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**The Role of Cathepsin L in Bactericidal Activity**

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## Abstract

The goal of this study was to analyze the role of cathepsin L (Ctsl) in innate immune response of mice to *Listeria monocytogenes* infection. *L. monocytogenes* is an important human pathogen that produces a varied immune response ranging from slight flu to severe meningitis. Differences in susceptibility to infection among individuals are often associated with genetic polymorphisms. Inbred mouse strains were used as a model to study effects of genetic polymorphisms on susceptibility to infection. A segment of chromosome 13 known as the L2 locus has been identified as the key locus that control differences in susceptibility of BALB/c (*l. monocytogenes* sensitive) and C57BL/6J (*l. monocytogenes* resistant) mice. Cathepsin L was identified as a candidate susceptibility gene based on its differential expression in macrophages from BALB/c and C57BL/6J mice. We created an expression construct to model differences in Cathepsin L expression found in vivo. This expression construct will be used to determine how differences in cathepsin expression affect response to infection with *L. monocytogenes*. Through the process of testing the expression vector, we were able to identify a novel mechanism of cathepsin regulation caused by DNA and LPS. Introduction of DNA had opposite effects on CTSL levels in BALB/c and C57BL/6J macrophages, which appeared to be controlled at the transcriptional level.

# Table of Contents

# Table of Figures and Tables

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# Background

Dr. Boyartchuks laboratory studies the genetic control of the innate immune response to infection with Listeria monocytogenes. The human population is genetically heterogeneous and as a result individuals display dramatic differences in their immune function. To understand the functional consequences of genetic polymorphisms the lab uses a mouse model of *L. monocytogenes* infection. Common mouse strains differ in their sensitivity to *L. monocytogenes* infection, with BALB/c being sensitive to infection and C57BL/6J being resistant to infection. Using genetic mapping a key locus termed L2 was identified in the middle of chromosome 13 that was responsible for the majority of phenotypic differences between these two strains. One of the genes encoded on the L2 locus is a cysteine protease known as cathepsin L. Cathepsins form a family of enzymes that are produced as inactive zymogens, and reside within the lysosome. There are 16 known cathepsins, which all work together to play important roles within the cell. Cathepsin L is an excellent potential candidate because it was show to play an important role in control of both innate and specific immune function.

## The Clinical Significance of Listeria Monocytogenes Bacterium

Listeria monocytogenes is a common microbe that thrives in a wide variety of environments, including soil, food and fecal matter. *L. monocytogenes* is a gram positive bacterium that is about 0.4-1.5 microns in length. (Vazquez, 2001. page 585) This pathogen is of considerable importance to humans because it can survive and reproduce in high salt concentrations, under low pH and at temperatures as low as 4 degrees Celsius (Vazquez, 2001. page 585). Many foods are packaged and stored in these conditions because they are intolerable to most pathogens except *l. monocytogenes.*

*L. monocytogenes* was associated with cases of food poisoning until an outbreak in the Maritime Province of Canada in 1981. Scientists were able to culture *L. monocytogenes* bacteria from the coleslaw associated with the outbreak. (Wing, 2002. s19) In the past 40 years, there have been an increasing number of outbreaks of food poisoning associated with *L.*

*monocytogenes* due to consolidation of the food service industry and centralized food processing. In 2008, the Center for Disease Control reported 1,600 cases of listeria and 260 fatalities. (Listerosis, 2011)The growing incidences of *L. monocytogenes* food poisoning in recent years make the study of this bacterium increasingly important.

Infection with *L. monocytogenes* commonly causes symptoms of gastroenteritis in healthy adults, and is associated with many point source outbreaks of food poisoning. However, there is a more virulent form of this infection known as listerosis. Listerosis is a serious concern for pregnant or immunocompromised individuals, where it can evade the immune system and cause serious complications. Listerosis is one of the most deadly bacterial infections known, with a 20-30% mortality rate, compared to food infection caused by salmonella, which has a 0.3% mortality rate. (Wing, 2002. s19). Infection with listerosis is one of the greatest concerns to those with compromised immune systems, especially since *l. monocytogenes* is such a widespread pathogen.

In immunocompromised adults who contract listerosis, the bacterial infection commonly follows a biphasic pattern. The first stage (3-10 days after exposure) consists of headache, vomiting, visual disorders, and general malaise (Vazquez, 2001. Page 586). The second stage typically presents itself as severe meningoencephalitis; a potentially fatal condition that occurs when the bacteria cause severe swelling of the brain and meninges. *L. monocytogenes* can be attributed to about 10% of community acquired bacterial meningitis, and in high risk groups (groups with compromised immune systems, like HIV positive patients) it is the most common cause of bacterial meningitis (Vazquez, 2001. Page 586).

Listeria Monocytogenes infection is also a serious threat during pregnancy. The infection is traditionally asymptomatic in the mother, but can invade the fetus via the placenta. This can result in a spontaneous abortion, a stillborn baby, or a baby born with a generalized infection,

known as granulomatosis infantiseptica (Vazquez, 2001. page 586). Since *L. monocytogene*s is such a common microbe, it presents a high risk to the health of pregnant women.

*L. monocytogenes* is of considerable interest to the scientific community not only because of its pathogenicity, but because it has become a commonly used intracellular parasite to study the immune system. *L. monocytogenes* has been used as a probe to help researchers discover many important aspects of the immune system, including the inability of antibodies to protect against infection produced by intracellular pathogens, the importance of activated macrophages in the elimination of intracellular parasites, and that the T cell is the macrophage activating element required or cell mediated immunity. (Vazquez, 2001) Many of the virulence factors that listeria utilizes are common to many other intracellular parasites.

In order to analyze the immune response to *L. monocytogenes,* scientists first needed to reproduce listeria infection in a mouse model. George Mackaness described experimental model of listerosis in mice in 1962 (Wing, 2002. S19) Mice cannot be infected with listeria the same way that humans can be due to differences in the E-cadherin receptor, which typically helps bring listeria into the bloodstream through the epithelial cell walls of the intestine. There is a single amino acid substitution in murine E-cadherin that prohibits intestinal uptake (Wing, 2002. S20). This means that mice cannot be infected with *L. monocytogenes* via the traditional route of ingestion. Scientists are able to combat this by simply infecting the mice intravenously, so the bacteria enter their bloodstream directly.

## *Mechanism of L. Monocytogenes Infection*

L. *Monocytogenes* traditionally infects humans in the food they consume, and enters the human body through the epithelial cells within the intestinal wall. Once the bacteria penetrate this barrier, they do not linger and replicate in the epithelial cells, but move deeper into the body through the bloodstream (Vazquez, 2001. page 590) As Listeria pass through the intestinal epithelial cells, they elicit an extreme immune response that produces symptoms of gastroenteritis in humans, which helps the body rid itself of the bacteria. The bacteria attempt to

avoid this by entering the bloodstream, where they can proliferate away from this attack. Immunocompromised individuals are more susceptible to listeria attack because their initial immune response is slower, so more bacteria are able to enter the bloodstream and proliferate.

Listeria bacteria that manage to enter the bloodstream are quickly brought to the liver, where they encounter both hepatocytes and kupffer cells, which are a a special kind of macrophage that capture bacteria and elicit an immune response. Studies have shown that most bacteria mainly multiply within the hepatocyte cells. (Vazquez, 2001. page 593) During the bacterial multiplication, the hepatocytes release special chemicals that bring neutrophils to the liver to destroy the bacteria and activate the adaptive immune system, usually five to seven days after initial infection.

### Cellular Response to L. Monocytogenes Bacterial Infection

When the *L. Monocytogenes* bacteria adheres to the surface of the cell, it activates receptors that can activate both the adaptive and innate immune system. There are a wide variety of transmembrane cell surface receptors, including TLR receptors, MHC II receptors (which activate the adaptive immune system), and inflammatory receptors that activate the complement system. These receptors tell the cell what to do with the bacteria as it enters the cell through the process of phagocytosis.

*L. Monocytogenes*, like all other bacteria, must enter the cell through the phagocyotic vesicle, which is important because it keeps the bacterium compartmentalized. Meanwhile, the cellular receptors present recognize that *L. Monocytogenes* as a threat and activate various immune biochemical pathways to eliminate it. Compartmentalization is one of the most important strategies of the innate immune system. Compartmentalization helps the cell limit bacterial growth because creates a physical barrier from the rest of the cell, which helps limit nutrient growth and gives the innate and adaptive immune system a place to focus their attack.

The phagosome eventually fuses with a lysosome, creating an acidic environment that is extremely damaging to bacteria. In addition, the lysosome contains an assortment of lipases, nucleases and proteases, which degrade lipids, nucleic acids, and proteins. (Radtke, 2006. Page 1721) Together, these chemicals can degrade intracellular pathogens.

One of the most powerful mechanisms that the lysosome uses to combat *L. Monocytogenes* infection is the use of reactive nitrogen and oxygen intermediates. Phagocyte oxidase uses NADPH to create a superoxide that can destroy bacterial cells. Mutations in phagocyte oxidase are linked to recurrent bacterial and fungal infections (Radtke, 2006. Page 1723). Another important enzyme is inducible nitric oxide synthase (iNOS), which is stimulated by bacterial infection. This enzyme produces nitric oxide, which can also react with bacteria. Reactive nitrogen and oxygen intermediates also prevent *L. monocytogenes* bacteria from escaping from the vacuole (Radtke, 2006, page 1723). This shows the importance of these reactive intermediates to the host's innate response to bacterial infection.

### Virulence factors

Listeria monocytogenes has a number of unique virulence factors that help it evade the adaptive and innate immune system and survive and multiply within the cell. The virulence factor of most interest to this project is known as listeriolysin O. This molecule becomes activated by in a drop in pH (4.5-6.5), which is the point in which the phagosome would merge with a lysosome and the innate immune system could mount an attack. (Vazquez, 2001. Page 598) LLO is able to form pores within the phagosome that allow the bacteria to escape to the cytosol, where they can replicate freely. In addition, another virulence factor known as ACTa creates an actin tail that propels the bacterium into adjacent cells without stimulating the immune system. (Wing, 2002. Page 185) These virulence factors help listeria proliferate within the liver.

## Genetic Polymorphisms of the Innate Immune system

Once bacteria enter the cell, there are a number of different biochemical pathways triggered that activate both the innate and the adaptive immune system. Since these pathways are so complicated and involve a wide variety of proteins and molecules, there is a lot of variation between how different individuals respond to infection. These differences are generally caused by genetic polymorphisms in the genes that control the production and regulation of molecules that are involved in immune response. These polymorphisms are studied using different inbred mouse model systems. These mouse models are used because different inbred strains show a wide range of sensitivities to infection. (Garifulin, 2007. Page 187) Using modern molecular biology resources, it is possible to discern the entire genetic map of these mice and identify genetic polymorphsims between the strains. These polymorphisms can then be linked to differences in immune response.

### Innate Immune Polymorphisms that control response to L. Monocytogenes Bacteria

There is a dramatic difference in the immune response to *L. Monocytogenes* in two different mouse strains known as the C57BL/6J and the BALB/c strains. C57BL/6J mice were able to survive infection with *L. monocyogenes* bacteria indefinitely, whereas BALB/c succumbed to infection within 72 hours. Through quantitative genetic backcrossing of these two strains, the exact genetic polymorphisms that cause this differential susceptibility were associated with a region on chromosome 5 known as the Listr1(L1) locus, and a region on chromosome 13 known as the Listr 2(L2) locus. (Boyartchuk, 2001, page 159).

One or more of the genes in this region that regulate and code for immune proteins are responsible for differential immune response. At this time the exact gene and mechanism are unclear. The Boyartchuk laboratory has been working to identify proteins encoded by genes within these loci that could be responsible. The candidate gene analyzed in this report was a

cysteine protease known at as Cathepsin L. The gene for this protein is located on the L2 locus of chromosome 13, which made it a prime candidate responsible for differential susceptibility.

## Cathepsins

Cathepsin L is a part of a larger group of proteases that degrade proteins by breaking apart peptide bonds. There are 11 known cysteine proteases (cathepsins B, C, F, H, K, L, O, S, V, X W), as well as 2 aspartic proteases (D and E), and 1 serine Protease (G). (Zavasnik, 2006. page 349). Cathepsins are usually about 30kd in size and are composed of disulfide linked heavy and light chains (Turk, 2001. Page 4361) These proteins have slight difference in their amino acid composition and length, but all evolved from the same ancestral gene and use a similar mechanism for protein degradation. Most cathepsins, including Cathepsin L, are endopeptidases, which break bonds of amino acid bonds internally as opposed to breaking the terminal end.

Cathepsins are found in high concentrations (up to 1 mM) inside of lysosomes, because they work best in a slightly acidic reducing environment. (Turk, 2001. page 4631). They are first created in the mannose-6 receptor pathway, then brought to lysosomes via the golgi bodies. In the lysosome, they work to degrade amino acids internally. Many cathepsins are very similar and seem to have redundant roles within the cell, but knockout experiments have shown scientists the important role of each cathepsin. (Turk, 2001. page 4631). They often work together and complement each other because they serve a wide variety of functions within the cell.

Cathepsins are produced as “zymogens”, or inactive proteins. They are produced with a “pro” region, which needs to be cleaved at the N-terminal peptide in order to be activated. The zymogen can be cleaved by other proteases (including pepsin or cathepsin D), or by autocatalytic cleavage at a low pH. (Turk, 2001. page 4629). In this manner, expression of active cathepsin can be highly regulated.

Cathepsins are inhibited by cystatins, stefins, and tyropins, which prevent substrate hydrolysis through competitive inhibition. These proteins bind to the target protein instead of cathepsins, which block cathepsin from binding to the site and prevents substrate hydrolysis and cleavage of the protein (Zavasnik, 2006. page 351). Cathepsins are also inactivated when they escape the lysosome, because of the higher pH in the cytoplasm.

The reason that cathepsins are so highly regulated is because of their high concentrations in the cell and their involvement in many different biochemical pathways. Mutated forms of cathepsin B, H and L have been shown to degrade extracellular membranes and activate enzymes that have been linked to cancer development. Overexpression of cathepsins has been shown to lead to arthritis, cancer, neurological disorders, osteoporosis, and many lysosomal storage diseases (Turk, 2001. page 4632) This shows that careful regulation of cathepsin levels are crucial for many biochemical reactions within the human body.

### The role of cathepsins in response to *L. Monocytogenes*

Cathepsins play an important role in many immune processes, and play a direct role in the degredation of bacteria. Cathepsin D has been directly linked to resistance to *l. monocytogenes* infection. Using a series of knockdown experiments, researchers determined that CTSD (-/-) mice had ten fold more bacteria then their Ctsd (+/+) counterparts. (Del Cerro, 2006. Page 13231) The study went on to show that Ctsd deficient cells were more susceptible to *l. monocytogenes* and that Ctsd controlled the growth of listeria monocytogenes within phagosomes.

This study showed the role of Cathepsin D in the inactivation of the LLO within the lysosome through in vivo LLO degradation analysis. In Ctsd (+/+) mice, 30% of the bacteria was located in phagasomes, and 70% was located in the cytosol, compared to Ctsd (-/-) mice, which had 20% of the bacteria in the cytoplasm and 90% within the cell. (Del Cerro, 2006. Page 1323) As a result of decreased Ctsd levels, *l. monocytogenes* was able to escape the innate mechanisms of the phagosomes and escape to the cytoplasm. This can be attributed to the

virulence factor LLO, so the researchers drew the conclusion that Ctsd was able to directly target the virulence factor, and prevent bacterial escape into the cytoplasm.

This study was groundbreaking because it identified a new innate mechanism that cells used to combat listeria infection. Since many cysteine proteases serve redundant and similar roles, this means that other cysteine proteases, including cathepsin L, could use a similar mechanism to target bacteria within the cytosol.

## Cathepsin L

Cathepsin L(also known as Ctsl or Catl) is one of the 11 endopeptidase cysteine proteases found within the lysosome. It is structurally very similar to other cysteine proteases. It is produced as a monomeric protein, but through post translational processing it is broken into two parts (a heavy and light chain) that are bound together through disulfide bonds. In between these chains is a cleft which contains the active site. The active site of cathepsin L is unique amongst the cathepsins because it has a chemical structure that favors aromatic proteins (specifically phenylalanine) and cleaves proteins at the second position before the cleaved bond. (Reiser, 2010. Page 3422) Cathepsin L is an important protease that plays an integral role in many biochemical pathways, including many immune pathways.

### The role of Cathepsin L in MHCII processing

In order for *L. Monocytogenes* bacterium to activate the innate immune system, they must first come into contact with antigen presenting cells, which include macrophages and dendritic cells. These cells are unique because their surface is coated in major histocompatibility complex II (MHC II) molecules. When bacteria are phagocytized by antigen presenting cells and lysed, their degraded proteins bind with developing MHC II molecules. As these MHC II molecules develop and move to the cell surface, they present the foreign antibody at the cell surface. (Kuby, 2000. Page 210)

T cells can only recognize these foreign antigens when they are bound to the MHC II molecules. When T cells bind to the foreign antibody, they grow rapidly to produce a large number of CD4+ cells, which help identify cells producing the foreign protein, as well as CD8+ cells, which directly lyse the foreign cells. This results in a strong, highly specific bacterial attack.

MHC II molecules are assembled within the endoplasmic reticulum using a chaperone molecule known as the invariant chain, which helps the MHC II protein fold correctly (Beers, 2003. Page 169) Once the protein is properly folded, the invariant chain is released, and mature MHC II molecules are produced. Cathepsin L is important to this process because it binds to the invariant chain, which removes it from the MHCII molecule.

The role of cathepsin L in processing the invariant chain was studied using cathepsin deficient mice. These Ctsl deficient mice had an accumulation of the unprocessed of Ii, as well as deficiencies in selecting CD4+ cells. (Zavasnik, 2006. Page 352). Since II is essential for the processing of MHC II molecules, which help select CD4+ cells, these deficiencies can be directly attributed to underexpression of CTSL.

### The role of Cathepsin L in Caspase-1 activation

There are wide variety of different proteases besides cathepsins that are involved in the innate immune system, including caspases. Caspase-1 is involved in a wide variety of both adaptive and innate immune pathways. Caspase-1 activates both IL-1β and IL-18, which are pro inflammatory cytokines that can result in inflammation and cell death via apoptosis. Deficiencies in Caspase-1 have been directly linked to increased susceptibility to listeria infection (Tsuji, 2004. Page 335). Caspase-1 activation is important to immune response to *L. Monocytogenes.*

Like cathepsins, caspase-1 is produced as a zymogen and needs to be cleaved to be activated. There are a number of different pathways that cause the cleavage of pro-caspase-1 into its active form. These pathways can be triggered by endotoxins, flagellin, DNA, RNA, and crystals. Procaspase-1 is converted to caspase when it interacts with a series of proteins known as an inflammasome.(Elliot, 2009. Page 6546). When the one of the inflammasome proteins is activated, procaspase-1 is converted. The NLRP3 inflammasome is the most well-known inflammaosome that activates Caspase-1,and is activated by a wide variety of compounds.

Caspase-1 activation has been linked to cathepsin-L in circumstances involving lysosomal damage. Lysosomal damage caused by cholesterol crystals caused the rupture of the phagosomal membrane and the introduction of Cathpesin-L and Cathepsin-B into the cytosol. This caused the activation of the NLRP3 inflammasome, which cleaves procaspase-1 into its active form(Duewell, 2009. Page 1258) This mechanism indicates that cathepsin-L indirectly causes activation of caspases-1.

### The role of Cathepsin L in the Complement system

When *L. Monocytogenes* bacteria adhere to the surface of cells, they activate a branch of the innate immune system known as the complement pathway. This pathway consists of a group of about 20 enzymes which are synthesized in the liver and circulate in the blood as "pro enzymes". (Kuby, 2000. Page 330) They cleave each other to activate the system in a biochemical cascade, where each factor is dependent upon the other factors in a highly regulated fashion.

The complement system is activated through either the classical or alternative pathway. In the classical pathway, an antibody binds to an antigen to form an immune complex. This exposes a binding site for the C1 component, which then activates C2, C3 and C4 which results in cleavage and production of C5b. Complement protein C3 contains a thioester bond that can spontaneously hydrolyze at a slow rate and activate the alternate complement pathway. This hydrolysis yields C3b, which binds to foreign surfaces like bacterial cell walls. When C3b binds

to a surface, it can generate more C3b to produce a high concentration of C3b protein on bacterial cell walls. (Kuby, 2000. Page 336) Both of these pathways result in cleavage of C3 and C5, which is the start of the bacterial attack.

In the next step of the biochemical cascade, C5b cleaves C6, C7, C8 and activates C9. These proteins join together in a ring structure called the membrane attack complex (or MAC) that perforates bacterial cell walls and lyses the cell. (Kuby, 2000. Page 338) Since *l. monocytogenes* is gram negative, the cell wall is too thick for the MAC to puncture, but the complement system still plays a crucial defensive role. The complement proteins activate other parts of the innate immune system, including an inflammatory response and secretion of immunoregulatory molecules, as marking bacteria for phagocytosis by macrophages through opsonization (Kuby, 2000. Page 329) Activation of the complement system is important for defense against pathogenic bacteria.

Expression of the C5 and C3 complement protein in the BALB/c and C57Bl/6J mice strains have been directly linked to differential immune response to *L. monocytogenes* bacterial infection. The importance of the activation of the complement system to *L. Monocytogenes* infection was first studied using C57BL/6J mice. This strain was compared to a strain known as A/J congenic mouse strain, which has a frameshift mutation in the gene encoding C5, which prevents it from producing the protein. C57BL/6By mice had a much higher bacterial clearance and survival rate in response to listeria monocytogenes. (Gervais, 1985. Page 2057) This study proved the importance of the complement proteins in resistance, and polymorphisms in these genes could result in differential susceptibility.

Both the gene that codes for C5 and the protein itself are identical in C57BL/6J and BALB/c mice. However, C5 is induced later following infection in BALB/c mice compared to their C57BL/6J counterparts (Patel, 2008. Page 1138) This indicates that C5 is differentially expressed, and controlled by another regulatory element. Since the complement system is

activated by a biochemical cascade, C5 activation is dependent upon upstream compounds, including C3.

Differences in expression of C3 protein could delay the induction of C5. Differential expression of C3 was found between the two mouse strains. Following infection with the trypanosome congolense bacteria, C3 protein levels were slightly higher in C57BL/6J mice. BALB/c mice had higher initial levels of C3, but declined rapidly compared to the other strains measured. (Otesile, 1991. Page 960) This study indicates that there are differences in the expression of C3 protein between the two mouse strain. This differential expression may be caused by polymorphisms in C3, or another protein.

Cathepsin L interacts with the complement system through the C3 member, so it may be responsible for differential activation or deactivation of the system by cleaving C3b. (which cleaves C5) Overexpression of procathepsin-L in melanoma cells caused cleavage of C3b and deactivation of complement system. These tumor cells were 60% more resistant to complement mediated lysis through the membrane attack complex. (Frade, 1998. Page 2736) This study demonstrated the relationship between the complement system and procathepsin-L, and suggests that procathepsin-L may deactivate the innate immune system by cleaving the C3 member.

## Therapeutic implications

Cathepsin L plays many important roles in the active and innate immune system, and differences in cathepsin expression may play a major role in differential immune response to infection with *L. Monocytogenes*. Identifying the functions of cathepsin L and how differential expression effects immune response is important because once an imbalance in a pathway is identified, drugs and therapies can be designed to correct the problem and restore biochemical homeostasis. Mice knockout models are useful tools to study the importance of cathepsin L because murine cathepsin L and human cathepsin L are virtually homologous. Human cathepsin L is coded by a gene on chromosome 9, whereas mouse cathepsin L, which is coded by a gene on chromosome 13.(Reiser, 2010. Page 3423) There are differences in expression levels of

cathepsin L based on transcriptional differences, and more analysis of how genetic polymorphisms alter cathepsin L expression in vivo is necessary in order to understand the role of this cathepsin in disease and target therapies around it.

Analysis of the role of cathepsin L in the innate immune system is important because it could reveal the role of this cathepsin in previously known biochemical pathways, such as the complement pathway or the MHC II pathway. It could also lead to the discovery of a new mechanism that the innate immune system uses to combat disease, similar to the mechanism discovered by Del Cerro-Vadillo in 2006. Cathepsin L was studied in the context of listeria monocytognes infection because this bacteria is not only extremely common and an emerging health problem, but one of the most commonly used probes to understand immune mechanisms. These studies could identify weaknesses in the innate immune system, which could be corrected in order to help human's combat bacterial pathogens by strengthening their innate immune response.

# Methods

## Creation of Overexpression Construct

In order to achieve high levels of Ctsl expression in macrophage –like cell lines, we created an overexpression construct by inserting the cathepsin L cDNA into pCI-neo mammalian expression vector. The first step in this process was PCR amplification and processing of the cathepsin L cDNA. A construct containg a full length Ctsl cDNA in a pYX-Asc vector was obtained from Open Biosystems (catalog # MMM1013-9497592)

### Amplification and processing of cathepsin L gene

Ctsl cDNA was amplified from the pYX-Asc construct using a polymerase chain reaction. During this amplification, restriction sites were added to the ends of the gene that could be used to insert it into the PCI Neo vector. The 5' primer complemented 26 nucleotides to the left of the ATG start sequence of the gene, and also contained the complementary code for the XhoI restrictase recognition sequence. The primer sequence was 5'-ATCTCGAGATGAATCTTTTACTCCTTTTGGCT-3'. The 3' primer complemented 25 nucleotides to the right of the end of the coding region, and contained the complementary code for the XhoI restriction enzyme. The sequence was 5' ATTGCGGCCGCTCAATTCACGACAGGATAGCTG 3' These primers were designed and ordered from UMass Medical school "primer3plus" primer design tool, and synthesized using the Umass Med CORE facilities. The oligonucleotides were resuspended in water and then mixed to a final concentration of 5 µg/µl.

To amplify the cathepsin L cDNA a polymerase chain reaction was set up. This contained 50 ng of the original a pYX-Asc construct was combined with 10x HF buffer (3 µl), 3 µl of the primer mix (5 µg/µl stock), 0.6 µl of DNTP (10 µg/ul stock), and 0.3 µl of TAQ polymerase. 22.1 µl of water was added to bring to a final volume of 30 µl. The product was

vortexed and amplified using a peltier thermal cycler. The initial denaturation step occurred for 2 minutes at 98°C, then the reaction underwent 25 cycles of denaturation for 20 seconds at 98°C, annealing for 30 seconds at 62°C, and extension for 45 seconds at 72 °C. This was followed by a final extension for 10 minutes at 72°c. The product was run on a 1% agarose gel for approximately 45 minutes to ensure PCR amplification took place. The PCR product was processed and concentrated using a promega Wizard SV Gel and PCR Clean-Up System. The concentration of DNA was measured using a thermo scientific Nanodrop spectrophotometer.

### Restriction Digest of the PCI Neo vector and CTSL PCR Product

Next, both the pCI-neo vector and the CTSL cDNA PCR product were cut using restriction enzymes to expose their restriction sites. Four separate restriction reactions were set up for the pCI-neo vector; product cut with both enzymes, product cut with each individual enzyme, and uncut product. A master mix was created consisting of 2 µl of the stock plasmid, 12 µl of 10X NEB Buffer 4, 1.2 µl of BSA (10 µg/µl), and 104.8 µl of $H_20$. 29 µl was aliquoted into each reaction, and 0.5 µl of each restriction enzyme or an equal volume of water was added, bringing the total volume to 30 µl. These samples were heated at 37°c for 1.5 hours, and then the restriction reactions heat inactivated by incubating at 65°c for 20 minutes. 10 µl of the final product was loaded on a 1% agarose gel and run for approximately 45 minutes to verify that the product was successfully digested.

A restriction digest of the insert was also performed to generate compatible ends to the vector. In this restriction digest, 10 µl of the PCR product was combined with 3 µl of 10X NEB buffer 4, 0.3 µl of of BSA (10 µg/µl), and 16.7 µl of water to bring to a final volume of 30 µl. The mixture was vortexed, then 0.5 µl of NotI restriction enzyme and 0.5 µl of XhoI restriction

enzyme. The sample was heated at 37°C for 1.5 hours, then the restriction enzymes were heat inactivated by incubating at 65°C for 20 minutes.

To ensure that the PCR product had been cut, two separate ligation reactions were created for both the digested insert and the undigested insert. One sixth of the restriction digest product (5 µl) was combined with 1 µl of promega T4 ligation buffer and 0.5 µl of promega T4 ligase. The reaction was brought to a final volume of 10 µl with 3.5 µl of water. An identical ligation of the uncut plasmid that did not undergo a restriction digest occurred by combining 1.5 µl of the uncut plasmid with 1 µl of T4 ligase and 0.5 µl of T4 ligase. The reaction was brought to a final volume of 10 µl with 7 µl of water. Both products were ligated overnight at 16 °C. The products were run for approximately 45 minutes on a 1% agarose gel to verify that the restriction digest worked.

### Ligation of PCI Neo and Cathepsin L and Bacterial Transformation

The inset and the vector now had compatible sticky ends and could be ligated together. Two ligation reactions were prepared, one with the insert and one using water instead. Each of these ligation reactions consisted of 4 µl of the cut PCI Neo vector, 5 µl of the construct (or 5 µl of water), 1 µl of T4 ligase buffer, 0.5 µl of T4 ligase, and 0.5 µl of water. This reaction was incubated for 1 hour at 25°C.

To screen for cells that contained the correctly ligated construct, the ligation reactions were transformed into competent E. Coli DH5α. Commercially available cells were slowly defrosted from -80°C stock before transformation. 5 µl of the ligation product was added to 50 µl of competent cells and incubated on ice for 30 minutes. After this incubation, cells were heat shocked for 20 seconds in a 42° C, then put on ice again for 2 minutes. The cells were then added to 950 µl of pre warmed LB broth and incubated at 37° C for 1 hour. 200 µl of the cells

were then plated on LB amp plates overnight. The next day, the ligated containing pCI-neo and insert were compared to the water control, and colonies were selected from both plates. These colonies were removed from the plate and transferred to polysterene culture test tubes containing 2 ml of LB broth with ampicillin. These tubes were incubated overnight at 37 °C.

### Screening of bacterial colonies and processing of construct

The colonies that were produced from these samples were screened through a restriction digest to determine if they contained the insert. DNA was isolated from these cells using an alkaline lysis protocol. Half of the solution was transferred to eppendorf tubes and centrifuged for 1 minute at 8,000G. The supernatant was removed and 110 µl of STETL (8% Sucrose, 5% Triton X-100, 50mM EDTA, 50mM Tris pH 8.0 0.5 mg/ml lysosyme) buffer was added and samples were vortexed. The samples were incubated for 1 minute at 95-100°C, then centrifuged at 16000G for 10 minutes. The pellet of cell debris was removed from the supernatant and discarded using pipette tips, then 110 µl of isopropanol was added to the samples and vortexed. The samples were then spun in a microfuge tube for 10 minutes at 16000G. The supernatant was removed and cells were washed by adding 400 µl of 70% ethanol. The samples were spun in a centrifuge at 16,000g for 1 minute, then the supernatant was removed. The pellet was resuspended in 40 µl of endonuclease free water. The colonies were then cut in a restriction digest using the same restriction enzymes previously used to screen for colonies that contained plasmids with the vector and insert successfully ligated. The digested DNA was separated on a 1% agarose gel for approximately 45 minutes.

Colonies that contained the construct were selected from and a small volume of these bacteria were spread onto LB amp plates using a pipette tip and incubated overnight at 37°c. Three colonies were selected and amplified overnight in 2 ml of LB broth, then colonies were

digested using the same restriction digest protocol as before and analyzed for the presence of the plasmid.

One of these clones was amplified in 100 ml of LB broth + ampicillin overnight. The next day, a Qiagen EndoFree plasmid maxi kit (catalogue number 12362) was used to isolate the plasmid DNA from this preparation. The plasmid DNA was resuspended in 1 ml of endofree water. The concentration of DNA was measured using a thermo scientific Nanodrop spectrophotometer.

## Cell Culture Techniques

Analysis of cathepsin L expression was carried out in vitro using several different cell lines. Different immortalized macrophage cell lines were used for experiments, including RAW264 and ZBM2 macrophages, as well as primary macrophages derived from mice in the lab. All cell culture experiments were carried out in a cell culture hood using sterile, appropriate techniques. The different cell culture mediums and unique culture conditions used for each cell line can be seen in Table 1.**Error! Reference source not found.** Cell culture medium was changed every 3-4 days and cells were split every week. Frozen stock solutions of cells were prepared in Nalgen cryovial tubes with 10% DMSO solution.

## Transient Transfection using Fugene HD reagent

The construct of interest was inserted into cell lines using FuGENE HD transfection reagent. The night before transfection, cells were counted and 500,000 cells/well were plated on 12 well plates. The next morning, cell medium was changed before transfection. An 8:2 ratio of fuGENE HD to DNA solution was prepared in 100 µl of DMEM, so 8 µl of fugene reagent was used for every 2 µg of DNA. When cells were cotransfected with GFP to monitor transfection efficiency, 1.8 ug of plasmid DNA was mixed with 0.2 ug of GFP plasmid DNA. The negative

control used for transfection experiments was the pCI-neo vector alone. This solution was incubated at room temperature for at least 15 minutes, then 50 µl was added to each well in a dropwise fashion.

## Transient Transfection using Lipofectamine

Primary cell lines were also used for transfection experiments, which were derived from Bone marrow of BALB/c and C57BL/6J mice by other lab members and of differentiated for 7 days in the presence of M-CSF. These cells were plated in 12 well plates at a confluency of 500,000 cells/well. The next day, the medium was changed to antibiotic free medium so that the cells would be ready for transfection. The transfection reagents were prepared individually for each transfection. Each component of the transfection reaction was initially prepared separately; 2.5 µg of DNA (or 2.5 µg of water as a negative control) was added to 50 µl of DMEM in 1 eppendorf tube, and in a separate eppendorf tube 2.5 µl of fugene HD was added to 50 µl of lipofectamine reagent. The tubes were incubated for 5 minutes, then the entire volume of 1 tube was transferred to the other tube, bringing the final volume to 100 µl, and the components were mixed by vortexing. These tubes were incubated for 20 minutes at room temperature, and then the entire volume (100 µl) was added drop wise to each well individually.

## LPS Treatment

To determine if Ctsl expression could be caused by endotoxins present in DNA treatments, RAW264 cells were treated with LPS. Cells were plated to achieve confluence, and 1 µg of LPS was added to 1 ml of cell culture medium. Cell lysates were collected immediately after treatment, then again 24 hours later.

## RNA Analysis

RNA was isolated from cells and analyzed to evaluate if the insertion of the construct was causing increased expression of the mRNA that coded for the cathepsin L protein. RNA was isolated using TRIzol reagent and DNAse treated. Next, RNA levels were analyzed using RT-PCR.

### RNA isolation

24 hours following transfection, RNA was isolated from cells using TRIzol reagent protocol. RNA samples were resuspended in 20 ul of RNASE free water and concentrations were determined using a Thermo scientific nano-drop 2000. The concentration of RNA in each samples was normalized by combining 1 µg of from each of these samples with an appropriate volume of water to bring the total volume of 15 µl. A DNase treatment was prepared by creating a master mix that consisted of 2 µl of RDD, 0.6 µl of DNase, 0.4 µl of RNase inhibitor, and 2 µl of water per reaction. 5 µl of the master mix was aliquoted to each sample. The samples were incubated at 37°C for 30 minutes, then 70°C for 10 minutes using a Peltier Thermal Cycler. The volume of these samples was then brought to 50 µl with RNASE free water, creating a final RNA concentration of 0.02 µg/ul.

### RNA Analysis using RT-PCR

Quantification of relative RNA levels was determined by using RT-PCR analysis. In this RNA analysis, concentration could be determined based on when sample fluorescence due to ROX crossed a threshold level (Ct). RNA concentrations were further normalized using the housekeeping gene RPS 17. Technical duplicates were performed for wells containing primers for our gene of interest as wells containing primers for the housekeeping gene. In addition, a control well containing primers for our gene of interest was used that had no reverse transcriptase in it, which verified if there was contamination from genomic DNA.

For both the RPS 17 primer control wells, the Cathepsin L primer wells, and the no reverse transcriptase wells, a "master mix" was created that contained every component of the RT-PCR but the DNA. This master mix contained 10 µl of 10x buffer, 0.8 µl of the primer used, 0.1 µl of Rnase inhibitor, 0.4 µl of ROX, 0.025 µl of SSII-reverse transcriptase (except for the no RT control), and 3.675 µl of water per individual well. 15 µl of this master mix was aliquoted out to each well, where it was combined with 5 µl of the DNA of interest. Samples were carefully sealed and vortexed.

The RT-PCR reaction as performed using a Applied Biosystems 7300 real time PCR system. In this thermocycling procedure, samples were heated to 50°C for 20 minutes so that the primers could anneal to the RNA and the reverse transcriptase could extend the DNA, then heated to 95 °C for 2 minutes to denature the strands. Next, the samples were heated to 60°C for 1 minute so that the primers could anneal to the DNA and the DNA could be replicated again, then heated at 95°C for 15 seconds for the two strands to separate. The last 2 steps were repeated 35 times, then a dissociation stage was added to ensure that the strands of RNA all had the same melting temperature.

RNA concentrations were derived by setting standards and dilutions within the machine. The machine was able to use this to calculate concentrations based on the cycle it took to reach relative fluorescence to this standard based on CT values. These levels were divided by the relative concentrations of RPS17 to ensure that baseline RNA levels were equal. Next, all RNA concentrations were divided by the lowest RNA value to obtain relative RNA concentrations.

## Protein Analysis

The ability of the construct to produce Ctsl protein was determined through SDS-PAGE. Protein levels were normalized by cell counting prior to transfection.

## Protein isolation

Twenty four hours after transfection, wells were washed two times with cold PBS, then cells were lysed in 50 µl of cell lysis buffer (0.4% triton 100x plus 5mM EDTA in PBS). These samples were treated with 4x laemelli buffer (250 mM tris-pH 6.8, 40% glycerol, 8% sds, 0.04% bromphenol blue). Samples were heated at 95°C for 2 minutes, and then centrifuged at 10,000 G for 10 minutes. Samples were occasionally refrozen and reheated to fracture DNA and reduce the viscosity if necessary. Samples were stored at 4°C.

## Protein analysis using SDS-PAGE

Protein expression of Cathepsin L was analyzed through SDS-PAGE using a mini-gel chamber setup. A 15% polyacrylamide resolving gel was prepared from 30% acrylamide mix, 1.5 Molar Tris (pH 8.8) and 10% SDS. A 5% stacking gel was added to the top, prepared from 30% acrylamide mix, 1.0 Molar Tris (pH 6.8) and 10% SDS. Once the gel hardened, the running chamber was assembled and filled with running buffer. The running buffer was prepared from water and a 10% stock solution (72 grams Tris, 15 grams glycine, and 5 grams SDS in 500 ml $H_20$). About 15 µg of sample was loaded into each well, and 15 µl of Bio-Rad Kaleidoscope protein standard was loaded as a marker. The samples were run at 100 volts until the bromphenol blue from the samples was at the bottom of the well. Transfer buffer was prepared from a 10x stock solution (72 grams Tris, 15 grams glycine in 500 ml $H_20$) with 10% MetOH in water. The transfer gel tray was assembled so that the protein ran from the gel (negative end) to the immobolin-P transfer membrane (positive end). The transfer gel was run at 100 volts for approximately 75 minutes.

After transfer, the transfer paper was washed 3x for 5 minutes with PBs with 10% tween 20 solution (PBS-T). The paper was blocked for 45 minutes with 5% nonfat milk in PBST, then washed 3x 5 minutes each with PBS-T. The primary incubation took place overnight at 4°C

with a 250X dilution of goat polyclonal Cathepsin L igG (santa cruz biotechnology-sc-6498). The sample was washed three times for 5 minutes with PBS-T. The secondary incubation took place for 45 minutes at RT with a 50,000X dilution of donkey anti goat IgG (santa cruz biotechnology sc-2020). The membrane was resolved onto x-ray film using HRP solution.

# Results

## Preliminary Research

Cathepsin L was selected as a candidate gene to analyze based on its location within the L2 locus of chromosome 13. Cathepsin L protein expression was analyzed in both BALB/c and C57BL/6J mice. The sequence of the cathepsin gene, as well as the 5' and 3'UTR, were compared in both mouse strains to identify polymorphisms. The differential protein expression and polymorphisms in the 3' UTR confirmed that cathepsin expression was altered in the two mouse strains and gave credence to the hypothesis that it was a candidate gene responsible for differential susceptibility caused by the L2 locus.

### Cathepsin Expresion in BALB/c and C57BL/6J cells

Cathepsin L protein expression was measured in BALB/c and C57BL/6J macrophages before and after infection with *L. monocytogenes* bacteria. There were significantly higher protein levels in C57BL/6J mice compared to BALB/c mice. (see Figure 1) There appeared to be no differences in expression caused by infection with bacteria. This experiment revealed that cathepsin was differentially expressed in these strains, but the cause of this expression was unknown.

### Differences in 3' UTR

To determine the cause of this differential expression, the cathepsin L gene, as well as the 3' and 5' UTR was sequenced in both BALB/c and C57BL/6J mice. The sequences of BALB/c

("C"), and C57BL/6J mice ("B") were compared by using NCBI BLAST software (see Appendix 3) No polymorphisms were identified in the 5' UTR and the coding region, however there were significant differences identified in the 3' UTR. There were 8 different single nucleotide polymorphisms between the two strains ( 332 (A->C), 339 (C-T), 364 (A-T), 439 (T-C) 446 (T-A), 463(G-A), 479 (T-A), 748 (C-T)), and the deletion of a T in BALB/c at nucleotide 544. These polymorphisms could alter post transcriptional regulation and explain the differential expression of cathepsin L.

## Creation of Overexpression Construct

The next step was to determine the correlation between cathepsin L protein and susceptibility or resistance to infection. A plasmid construct was designed that would cause overexpression of cathepsin L in BALB/c derived cells, so that the effect of increased expression on response to bacterial infection could be analyzed in vitro. This construct was created by inserting the Ctsl cDNA region into the mammalian expression vector pCI-neo downstream of its CMV promoter. (see Figure ) This construct could then be transfected into cells to cause overexpression of CTSL.

### Amplification and processing of cathepsin L gene

The first step in this process was to amplify the coding region of the cathepsin L cDNA. This DNA was obtained from OpenBiosystems, where it was encoded in a a pYX-Asc vector . The cathepsin L cDNA inserted into this nonexpression vector contained about 73 base pairs of the 5' UTR and about 314 base pairs of the 3' UTR. The coding region of cathepsin L contained 5 restriction sites, which were identified using the VectorNTI program. (Figure 2). These sites were avoided when determining potential restriction sites that could be used to insert the cDNA into the vector, since restriction digests would cleave the insert. The NotI and XhoI restriction sites were selected to insert this cDNA into the vector, and primers were designed to amplify the

coding region and attach these restriction sites in the right orientation. None of the 3' or 5' UTR was included into the vector, since preliminary data had shown that this region contained polymorphisms that might affect protein expression.

These primers were used to amplify Ctsl cDNA from the pYX-Asc vector using a PCR, which was done in duplicate. To ensure that this PCR amplification worked, the product was screened on an agarose gel (see Figure 3). The gel shows a thick, strong band slightly above the 1 kb molecular marker, which matched the size of the amplification product (1.073 kb). The product was purified using promega Wizard® SV Gel and PCR Clean-Up System. 2.46 µg of DNA were resuspended in 40 µl of water to create a final concentration of .0614 µg/µl.

Next, the blunt ends of the PCR product were removed in a restriction digest using the NotI and XhoI restriction enzymes, which ensured that the restriction sites flanking the insert could be ligated with their complementary regions in the vector. The success of the restriction digest was verified by ligating both the plasmid cut by the restriction digest and the uncut plasmid. Without the sticky ends of the restriction digest, the plasmid would not be able to ligate together, so it would be the same size as the uncut plasmid. The cut plasmid would be able to ligate together at many different places and show a smear of differently sized fragments on the gel. The results of the gel showed that the ligated plasmid had many different segments, representing the fragments that had ligated together (see Figure 4). The restriction digest of the insert was successful.

### Processing of The PCI Neo Vector

The pCI-neo mammalian overexpression vector was used as the plasmid that would be inserted into cells to cause overexpression of the gene of interest. The pCI-neo virus contains a CMV promoter which constitutively promotes expression of DNA downstream. Directly

downstream of this region was a polylinker region (shown in box in Figure ) This region contained a number of restriction sites not present anywhere else within the vector. These sites could be cut to insert exogenous DNA into the vector. The NotI and XhoI sites were selected because these sites were not present in our insert, so they would not be cut during subsequent digests.

The pCI-neo vector was cut using the NotI and XhoI restriction enzymes to create a linear fragment of DNA. The success of the restriction digest of the vector was tested by comparing the vector cut at both ends and the vector cut with each individual restriction digest to an uncut vector (see Figure 5) If one of the restriction enzymes did not work, the product would be the same size as the uncut vector. The results of the gel show that the restriction reaction was successful.

### Assembly of Construct

Next, the digested insert and the digested pCI-neo vector were ligated together using T4 ligase. The ligation product was transformed into E. Coli DH5α to create colonies that could be screened for the construct of interest based on the presence of the ampicillin resistance gene in the pCI-neo plasmid. The colonies were grown overnight on ampicillin plates, so only colonies that contained the plasmid could survive. Several of these colonies were selected to determine if they contained the construct full construct containing the cathepsin L cDNA within the vector. These samples were lysed using an alkaline lysis protocol and the DNA was cut using restriction enzymes to determine if the cathepsin L plasmid had ligated into the vector within any of the colonies. The digested fragments were analyzed on a 1% agarose gel (see Figure 6) This gel shows that colony three contains a cut fragment at about 1 kb, which corresponds to the size of the gene of interest.

Three samples of colony three were grown overnight to ensure that this colony faithfully carried the insert. The samples of the DNA from these colonies was analyzed using the same restriction digest enzymes as previously described, and the digest project was separated on a 1% agarose gel. The results of this gel show that the correct plasmid was selected (see Figure 7). Next, a large scale preparation of plasmid DNA was performed using a Qiagen EndoFree Plasmid Maxi Kit. 0.516 ug of DNA was purified and resuspended in 1 ml of water, creating a final concentration of 0.517 µg/ml.

To ensure that the maxi prep had correctly purified the plasmid of interest, a final restriction cut of the plasmid was performed, and the samples were analyzed on a 1% agarose gel. (see Figure 8) When cut with both restriction enzymes showed a fragment at approximately 1 kb, which corresponded to size of the insert. This shows that the ctsl cDNA was inserted into the vector, and it was faithfully conserved after multiple cloning steps.

## Analysis of Expression of Construct in Various Cell lines

The final construct was a purified circular plasmid that could be transfected into cells, which would cause the cellular machinery to transcribe the area downstream of the CMV and produce protein from the insert. Transfection of cells with this construct would allow us to stimulate differential regulation so it could be studied in the context of *L. monocytogenes* infection. To ensure that the construct worked, it was transfected into different cell lines using fuGENE HD transfection reagent, and Ctsl protein and mRNA levels were measured using SDS-PAGE and RT-PCR .

### Transient transfection in 293 T Cells

To determine if the construct could cause expression of cathepsin L protein, it was tested using 293T cells. These cells are derived from human kidney cells, so they are inappropriate for

analysis of murine immune response. However, these cells have a high transfection efficiency, so they could be used to determine if the construct worked or not. Cells were plated in 12 well plates and grown overnight to achieve confluency, then transfected with fuGENE reagent at a 2:8 ratio. 24 hours later, cells were lysed in Triton-X buffer. Lysate proteins were separated using SDS-PAGE, transferred to a PVDF membrane, and probed for cathepsin L using goat polyclonal Cathpesin L igG and donkey anti goat IgG. (see Figure 9). Protein levels were normalized through cell counting. The results of this blot show dramatic overexpression of cathepsin levels in the plasmid consisting of the pCI-neo vector with the cathepsin L insert when compared to the vector alone and untransfected samples.

### Transient transfection in Raw 264 Cells

Since the construct appeared to work in 293T cells, it could be transfected using our cell line of interest: RAW264 cells. These cells are derived from BALB/c macrophages, which the lab had previously shown had low basal levels of CTSL. Increasing the levels of cathepsin L protein in this cell would allow the lab to determine the direct effect of Ctsl protein on the response of these macrophages to *L. monocytogenes* infection.

Transfection using the construct should increase the amount of Ctsl RNA in cells a significant amount, so RNA levels of untransfected cells, cells transfected with the pCI-neo vector, and cells transfected with the construct were measured from RNA harvested 24 hours after transfection. Quantification was done using RT-PCR software and RNA levels were standardized using the housekeeping gene RPS17. Relative levels can be seen in Figure 10. Untransfected cells had relative RNA concentrations of 1.88 compared to the vector alone (1.49) and the construct (1.51). Factoring in standard deviations, there did not appear to be any increase in RNA levels after transient transfection.

Protein levels of cells transfected with the construct were also analyzed to determine if the presence of the construct caused additional protein production. The cells were transfected and proteins were analyzed in an identical manner to the 293T cells. The results of this SDS-PAGE can be seen in Figure 11. The cells that were transfected with vector alone appeared to have slightly lower levels of CTSL, but not an appreciable amount. Subsequent transient transfections also yielded little overexpression of CTSL in the construct.

### Cathepsin L overexpression in RAW264 cells

After several independent transfections of raw 264 cells were performed, it became clear that the construct could not cause transient overexpression in RAW264 cells. We then tried to optimize the transfection reaction by adding more controls to determine what part of the reaction was causing endogenous expression of cathepsin. The construct used in this experiment was a construct that had been previously designed by the lab (it contained CTSL as well, but was built using different restriction sites by a pervious lab member).

Cathepsin L protein levels were compared in cells that remained untransfected (lane 6), cells that had Opti-MEM added(lane 1), cells that had opti-MEM and fuGENE added (lane 2), cells that had pCI-neo and Opti-MEM(lane 3), and cells that were transfected with pCI-neo, opti-MEM and fuGENE (lane 4), and cells transfected with the construct, pCI-ne, and fuGENE, (lane 5)(Figure 12). This experiment revealed that the presence of extracellular DNA or cytosolic DNA increased cathepsin L protein expression.

Since it was unclear if this DNA was purified using a Qiagen kit, it was possible that the DNA contained endotoxins which could trigger an immune response. To determine if endotoxins could stimulate Ctsl, RAW264 cells were treated with 1 ug/ml LPS and protein was harvested after initial LPS treatment and then 24 hours later. These samples were separated

using SDS-PAGE, transferred to a PVDF membrane, and probed using the same antibodies for Ctsl as previously described. (see Figure 13). After 24 hours, there was significant stimulation of CTSL caused by LPS. This revealed that the presence of endotoxins could stimulate cathepsin L.

Our construct was purified using a Qiagen endofree kit, so it should not contain any endotoxins that would stimulate cathepsin L. We used an endo free pCDNA plasmid to determine if addition of "clean" DNA would also stimulate cathepsin L. Cells were transfected with either water or pCDNA, and RNA samples were harvested 24 hours later to determine if the Ctsl was being stimulated at a transcriptional level, or if this stimulation was the result of another immune mechanism that caused overexpression of cathepsin L protein without causing additional translation of RNA.

RNA was isolated from these cells and the relative RNA levels were analyzed using RT-PCR. Untransfected cells had relative Ctsl mRNA levels of 1.16 compared to 2.10 in cells transfected with just water, indicating a twofold increase in expression levels caused by the act of transfection. In cells transfected with the pCDNA vector, mRNA levels were about 5.49 compared to the other cells. This can be seen in figure Figure 14. This experiment showed that the presence of endofree DNA caused almost a three fold increase in the amount of cathepsin L mRNA expression.

## DNA stimulation & Cathepsin L expression in various cell lines

The results of transfection experiments suggested that endotoxins, as well as either cytosolic or extracellular DNA had an effect on cathepsin L expression. There are several receptors, both intracellular and in the cell membrane, that could trigger cathepsin L expression. We analyzed the extent of this expression in both BALB/c and C57BL/6J derived cell lines and

primary cells to determine if this mechanism was dependent upon the level of DNA, and if the mechanism differed between the cell lines.

### DNA stimulation of Raw264 Cells (BALB/c derived)

In previous experiments, the addition of plasmid DNA had been shown to increase cathepsin L mRNA levels. In order to determine if this consistently caused increased protein production, as well as to determine the extent of the sensitivity to plasmid DNA concentration, cells were retransfected using a serial dilution of plasmid DNA. Cell numbers were normalized through cell counting, then the next day cells were transfected using fuGENE HD reagent in an 8:2 fuGENE to DNA ratio. The amount of DNA varied from 3 µg to 1 µg to 1/3 µg to 0 µg (water was used instead). These values were chosen because they were slightly above the normal amount of DNA added (2µg). 24 hours after transfection, cells were lysed and protein levels were analyzed using the same SDS-PAGE as previously described.

This experiment was independently performed on two separate occasions, and both experiments yielded similar results, which can be seen in Figure 15 and

Figure 16. Cathepsin L levels were very low in the presence of water and fuGENE reagent alone. Addition of DNA caused a sharp increase in Ctsl protein expression. This increased expression appeared to be stimulated by even low levels of DNA, and did not seem dependent on the amount of DNA added.

### DNA stimulation of ZBM2 cells (C57BL/6J derived)

Cathepsin L expression caused by DNA response was also analyzed in ZBM2 cells, which are derived from C57BL/6J mice. These mice are more resistant to infection with L. monocytogenes, and preliminary results showed they had higher basal levels of cathepsin L.

These cells were transfected and protein levels were analyzed in identical manner to the RAW264 cells.

Two independent transfections were performed that yielded similar protein expression results, which can be seen in

Figure 18 and Figure 19. The initial levels of cathepsin L were reduced when additional DNA was added. Unlike RAW264 cells, cathepsin L levels were reduced in a gradient fashion corresponding to the level of DNA added, with almost no Ctsl protein expressed when 3µg/ul of DNA were added. These cells also contained both the pro and processed form of cathepsin L, which is indicated by two bands. The protein expression patterns of the ZBM2 cells completely contradicted the results from the RAW264 cells.

### DNA stimulation of Primary Macrophages

The differences in these immortalized cell lines were striking, so we decided to investigate if the differences in cathepsin L expression caused by the addition of DNA could be replicated in primary cells derived directly from BALB/c and C57BL/6J mice. Bone marrow cells were harvested from mice and differentiated for 7 days in the presence of M-CSF. Following differentiation, these cells were transfected with 2.5 µg of DNA (or water as a negative control) using Lipofectamine-2000 transfection reagent. The next day, cells were lysed and protein levels were analyzed using SDS-PAGE electrophoresis.

The level of cathepsin L in these samples can be seen in Figure 19. Basal cathepsin L level stimulated with a water control was much higher in C57BL/6J mice, which matched previous lab data. There appeared to me more of the processed form in C57BL/6J mice, whereas BALB/c mice had an even distribution of the pro and processed form. The addition of DNA

significantly reduced Ctsl levels in C57Bl/6J mice, which mimicked the results of the ZBM2 transfections. The addition of exogenous plasmid DNA did not seem to affect cathepsin L levels in BALB/c primary cells.

## Discussion

Cathepsin L was identified as a potential candidate gene responsible for differential susceptibility to *L. monocytogenes* based on its location on the L2 locus. Protein levels of cathepsin L in BALB/c and C57BL/6J mice were analyzed before and after infection. Cathepsin L expression was significantly higher in C57BL/6J mice, which are resistant to infection. The cathepsin expression was not affected by infection with listeria, indicating that there is always a higher basal level of cathepsin present in macrophages that is not stimulated by bacterial infection.

To determine the cause of differential protein expression, the genomic sequences of BALB/c and C57BL/6J mice were compared using NCBI BLAST software. There were no differences of the 5' UTR or the coding region, but there were 8 single nucleotide polymorphisms and 1 deletion in the 3' UTR of these mice. Since the 3' UTR can control post transcriptional regulation, differences in this region could be responsible for differential expression of cathepsin L. These results confirmed that cathepsin L was a candidate gene that could be the cause of differential immune response in BALB/c and C57BL/6J mice.

A mammalian overexpression construct was created using the pCI-neo overexpression vector. This construct was able to successfully overexpress cathepsin-L in 293-T cells. Our goal was to use this construct in BALB/c derived cell lines to determine how increased cathepsin production affected response to *L. monocytogenes* infection. RAW264 macrophages (immortalized cell line derived from BALB/c mice) were selected for these experiments because of examples of successful transfection using fuGENE reagent in primary literature and in previous laboratory experiences.

To our surprise, there were no changes in cathepsin L expression at the transcriptional or translation level as judged through RT-PCR and western analysis after transient transfection. The construct that had proven successful in other cell lines could not be expressed in these macrophages. This could be attributed to the low transfection efficiency of these cells, which could be observed when cells were cotransfected with GFP. If only a small amount of cells were expressing the plasmid, it would be difficult to detect these differences.

A solution to this problem is the creation of a stable cell line, which would only contain cells that had spontaneously inserted the construct into their genomic DNA. This cell line was started by another member of the lab using the construct I created, but, like the transient transfections, it showed no RNA overexpression. Two other constructs created by other lab members prior to the start of this project also were unable to transiently or stably cause Ctsl overexpression.

The problems associated with creating a Ctsl overexpression construct had been attributed to problems caused by including sections of the untranslated region, or the use of a weaker expression vector (pCDNA3). However, after this expression construct was also unsuccessful in this cell line, evidence was mounting that Cathepsin L expression may be regulated in macrophages, and an unknown mechanism related to the transfection process was affecting this regulation. Since cathepsin L plays a role in many biochemical pathways, it is known to be highly regulated. Cathepsin L RNA levels could be regulated in macrophages through siRNA silencing, in order to keep the inflammatory immune system in a balance.

An experiment designed to identify transfection conditions that affected cathepsin L protein expression showed that the addition of extracellular or cytosolic DNA (introduced

through fuGENE) caused increased cathepsin L expression in RAW264 cells. This increased expression could be caused by endotoxins present in the DNA preparation. Since plasmid DNA was grown and isolated from E. Coli bacteria, it could contain endotoxins such as LPS (lipopolysaccharide) that were present in the cell membrane. These endotoxins are known to stimulate immune and inflammatory processes. LPS treatment of RAW264 cells revealed that the addition of LPS directly stimulated Ctsl protein expression.

The cell membrane of different cells, including macrophage membranes, contains receptors that recognize endotoxins, and initiate an immune response. The transmembrane receptor TRL4 has been binds to LPS and stimulate the NF-kB family of transcription factors (Chow, 1999, page 10689) These transcription factors cause production of IL-1β, IL-6, and IL-18, which are cytokines that cause immune response and apoptosis. (See Figure 20). It appears that activation of the TRL4 receptor could directly or indirectly increase Ctsl protein levels. It is unclear if the activation of the receptor itself, the transcription factors, or the increased levels of inflammatory cytokines cause this activation. One possible candidate is IL-6, which has been proven to increase Ctsl mRNA levels in a concentration dependent manner (Gerber, 2001, page 11). The addition of LPS and subsequent increase in IL-6 levels could be responsible for increased Cathepsin L expression.

This increased Cathepsin L expression would not be a factor for the innate immune response to *L. Monocytogenes* infection, since it is a gram positive bacterium that does not contain LPS. Genetic differences in this mechanism would not alter immune response to L. Monocytogenes. However, this experiment was important to the lab because it revealed the importance of preparing DNA using an endofree kit. The Qiagen endofree kit guarantees less then 0.056 ng LPS/ µg DNA purified (EndoFree Plasmid Maxi Kit, 2003). This is significantly

less LPS then used in previous LPs experiment. This experiment should be done again using a diluted amount of LPS comparable to the maximum amount found in an endofree kit to ensure that this level of LPS could not cause appreciable Ctsl expression. In addition, the endotoxin levels of the construct should be measured using LAL (Limulus Amebocyte Lysate) to ensure that these levels are low.

Transfection of RAW264 cells with an empty mammalian overexpression vector (pCDNA3) caused increases in Ctsl mRNA expression. This indicated that differential cathepsin L expression was not associated with the gene carried in our vector, and that addition of plasmid DNA caused increases in Ctsl transcription. Since these cells were transfected with the plasmid, it is unclear if the DNA causing increased transcription originated from the extracellular environment or from within the cytosol. Future experiments are necessary to determine the origin of this DNA.

Extracellular DNA could be phagocytized into the cell, where it could interact with the TRL9 receptor, which is located in the phagosome and is responsible for trafficking endosomal compartments. (Mayer, 2010, page 264.) The activation of the TRL9 receptor also activates the NF-kB receptor, and could cause increased transcription of Cathepsin L through the production of IL-6 or another pro inflammatory cytokine. (see Figure 21)

The transfection reaction also introduced DNA into the cytosol of macrophage cells, which could activate an alternate pathway. The AIM2 receptor binds to cytosolic DNA as well as the ASC protein and pro caspase-1 to form an inflammasome. (Hornung, 2009, page 415). This inflammasome cleaves the pro form of caspase-1 to produce an activated form of caspase protein. The activated caspase-1 protein causes inflammation and apoptosis.

The activation of the AIM-2 receptor could also be the cause of increase cathepsin L protein production. The increase in Cathepsin L protein production could lead to lysosomal damage, which would stimulate another inflammasome known as NLRP3, which is activated by lysosomal damage and the translocation of cathepsin into the cytoplasm. (Duewell, 2010, page 1357) This inflammasome also activates and cleaves caspase-1 producing an active form of caspase that can lead to inflammation and apoptosis. This pathway suggests that increased cathepsin L production could stimulate caspase-1 via the NRLP3 receptor. (see Figure 22)

Caspase-1 has proven to be an important factor for resistance to *l. monocytogenes* infection, and cathepsin L could play a role in caspase-1 activation by stimulating the NRLP3 receptor. In the context of listeria infection, dsDNA from listeria could activate this pathway. The next step in this research needs to be to determine if extracellular or cytosolic DNA is responsible for cathepsin L expression, and further analysis of the mechanism responsible needs to take place to determine the exact location of the pathway that causes Ctsl mRNA production.

Cathepsin L is clearly involved in a number of biochemical pathways within RAW264 cells. These pathways are important to identify when working with cells during transfection reactions, but the exact mechanisms may not have any effect on susceptibility. It was previously known that Cathepsin L had differential basal expression in these cells, so Cathepsin L expression was analyzed in the presence of plasmid DNA. These experiments were important because it showed that this pathway was significantly different within the two mouse strains, and gave a broader context for these results.

Cathepsin L levels were higher in C57BL/6J primary macrophages and immortalized cells (ZBM2) then BALB/c primary macrophages and immortalized cells (RAW264). These

results confirmed the results found in preliminary experiments. The addition of DNA caused complete suppression of Ctsl protein in C57BL/6J primary cells, and a gradual suppression proportional to DNA concentration in C57Bl/6J immortalized cell lines. In complete contrast, the addition of exogenous DNA caused no change in Ctsl protein expression in primary BALB/c cells, and increased Ctsl protein expression in BALB/c derived immortalized cells.

These results were provocative because they unveiled a fundamental difference in the pathway of dsDNA recognition and activation of cathepsin L transcription and translation within the two mouse strains. Our initial hypothesis that increased Ctsl expression in C57BL/6J macrophages was responsible for their increased resistance to listeria. The differing immune mechanism induced by dsDNA indicates that Ctsl expression is multifaceted and differentially controlled within these strains. More analysis of this mechanism is necessary to identify specific components that are different within the strains. These experiments involving dsDNA need to be put into the context of *L. monocytogenes* infection. The pathway that is activated by plasmid dsDNA may also be activated by bacterial DNA, or another element.

## Concluding remarks and Future experiments

Cathepsin L is a candidate gene responsible to differential susceptibility to *L. Monocytogenes* based on its location on the L2 locus, differential expression in BALB/c and C57BL/6J mice, and polymorphisms in the 3' UTR. Through this project, I was able to successfully create a mammalian overexpression construct that could increase cathepsin L protein production in vitro. However, the effectiveness of this construct is limited because multiple experiments have shown significant problems with overexpression in macrophage cells.

These problems may be attributed to the fact that cathepsin L is involved in many immune pathway, and its regulation is multifaceted. Initial experiments indicate that LPS may

effect Ctsl overexpression through the TRL9 receptor. This is not a factor in L. Monocytogenes infection, but is important in transfection experiments because DNA used must be endofree. Endotoxin levels of the construct need to be tested using LAL, and RAW264 cells should be stimulated using an equivalent amount of LPS to ensure that endotoxins will not affect cathepsin l expression during in vitro experiments.

Cathepsin L expression in RAW264 macrophages is also affected by the presence of either extracellular DNA through the TLR9 receptor, or cytosolic DNA through the AIM2 receptor. Experiments may need to be redesigned to use significantly less DNA, however even small amounts of DNA caused Ctsl expression. Another BALB/c derived macrophage cell line may need to be used to conduct overexpression experiments, for example J774.1 macrophages. Alternatively, the effect of Ctsl overexpression on *L. monocytogenes* infection may need to be studied in a completely new context using knockdown experiments. Since Ctsl expression is initially higher in C57BL/6J derived cell lines, siRNA could be designed to suppress or silence Ctsl expression. The effect of these diminished Ctsl levels during L. monocytogenes infection could be analyzed to determine if decreased expression of Ctsl caused more susceptibility to *l. monocytogenes.*

The biochemical pathways that respond to the presence of extracellular or cytosolic dsDNA appear to be differentially regulated in BALB/c and C57BL/6J mice. The presence of extracellular DNA causes no change in CTSL expression in BALB/c cells, and dramatically reduces Ctsl levels in C57BL/6J cells. This differential expression needs to be studied in more detail determine if cytosolic or extracellular DNA is responsible, and to pinpoint the exact component of the mechanism that governs differential Ctsl stimulation. This expression also

needs to be studied in the context of L. Monocytogenes infection, to determine if differential regulation of this pathway is responsible for differences in susceptibility.

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# Appendix 1: Tables

Table 1: Cell lines used and culture medium and conditions

| Cell Line | Origin of line | Culture Medium | Culture vessel used |
|---|---|---|---|
| **RAW264** | BALB/c Derived | DMEM+10% FBS+ P/S | Adherent cells: cell culture plate |
| **ZBM2** | C57BL/6J Derivied (primary) | DMEM+10% FBS+10% LCCM+ P/S | Adherent cells: Sterile petri plate |
| **293T** | Human Embryonic kidney cells | DMEM+10% FBS+ P/S | Adherent cells: cell culture plate |
| **Primary BALB/c and C57BL/6J cells** | Derived from bone marrow of mice in lab | DMEM+10% FBS+10% LCCM+ P/S | Adherent cells: cell culture plate |

## Appendix 2: Figures

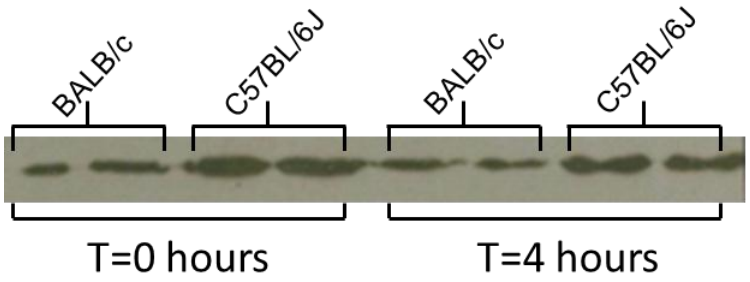


**Figure 1: Ctsl expression in primary macrophages following transfection**

Western blot showing cathepsin L levels before infection (T=0) and 4 hours after infection (t=4) in both BALB/c and C57Bl/6J macrophages.

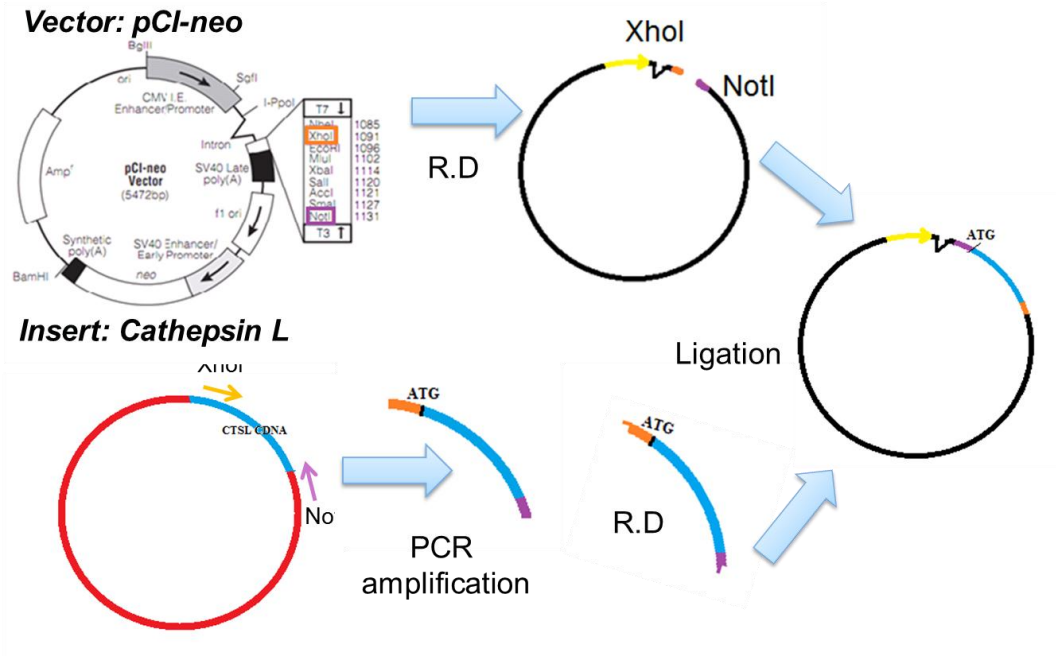


**Figure 2: experimental Design of overexpression Vector**

The pCI-neo vector was digested using XhoI and NotI, which created a linear DNA fragment. The cathepsin L cDNA was amplified in a PCR reaction using primers that included the XhoI and NotI restriction sites. After a restriction digest to create sticky sites compatible with the ends of the vector, the insert and the vector were ligated together to form a complete plasmid.

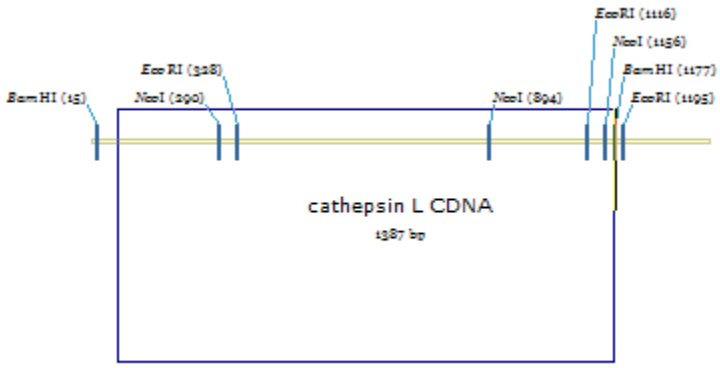


**Figure 2: Restriction sites with Ctsl cDNA**

The Vector NTI analysis of the Ctsl cDNA within the pYX-Asc vector. The blue box contains the coding region, and the restriction sites are indicated.

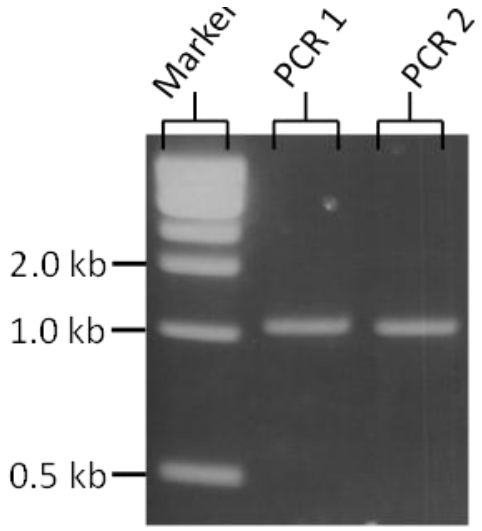


**Figure 3: PCR amplification of Ctsl cDNA**

Agarose gel loaded with 1 kb marker (lane 1), and DNA from PCR amplification of pYX-Asc construct from openbiosystems (lanes 2 and 3)

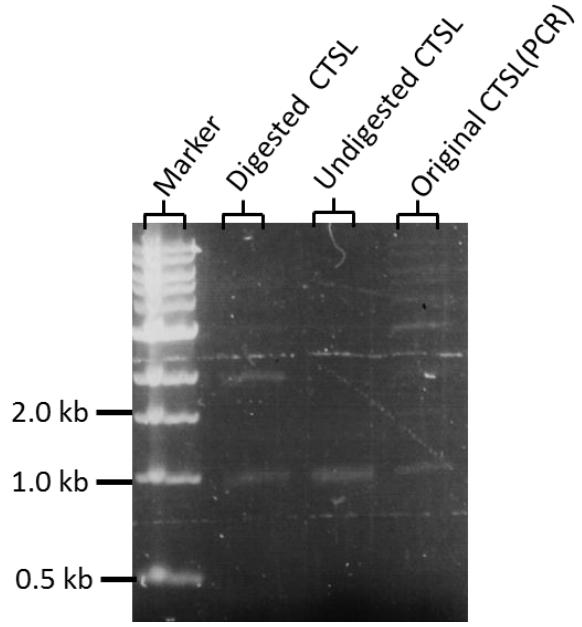


**Figure 4: ligation of digested and undigested Ctsl**

Agarose gel loaded with 1 kb marker (lane 1), Ctsl pcr product digested with XhoI and NotI enzymes and ligated (lane 2), undigested Ctsl PCR product following ligation (lane 3), and the original PCR product (lane 4)

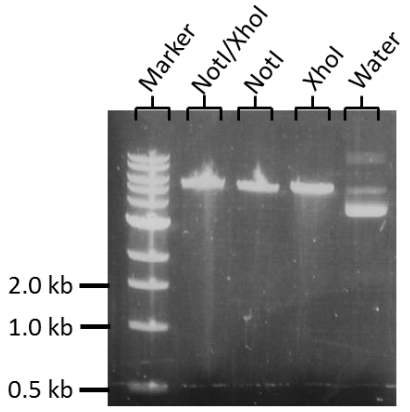


**Figure 5: Restriction digest of pCI-neo**

Agarose gel loaded with 1 kb marker (lane 1), pCI-neo vector digested with NotI and XhoI (lane 2), pCI-neo vector digested with notI (lane 3), pCI-neo vector digested with XhoI (lane 4), and undigested vector (lane 5)

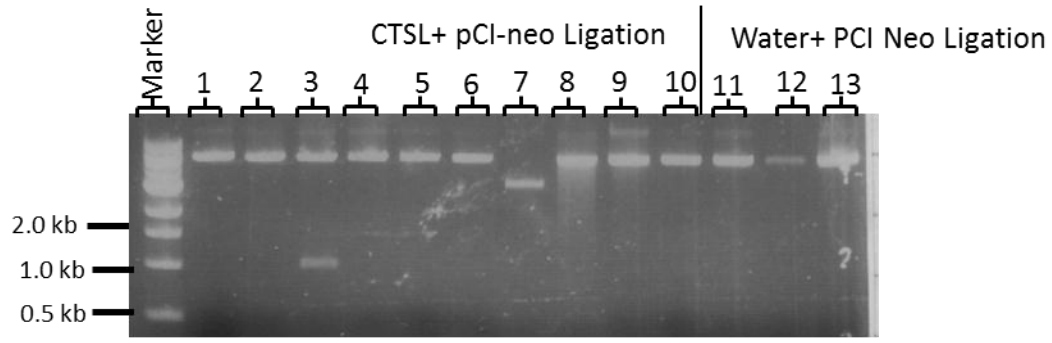


**Figure 6: Restriction digest of colonies**

Agarose gel loaded with maker (lane 1), DNA of colonies transformed with construct and digested using XhoI and NotI (lanes 2-10), and DNA of colonies transformed with pCI-neo and digested using XhoI and NotI (lanes 12-15)

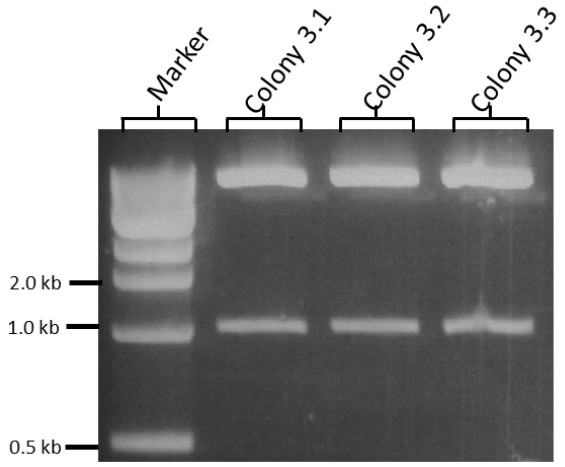


**Figure 7: restriction digest of clones isolated from colony 3**

Agarose gel loaded with marker (lane 1), DNA of clones isolated from colony three and digested using XhoI and NotI (lanes 2-4)

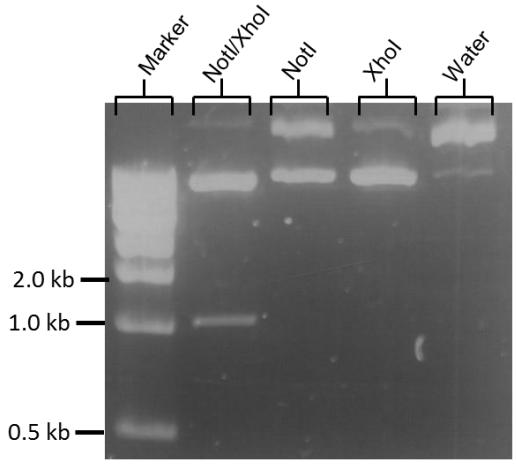


**Figure 8: Restriction digest of Qiagen endofree prep product**

Agarose gel loaded with 1 kb marker (lane 1), construct vector digested with NotI and XhoI (lane 2), construct digested with notI (lane 3), construct digested with XhoI (lane 4), and undigested construct (lane 5)

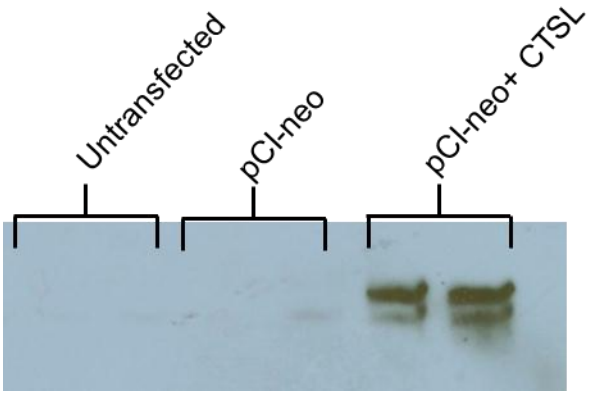


**Figure 9: Ctsl levels in 293 T cells following transfection**

Western analysis of Ctsl levels in 293T cells 24 hours after infection. Lanes 1 and 2 are untransfected cells, 3 and 4 are cells transfected with pCI-neo vector, Lanes 5 and 6 are cells transfected with construct.

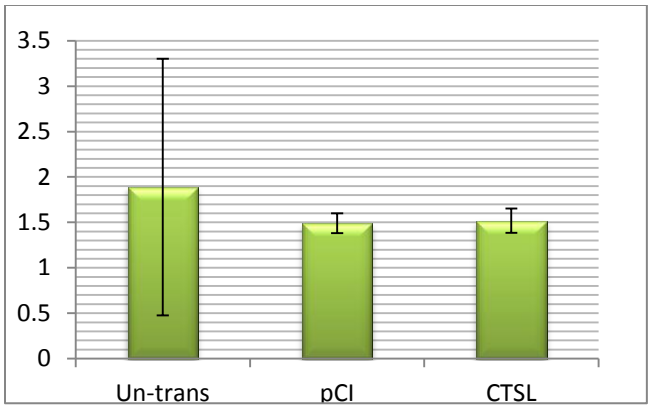


**Figure 10: RNA levels of RAW264 cells following transfection**

RT-PCR analysis of RAW264 mRNA levels. mRNA was isolated using TRIzol 24 hours after transfection. The first bar shows relative RNA levels of untransfected cells, the second bar shows relative RNA levels of cells transfected with pCI-neo vector alone, and the third bar shows relative RNA levels of cells transfected with the construct.

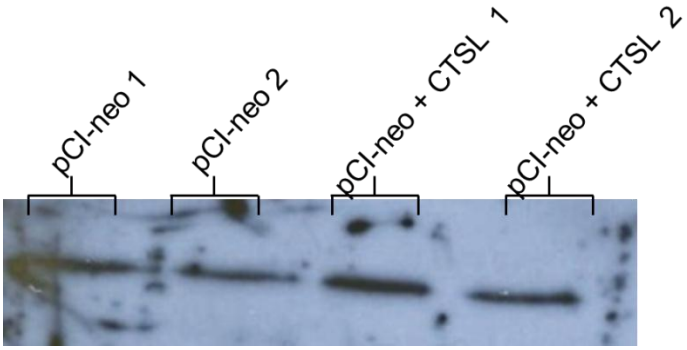


**Figure 11: Protein levels of transfected RAW264 cells**

Western analysis of Ctsl levels in RAW264 cells 24 hours after infection. 1 and 2 are cells transfected with pCI-neo vector, and Lanes 3 and 4 are cells transfected with construct.

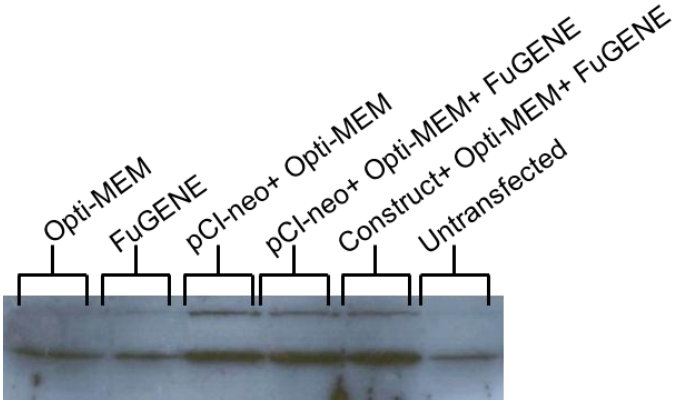


**Figure 12: Ctsl levels in different RAW264 Tranfection conditions**

Western analysis of Cathepsin L expression in RAW264 cells 24 hours after transfection in order to optimize transfection conditions. Lane 1 shows protein expression in cells with Optimem added, lane 2 shows protein expression in cells with FuGENE added, Lane 3 shows protein expression in cells with just pCI-neo vector added, lane 4 shows protein expression in cells transfected with pCI-neo vector using fuGENE, lane 5 shows protein expression in cells transfected with another Ctsl construct, and lane 6 shows untransfected cells.

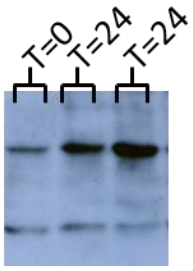


**Figure 13: LPS treatment of RAW264 cells**

Western analysis of Cathepsin L expression in RAW264 cells. Lane 1 shows expression in cells before LPS treatment (T=0), and lanes 2 and 3 shows expression in cells 24 hours after treatment with 1 µg/ml of LPS (T=24)

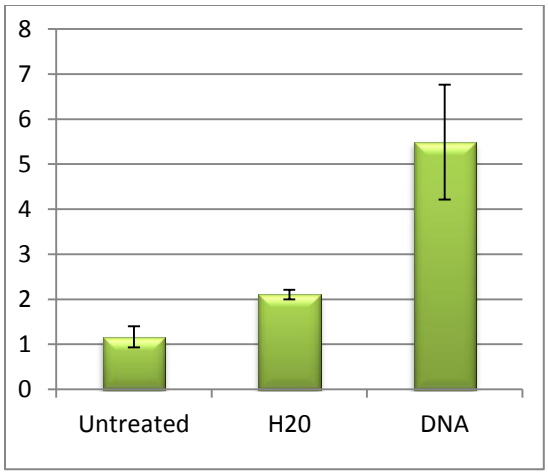


**Figure 14: Ctsl mRNA in RAW264 following transfection**

RT-PCR analysis of RAW264 mRNA levels. mRNA was isolated using TRIzol 24 hours after transfection. The first bar shows relative RNA levels of untransfected cells, the second bar shows relative RNA levels of cells transfected with water, and the third bar shows relative RNA levels of cells transfected with pCDNA.

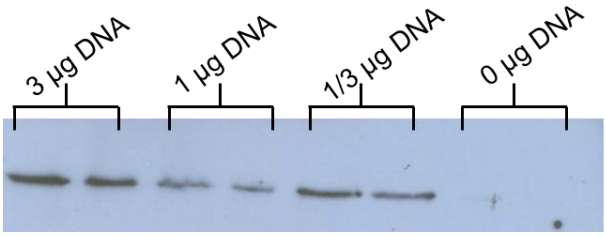


**Figure 15: Trial 1 Raw264 Ctsl expression following DNA stimulation**

Western analysis of CTSL levels in RAW264 cells 24 hours after transfection. Lanes 1-2 show transfection with 3 µg of DNA, lanes 3-4 show transfection with 1 µg of DNA, lanes 5-6 show transfection with 1/3 µg of DNA, and lanes 7-8 show transfection with 2 µl of water.

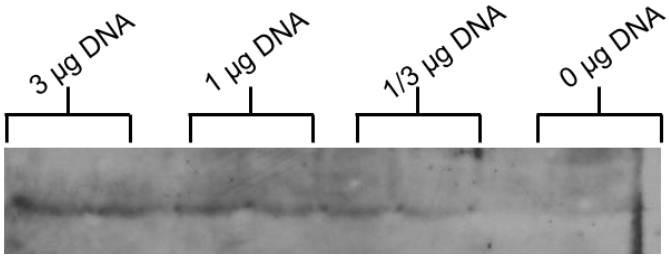


**Figure 16: Trial 2 RAW264 Ctsl expression following DNA stimulation**

Western analysis of CTSL levels in RAW264 cells 24 hours after transfection. Lanes 1-2 show transfection with 3 µg of DNA, lanes 3-4 show transfection with 1 µg of DNA, lanes 5-6 show transfection with 1/3 µg of DNA, and lanes 7-8 show transfection with 2 µl of water.

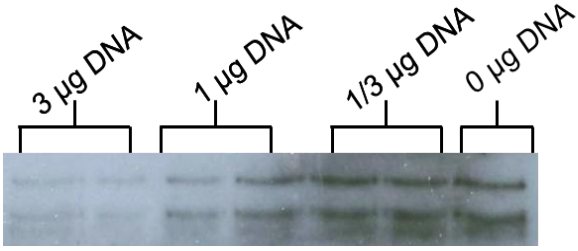


**Figure 17: Trial 1 ZBM2 Ctsl expression following DNA stimulation**

Western analysis of CTSL levels in ZBM2 cells 24 hours after transfection. Lanes 1-2 show transfection with 3 µg of DNA, lanes 3-4 show transfection with 1 µg of DNA, lanes 5-6 show transfection with 1/3 µg of DNA, and lanes 7-8 show transfection with 2 µl of water.

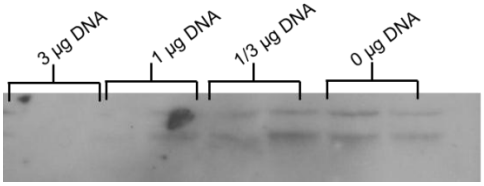


**Figure 18: Trial 2 ZBM2 Ctsl expression following DNA stimulation**

Western analysis of CTSL levels in ZBM2 cells 24 hours after transfection. Lanes 1-2 show transfection with 3 µg of DNA, lanes 3-4 show transfection with 1 µg of DNA, lanes 5-6 show transfection with 1/3 µg of DNA, and lanes 7-8 show transfection with 2 µl of water.

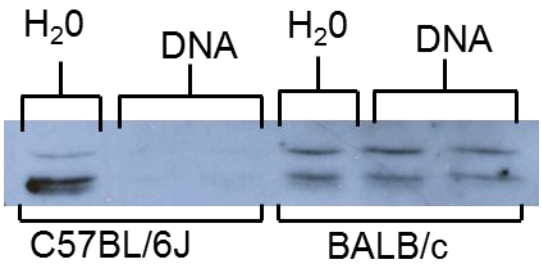


**Figure 19: Primary cell Ctsl expression following DNA stimulation**

Western analysis of CTSL levels in primary macrophages 24 hours after transfection. Lanes 1-3 are cells derived from C57BL7 mice, and lanes 4-6 are derived from BALB/c mice. Lanes 1 and 4 were transfected with 2 µl of water, and lanes 2-3 and 5-6 were transfected with 2µg of DNA.

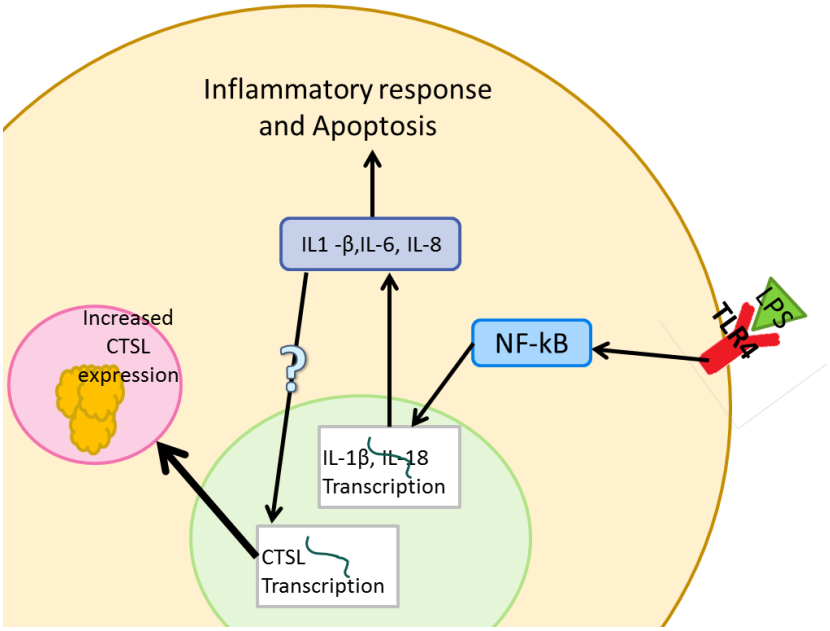


**Figure 20: Innate Immune response to LPS treatment**

Schematic of the immune response to LPS treatment. LPS stimulates the TLR4 receptor, which activates the NF-kB transcriptome, which leads to the transcription and translation of inflammatory cytokines. The Il-6 cytokine stimulates Ctsl transcription. This is one possible mechanism leading to increased Ctsl expression.

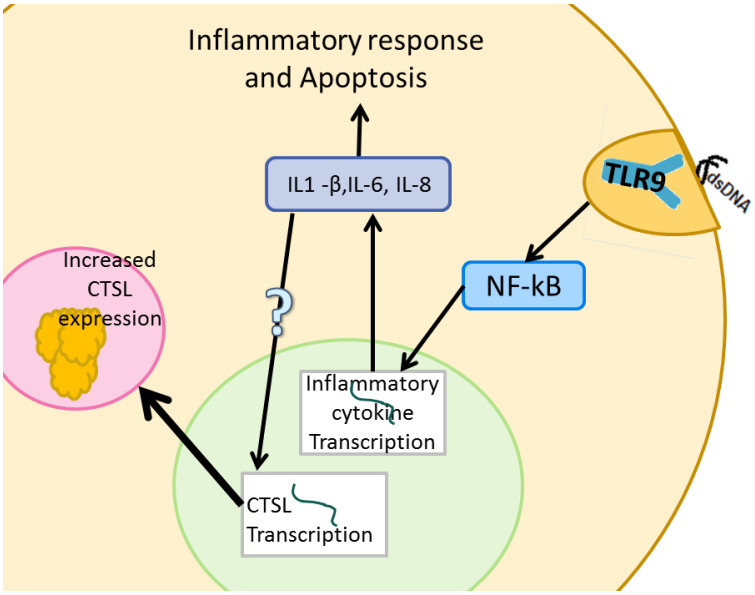


**Figure 21: Innate immune response to extracellular dsDNA**

Schematic of the immune response to extracellular DNA. DNA stimulates the TL9 receptor, which activates the NF-kB transcriptome, which leads to the transcription and translation of inflammatory cytokines. The Il-6 cytokine stimulates Ctsl transcription. This is one possible mechanism leading to increased Ctsl expression.

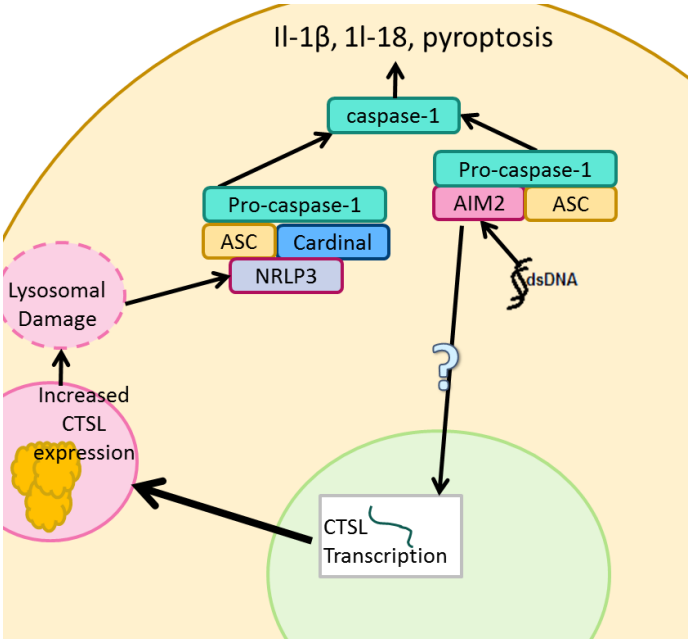


**Figure 22: Innate immune response to cytosolic dsDNA**

Schematic of the immune response to cytosolic DNA. Cytosolic DNA stimulates AIM2, which activates the AIM2 inflammasome, which causes caspase-1 activation. This could also lead to increased Ctsl transcription and increased Ctsl protein levels. These increased levels could introduce Ctsl to the cytoplasm or or cause lysosomal distress. Ctsl can activate the NRLP3 inflammasome, which also causes caspase-1 activation.