the green channel due to uncleaved substrate as described in Chapter 2. Analysis of cathepsin activity in a macrophage cell line performed with and without compensation revealed that the compensation settings did not alter the red fluorescence of the product (data not shown). Optimal substrate concentrations were determined by serial dilutions of each substrate for both murine spleen cells and macrophage cell line. Assays were performed in PBS and medium, and mean fluorescence intensities (MFI) were compared. Cells incubated with substrates in medium had slightly lower fluorescence intensity compared to those in PBS (data not shown), although this was not significantly different. Cat S substrate concentrations ranged from 6 µM to 36 µM (Figure 4A), and fluorescence profiles displayed unimodal distribution. Red fluorescence intensity, which measured product formation, shifted to higher values with increasing substrate concentrations, and maximal signal was achieved at 24 µM. In contrast, green fluorescence intensity from the uncleaved substrate did not change (data not shown). Cat B activity was assayed using substrate concentrations from 0.4 µM to 40 µM, and 6 µM was selected for subsequent experiments (data not shown). Cat L activity was assessed in spleen cells with substrate concentrations ranging from 1.8 µM to 36 µM (data not shown) and in macrophage cell line J774 with substrate concentrations ranging from 1.8 µM to 6 µM (Figure 4B). Cells incubated in the absence of substrate had a MFI of 3.8, and, again, fluorescence intensity increased with higher substrate concentrations. Cat L substrate at 6 µM was used in
Figure 4. Optimal cathepsin substrate concentrations and time course of product formation.

Cells were incubated with the indicated substrate concentrations at 37°C for 1 h, washed, and red fluorescence intensity (FL3-H) was measured by FCM. Counts are number of cells per channel. (A) Cat S activity in murine spleen cells. (B) Cat L activity in J774 macrophage cell line. Negative denotes cells incubated without substrate. (C and D) Spleen cells were incubated with 6 µM Cat L substrate at 37°C for the indicated time periods, washed, and analyzed by FCM. Histograms are representative of 3 separate experiments. Reproduced with copyright permission.
subsequent assays.

**Time Course of Product Formation and Evaluation of Product Leakage**

I also determined the time kinetics of product formation, and evaluated the possibility that the fluorescent product may leak from cells. Separate spleen cell samples were incubated with Cat L substrate for 15 to 75 min. At each time point, cells were washed twice in PBS, fixed, and analyzed by FCM. At 15 min, the MFI was 29 and increased to 44 by 45 min (**Figure 4C**). At 75 min, the MFI was 40, which was not significantly different from that at 45 min. Similar results were observed for Cat B activity (data not shown). A 45-min incubation was utilized for all following assays. In a separate experiment, the incubation time period was extended to 3 h to detect any major changes of the cresyl violet product in cells. Cells incubated with Cat L substrate from 1 to 3 h had MFI values of 25 that did not change (**Figure 4D**). Analogous findings were also seen for Cat B activity (data not shown). No significant product loss from cells was detected indicating that additional incubation time required for immunofluorescence staining would not affect measurements of cathepsin activity.

**Increased Cathepsin Activity in LPS-Stimulated Macrophages**

The macrophage cell line was stimulated with LPS for 48 h, and then assayed for Cat B, L and S activities (**Table 3**). Fluorescent profiles of LPS-stimulated macrophages shifted to higher values for all the cathepsins. LPS treatment caused a significant increase in red MFI of cells for Cat B, L, and S, indicating that the substrates were not limiting.
Table 3
Comparison of flow cytometer and cell lysate assays.

<table>
<thead>
<tr>
<th>Cathepsin</th>
<th>Flow Cytometer Assay</th>
<th>Cell Lysate Assay</th>
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<tbody>
<tr>
<td>B</td>
<td>2.00 ± 0.90*</td>
<td>4.28 ± 1.56**</td>
</tr>
<tr>
<td>L</td>
<td>2.48 ± 0.96**</td>
<td>4.56 ± 1.91***</td>
</tr>
<tr>
<td>S</td>
<td>3.28 ± 1.14*</td>
<td>3.51 ± 0.58***</td>
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Macrophages were stimulated with 10 µg/ml of LPS for 48 h. Indicated cathepsin activities were assayed in cells with MR substrates by FCM, or in cell lysates with AMC substrates. Data represent the mean ± SD from 3 or more separate experiments. Fold increase was calculated by dividing the fluorescence measurements for LPS-stimulated cells by the values for medium control cells. LPS vs. medium: *$P < 0.05$; **$P < 0.01$; ***$P < 0.0001$. Reproduced with copyright permission.
Similar results were obtained when these cathepsin activities in cell lysates were assayed using AMC substrates. Substrates for the cell lysate assay have the same amino acid target sequence as the MR substrates, but the leaving group was AMC. Analogously, proteolytic activities of Cat B, L, and S within cellular lysates were significantly increased after LPS stimulation (Table 3). Enzymatic activity of all three cathepsins was significantly higher in LPS-stimulated cells compared to medium control cells using both assays.

Comparison of Product Fluorescence in Live and Post-Fixed Spleen Cells

Fluorescent product values for Cat B, L and S in cells post-fixed with 0.5% paraformaldehyde were compared to those for live cells. Spleen cells were incubated with MR Cathepsin substrates, washed twice and either analyzed as live cells in PBS or fixed in paraformaldehyde before analysis. When Cat B product fluorescence was evaluated in live cells the MFI was 24, almost half that in post-fixed cells with a MFI of 42 (Figure 5A). No significant difference for fluorescence values between live and post-fixed cells was observed for Cat L and S (Figure 5B) and C). In the absence of substrate, there was no difference in red autofluorescence between live cells and fixed cells, both having MFI values of 1.6 (Figure 5A). Fixed cells stored in a refrigerator for 14 days also had no significant decrease in red product fluorescence intensity (data not shown). Fluorescent products did not diffuse out of post-fixed cells, indicating cell fixation after immunofluorescence staining would not underestimate cathepsin activities.

Selectivity of Cathepsin Assays
Figure 5. Cathepsin activities in viable and post-fixed spleen cells.

Viable spleen cells in duplicate were incubated for 45 min with or without cathepsin substrate. Cells were washed and analyzed as live cells. Cells in parallel were washed and then fixed in paraformaldehyde before analysis. (A) Cells incubated with or without 6 μM Cat B substrate. Lv Neg denotes negative control of live cells. Fx Neg denotes negative control of fixed cells. (B) Cells incubated with 6 μM Cat L or (C) 24 μM Cat S substrates. Reproduced with copyright permission.
Although the MR substrates have the same peptide sequences as those utilized for cell lysate assays, reaction conditions with cell lysates are manipulated to favor cysteine cathepsin activity (Barrett, 1981; Kinschke, 1994), which is impossible to do with viable cells. Selectivity of the assays, in addition to the amino acid target sequence of the substrates was further assessed by pre-incubating spleen cells with cell permeable cysteine cathepsin inhibitors before substrate addition. Splenocytes were sensitive to the toxicity of inhibitors, especially the Cat L selective inhibitor. Due to the splenocyte sensitivity, 5-CFDA, AM was used as a cell viability marker. 5-CFDA, AM and cathepsin substrates were incubated for an additional 30 min after pre-incubation with the indicated inhibitor. All cells were washed twice and suspended in PBS for analysis as live cells. Analysis was performed on green fluorescent 5-CFDA, AM+ viable cells, and cell viability was ≥ 70% in the representative experiments shown in Figure 6. Spleen cells were incubated with E-64d, a general cysteine cathepsin inhibitor (Barrett, 1981), before addition of Cat B substrate and 5-CFDA, AM. Pre-treatment of cells with E-64d decreased red MFI by 37% compared with cells incubated with the substrate alone (Figure 6A). Similarly, cells were incubated with Cat L selective inhibitor, Z-Phe-Phe-CHN₂ prior to substrate addition (Kinaschke, 1994). This inhibitor diminished MFI by 44% (Figure 6B). Likewise, Cat S selective inhibitor, Z-Val-Val-Nle-CHN₂ (Shaw, 1993), decreased MFI by 65% (Figure 6C). Higher inhibitor concentrations caused substantial cell death (< 60% viable cells) and were excluded from analysis.

To confirm the selectivity of the cathepsin assays, inhibitors were utilized in conjunction with non-corresponding substrates. Cat L selective inhibitor marginally
Figure 6. Selectivity of cathepsin assays.

Spleen cells were incubated without or with a cysteine cathepsin inhibitor, and then with a cathepsin substrate and cell viability marker 5-CFDA, AM for an additional 45 min. Cells were washed and analyzed by FCM. Graphs are the product fluorescence profiles of viable 5-CFDA, AM$^+$ cells. (A) Pre-incubation for 3 h without and with E-64d followed by Cat B substrate. (B) Incubation for 30 min without and with Z-Phe-Phe-CHN$_2$ and then with Cat L substrate. (C) Pre-incubation for 30 min without and with Z-Val-Val-Nle-CHN$_2$ followed by Cat S substrate. (D) Pre-incubation without or with Cat L selective inhibitor Z-Phe-Phe-CHN$_2$ followed by Cat B selective substrate. (E) Pre-incubation without or with Cat L selective inhibitor followed by Cat S selective substrate. (F) Pre-incubation without or with Cat S selective inhibitor Z-Val-Val-Nle-CHN$_2$ followed by Cat B selective substrate. (G) Pre-incubation without or with Cat S selective inhibitor followed by Cat L selective substrate. (H) Macrophage cell line was incubated with or without the indicated inhibitor (Inhib), followed by a selective cathepsin substrate conjugated to AMC. Fluorescence was measured with a spectrofluorophotometer. Percent inhibition was calculated as described in Chapter 2. Values are the mean ± SD from three separate experiments. * Cells with inhibitor vs. cells without inhibitor: $P < 0.01$. Reproduced with copyright permission.
affected the MFI of cells incubated with Cat B and S substrates (2.5% and 19% decrease, respectively) (Figure 6D and E), in contrast with Cat L substrate (see Figure 6B). Cat S selective inhibitor did not diminish MFI of cells using Cat B and L substrates (Figure 6F and G), unlike the results with Cat S substrate (see Figure 6C). Nonspecific substrate cleavage appeared to be minimal.

Because the inhibitors did not completely decrease MFI to background MFI of cells incubated without substrate, cathepsin activity in a viable macrophage cell line was assessed using AMC conjugated substrates and fluorescence was measured using a spectrofluorophotometer. Cells pre-incubated with E-64d had a 26% reduction in fluorescence units for Cat B activity (Figure 6H). Cat L activity dropped by 41% in cells pre-incubated with the Cat L inhibitor. The Cat S selective inhibitor decreased fluorescent product formation by 61% (Figure 6H). These results parallel those observed for the MR substrates including the rank order of the degree of inhibition.

Cathepsin Assay Combined with Immunofluorescence Staining

Cathepsin activity in splenic cell subpopulations was evaluated using immunofluorescence staining with two-color flow cytometric analysis. mAb recognizing cell lineage-specific molecules were employed to distinguish three cell types. Spleen cells were incubated with a Cat Substrate followed by immunofluorescence staining. Figure 7 illustrates representative contour plots. Although all nucleated splenocytes had cathepsin activities, Mac-1+ macrophages, B220+ B cells and Thy1.2+ T cells were easily identified. Incubation of spleen cells with the substrates did not change the percentages of the three cell types based on immunofluorescence staining (data not shown). Cells positively
Figure 7. Cathepsin assays combined with immunofluorescence staining compared to cathepsin assays of purified cells.

Spleen cells were incubated with a cathepsin substrate, followed by immunofluorescence staining for cell lineage-specific cell surface molecules. Contour plots were generated by two-color FCM. (A) Cat B activity (FL3-H) vs. Mac-1 expression (FL1-H). (B) Cat L activity (FL3-H) vs. B220 expression (FL1-H). (C) Cat S activity (FL3-H) vs. Thy1.2 expression (FL1-H). Boxes designate cells expressing the respective cell surface molecules. (D) Cat L activity in purified splenic macrophages vs. gated Mac1+ cells from unpurified spleen cells. (E) Cat L activity in purified splenic B cells vs. gated B220+ cells from unpurified spleen cells. Reproduced with copyright permission.
staining for these molecules as denoted by boxes in Figure 7 were analyzed for the fluorescent cleavage product (Table 4). MFI for Cat B, L and S activities did not significantly differ between Mac1+ cells and B220+ cells, however B220+ cells had a trend of lower values. Thy1.2+ cells had significantly lower MFI values for all three cathepsins when compared to Mac1+ cells.

To assess whether immunofluorescence staining influenced product fluorescence, splenic macrophages and B cells were purified by negative selection using Ab- and complement-mediated cytolysis and assayed for cathepsin activities. Fluorescence profiles for Cat L activity in the enriched cells were compared to the profiles for cells gated on green positive staining for Mac1 or B220 molecules. Red fluorescence intensity due to product formation for purified macrophages showed no significant difference from that for gated Mac1+ cells from unfractionated splenocytes (Figure 7D; 48 MFI for purified cells vs. 58 MFI for stained cells). Similarly, MFI values were comparable for purified B cells and gated B220+ cells (Figure 7E; 56 MFI for purified cells vs. 58 MFI for stained cells). Similar results were obtained for Cat B and S product profiles of isolated macrophages versus Mac1+ cells, and purified B cells versus B220+ cells (data not shown). This protocol measured cathepsin activities in cell subpopulations without the need for cell purification.

Discussion

The assay I developed has the distinct advantage of assessing activity of Cat B, L and S in live cells on a per cell basis using a low number of cells and could be easily
Table 4

Cathepsin activities in spleen cells expressing Mac-1, B220, and Thy1.2 molecules.

<table>
<thead>
<tr>
<th>Cathepsin</th>
<th>Mac 1⁺ cells</th>
<th>B220⁺ cells</th>
<th>Thy1.2⁺ cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>B</td>
<td>315 ± 76</td>
<td>204 ± 73</td>
<td>141 ± 55*</td>
</tr>
<tr>
<td>L</td>
<td>80 ± 15</td>
<td>67 ± 12</td>
<td>31 ± 5*</td>
</tr>
<tr>
<td>S</td>
<td>170 ± 31</td>
<td>142 ± 54</td>
<td>57 ± 14*</td>
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</table>

Cells were incubated with a cathepsin substrate followed by immunofluorescence staining (see Figure 3 legend). Values are MFI ± SD for cathepsin activity in cells expressing the indicated molecules from five separate experiments. *Thy 1.2⁺ cells vs. Mac1⁺ cells: $P < 0.05$. Reproduced with copyright permission.
adapted for the clinical laboratory setting. Peptide substrates coupled to cresyl violet are routinely utilized for confocal microscopy and have been infrequently applied to FCM. Confocal microscopes and dual laser flow cytometers employ a helium neon laser to excite cresyl violet at 543 nm. Single argon laser flow cytometers are very prevalent, and this laser excites at 488 nm, which is below the peak excitation wavelength for the cresyl violet leaving group (Boonacker, 2001). Despite this potential problem, cathepsin activity inside cells was readily quantitated, and measurements depended on substrate concentration. Red fluorescence values increased, whereas green fluorescence measurements were constant, indicating higher product formation with increasing substrate concentrations. Key to measuring substrate cleavage using an argon laser was appropriate compensation settings. Furthermore, LPS stimulation of a macrophage cell line significantly increased red fluorescence values for Cat B, L and S, suggesting that the substrates were not limiting. The higher product measurements probably reflected increased proteolytic activities inside stimulated macrophages. This was confirmed by performing cell lysate protease activity assays for Cat B, L and S. Activity of all three cathepsins within cell lysates was significantly increased in LPS-stimulated cells, which correlated with results obtained by the FCM assay. Although there were slight differences in the fold increase values between the two assays, this could possibly be attributed to product leakage from live cells, while products accumulate in the cell lysate assay. Furthermore, higher values for Cat B and L lysate assays also indicate reaction conditions probably drive activation of precursor Cat B and L. Increased activity in the Cat S lysate assay, which is performed under neutral conditions, was not observed, and mature Cat S retains 70% maximal activity. Although
cleavage products could accumulate, precursor Cat S would not be activated at neutral pH. Potential precursor activation in the cell lysate assays may account for the disparate rank order of cathepsin activity between the assays. The possibility that the disparate rank order is due to technological differences between the instruments is highly unlikely. When AMC-coupled substrates, which were used for cell lysate assays, were adapted for live cells, the rank order was identical. These results demonstrate the ability to use these substrates with single argon laser flow cytometers to quantify intracellular cysteine cathepsin activity.

Time courses indicated a 45-min incubation was sufficient to assess cysteine cathepsin activity accurately. Incubations from 1 to 3 h showed no significant decrease in measurements or quenching of the fluorescent product. These findings indicate that steady-state conditions were stable during this time frame. Steady-state conditions occurred earlier than that for cell lysate assays (Barrett, 1981; Kinschke, 1994). Time kinetics for the whole cell assay is more complex than that for cell lysate assays. For the former assay, substrates must be internalized by live cells and enter the proper organelles where functional cysteine cathepsins reside. The fluorescent product can then diffuse from cells (Boonacker, 2001). In contrast, cell lysate assays depend only on the reaction rate. In addition, unlike substrates for cell lysate assays, cresyl violet is bi-substituted with peptides. However, pseudo first-order rate kinetics can be achieved if less than 15% of the substrate is cleaved (Boonacker, 2001). Immunofluorescence staining requires an additional incubation of 1.5 h, and the time course determined that the extended incubation time would have no effect on assessing cysteine cathepsin activity.
Cysteine cathepsin activity was assessed in live or post-paraformaldehyde fixed cells. There was no significant difference in Cat L and S measurements in live or post-fixed cells. Although cresyl violet product diffuses from living cells (Boonacker, 2001), paraformaldehyde fixation of cells apparently prevented product loss. Fixed cells were stored for 14 days without a significant decrease in product measurements. However, Cat B values were lower for live cells than post-fixed cells, which could not be attributed to product leakage from live cells or the process of fixation. The reason for the lower fluorescence in live cells remains unclear, but this result occurred only with the Cat B substrate. Hence, comparisons of Cat B measurements with other cathepsins in post-fixed cells need to consider this property of the Cat B substrate. Overall, these findings indicate that cells can be fixed and stored before analysis without a loss of product.

Selectivity of substrate cleavage by a particular cathepsin was assessed by the use of cell permeable general and selective inhibitors. However, the inhibitors were problematic due to their toxicity to spleen cells, which is not an issue for cell lysate assays. Therefore, 5-CFDA, AM was a marker for viable cells, which were gated by green fluorescence to exclude dead cells. Pre-incubation of cells with cathepsin inhibitors significantly decreased fluorescence intensity using the appropriate substrates, indicative of lower proteolytic activity. Fluorescence intensities did not drop to baseline levels of cells incubated in absence of substrate. Similar results were also obtained using AMC-conjugated substrates with live cells, and measuring fluorescence with a spectrofluorophotometer. The AMC-conjugated substrates are routinely used to measure cathepsin activity in cell lysates (Barrett, 1981; Kischke, 1994). Hence, the inability to
reduce fluorescence intensities to baseline levels cannot be attributed to a problem with cresyl violet-conjugated substrates or FCM measurements. Most likely, inhibitors did not reach sufficient concentration levels within organelles of live cells to inactivate cysteine cathepsins completely. Selectivity of the assays was further investigated by evaluating non-selective cleavage. The Cat L selective inhibitor minimally decreased product formation from Cat B substrate, which correlates with the extremely low affinity of the Cat L selective inhibitor for Cat B (Barrett, 1981). The Cat L selective inhibitor impaired Cat S substrate cleavage, but not to the extent seen with Cat L substrate. These findings agree with the reported cleavage of the Cat S substrate by Cat L due to a low affinity interaction (Brömme, 1989). Furthermore, the Cat S selective inhibitor had no effect on product formation from Cat B or L substrates, which is in agreement with a previous report using purified enzymes (Shaw, 1993). Taken altogether, non-specific cleavage was minimal, and the selectivity of this assay parallels that reported for cell lysate assays (Barrett, 1981; Brömme, 1989; Kirschke, 1994; Shaw, 1993). Additional experiments with cells from Cat B, L or S genetically deficient mice would elucidate the exact degree of selective substrate cleavage.

Peptide substrates coupled to rhodamine 110, an analogue of fluorescein, are non-fluorescent and have been utilized with live cells for FCM. Upon peptide cleavage, the leaving group emits with a spectrum similar to fluorescein (Boonacker, 2001). These substrates have disadvantages compared to cresyl violet-conjugated substrates. The product is positively charged and accumulates within mitochondria (Boonacker, 2001). Due to intracellular product accumulation, short incubation times and low substrate
concentrations are typically employed for live cell measurements (Boonacker, 2003; Ulbricht, 1995). In contrast, intracellular cresyl violet product localizes to the site of active proteases (Boonacker, 2001; Boonacker 2003). Substrate cleavage specificity is influenced by properties of fluorophores in synthetic substrates in addition to the peptide sequence. Rhodamine 110-coupled peptide substrates for Cat B and L, and dipeptidyl peptidase IV exhibit more promiscuous cleavage than substrates with the same amino acid sequence conjugated to AMC, 4-methoxy-b-naphthylamide or cresyl violet (Boonacker, 2003; Ulbricht, 1995).

Spectral overlap was a concern due to the emission spectrum of cresyl violet perchlorate (Boonacker, 2001; Du, 1998). Fluorescein emits in the red channel to a very low degree. To avoid false measurements, compensation settings were determined to subtract signals in FL-1 channel. I also used a Beckman Coulter Cytomics FC 500, and similar results were observed. The cell surface molecule detected by immunofluorescence staining must be highly expressed, because spectral compensation decreases the sensitivity of detectors to measure signals. Since many cell types, including tumor cells, highly express at least one cell surface molecule, this disadvantage is minimal. I assessed the activity of each cathepsin in spleen cells expressing Mac-1, B220, and Thy1.2 molecules. The percentages of cells staining positively for these molecules were not influenced by the substrates. Mac-1$^+$ macrophages had the highest activity of all three cathepsins followed by B220$^+$ B cells, while Thy1.2$^+$ T cells had the lowest cysteine cathepsin activity. Without immunofluorescence staining, spleen cells showed unimodal fluorescent product profiles, which masked the differences between T cells and B cells. Immunofluorescence
staining clearly revealed that product measurements in T cells were half to one third of those in macrophages and B cells. Cathepsin protein levels have not been measured in these different cells types however, Cat B and S were highly active in Mac-1 and B220 expressing cells, which correlate with their function as antigen-presenting cells (Berdowska, 2004; Chapman, 2006; Honey, 2003). Fluorescent product formation in purified macrophages and B cells was equal to that obtained in unpurified cells gated for Mac1⁺ or B220⁺ expression. The combination of this new cathepsin assay with immunofluorescence staining allows one to assess cathepsin activity in multiple cell types without the need for cell purification, which could be useful for diagnostic and prognostic tests.

To determine the role of cysteine cathepsins in physiological and pathophysiological processes, methods to measure proteolysis in individual viable cells are needed. I developed a novel assay to measure Cat B, L and S activities in live cells on a per cell basis utilizing FCM. This method is fairly rapid and can be performed with a low number of cells. This technique was combined with immunofluorescence staining allowing us to determine proteolytic activity in distinct cell types within a heterogeneous cell population. Cells were fixed and stored before analysis without product loss. Therefore, this assay could be used to evaluate clinical cell samples from peripheral blood and various organs and easily adapted for high-throughput assessment. This method was employed for my subsequent studies investigating regulation of cathepsin activities.

Note: This chapter, in part, was published in Cytometry Part A, 71A:114-123, 2007.
Chapter 4: Toll-Like Receptor Regulation of Cysteine Cathepsins

Introduction

Previous studies investigating the effects of TLR-4 ligand, LPS, on cathepsins are inconclusive due to conflicting data. Studies with dendritic cells indicate that LPS alters the intracellular localization of active proteases within phagosomal and endosomal compartments (Lautwein, 2002; Lennon-Dumenil, 2002). However, these studies did not elucidate whether LPS affects overall cathepsin activity and expression. Pro-inflammatory cytokines TNF-α, IL-1β and IL-6 are rapidly induced upon LPS and PGN activation of multiple cell types, and these cytokines by themselves regulate cathepsins in several cell types (Bjorkbacka, 2004; Hirschfeld, 2001; Jones, 2001; Lautwein, 2002; Watari, 2000). Changes in cathepsin mRNA expression do not always correlate with protein levels or increased activity (Keyszer, 1998), because cathepsins are regulated on several levels. These studies demonstrate differential regulation of Cat B, L and S by cytokines and reveal differences among cell types. Few studies have focused on TLR regulation of these proteases in macrophages, despite the fact they have the highest expression of cathepsins in diseased tissues. Hence, the mechanisms by which these proteases are upregulated during inflammatory diseases are poorly understood.
In the previous chapter, Cat B, L and S activities in live cells were increased in response to LPS, however the impact of other TLR ligands on cysteine cathepsins have not been investigated. In this chapter, I examined the effects of multiple TLR ligands on cysteine cathepsins. This study focused on the regulation of cysteine Cat B, L and S activities in macrophages during inflammatory responses to both MyD88-dependent and -independent TLR ligands (Table 5), including TLR-2 ligand, PGN, TLR-4 ligand, LPS, TLR-3 ligand Poly I:C and formalin-killed C. parvum. Changes in mRNA expression of cathepsins and their endogenous inhibitors, cystatins, were measured at early and late time points. Increased cathepsin activities occurred in the absence of upregulated cathepsin gene expression, however cystatin C mRNA levels were decreased.

To distinguish between a primary LPS signaling effect or a secondary cytokine effect, a LPS non-responsive macrophage/microglial cell line was co-cultured with a LPS responsive macrophage cell line. LPS-stimulated macrophages activated LPS non-responsive bystander cells to upregulate cathepsin activity. In addition, cell-free culture supernatants from macrophages stimulated with LPS for 6 h increased cathepsin activities in LPS non-responsive macrophages, suggesting that cytokines produced early in response to TLR ligands are involved. Furthermore, LPS non-responsive cells were stimulated with culture supernatants pre-incubated with neutralizing anti-TNF-α or IL-1β antibodies. Neutralization of TNF-α and IL-1β resulted in differential effects on cysteine cathepsins. These results indicate that pro-inflammatory cytokines increased
Table 5

TLR ligands, receptors and pathways investigated.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Receptor</th>
<th>Response</th>
<th>MyD88 Dependent</th>
<th>MyD88 Independent</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPS</td>
<td>TLR-4</td>
<td>Classic <em>in vitro</em> Inflammatory Model</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>PGN</td>
<td>TLR-2</td>
<td>Similar to LPS</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>PIC</td>
<td>TLR-3</td>
<td>Robust IFN-β Production</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><em>C. parvum</em></td>
<td>TLR-2 &amp; other PRRs</td>
<td>Whole Organism</td>
<td>+</td>
<td>+*</td>
</tr>
</tbody>
</table>

+*; Involvement of other PRR.
cathepsin activities in response to LPS and may play a role in downregulation of cystatin C, an endogenous cysteine cathepsin inhibitor.

**Results**

**TLR Ligands Increase Cathepsin Activities** I investigated the effects of TLR ligands on cysteine cathepsin activity in multiple macrophage cell lines. To measure cathepsin activity inside live cells, cell permeable, selective peptide substrates conjugated with AMC or MR were utilized. Fluorescent AMC and MR products were measured by spectrofluorophotometry or flow cytometry, respectively, based on their spectral properties (Creasy, 2007). Studies in Chapter 3 employing these substrates revealed comparable results with the two assays (Creasy, 2007). When P388D1 (Figure 8A) and Clone 63 (data not shown) macrophages were stimulated with LPS for 24 h, activities of Cat B, L and S significantly increased with Cat L showing the greatest augmentation in P388D1 cells. A macrophage cell line from cannabinoid receptor subtype 2 (CB2) deficient mice had low CD14 expression, which is a coreceptor critical for LPS signal transduction. Therefore, I investigated their ability to respond to LPS. CB2<sup>−/−</sup> cells stimulated with LPS significantly increased IL-1β and MCP-1 production, indicating they are LPS responsive (data not shown). This cell line was utilized for studies in Chapter 5 and details on its characterization are presented there. LPS stimulation of CB2<sup>−/−</sup> macrophages for 24 h also significantly enhanced intracellular activities of Cat B, L and S (Figure 8B-D). Dose-response and time course studies revealed that cathepsin upregulation occurred by 18 h in cells incubated with 10 µg/ml LPS, with
Figure 8. LPS stimulation increases cathepsin activities in live macrophages.

Macrophages were cultured with or without 1 µg/ml LPS for 24 h, and live cell cathepsin activities were assessed by (A) spectrofluorophotometric or (B-D) flow cytometric assays. (A) Cysteine cathepsin activities in P388D1 macrophages. Data are representative of 3 or more separate experiments. LPS vs. medium: *P < 0.05. (B) Cat B, (C) Cat L and (D) Cat S activities in CB2$^{-/-}$ macrophage cell line.
average fold increase in MFI values for Cat B, L and S of 1.4, 1.8 and 1.4, respectively. LPS at concentrations as low as 0.3µg/ml caused cathepsin activities to increase at 24 h however, more significant increases were observed with doses of 1 µg/ml or higher (Appendix, Figure S1). Cat B, L and S activities significantly increased at 24 h in CB2−/−, P388D1 and Clone 63 macrophages cultured with 1 µg/ml LPS. Activity remained increased at 48 h, however longer time points were not analyzed due to cell loss that occurs with extended incubation times. To insure increased fluorescence was due to enhanced cathepsin activity within acidic intracellular organelles, the ImageStream® Cell Analysis System was employed. This system combines FCM and fluorescent microscopy allowing one to examine localization of fluorescent molecules, as well as quantitative changes (Beuma, 2006). As shown in Figure 9, fluorescent product generated by cleavage of Cat S MR-conjugated substrate in LPS-stimulated cells remained localized to acidic vesicles and paralleled results obtained using confocal microscopy (see Figure 3). Similar results were obtained for Cat B and L substrates, and similarity bright detail scores supported co-localization (data not shown). Red channel MFI of Cat B, L and S products in LPS-stimulated cells was compared to medium control values and increased from 63,833 to 89,259, 61,384 to 104,810, and 105,907 to 117,262, respectively.

I extended the study to include other TLR ligands which signal through MyD88-dependent or –independent pathways. Dose response studies were performed with PGN and Poly I:C to determine an effective dose (Appendix, Figures S2 and S3). P388D1 macrophages stimulated with 4 µg/ml PGN, a TLR-2 ligand, for 24 h significantly
**Figure 9.** Increased activity of Cat S in LPS stimulated cells is localized within acidic organelles.

Macrophages were cultured without or with LPS for 24 h, incubated with the MR-conjugated Cat S substrate and LysoTracker DND-26. Analysis of live cells was performed using the ImageStream® Cell Analysis System to detect fluorescent intensity and localization of intracellular red fluorescent hydrolyzed product from Cat S substrate and green LysoTracker DND-26.
increased Cat L and S activities compared with medium controls (Figure 10A). While Cat B showed a trend of increased activity this increase was not as significant as that observed for Cat L and S. Similar results were obtained when P388D1 cells were stimulated with 10 µg/ml Poly I:C, whereby only activities of Cat L and S were significantly increased (Figure 10B). To examine the effects of signaling through multiple PRR, cells were incubated with or without 10 µg/ml formalin-killed C. parvum for 48 h. Cat B and S were significantly upregulated, whereas Cat L was not affected (Figure 10C). To investigate the possible influence of phagocytosis of C. parvum on cathepsin upregulation, cells were incubated with 1-mm diameter polystyrene beads. The fold increase in MFI was 1.04 or less for the three cathepsins after phagocytosis of the beads, thus Cat B, L and S were not increased due to phagocytosis of the beads.

**TLR Ligands Induce Cathepsin Secretion**

Cells regulate their intracellular levels of cysteine cathepsins by secreting pro and active forms of the enzymes. During inflammation, cathepsins can degrade extracellular matrix components, however whether cleavage occurs intracellularly or extracellularly is not clear. I investigated whether LPS or PGN stimulation induced secretion of active cathepsins. Cells were incubated with 1 µg/ml LPS, 4 µg/ml PGN or medium containing 10 mM NH₄Cl/mannose-6-phosphate, which was a positive control, for 24 h. NH₄Cl promotes secretion of endosomal and lysosomal proteins, and mannose-6-phosphate prevents cellular re-uptake of secreted proteins by saturating mannose-6-phosphate receptors. Cathepsin activity in cell-free culture supernatants was
Figure 10. Differential impact of PGN, Poly I:C, and C. parvum stimulation on macrophage cathepsin activities.

P388D1 macrophages were incubated without or with (A) 4 µg/ml PGN or (B) 10 µg/ml Poly I:C for 24 h. (C) CB2⁺ macrophages were incubated with 10 µg/ml formalin-killed C. parvum for 48 h. Live cell cathepsin activities were assessed by spectrofluorophotometry (A&B) or flow cytometry (C). Data are the mean ± standard deviation from 3 or more separate experiments. Fold increase was calculated by dividing fluorescent values of stimulated cells by values of medium controls. PGN, Poly I:C or C. parvum vs. medium: *P < 0.05; **P < 0.01.
measured according to protocols for cell lysates as described in Chapter 2. Activity per live cell of Cat B, L and S increased in culture supernatants from LPS-treated cells (Figure 11). Similar results were obtained for culture supernatants from PGN-stimulated cells (data not shown).

**LPS Stimulation Decreases Cystatin C mRNA Expression**

Possible mechanisms for changes in cathepsin activity were investigated utilizing the LPS model system. To distinguish between direct effects of LPS signaling and indirect effects, mRNA expression was examined at early and late time points. RNA was isolated from cells stimulated with 1 µg/ml LPS for 6 h or 24 h, and semiquantitative RT-PCR was performed. Cystatin A mRNA was not detected in LPS-stimulated or medium control macrophages at either time point (data not shown). At the 6-h time point, no changes in transcripts of Cat B, L, and S or the endogenous inhibitors, cystatins B and C were observed (data not shown). As shown in Figure 12, at 24 h there was a slight increase in mRNA expression of Cat B and L, but not S, although the increase was not significant. Cystatin B mRNA expression was not altered at 24 h, however cystatin C transcript decreased noticeably (Figure 12). Semi-quantitative Real Time-PCR using SYBR GreenER™ Two-Step qRT-PCR confirmed the results obtained for Cat B, L and cystatin C mRNA (Appendix, Figure S4).

**Activation of LPS Non-responsive Bystander Cells**

Pro-inflammatory cytokines, such as IL-1β and TNF-α, are readily produced by macrophages activated by LPS and PGN (Jones, 2001; Hirschfeld, 2001), which may mediate increased cathepsin activities. EOC 20 macrophage/microglial cell line is
**Figure 11.** LPS induces cathepsin secretion.

CB2<sup>+</sup> macrophages were incubated in medium, 1 µg/ml LPS or 10 mM ammonium chloride/mannose-6-phosphate (+ secretion control) for 24 h. Secreted enzymatic activity per cell was measured in concentrated cell-free culture supernatants for (A) Cat B, (B) Cat L and (C) Cat S. Values represent the mean ± standard error from 3 separate experiments. *P < 0.05 compared with medium control.
Figure 12. Effects of LPS stimulation on mRNA expression of Cat B, L and S and cystatins B and C.

P388D1 Macrophages were cultured with or without 1 μg/ml LPS for 24 h. Cells were lysed and prepared for semiquantitative RT-PCR. Cat B, L, S, Cystatins (Cys) B and C and β-actin cDNA were amplified at different cycle numbers to maintain linearity. Top panels are representatives of 3 separate experiments. Bottom panels are mean ± SD of relative expression compared with β-actin.
derived from C3H/HeJ mice. These mice are LPS non-responsive due to a mutation within the cytoplasmic domain of TLR-4 but are responsive to PGN and Poly I:C (Takeuchi, 2000). As expected, EOC 20 cells cultured with LPS did not increase cathepsin activities (Figure 13). I investigated the ability of EOC 20 cells to increase cathepsin activities in response to PGN and Poly I:C. Similar to P388D1 cells (see Figure 10), EOC 20 cells significantly upregulated Cat L and S activities in response to PGN and Poly I:C (Figure 13). To investigate the role of cytokines, EOC 20 cells were co-cultured with LPS responsive P388D1 cells at a 1:1 ratio and stimulated with LPS for 24 h. Immunofluorescence staining was combined with the live cell cathepsin flow cytometric assay. Staining for MHC class I K\(^k\) molecule, which is expressed by EOC 20 cells, was used to distinguish the two cell types and perform individual assessment of cathepsin activities. Cells positive (EOC 20) and negative (P388D1) for H-2K\(^k\) were gated and analyzed for cathepsin activities in the same manner as shown in Figure 7. EOC 20 cells had increased activities of Cat B and L, but not S, when co-cultured with P388D1 cells in the presence of LPS (Table 6). P388D1 cells from the co-cultures displayed significant upregulation of Cat B and L relative to medium control cells with a minimal fold increase of 1.7.

**TNF-\(\alpha\) and IL-1 Exert Differential Effects on Cathepsins**

Increased Cat B and L activities in bystander EOC 20 cells suggest LPS-stimulated P388D1 cells secreted cytokines leading to the increase. However, Cat S activity did not increase in either cell line, which may be due to cell competition. To
Figure 13. EOC 20 microglial cells are LPS non-responsive but upregulate cathepsin activity in response to PGN and PIC.

EOC 20 cells were incubated without or with 1 µg/ml LPS, 4 µg/ml PGN or 10 µg/ml Poly I:C for 24 h and assessed for live cell Cat B, L and S activities by flow cytometry (top panels) or spectrofluorophotometry (bottom panels). Data is representative of 3 or more separate experiments. Fold increase was calculated as described in Figure 9. *P < 0.05 compared with medium control.
Table 6

Bystander upregulation of cathepsin activities in LPS non-responsive cells.

<table>
<thead>
<tr>
<th></th>
<th>P388D1 Med</th>
<th>P388D1 LPS</th>
<th>EOC Med</th>
<th>EOC LPS</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cat B</strong></td>
<td>113 ± 17</td>
<td>195 ± 32</td>
<td>217 ± 20</td>
<td>296 ± 11*</td>
</tr>
<tr>
<td><strong>Cat L</strong></td>
<td>55 ± 10</td>
<td>121 ± 58</td>
<td>65 ± 3</td>
<td>155 ± 117</td>
</tr>
<tr>
<td><strong>Cat S</strong></td>
<td>128 ± 22</td>
<td>146 ± 24</td>
<td>138 ± 17</td>
<td>118 ± 6</td>
</tr>
</tbody>
</table>

EOC 20 cells were co-cultured with P388D1 macrophages in medium with or without 1 µg/ml LPS for 24 h. Cells were incubated with a cathepsin substrate followed by immunofluorescence staining to detect MHC class I Kk on EOC 20 cells. Numbers represent MFI values of Cat B, L and S activities in P388D1 and EOC 20 medium (Med) control and LPS treated cultures. Data are the mean ± SD of 3 separate experiments. Medium vs. LPS: *P < 0.05.
further investigate cytokines’ role in enhanced cathepsin activity, P388D1 macrophages were cultured in medium with or without LPS for 6 or 24 h. Then, EOC 20 cells were incubated with P388D1 cell-free culture supernatants for 24 h and cathepsin activities were assessed using live cell AMC assays. The 6-h culture supernatants significantly increased activities of the three cathepsins in EOC 20 cells (Figure 14). In contrast, 24-h culture supernatants had no effect on cathepsin activities in EOC 20 cells, indicating that early cytokines enhanced cathepsin activity (Figure 14).

TNF-α is rapidly released from LPS-stimulated cells. Other pro-inflammatory cytokines, such as IL-1β, are also secreted from TLR-activated macrophages. To investigate the role of TNF-α and IL-1β, 6-h culture supernatants were incubated with various concentrations of neutralizing anti-TNF-α or anti-IL-1β mAb, or correlating concentrations of mouse IgG as a negative control. EOC 20 cells were then incubated with antibody-treated culture supernatants for 24 h. Preliminary results revealed P388D1 culture supernatants containing higher concentrations of neutralizing TNF-α mAb inhibited upregulation of Cat B in EOC 20 cells when compared to IgG treated controls (Figure 15). Cat L activity was only slightly reduced by neutralizing TNF-α or IL-1β mAb (Figure 15 & 16). Activity of Cat S was also not significantly affected when culture supernatants were treated with anti-TNF-α or anti-IL-1β mAb, suggesting other cytokines may be responsible for it’s regulation (Figure 15 & 16). Neutralization of IL-1β selectively inhibited upregulation Cat L activity but appeared to have no effect on Cat B activity compared to IgG treated controls (Figure 16). These results
**Figure 14.** P388D1 culture supernatants increase cathepsin activities in EOC 20 cells.

P388D1 macrophages were cultured in medium or 1 µg/ml LPS for 6 or 24 h. Cell-free culture supernatants were harvested, concentrated, added to EOC 20 cell cultures and incubated for another 24 h. EOC 20 cells were harvested and live cell AMC cathepsin assays were performed. Fold increase was calculated by dividing the fluorescence values for LPS-stimulated culture supernatants by the values for medium control culture supernatants. LPS vs. medium: *$P < 0.05$; **$P < 0.01$. 
Figure 15. Neutralization of TNF-α in P388D1 culture supernatants reduces increased activity of Cat B and L in EOC 20 cells.

P388D1 macrophages were cultured in medium or 1 µg/ml LPS for 6 h. Cell-free culture supernatants were harvested, concentrated, and treated with the indicated concentrations of anti-TNF-α monoclonal Ab or controls were treated with 3 µg/ml IgG prior to addition to EOC 20 cell cultures EOC 20 cells were incubated for 24 h, harvested and live cell AMC cathepsin assays were performed. Data are the mean ± the standard deviation of three separate experiments. Medium IgG vs. LPS IgG: *P < 0.05. LPS IgG vs. LPS anti-TNF-α: *P < 0.05.
**Figure 16.** Neutralization of IL-1β reduces increased activity of Cat L but not Cat B and S in EOC 20 cells.

P388D1 macrophages were cultured in medium or 1 µg/ml LPS for 6 h. Cell-free culture supernatants were harvested, concentrated, and treated with the indicated concentrations of anti-IL-1β monoclonal Ab or controls were treated with 20 µg/ml of IgG prior to addition to EOC 20 cell cultures. EOC 20 cells were incubated for 24 h, harvested and live cell AMC cathepsin assays were performed. Data are the mean ± the standard deviation of two separate experiments. Medium IgG vs. LPS IgG: *P < 0.05. LPS IgG vs. LPS anti-IL-1β: NS.
suggest differential regulation of these cathespins by TNF-α and IL-1β and indicate the involvement of other cytokines not investigated here.

**Discussion**

This study is the first one to examine the impact of other PRR ligands in addition to LPS on cysteine cathepsins. I utilized two live cell enzymatic assays to assess changes in cysteine cathepsin activities on a per cell basis in response to inflammatory PRR stimuli in culture. LPS consistently increased Cat B, L and S activities by 2- to 4-fold in several macrophage cell lines. In a similar manner, PGN, a TLR-2 ligand, and Poly I:C, a TLR-3 ligand, also enhanced proteolytic activity of Cat L and S. Lower levels of enhanced Cat B activity in response to PGN and Poly I:C suggests that increased Cat B activity is dependent on activation of both MyD88-dependent and -independent pathways, which occurs with only LPS. All together this study demonstrates cathepsins can be altered in response to both MyD88-dependent and -independent TLR ligands. In contrast, macrophages incubated with formalin-killed *C. parvum* increased activity of Cat B and S, but not L. *C. parvum* signals through TLR-2 as well as other PRR. Perhaps, *C. parvum* activated a pathway that negated a positive signal for upregulating Cat L. Phagocytosis alone could not explain the disparate results, because cells cultured with polystyrene beads had no change in cathepsin activity. This supports the idea that PRR signaling activated by *C. parvum* causes alterations in cathepsin activity.

Previous studies have shown a lack of correlation between altered cathepsin activity and mRNA expression (Keyszer, 1998). I observed a significant increase in
cathepsin activity in LPS stimulated cells, which occurred in the absence of increased cathepsin mRNA expression. Cystatins, endogenous inhibitors of cysteine cathepsins, are often inversely affected during inflammatory diseases. These data support this trend, showing decreased levels of cystatin C may contribute to the increased activity of cathepsins during the inflammatory response. Interestingly, the intracellular inhibitor cystatin B, was not altered during the LPS response, suggesting its role in regulation of these proteases during inflammation may be minute. Furthermore, changes in cystatin C mRNA expression occurred at later time points suggesting an indirect role for TLR signaling in regulating these proteases. Signaling through TLR induces production and secretion of pro-inflammatory cytokines, which have been shown to regulate cathepsin expression and activity (Watari, 2000; Fiebiger, 2001; Kitamura, 2005). The time at which changes in expression occurred in this study indicate pro-inflammatory cytokines produced in response to LPS lead to increased cathepsin activities.

TNF-α, IL-1β and IL-6 secretion rapidly occur in LPS-stimulated macrophages although increases in TNF-α typically occur quicker than IL-1β and IL-6. These pro-inflammatory cytokines have been reported to upregulate Cat B and S, although the results depend on the cell type (Jones, 2001; Fiebiger, 2001). P388D1 macrophages were LPS responsive, whereas EOC 20 cells were LPS non-responsive. In 24-h co-cultures, EOC 20 cells increase Cat B and L activities, indicating a secondary mechanism was involved in cathepsin upregulation. When this study was extended to examine the effects of culture supernatants from LPS-stimulated P388D1 cells on cathepsin activity of EOC 20 cells, the early cytokine response played a significant role
in the regulation of cathepsins. In contrast to co-culture experiments, upregulation of Cat S activity was observed in the culture supernatant studies. An unknown mechanism, which controls Cat S, may have occurred in co-cultures or co-culture conditions were not optimal for Cat S upregulation. EOC 20 cells are capable of binding LPS even though their signaling cascade is defective. Therefore, availability of LPS to P388D1 cells may have been limited in co-cultures. Neutralization of TNF-α and IL-1β had differential effects on Cat B, L and S. Cat L appears to be regulated by both TNF-α and IL-1β, whereas Cat B activity was decreased only when TNF-α was neutralized. Cat S was not affected by neutralization of either of these cytokines suggesting another cytokine(s), possibly IL-6, contributes to Cat S regulation. Activity levels of Cat L and S were not reduced to medium control values when a single cytokine was neutralized. This suggest TNF-α and IL-1β together or in conjunction with other cytokines control these proteases, possibly by decreasing the expression of cystatin C.

The extracellular microenvironment of inflamed tissues becomes acidic enough to allow cathepsins to retain activity (Gatenby, 2006). For this reason, secretion of active cathepsins from TLR-stimulated macrophages was also investigated. Both LPS and PGN activation of macrophages increased active cathepsins in culture supernatants by approximately 5-fold compared to cells incubated in medium alone, suggesting induced secretion of active proteases. Cell viability in the various cultures was comparable, and enzymatic activity was calculated per live cell. Thus, the likelihood that enhanced cathepsin activity in LPS and PGN culture supernatants was due to
release of proteases from dead cells is remote. However, the level of protease activity in culture supernatants was minimal when compared intracellular activity, and, therefore, the biological relevance of this secretion is questionable.

In conjunction, these studies show cathepsin activities were altered in response to both MyD88-dependent and –independent TLR ligands. TLR ligands are currently being investigated as possible vaccine adjuvants as well as for other immunotherapy applications. Understanding the regulation of these proteases by these ligands is important when considering their use for immunotherapy. Furthermore, macrophages activated by TLR ligands caused cathepsin activities to increase in non-responsive bystander cells increasing their role in pathogenesis. Culture supernatants from 6-h LPS-stimulated macrophages increased cathepsin activity in LPS non-responsive macrophages, which was diminished by neutralization of TNF-α and IL-1β. LPS-stimulated macrophages had decreased levels of cystatin C mRNA, which may have contributed to increased proteolytic activity. However, cystatin C also regulates Cat S, which was not affected by neutralization of TNF-α or IL-1β suggesting the involvement of other mechanisms for Cat S. Altogether these studies suggest TLR antagonists and other therapeutic agents targeting pro-inflammatory cytokines involved in cathepsin regulation may be useful in controlling cathepsins during chronic inflammatory diseases.
Chapter 5: Cannabinoid Inhibition of Cysteine Cathepsin Upregulation During an Inflammatory Response

Introduction

Marijuana has long been used for medicinal purposes, dating back to the Neolithic period, around 4000 BC (Benamar, 2006). THC along with endogenous cannabinoids, and synthetic cannabinoids derivatives are currently being investigated as possible therapeutic candidates. Cannabinoids, including THC, alter inflammatory responses by immune cells, in particular macrophages. Stimulation of cells with LPS is frequently used as a classical model for inflammation leading to NFκB activation and production of pro-inflammatory cytokines. In the previous chapter cysteine cathepsin activities were increased in macrophages stimulated with LPS and cytokines contributed to this increase. THC attenuates iNOS gene expression, NFκB activation, and cytokine production during macrophage response to LPS (Cabral, 2005; Jeon, 1996).

Cannabinoids also interfere with processing of intact antigens by macrophages and increase cathepsin D activity. Other proteases, such as MMP, are also modulated by cannabinoids (Blazquez, 2003; 2008). MMP and cathepsins play a key role in angiogenesis and tissue destruction and are similarly altered during inflammatory disease. These findings support the possibility that cannabinoids may affect cysteine cathepsins during inflammation.

This study investigated the ability of cannabinoids, THC and CP55940, to modulate cathepsins during an inflammatory response. To investigate this possibility,
P388D1 macrophages were pre-treated with THC or CP55940 prior to LPS stimulation and cathepsin activity was assessed using live cell enzymatic assays. THC and CP55940 reduced the levels of cathepsin upregulation in LPS-stimulated cells. Multiple studies investigating immune modulation by cannabinoids using LPS as a model for inflammation have been reported (Cabral, 2005; Klein, 2006; Jeon, 1996). However, the mechanisms by which cannabinoids mediate these effects are not well elucidated. LPS activation of macrophages requires cell surface expression of TLR-4 and its co-receptor CD14. Because cannabinoids interfere with multiple responses occurring in LPS-stimulated macrophages, the possibility that cannabinoids downregulate the surface expression of these molecules was investigated. Some studies have suggested the immunosuppressive effects of cannabinoids can be attributed to their ability to induce immune cells to undergo apoptosis (Guzman, 2005; Lombard, 2007). Therefore, the possibility reduced cathepsin activities were related to cell death was also examined.

Immune modulation by cannabinoids, including THC may be mediated through cannabinoid receptors CB1 and CB2. Immune cells primarily express CB2, however the expression of cannabinoid receptors can be altered upon cellular activation (Carlisle, 2002). In this study the mRNA expression of cannabinoid receptors CB1 and CB2 was examined in resting and LPS activated macrophages. P388D1 macrophages expressed only the CB2 receptor, suggesting the modulation of cathepsins by cannabinoids was CB2 receptor mediated. To further investigate the involvement of the CB2 receptor a macrophage cell line derived from CB2−/− mice was utilized. Cannabinoid modulation of cathepsins was absent in macrophages lacking CB2 expression. These results indicate
cannabinoids via the CB2 receptor interfere with increased cysteine cathepsin activities during an inflammatory response.

Results

**Cannabinoid Receptor Expression in P388D1 Macrophages**

Expression of cannabinoid CB1 and CB2 receptors in resting and LPS-stimulated P388D1 cells was assessed. P388D1 cells were incubated in medium without or with 1 µg/ml LPS for 6 or 24 h, and RNA was prepared. CB1 mRNA was not detected in resting or LPS-stimulated P388D1 cells at either time point (Figure 17) whereas CB1 transcripts were readily found in bone marrow macrophages from 129 mouse strain. In contrast, P388D1 cells expressed CB2 mRNA in both resting and activated states. However, the expression level of CB2 mRNA did not significantly alter upon LPS stimulation at either 6 or 24 h.

**THC Inhibits LPS-Induced Cathepsin Upregulation**

As shown in Chapter 4, macrophages upregulated cathepsin activities during the inflammatory response to LPS. After confirming P388D1 cells expressed the CB2 receptor, the ability of cannabinoids to modulate cysteine cathepsins in LPS-stimulated macrophages was investigated. P388D1 macrophages were pre-treated with vehicle or various concentrations of THC, followed by LPS stimulation for 48 h. Cells were harvested, and live cell cathepsin assays were performed using AMC-conjugated substrates as described in Chapter 2. As shown in Figure 18, LPS activation caused
Figure 17. Cannabinoid receptor expression in P388D1 macrophages.

P388D1 macrophages were cultured without or with LPS for 6 or 24 h. RNA was prepared for semi-quantitative RT-PCR. CB1, CB2 and β-actin cDNA were amplified at different cycle numbers to maintain linearity. Top panels are representatives of 3 separate experiments. Bottom panels are the mean relative CB2 expression compared with β-actin ± SD at the indicated time points. 129 bone marrow (BM) macrophages were used as a positive control for CB1 mRNA.
Figure 18. THC inhibits upregulation of cysteine cathepsins in LPS-stimulated macrophages.

P388D1 macrophage cell line was pre-incubated with various THC concentrations or 0.1% ethanol (vehicle) for 4 h at 37°C then incubated with 1 µg/ml LPS or medium for 48 h. Cathepsin activity in live cells was assessed using AMC-conjugated substrates as described in Chapter 2. Values represent mean ± SE from 3 or more separate experiments. Medium vehicle or LPS vehicle controls: *P < 0.05. Medium vehicle vs. medium THC: NS. LPS vehicle vs. LPS THC: *P < 0.05.
increased cathepsin activities in vehicle-exposed cells, in agreement with the results in the preceding chapter (see Figure 8). THC did not significantly change cathepsin activities in medium control cells (Figure 18). At 1 nM, THC significantly inhibited cathepsin enhancement in LPS-stimulated P388D1 macrophage cell line. Activities of Cat B, L and S were reduced to medium control levels. In general, higher THC concentrations were less inhibitory. Decreased cathepsin activities in THC-exposed cells were not due to loss of cell viability as assessed by trypan blue exclusion. THC is a partial cannabinoid receptor agonist. The influence of CP55940, a full agonist, on cathepsin activities was also examined. Unlike THC, CP55940 tended to increase cathepsin activities in medium control cells in 24-h cultures (Figure 19). Although CP55940 was less effective than THC at suppressing cathepsin upregulation in LPS-stimulated cells, decreased cathepsin activity was more dose-dependent and could not be attributed to cell death.

**Characterization of CB2\(^{-/-}\) Bone Marrow Macrophage Cell Line**

Because P388D1 cells only expressed the CB2 receptor, the role of this receptor was examined. To investigate the involvement of the CB2 receptor in modulating cathepsins during an inflammatory response, a bone marrow (BM) macrophage cell line from mice deficient for CB2 receptor was generated in collaboration with Dr. Howard Young at the National Institutes of Health, as described in Chapter 2. The cells expressed cell surface molecules typical of macrophages and lacked those expressed by DC (Table 7). Co-stimulatory molecules expressed by CB2\(^{-/-}\) macrophage cell line were fully functional, because co-stimulatory activity was inhibited by antibodies
Figure 19. CP55940 reduces cathepsin upregulation in LPS stimulated macrophages.

P388D1 macrophage cell line was pre-incubated with vehicle or the indicated concentrations of CP55940 for 4 h at 37°C then culture without or with 1 µg/ml LPS for 24 h. Cathepsin activity in live cells was assessed (see Figure 18 legend). Values are mean ± SE from 3 or more separate experiments. Medium vehicle vs. LPS vehicle: *P < 0.05. Medium vehicle vs. medium CP55940: NS. LPS vehicle vs. LPS CP55940: NS.
Table 7

CB2\textsuperscript{\textae} macrophage cell surface molecular expression.

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Expression Level</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mac-1</td>
<td>+++</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>+++</td>
</tr>
<tr>
<td>FcγR</td>
<td>+</td>
</tr>
<tr>
<td>CD14</td>
<td>Low</td>
</tr>
<tr>
<td>B7-1</td>
<td>Low</td>
</tr>
<tr>
<td>B7-2</td>
<td>+</td>
</tr>
<tr>
<td>CD48</td>
<td>++</td>
</tr>
<tr>
<td>DEC-205</td>
<td>Negative</td>
</tr>
<tr>
<td>CD11c</td>
<td>Negative</td>
</tr>
<tr>
<td>I-A\textsuperscript{\textae}</td>
<td>Negative</td>
</tr>
</tbody>
</table>

Immunofluorescence staining for cell surface molecules on CB2\textsuperscript{\textae} macrophages was analyzed by flow cytometry. +++ Very High; ++ High; + Med-High.
specific for B7-1, B7-2, CD48 and ICAM-1 (Personal Communication, K. McCoy). Due to their low CD14 expression, a co-receptor required for LPS responses, their ability to respond to LPS was investigated. CB2\(^{-/-}\) cells stimulated with LPS increased IL-1\(\beta\) and MCP-1 production, indicating they are LPS responsive (Personal Communication, K. McCoy). Fc\(\gamma\) receptors of CB2\(^{-/-}\) macrophages mediated phagocytosis of rat IgG-coupled carboxylated beads normally (Personal Communication, K. McCoy). Lack of CB2 mRNA expression was confirmed (Figure 20), and CB1 mRNA in resting and LPS-stimulated CB2\(^{-/-}\) cells was assessed after 24 h. Similar to P388D1 macrophages, CB1 mRNA expression was not detected in resting or LPS-activated CB2\(^{-/-}\) macrophages (Figure 20). Therefore, the CB2\(^{-/-}\) macrophage cell line expressed functional cell surface molecules required for phagocytosis, co-stimulation of helper T cells and LPS response, and lacked expression of cannabinoid CB1 and CB2 receptors.

**THC Inhibition Requires CB2 Receptor Expression**

The ability of THC to suppress cathepsin activities in LPS-stimulated CB2\(^{-/-}\) macrophages was investigated. These cells increased cathepsin activities in response to LPS (see Figure 8). As above, CB2\(^{-/-}\) cells were pre-incubated with THC or vehicle before LPS stimulation for 24 h, and live cell cathepsin assays were performed. THC did not inhibit upregulation of cathepsin activities in CB2\(^{-/-}\) cells during the LPS response (Figure 21). The lack of CB1 mRNA expression in both macrophage cell lines combined with the inability of THC to modulate cathepsin upregulation in CB2\(^{-/-}\) macrophages suggest a direct role for the CB2 receptor.
Figure 20. Lack of cannabinoid receptor expression in CB2<sup>−/−</sup> macrophages.

CB2<sup>−/−</sup> macrophages were cultured without or with 1μg/ml LPS for 24 h. RNA was prepared for semi-quantitative RT-PCR. CB2 and β-actin cDNA were amplified at different cycle numbers to retain linearity. Amplification of CB1 cDNA proceeded for 44 cycles. Data are representative of 3 separate experiments.
Figure 21. THC-mediated inhibition of cathepsin upregulation requires CB2 receptor expression.

CB2<sup>-/-</sup> macrophage cell line was pre-incubated with vehicle or various THC concentrations for 4 h at 37°C and then cultured without or with 1 µg/ml LPS. After another 24 h, cathepsin activity in live cells was assessed (see Figure 18 legend). Values represent mean ± SE from 3 or more separate experiments. Medium vehicle vs. LPS vehicle: *<i>P < 0.05</i>. Medium vehicle vs. medium THC: NS. LPS vehicle vs. LPS THC: NS.
THC Does Not Cause Apoptosis in P388D1 Cells

Some studies have reported that cannabinoids induce apoptosis in immune cells. The possibility THC causes macrophages to undergo apoptosis, thereby reducing their response to LPS was investigated. P388D1 cells were treated with various THC concentrations, vehicle or sodium arsenite as a positive control for 18 h. Cells were harvested, stained with FITC Annexin-V and analyzed by flow cytometry. As shown in Figure 22 THC did not induce apoptosis in P388D1 cells, even at a concentration 1000-fold higher than what significantly inhibited cathepsin upregulation in LPS-stimulated macrophages. Furthermore, viability assessments after 24 and 48 h in culture using trypan blue exclusion were performed for all above experiments, and average cell viability was greater than 90%, ruling out necrotic cell death as well.

THC Does Not Effect TLR-4 and CD14 Cell Surface Expression

LPS signaling requires TLR-4, MD2 and CD14 expression. The effect of THC on cell surface expression of these receptors and co-receptors was assessed. P388D1 cells were incubated with THC or vehicle for 4 h, and immunofluorescence staining was performed. The monoclonal antibody employed is specific for the functional TLR-4/MD2 complex. Cell surface expression of these molecules did not differ between vehicle- and THC-treated cells (Figure 22).

Discussion

This study is the first one to demonstrate that a cannabinoid influences cysteine cathepsins during an inflammatory response. LPS is the most extensively studied TLR
Figure 22. THC does not induce apoptosis in P388D1 macrophages.

P388D1 cells were incubated with 10 µM THC, 2 µM sodium arsenite as a positive control, or 0.1% ethanol for 18 h. Cells were incubated with FITC-Annexin V and analyzed by flow cytometry.
Figure 23. THC does not alter TLR-4/MD2 or CD14 surface expression.

P388D1 macrophages were incubated with vehicle or the indicated doses of THC for 4 h at 37°C. Cells were harvested and stained for TLR-4/MD2 or CD14 and analyzed by FCM.
ligand and is a classic model for inflammation. LPS significantly increased Cat B, L and S activities in P388D1 macrophage cell line. THC concentrations in the nM range inhibited cathepsin enhancement in LPS-stimulated P388D1 cells, but did not affect cathepsin activities in medium control cells. CP55940 also reduced the level of LPS-increased cathepsin activities, however the degree of suppression was less than that observed with THC, which was unexpected. THC is a partial agonist, whereas CP55940 is a full agonist. The results between may be related to the doses utilized. THC was most effective between 1 nM and 10 nM, while 100 nM was less inhibitory, suggestive of a biphasic response. A number of studies indicate higher concentrations of CP55940 are required to obtain inhibitory effects on macrophage function (Klein, 2006; Raborn, 2007). Perhaps, higher CP55940 concentrations than those examined may be most effective at inhibiting upregulation of cathepsin activities. Biphasic responses of immune cells are often observed with cannabinoids, although the mechanisms involved are not fully understood. Overall this study shows THC effectively inhibited the enhancement of cathepsin activities during inflammation.

Cannabinoids have been reported to induce apoptosis especially in cell lines. Live cell enzymatic assays were utilized to investigate the ability of cannabinoids to interfere with increased cathepsin activities during the inflammatory response. These assays exclude that decreased enzymatic activity was due to cell death. Furthermore, 10 mM THC did not induce apoptosis in P388D1 cells while the cells were sensitive to arsenite-mediated apoptosis. Therefore, apoptosis cannot account for cannabinoid downregulation of cathepsin activities.
Cannabinoids, including THC, may mediate immune modulation through cannabinoid CB1 and CB2 receptors (Schatz, 1997). While immune cells primarily express the CB2 receptor, various stimuli can alter the expression of these receptors. Stimulation of HL-60 cells with phorbol ester increases CB2 mRNA expression. RAW 264.7 cells do not express CB1 receptor in their resting state, which is consistent with multiple immune cell types. However, in response to LPS, CB1 mRNA is induced within hours in this macrophage cell line (Friedman, 1995). Furthermore, cannabinoid receptor expression on macrophages is downregulated in response to IFN-γ or LPS (Caslisle, 2002). The mRNA expression of CB1 and CB2 was assessed in resting and LPS-stimulated P388D1 cells at 6- and 24-h time points. LPS-stimulation did not cause any significant difference in CB2 mRNA expression. Furthermore, there was no detectable CB1 mRNA in resting or LPS-stimulated P388D1 cells indicating that the inhibitory effect observed with THC was not mediated through the CB1 receptor. Utilizing a macrophage cell line deficient for CB2 receptor, the role of this receptor was investigated. Previous studies characterizing the CB2−/− mice demonstrate a lack of cannabinoid-mediated immune modulatory effects in these mice (Buckley, 2000). A macrophage cell line was generated from these mice, and characterization of this cell line revealed it expressed surface molecules characteristic of macrophages and lacked molecules expressed on DC. These cells were also capable of multiple macrophage functions, including phagocytosis and LPS response to secrete cytokines. CB2−/− macrophages not only lacked mRNA expression of CB2, but also CB1, even after stimulation with LPS. Analogous to P388D1 cells, LPS augmented cathepsin activities in CB2−/− cells. However, the inhibitory effect of THC was not observed in LPS-
stimulated CB2−/− macrophages. This finding indicates the CB2 receptor is required for modulation of cathepins by THC during an inflammatory response.

Cannabinoids interfere with multiple macrophage functions. For example, alveolar macrophages isolated from chronic marijuana users are compromised in their ability to produce pro-inflammatory cytokines, such as TNF-α and IL-6, in response to LPS stimulation (Baldwin, 1997). In addition, macrophage cell line RAW 264.7 treated with THC has attenuation of LPS-stimulated iNOS gene expression, which is mediated by inhibition of NF-κB activation (Jeon, 1996). No studies to date have investigated the possibility that THC alters cell surface expression of receptors and co-receptors required for LPS responses. In my study, THC did not change the expression of active TLR-4/MD2 complex or CD14, a co-receptor for LPS. However this does not rule out the possibility that THC affects events downstream in the TLR-4 signaling cascade, thereby leading to the decreased activities observed in THC-treated cells stimulated with LPS. TLR-4 signaling leads to the rapid activation of NF-κB. This transcription factor is a key regulator of cytokine production and, therefore, cannabinoid modulation may be due to a decrease in pro-inflammatory cytokines, which control these proteases.

These results indicate THC via the CB2 receptor interfered with increased cysteine cathepsin activities during an inflammatory response. The inhibitory effect exerted by THC could not be attributed to apoptosis or alteration of cell surface receptors required for LPS response. Similar to MMP, cysteine cathepsins contribute to pathological tissue destruction and tumor angiogenesis. These findings support the possibility of CB2
receptor-selective agonists as therapeutic agents for chronic inflammatory diseases to prevent cathepsin involvement in pathological tissue destruction.
CHAPTER 6: DISCUSSION

TLR Regulation of Cysteine Cathepsins

Cysteine cathepsins participate in joint destruction in RA, tumor angiogenesis, formation and instability of atherosclerotic plaques, and pathological tissue destruction in multiple other chronic inflammatory diseases (Berdowska, 2004). Regulation of these proteases during inflammation is not well elucidated. Although cell activation by TLR ligands contributes to the pathology of chronic inflammatory diseases, the impact of TLR ligands other than LPS on cathepsins has not been investigated. I hypothesized that stimulation through TLR’s would augment cysteine cathepsin activities, which would be an integral part of an inflammatory response. In my studies, LPS, PGN and Poly I:C increased cathepsin activities in macrophages. This increase was, in part, mediated by IL-1β and TNF-α, pro-inflammatory cytokines. Decreased cystatin C gene expression accompanied increased cathepsin activities in LPS-stimulated macrophages. Hence, diminished expression of this endogenous protease inhibitor may contribute to the enhanced cathepsin enzymatic activity.

LPS signals through TLR-4 and activates both MyD88-dependent and –independent signaling cascades (Takeda, 2004). This stimulus increased activity of Cat B, L and S in various macrophage cell lines. MyD88-dependent and –independent ligands regulated the three cathepsins differently from LPS. PGN stimulus through TLR-2 is
strictly MyD88-dependent, whereas the Poly I:C signal via TLR-3 is independent of MyD88 (Trinchieri, 2007). Both ligands significantly increased Cat L and S activities, however enhancement of Cat B activity was minimal. Hence, stimulated Cat L and S activities occurred by either pathway. LPS and PGN stimulation enhanced Cat B activity, however LPS was much more effective. These findings imply that more significant increases in Cat B activity may require activation of both MyD88-dependent and - independent pathways. Furthermore, Cat B upregulation does not occur through activation of only the MyD88-independent pathway.

Multiple cytokines induced by TLR activation regulate cysteine cathepsins, however, the outcome depends on the cell type and these findings are described in detail in Chapter 1 (Chae, 2007; Honey, 2001; 2003; Lemaire, 1997). Very few studies have investigated cytokine regulation of these proteases in macrophages. There are significant differences in macrophage cytokine production in response to various TLR ligands. LPS rapidly induces gene expression of IL-1β, TNF-α, IL-6, IL-12 and IFN-γ in macrophages (Hirschfeld, 2001; Bjorkbacka, 2004; Jones, 2001; Kawai, 2001). All of these cytokines affect can cathepsin activity or expression. Secretion of IL-1β, TNF-α, and IL-6 also occurs in response to TLR-2 ligands by macrophages, however cytokine levels are lower, particularly IL-1β and IL-6, compared with those after LPS stimulation. (Hirschfeld, 2001; Jones, 2001; 2005). The duration of increased cytokine gene expression is also shorter when signaling through TLR-2, and induction of IL-12 and IFN-γ are not usually observed (Hirschfeld, 2001). Similar to TLR-2, TLR-3 induces low-level production of IL-1β, TNF-α, and IL-6 but causes a robust IFN response. My studies showed antibody
neutralization of TNF-α in culture supernatants from LPS-stimulated P388D1 cells completely prevented upregulation of Cat B activity in LPS-non-responsive EOC 20 cells demonstrating an obligatory dependency on TNF-α. Therefore, reduced TNF-α production in response to PGN and Poly I:C may not reach a threshold sufficient to induce Cat B upregulation and may be responsible for the lower levels of increased Cat B activity in response to these TLR ligands. Antibody neutralization of TNF-α or IL-1β in P388D1 culture supernatants significantly reduced the increased Cat L activity in EOC 20 cells, but neither antibody alone decreased Cat L proteolysis to medium control level. I predict neutralization of both cytokines simultaneously would have a greater effect on Cat L activity. Results with Cat S were distinct. In contrast to Cat B and L, Cat S activity was not affected by antibody neutralization of TNF-α or IL-1β. Therefore, Cat S upregulation was not dependent upon on these two cytokines. Other cytokines, such as IL-6 and IFN-γ, IFN-β which were not investigated here, could contribute to Cat S upregulation. While the exact role of individual cytokines was not completely delineated, TNF-α and IL-1β are clearly important for increased Cat B and L activities in macrophages during an inflammatory response. Furthermore, there is differential regulation of Cat B, L and S by MyD88-dependent and –independent TLR ligands.

I originally predicted cathepsin upregulation would be MyD88-dependent because of vigorous cytokine production. Studies indicate pro-inflammatory cytokines regulate cathepsins, and the same cytokines are produced in a MyD88-dependent manor upon TLR stimulation (Bjorkbacka, 2004). Poly I:C, which tranduces a MyD88-independent signal, induces a low level of pro-inflammatory cytokine production in comparison to LPS. Based
on my premise, Poly I:C would have little effect on these proteases. Unexpectedly, Cat L and S activities in P388D1 cells were highest in response to Poly I:C with average fold increases of 4.4 and 3.6, respectively. Perhaps, Poly I:C stimulation induces a different mechanism for enhanced cathepsin activity from that after LPS activation. Unlike LPS and PGN, Poly I:C causes a robust IFN-β response (O’Neil, 2002; 2006; Trinchieri, 2007). Interferons may play an important role in the regulation of these proteases and possibly compensate for the low levels of TNF-α and IL-1β produced in response to Poly I:C. Recently, it was demonstrated IFN production by macrophages in response to Poly I:C leads to the induction of other IRF-dependent gene products, such as IL-27 (Pirhonen, 2007). IL-27 increases cell surface expression of MHC class I and II molecules, and might contribute to the regulation of cathepsins (Feng, 2007). IFN-γ increases Cat L expression in macrophages, however an IRSE element has not been identified in the Cat L promoter (Lah, 1995). IFN-γ also increases Cat S expression in multiple cells types (Zheng, 2005; Strom, 2002). IFN production in response to Poly I:C leads to the activation of IRF-1, which regulates Cat S expression in lung epithelial cells (Pirhonen, 2007; Storm, 2002). In my studies, mRNA expression of Cat L and S was not altered in response to LPS, however changes in cathepsin gene expression in cells stimulated with Poly I:C were not investigated. In contrast to Cat L, IFN-γ decreases Cat B mRNA expression in macrophages, although activity was not measured in this study (Liuzzo, 1999). This result raises the possibility IFN-γ counteracts the low level production of TNF-α in Poly I:C stimulated cells, thereby preventing changes in Cat B activity. Future studies would investigate the effect neutralizing Ab to IFN-α, IFN-β, IFN-γ and IL-27 exert on cathepsin
activities in macrophages stimulated with Poly I:C. These studies indicate the disparity in cytokine production that occurs in response to the various TLR ligands regulate cysteine cathepsins in a differential manor.

I hypothesized both direct and indirect mechanisms of TLR signaling would regulate cysteine cathepsins. Transcription factors, such as NFκB, AP-1, SP-1 and IRF are activated by TLR signaling cascades, and these transcription factors have been shown to regulate cysteine cathepsins. The Cat B promoter region contains several SP-1 sites and a NFκB binding site (Bien, 2003; Quian, 1991). IFN-γ alters mRNA expression of Cat B in microglia, primary alveolar macrophages and macrophage cell lines, however an ISRE has not been identified in the Cat B promoter (Liuzzo, 1999). The promoter region for Cat L contains two AP-2, a SP-1 cluster and a cAMP response element (Ishidoh, 1989). The 5′ untranslated region of the human Cat S gene contains two SP-1 sites, an ISRE site and at least 18 AP1 sites (Shi, 1994). Therefore, I expected to observe some direct effects mediated through TLR signaling cascades. However, the results did not indicate direct effects. The earliest time point at which increases in cathepsin activities were detected was 18 h, supporting the involvement of a secondary mechanism. In addition, the levels of increased activity in EOC 20 cells treated with P388D1 culture supernatants were almost identical to the levels of TLR ligand-stimulated cells. Furthermore, neutralization of TNF-α completely inhibited the upregulation of Cat B. The gene expression of cathepsins and their endogenous inhibitors, cystatins, was not altered in cells stimulated with LPS for 6 h, further supporting the lack of direct TLR signaling effects. These results indicate the effects of TLR signaling on cysteine cathepsins are primarily indirect and mediated by
cytokines.

The increase in cathepsin activities in LPS-stimulated cells may be partially mediated by decreases in cystatin C mRNA expression. LPS stimulation of cells decreased cystatin C expression, but did not significantly change cathepsin mRNA expression. Hence, cytokines did not affect cathepsin gene expression. While neutralization of TNF-α and IL-1β reduced upregulation of Cat B and L activities, there was no effect on Cat S. Yet, cystatin C inhibits all three cathepsins. Thus, the lack of cytokine neutralization affecting Cat S suggests that these cytokines may not regulate cystatin C. This also raises the possibility that mechanisms other than decreased cystatin C lead to increases in cathepsin activity. Although cystatin C is a high affinity endogenous inhibitor of cathepsins, it is primarily active in the extracellular matrix (Kopitar-Jerala, 2006). Studies investigating changes and localization of cystatin C in DC cells indicate cystatin C is not localized to lysosomal vesicles and furthermore do control antigen presentation (Zavasnik-Bergant T, 2005; Sukkari, 2003). In contrast, other studies show decreased levels of cystatin C increase cathepsin S activity and control intracellular levels and trafficking of MHC class II in DC (Boes, 2005; Kitamura, 2005; Pierre,1998). Images generated from the ImageStream® Cell Analysis System demonstrated cathepsin substrate products remained localized within intracellular acidic vesicles in LPS-stimulated cells. Therefore, the significance of decreased cystatin C mRNA expression on intracellular cathepsin activities in LPS-stimulated cells remains unclear. Furthermore, while cystatin C was reduced the level was not statistically significant, supporting a minimal role for cystatin C in the regulation of cathepsins in macrophages responding to LPS.
LPS and PGN increased the extracellular activity of cathepsins. Decreases in the level of cystatin C correlated with increased cathepsin activities in culture supernatants from TLR-stimulated cells. Furthermore, the secreted activity of Cat B and S from cells treated with 10 mM ammonium chloride/mannose-6-phosphate was lower than that observed for LPS-stimulated cells. Several mechanisms may contribute to this phenomenon. Cathepsins are regulated on multiple levels, and protein expression was not investigated in these studies. LPS may increase translation of these enzymes, thereby increasing the overall protein levels. The post-transcriptional regulation of cathepsins has not been extensively studied and the effects LPS may exert remain unknown. One way cathepsins regulate their intracellular levels is by enzyme secretion (Chapman, 2006; Honey, 2003). Therefore, an overload of proteases in intracellular vesicles would lead to the secretion of these enzymes. LPS stimulation could also cause the acidification of the culture medium, thereby promoting enzyme activation. The extracellular microenvironment of inflamed tissues can be acidic enough to allow cathepsins to remain active (Gatenby, 2006). Alternatively, while LPS decreased cystatin C gene expression, this probably did not occur in cells treated with ammonium chloride/mannose-6-phosphate, thereby allowing cystatin C to retain its regulation of secreted cathepsins. Most likely, a combination of these mechanisms contributes to the increased extracellular cathepsin activities. The level of extracellular enzymatic activity is nominal compared to intracellular levels. However, these studies were performed at 24 h and did not investigate the extracellular accumulation of these proteases over an extended time, which would be more representative of chronic inflammation. These results suggest LPS decreases cystatin C
gene expression contributing to increases in extracellular activity of cathepsins, thereby promoting their involvement in tissue destruction.

Regardless of the stimulus utilized, the fold-increase in Cat B activity is the lowest of all three cathepsins. It has been suggested Cat B is not highly active in macrophages, however the level of gene expression was comparable to that of Cat L and S. LPS alters the endosomal/lysosomal location of these proteases, however this effect in the context of other TLR ligands has not been found. (Lautwein, 2002). Recruitment of cathepsins to more acidic endosomal compartments promotes enzyme activation, which may not occur with other TLR ligands. TLR-4 is internalized upon ligand binding and acidification of endosomal compartments is required for ligand-receptor disassociation, which would activate cathepsins (Kobayashi, 2006). Furthermore, a recent study indicates endocytosis of TLR-4 is required to initiate MyD88-independent signaling cascades (Kagan, 2008). Although fluorescence generated by LysoTracker DND-26 was higher in LPS-activated cells, it could not be determined if changes in acidity, or increases in the size or number of acidic vesicles contributed to the increase in cathepsin activities. It is likely multiple mechanisms, including changes in localization within acidic compartments, contribute to the differential regulation of these proteases during the response to TLR ligands.

TLR agonists and antagonists are also currently proposed for various immunotherapies. TLR agonists are currently under investigation for use as vaccine adjuvants to promote immune response. Cathepsins are upregulated in a number of diseases and TLR activation of cells can alter the severity of disease states. These studies indicate patients with chronic inflammatory diseases involving the upregulation of
cathepsins may not be suitable candidates for vaccines using TLR agonists. On the other hand, the increase in cathepsin activities in TLR-activated cells appears to be primarily mediated by cytokines. A number of cytokines are currently targeted for drug therapy, including TNF-α and IL-1β. Both neutralizing antibodies, soluble receptors and receptor antagonists are being utilized to treat chronic inflammatory diseases, such as RA. Cathepsins are increased in the synovial joints from patients with RA. An interesting study would be to investigate whether patients undergoing anti-cytokine therapy have decreased levels of cysteine cathepsins compared to patients receiving alternative therapies. Altogether, these studies indicate anti-cytokine therapies could be useful in preventing the upregulation of cysteine cathepsins that occurs in TLR-activated cells.

**Cannabinoid Inhibition of Cysteine Cathepsin Upregulation**

Cannabinoids, including the major psychoactive component of marijuana, THC, modulate a multitude of immune responses. LPS is often used as a classical model of inflammation. LPS-stimulated P388D1 macrophages have increased cysteine cathepsin activities. P388D1 macrophages lacked mRNA expression of the CB1 receptor however expressed CB2 mRNA in resting and LPS-activated states. LPS-induced upregulation of cathepsins was reduced by cannabinoids THC and CP55940. The effects of THC could not be attributed to cell death or downregulation of the receptors required for a LPS response. Utilizing a macrophage cell line genetically deficient for the CB2 receptor, the role of this receptor was investigated. Cannabinoid modulation of cysteine cathepsins was absent in CB2−/− macrophages. These results suggest a CB2 selective agonists could be used as a possible therapeutic to control cysteine cathepsin upregulation during inflammation.
LPS significantly increased Cat B, L and S activities in P388D1 macrophages. THC and CP55940 reduced the level of increased cathepsin activities, however CP55940 was less effective than THC. THC has lower CB1 and CB2 receptor affinities than CP55940 and acts as a partial receptor agonist. CP55940, HU-210, and WIN55212 have high affinities for both cannabinoid receptors, acting as full agonist. A biphasic effect was observed when investigating the effect of THC on the modulation of cysteine cathepsins. Biphasic effects have been observed in a number of studies. For example, TNF-α production is inhibited by nM concentrations of THC but stimulated by μM concentrations (Pertwee, 2005). Biphasic effects are also observed in immune modulation of cannabinoids on macrophage co-stimulatory activity and antigen processing (Chuchawankul, 2004; Matveyeva, 2000). Cannabinoids also produce biphasic inhibition of cAMP as well as behavioral and stress related responses (Little, 1991; Viveros, 2007). In terms of THC, a possible reason for a biphasic effect is that THC at μM concentrations can behave as a CB2 receptor antagonist rather than as an agonist (Pertwee, 2008). Differential effects among the various cannabinoid agonists are also reported in several studies (Cabral, 2005; Pertwee, 2005). For example, THC and anandamide inhibit pro-inflammatory cytokine production by peripheral blood monocytes, however increases in IL-10 production are specific to THC and do not occur with anandamide (Cabral, 2005). The inhibition of nitric oxide production by RAW264.7 macrophages occurs with palmitoylethanolamide, WIN55212, and CP55940, however CP55940 is less effective and requires higher concentrations (Ross, 2000). The necessity for higher concentrations of CP55940 have also been observed in studies investigating the effects of cannabinoids on macrophage
chemotaxis and pro-inflammatory cytokine production by LPS-stimulated microglial cells (Klein, 2005; 2006; Raborn, 2007). In my studies, CP55940 decreased cathepsin upregulation in LPS-stimulated cells but complete inhibition was not observed. Although LPS stimulation did not alter expression of CB2 receptor mRNA, receptor-coupling efficiency may have been enhanced. This would lead to increased potency of THC, a partial agonist, while not affecting the full agonist CP55940 potency.

Multiple reports have indicated the immune modulatory effects of cannabinoids may be due to the induction of apoptosis or inhibition of cell proliferation (Guzman, 2005). Cell proliferation of P388D1 cells was abolished when cells were cultured with LPS. Cell numbers from medium control cultures treated with cannabinoids were only slightly lower than vehicle control cells, indicating the effect on cell proliferation was minimal. Interestingly, cannabinoid treatment of cells appeared to impair the LPS-induced inhibition of cell proliferation. Cannabinoid receptor signaling cascades can lead to the activation of PI3K/Akt, which promotes cell survival (Guzman, 2005). The significance of this pathway is discussed in greater detail below, however it may contribute to the proliferation observed in cannabinoid-treated, LPS-stimulated cells. Long-term cannabinoid exposure of glioma cells and other cancer cell lines induces apoptosis. The induction of apoptosis in glioma cells is partially mediated by the accumulation of ceramide (Guzman, 2005). Cannabidiol induces apoptosis in primary lymphocytes, which is associated with oxidative stress and activation of caspase-8 (Wu, 2007). Cannabinoid-induced apoptosis of human lung cancer cells, H460, colorectal cancer cells and Jurkat T cells are mediated through mitochondrial pathways involving the induction of pro-apoptotic Bcl-2 family member,
BAD (Anthansiou, 2007; Greenhough, 2007; Jia, 2006). In the majority of these studies, 10 µM or higher THC concentrations are required for apoptosis. The possibility that cell apoptosis contributed to the effects of cannabinoids on cysteine cathepsins was investigated. THC did not induce apoptosis of P388D1 macrophages, even at concentrations as high as 10 µM. Furthermore, cell viability utilizing trypan blue exclusion indicated necrotic cell death also did not occur. Overall, my results indicate cell proliferation, apoptosis and necrosis were not involved in THC-mediated modulation of cathepsin activities.

Studies have shown the immune modulating effects of cannabinoids can be mediated through cannabinoid CB1 and/or CB2 receptors. CB1 receptor is predominantly expressed in cells of the CNS, whereas immune cells primarily express the CB2 receptor (Bouaboula, 1993; Matsuda, 1990; Munro, 1993). This study investigated the role of CB1 and CB2 receptors in cannabinoid modulation of cysteine cathepsins. P388D1 cells lacked CB1 mRNA expression in resting and LPS-activated states, however CB2 mRNA was readily detected. The lack of CB1 receptor expression in P388D1 cells suggested the inhibitory effects of THC were mediated through the CB2 receptor. A number of cannabinoid immune modulating effects are mediated through the cannabinoid receptors. Inhibition of LPS-induced nitric oxide production in RAW264.7 macrophages by WIN55212 is attenuated by the CB2 receptor selective antagonist SR144528 (Ross, 2000). In contrast, the effects mediated by palmitoylethanolamide are inhibited by CB1 receptor antagonist, SR141716A (Ross, 2000). In murine models of endotoxic shock, cannabinoids are protective (Klein, 2005; Cabral, 2005). Furthermore, fever induced in mice by
intraperitoneal LPS injection is attenuated by WIN55212 but is ablated by treatment with CB1 and CB2 receptor antagonists (Benamar, 2007). These studies indicate both CB1 and CB2 are involved in cannabinoid modulation of *in vivo* LPS responses. The specific roles for cannabinoid receptors in macrophage LPS responses *in vitro* have not been extensively studied. Some studies investigating receptor specific effects of cannabinoids on macrophage functions emphasize a role for the CB2 receptor. Chemotaxis of macrophages in response to RANTES is inhibited by THC and CP55940. SR144528 reversed the effects of CP55940, furthermore the inhibitory effects of THC were not observed in CB2\(^{-/-}\) macrophages (Raborn, 2007). Co-stimulatory activity of peritoneal macrophages is inhibited by THC, which is also reversed by SR14528 and absent in cells from CB2\(^{-/-}\) mice (Buckly, 2008; Chuchawankul, 2004). Interestingly, THC inhibits glioma cell invasion by downregulating MMP-2, which is reversed by the CB2 receptor antagonist (Blazquez, 2008). As mentioned previously, MMP and cathepsins are both involved in tumor angiogenesis and tissue destruction. Upon further investigation into the role of CB2 in cannabinoid modulation of cysteine cathepsins, I discovered THC did not alter cathepsin activities in macrophages lacking CB2 receptor expression due to a genetic deficiency. The CB2\(^{-/-}\) macrophages also lacked expression of CB1 mRNA in resting or LPS-activated states. The absence of CB1 expression in P388D1 cells combined with the lack of cannabinoid modulation in CB2\(^{-/-}\) macrophages indicates THC modulation of cysteine cathepsins in LPS-stimulated macrophages is CB2 receptor-mediated. Furthermore, this implies CB2 receptor expression is required for the modulation of cathepsins by cannabinoids during an inflammatory response.
Cannabinoids alter a variety of responses typical of LPS-stimulated immune cells. LPS-activated splenocytes treated with THC produce decreased levels of type I IFN (Klein, 2005), and treatment of these cells with WIN55212 or HU-210 also decreases TNF-α production (Klein, 2005). Endogenous cannabinoids, AEA and 2-AG as well as THC, decrease IL-6 production by LPS-activated J774 macrophages (Cabral, 2005). Alveolar macrophages isolated from chronic marijuana users have decreased IL-1β, TNF-α and IL-6 production upon stimulation with LPS (Baldwin, 1997). THC decreases TNF-α production by LPS-stimulated RAW264.7 macrophages by altering the conversion of TNF-α to the secreted form (Klein, 2005; Cabral, 2005). Due to the multitude of LPS responses altered by cannabinoids, I investigated the effects of THC on cell surface expression of the LPS receptor and co-receptor, TLR-4 and CD14. THC did not alter the cell surface expression of the functional LPS receptor, TLR-4/MD2, or co-receptor, CD14. This rules out the possibility THC-induced downregulation of the LPS receptor molecules is responsible for the immune modulating effects observed in these studies. However, THC may interfere with the downstream signaling cascade.

Attenuation of LPS-induced iNOS production in RAW264.7 macrophages by THC is associated with decreased NFκB activation (Jeon, 1996). NFκB is a master regulator of pro-inflammatory cytokines and is rapidly activated by TLR-4 signaling cascade (Chapter 1, Figure 1). TNF-α production by LPS-stimulated macrophages is NFκB-dependent. TNF-α and IL-1β contributed to the increased activity of Cat B and L in macrophages. Therefore, cannabinoid receptor signaling may interfere with TLR-4 signaling cascades leading to NFκB activation, ultimately inhibiting the production of pro-inflammatory cytokines.
cytokines involved in regulating cathepsins (Figure 24). Alternatively, PI3K and MAPK activation are associated with cannabinoid receptor signaling (Chapter 1, Figure 2) (Howlett, 2005; Sanchez, 2003). The MAP kinases, ERK1/2 and JNK1/2, are activated by both TLR-4 and cannabinoid receptor signaling cascades. The specific role of these kinases in the LPS responses is not clear. However, they may promote an anti-inflammatory response that occurs to control inflammation in LPS-activated cells. This raises the possibility MAPK activation by cannabinoids may increase anti-inflammatory mediators, which decreases LPS-induced inflammatory responses. Recently a critical role for the p110β subunit of PI3K was identified in LPS-induced nitric oxide production by RAW264.7 cells (Tsukamoto, 2008). Genetic deficiency of p110β leads to a loss of Akt activation by PI3K and increases production of nitric oxide and IL-12 by LPS-activated macrophages. The p110β subunit of PI3K is involved in the negative regulation of LPS activation. While Gβγ subunits commonly associate with PI3Kγ, the p110β subunit is also activated by Gβγ subunits (Tsukamoto, 2008). Therefore, cannabinoid regulation of LPS responses may involve the activation of negative feedback regulators, such as p110β.

This study is the first report indicating cannabinoids can modulate cysteine cathepsins during an inflammatory response. THC has been used to treat pain, asthma, glaucoma, AIDS, cancer and a number of other chronic inflammatory diseases (Benamar, 2006). LPS activation of macrophages leads to the production of pro-inflammatory
Figure 24. Model of inflammatory regulation of cysteine cathepsins.

Binding of LPS induces TLR-4 receptor dimerization and the association of adaptor molecules MyD88 and TRAM/TRIF. This in turn leads to the activation of TBK1, IKK and MAPK signaling cascades and ultimately the activation of transcription factors NFκB, AP-1, and IRFs, which promote the production of pro-inflammatory cytokines. Pro-inflammatory cytokines produced in response to LPS increase cathepsin activities and decrease levels of cystatin C. THC binding to the CB2 receptor induces the disassociation of G protein subunits. The Gα subunit interacts with andenyl cyclase, decreasing cAMP levels. The βγ subunit activate PI3K and MKKs, leading to a reduction in pro-inflammatory cytokine production, thereby preventing increased cathepsin activities.
cytokines that contribute to increases in cysteine cathepsin activities. Anti-cytokine therapies are currently utilized to treat some chronic inflammatory diseases, however they are not suitable for all patients. These results indicate THC via the CB2 receptor interfered with increased cysteine cathepsin activities during an inflammatory response. A number of effects cannabinoids exert on inflammatory responses are mediated through the CB2 receptor. Due to the lack of psychoactive effects, CB2 receptor-selective agonists are currently being investigated as therapeutics to treat chronic inflammatory diseases. These findings support the possibility of CB2 receptor-selective agonists as therapeutic agents for chronic inflammatory diseases to prevent cathepsin involvement in pathological tissue destruction.
Literature Cited


Appendix

This Chapter contains supplemental figures.
Supplemental Figure 1. LPS increases cathepsin activity in a dose dependent manor.

Macrophages were cultured in medium with or without the indicated doses of LPS for 24 h. Cells were harvested, incubated with the cathepsin selective MR conjugated substrates and analyzed by FCM. Histograms are representative of two separate experiments.
Supplementary Figure 2. Enhanced cathepsin activities correlate with increasing concentrations of PGN.

Macrophages were incubated in medium without or with the indicated doses of PGN for 24 h. Cells were harvested, washed, viability was assessed and cathepsin activities were measured using AMC conjugated substrates. Data are the mean ± SD from 3 or more separate experiments.
Supplementary Figure 3. Analysis of cathepsin upregulation in response to various doses of Poly I:C.

Macrophages were incubated in medium with or without various doses of Poly I:C for 24 h. Cells were harvested and cathepsin activities were assessed using AMC conjugated substrates. Data are mean ± SD of fold increase over medium control from two separate experiments.
Supplementary Figure 4. Semi-quantitative Real Time-PCR analysis of Cat B, L and cystatin C mRNA expression in macrophages cultured without or with LPS.

Macrophages were incubated without or with 1 µg/ml LPS for 24 h. Cells were harvested and RNA isolation was performed. Quantitative Real Time-PCR using SYBR GreenER™ Two-Step qRT-PCR kit was performed. The BIO-RAD iCycler was utilized for amplification and analysis. Data are relative expression normalized to β-actin from 3 separate samples run in unison without triplicates of each sample.
VITA

Blaine Madison Creasy was born November 21, 1977 in Salem, VA. She graduated from at Liberty High School in Bedford, VA in 1995. She attended Central Virginia Community College from 1998 to 2000 and earned an associates degree in general studies. She then attended James Madison University where she studied Biology and graduated with a B.S. in 2002 with biological honors. In 2003 she joined the Ph.D. program at Virginia Commonwealth University. Throughout the years she has received multiple scholarships and awards to support her education.