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LIPASE ACTIVITY IN THE MURINE ACQUIRED IMMUNE DEFICIENCY SYNDROME (MAIDS) MODEL

by

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ABSTRACT

Murine acquired immune deficiency syndrome (MAIDS) is a retrovirally induced disease caused by exposure to the murine leukemia virus (MuLV) and is used to study human AIDS. Two strains of mice are used in the model system; the BALB/c mice, which are resistant to the progression of MAIDS and the C57BL/6 mice, which are susceptible and progress to MAIDS with time. A DNA microarray analysis was used to recognize differentially expressed genes in lymph nodes and spleen at three and seven days post infection in both strains. Pancreatic lipase was found to be differentially expressed over 100 fold in the spleen at three days post infection and is thought to be resistance-associated.

To investigate the activity of pancreatic lipase, a colorimetric assay was used to compare spleen lysates from naïve, three day and seven day infected mice. Results suggest higher levels of activity in the naïve and infected BALB/c mice compared to C57BL/6 animals. Large biological variability in the lipase activity within each condition suggests that the trend is not statistically significant. Therefore, it is unlikely that pancreatic lipase is playing an essential role in the recovery of the BALB/c mice.
INTRODUCTION

Immune system

The vertebrate immune system is composed of cells and mechanisms dedicated to the recognition and elimination of foreign pathogens if the chemical and physical barriers protecting the body are breached. The innate immune response begins when a pathogen is recognized by effector cells in the infected region because of foreign structures, such as peptidoglycan and lipopolysaccharides, which are absent from healthy cells (Davies, 2008). Innate immune cells, including macrophages and neutrophils, begin a generalized and rapid response leading to inflammation, the secretion of acute-phase proteins, and the initiation of the complement cascade (Davies, 2008). If the innate immune response cannot effectively eliminate the pathogen in the local tissue, the adaptive immune response is stimulated to provide a more specialized defense against the pathogen.

The adaptive immune response is initiated when antigen and lymphocytes encounter each other in the secondary lymphoid organs (Medzhitov and Janeway, 1997). Secondary lymphoid tissues, including the spleen and lymph nodes, have functional variations to cover the different ways antigen travels through the body. The spleen is a filter that removes dead cells and foreign pathogens from the blood. The lymph nodes, located throughout the body, are initial contact sites for the recognition of the antigen by lymphocytes. Lymphocytes enter the lymph
node through blood vessels, known as high endothelial venules, while antigen is carried through the lymphatic vessels by antigen presenting cells (APC) such as dendritic cells, macrophages, and activated B cells (Janeway, 2001).

The two small lymphocytes active in the adaptive immune response are B lymphocytes (B cells) and T lymphocytes (T cells). Both B and T cells develop in the bone marrow; however, B cells undergo maturation in the bone marrow while T cells finish maturation in the thymus. Mature B cells recognize complete antigens and molecules using their immunoglobulin receptors. When an antigen binds with the B cell receptor, the B cell begins to proliferate and differentiate into plasma cells which in turn secrete antibodies (Parham, 2005). Higher affinity, antigen-specific B cells can be activated by stimulation from follicular dendritic cells (Udono et al., 2007). The antibodies produced by these B cells can then travel throughout the body to mark or neutralize antigens. Furthermore, the binding of an antibody with antigen signals to circulating macrophages to ingest and degrade the opsonized complex (Davies, 2008).

While B cells recognize complete antigens, T cell receptors recognize peptides of an antigen presented by a membrane glycoprotein called the major histocompatibility complex molecule (MHC) (Parham, 2005). The genes coding for MHC are highly polymorphic in most vertebrates and determine the recognition of self versus nonself (Penn and Ilmonen, 2005). The MHC genes of the mouse, called the Histocompatibility-2 complex (H-2), are tightly clustered on chromosome 17 (Melvold, 2005). Due to low rates of recombination in mice, the
allele combinations tend to be transmitted through the generations on each member of the chromosome pair (Melvold, 2005).

Three class I loci, which are equivalent to HLA-A, B, and C in humans, encode for single chain proteins that are highly polymorphic and are used to present to CD8+ T cells (Melvold, 2005). The peptides presented to the CD8+ T cells (cytotoxic T cells) are shorter length peptides derived from proteins in the cytosol or from pathogens that replicate intracellularly (Penn and Ilmonen, 2005). After activation, cytotoxic T cells travel to the site of infection and induce apoptosis in targeted cells.

There are two different chains that make up the Class II loci, α and β, which are equivalent to the DR, DP, and DQ loci of the HLA complex in humans (Melvold, 2005). B cells, macrophages, monocytes, and dendritic cells express these chains in a dimer formation in order to present larger, exogenous peptides to CD4+ T cells (Melvold, 2005). There are two types of CD4 T cells, also referred to as helper T cells, which serve distinct functions. CD4 T_H1 cells travel to the infected tissue and produce cytokines which activate macrophages to digest the extracellular pathogens. Meanwhile, CD4 T_H2 cells remain in the secondary lymphoid tissue in order to stimulate B cells bound with antigen to differentiate into higher-affinity antibody-secreting cells (Parham, 2005). Allelic variations of these genes allow for a larger quantity of possible combinations of H2-peptide complex which can be recognized by T cells and used to fight off pathogens.
If the adaptive immune response is successful, the pathogen can be eliminated from the body and memory cells remain to provide protective immunity against the pathogen in the future. However, if the pathogen is able to evade the adaptive immune response the results can range from chronic illness or disease to death.
AIDS and HIV

In 2007, the World Health Organization reported that approximately 33 million people world-wide are infected with human immunodeficiency virus, the virus which is known to induce acquired immunodeficiency syndrome (AIDS) (World Health Organization Report on the Global AIDS epidemic, 2008). Although there are two isolates of the virus, HIV- type 1 is the most common agent of AIDS found throughout the world (Fanales-Belasio et al., 2010). HIV is a lentivirus, a type of retrovirus, which causes a slow progression of disease (Parham, 2005). HIV is contracted by transmission of the free virus through unprotected sexual intercourse, intravenous drug use, or fluid exchange with an infected individual. The provirus, a virus that can use the transcription and translation mechanisms in the host cell to make infectious virions, can also be transmitted when carried by infected CD4 T cells, macrophages, and dendritic cells (Janeway, 2001).

After direct contact with HIV, the virus begins to infect dendritic cells and CD4 lymphocytes through receptor-dependent mechanisms allowing the virus to spread through the blood to the lymph nodes (Fanales-Belasio et al., 2010). After seroconversion, anti-HIV antibodies appear in the blood as a result of B cells trying to form a specific immune response against HIV (Fanales-Belasio et al., 2010). During this symptomatic phase, individuals will experience flu-like symptoms that can last approximately fourteen days (Fanales-Belasio et al., 2010). If their serum is tested during this stage, there is an evident decline in the
circulating number of CD4 T cells associated with the high viral levels of HIV (Fanales-Belasio et al., 2010).

Following the acute infection, a phase of clinical latency begins with constant infection and replication of the virus in CD4 T cells leading to increased cell death and destruction of lymphoid architecture (Fanales-Belasio et al., 2010). Clinical latency ends when the number of CD4 T cells is so low that an immune response cannot be mounted against any foreign pathogen (Parham, 2005). This marks the onset of AIDS, after which, opportunistic infections by viruses or bacteria can result in life-threatening illness and death. In addition, patients with AIDS are more likely to develop cancers such as lymphomas and complex neurological symptoms (Andrews and Koup, 1996).

Although the majority of individuals infected with HIV progress to AIDS, some individuals seroconvert but maintain their CD4 T cell counts with low levels of virus in their blood (Parham, 2005). An even smaller number of individuals remain seronegative and disease-free. Investigation of the immune systems of these groups could lead to advances in the prevention and treatment of AIDS.
MAIDS model system

The murine acquired immune deficiency syndrome (MAIDS) is a retrovirally induced disease caused by exposure to the LP-BM5 isolate of murine leukemia virus and is an animal model system to study human AIDS. Clinical manifestations caused by infection with the murine leukemia virus (MuLV) are similar to those found in AIDS; however, MuLV is a type-C retrovirus rather than a lentivirus (Mosier et al., 1996). Unlike HIV, which primarily infects macrophages and CD4 T cells, MuLV infects B cells, macrophages, and a few T cells (Mosier et al., 1996). However, research using nude mice infected with MuLV has shown the importance of both CD4 T and B cells to the progression of MAIDS (Jolicoeur et al., 1991). Without these two cell types present, the infected mice did not develop MAIDS (Jolicoeur et al., 1991).

The function of T cells and B cells appears to be significantly altered after infection with MuLV (Jolicoeur et al., 1991). After infection with LP-BM5, an initial increase in CD4 T-cell-dependent polyclonal B-cell activation has been observed which results in an increase in the concentrations of IgG1, IgM, and IgE in the serum (Mosier et al., 1996). With prolonged stimulation, T cells become unresponsive leading to T cell anergy (Mosier et al., 1996). Following T cell anergy, the quantity of immunoglobulin-producing B cells begins to decrease with a noticeable reduction in T and B cells (Jolicoeur et al., 1991). The reduction in T cells can be explained by an increased expression of the Fas antigen, an antigen known to cause high rates of apoptosis, on T cells (Mosier et al., 1996). In some
strains of mice, B cell lymphomas begin to develop as early as six weeks after infection, with animal death approximately 11 weeks later (Mosier et al., 1996).

MAIDS susceptibility, however, was found to differ among strains of mice (Hartley et al., 1989). Some mice were susceptible to the development of MAIDS after infection with MuLV, yet others were resistant to the progression of the disease. C57BL/6 (BL/6) was determined to be a highly susceptible strain. After infection with the virus, BL/6 began to show symptoms of polyclonal B cell activation, T cell stimulation, and general immunodeficiency as early as 3-4 weeks after infection. There was also a marked increase in the weight of the peripheral lymph nodes and spleen (Hartley et al., 1989). It was noted that death would occur suddenly due to complications caused by lymph node enlargement and lymphoid infiltration of lungs, kidneys, and the central nervous system. However, resistant strains such as BALB/c were able to respond to the infection, prevent progression to MAIDS, and have protective immunity to future infections with MuLV (Hartley et al., 1989). Therefore, this study suggests that susceptibility to MAIDS is determined by the genetic background of the strain.

The association of H-2 haplotypes with resistance or susceptibility to MAIDS induced by LP-BM5 infection was further investigated (Makino et al., 1990). Previous research suggested that in addition to this locus, other non-MHC linked loci interacted with retroviral infection leading the authors to explore the effects of MHC and non-MHC genes by investigating different mice strains. It was discovered that variations of the H-2 loci could determine susceptibility or
resistance to the disease in a few different genetic backgrounds. To test the pattern of inheritance, resistant and susceptible strains were crossed with results suggesting that susceptibility to MAIDS is dominant. Furthermore, the authors concluded that the H-2 loci, in addition to loci outside of the MHC region, appear to control replication of the ecotropic virus.
Previous studies

A review of the benefits of cDNA microarrays in bio-medicine described the technique as a large-scale transcriptome analysis which uses RNA extracted from different cell cultures to compare expression levels (Lamartine, 2006). The RNA is reverse transcribed into cDNA and the two cultures are independently labeled with red or green markers. The cDNAs are then mixed together and put onto a matrix of single-stranded DNA probes and incubated so the cDNA can hybridize on the probe spots. Fluorescent images are scanned and read by superimposing the two colors. Knowing that the amount of fluorescent DNA is proportional to the amount of mRNA allows for the gene expression to be calculated.

A 2008 microarray analysis compared the transcriptome patterns of differential expression between BALB/c and C57BL/6 (BL/6) mice at 3 and 7 days post MuLV infection in secondary lymphoid tissues (Tepsuporn et al., 2008). A gene was labeled as susceptibility-associated if, after MuLV infection, there was a higher expression in BL/6 mice compared to mock infected controls. However, if after infection there was an increase in expression in the BALB/c animal, the gene was labeled as resistance-associated. The greatest amount of overall differential expression was shown to be at 3 days post-infection with a total of 176 differentially expressed genes. Interestingly, pancreatic enzymes associated with digestion were determined to be resistance-associated genes at 3
days post infection. These included pancreatic lipase, pancreatic colipase, and elastase.

Pancreatic lipase was differentially expressed with a resistance-associated pattern in both the lymph nodes and spleen with a fold change of 160.1 and 108.0, respectively. However, pancreatic colipase, the co-enzyme that is thought to be required for the activation of pancreatic lipase, was found to be differentially expressed only in the spleen with a fold difference of 119. High expression of this digestive enzyme and its cofactor in the spleen after MuLV infection may suggest a potential role of pancreatic lipase in the innate immune response to MuLV.
Pancreatic lipase

Pancreatic lipase (Pnlip) is an enzyme in the lipase gene family which is secreted by acinar cells of the pancreas and is used for fat metabolism in the small intestine (Lowe 1997). Pancreatic lipase has a preference for the hydrolysis of ester bonds in triglycerides and acylglycerols over those found in phospholipids and galactolipids (Lowe, 2002). It is thought that this preference for substrates is due to the unique structure of the enzyme.

The structure of pancreatic lipase in humans contains a catalytic triad of Ser-His-Asp found beneath a lid domain which appears to prevent access to the substrate site creating an inactive form of the enzyme (Whitcomb and Lowe, 2007). It has been hypothesized that the lid remains closed until the enzyme recognizes an oil-water interface at which point the enzyme becomes active (Whitcomb and Lowe, 2007). The quality of this interface between the substrate and the aqueous phase, as well as the concentration of the substrate, determines the amount of enzymatic activity (Tietz and Shuey, 1993). When the lid domain is in the open position, as shown in Figure 1, the substrate can reach the active site of pancreatic lipase. The stabilization of the lid domain in the open position occurs through binding with colipase, an enzyme required to restore the enzymatic activity of pancreatic lipase when inhibited by bile salts, phospholipids, and proteins, at the C-terminal domain in the presence of the oil-water interface (Lowe, 2002). Recent results suggest that the size and polarity of the lid domain of pancreatic lipase could be a factor in determining substrate specificity (Lowe,
2002). Though crystalline structures for pancreatic lipase are based on human pancreatic lipase, other models such as rat and porcine support the conservation of the lid domain and the catalytic triad (Lowe, 2002).

Early studies of pancreatic serum samples using isoelectrofocusing profiles found the presence of three peaks of lipolytic activity (Kurooka and Kitamura, 1978). The presence of three peaks suggested that in pancreatic serum samples there were three structures similar to that of pancreatic lipase. Fifteen years later, lipase linkage mapping in mice revealed a duplication of an ancestral gene resulting in a gene cluster on mouse chromosome 19 which contained genes for pancreatic lipase and two pancreatic lipase-related proteins (Warden et al., 1993). Lipase mapping in humans supported the presence of the same gene cluster on chromosome 10q26 (Warden et al., 1993). Analysis of one related protein, pancreatic lipase related protein 1, has shown a lack of activity against substrates used by pancreatic lipase (Lowe, 2002). Though present in pancreatic secretions of both humans and mice, the exact substrate, co-factor requirements, and function remain unknown (Giller et al., 1992).

Research on pancreatic lipase related protein 2 in mice has revealed interesting structural and substrate similarities to pancreatic lipase with a potential function in the immune response (Lowe, 2000). The initial observation of a lipase acting as a cytokine was found as a response to fatty acid production during the cell-mediated immune response in a model of cancer (Okada and Cyong, 1979).
Figure 1. The crystal structure of human pancreatic lipase (PTL) in the closed and open conformations with colipase binding at the C-terminus domain (Image adapted from Lowe, 2002).
A more recent study suggested a potential role of interleukin-4 in inducing lipase production from cytotoxic T-lymphocytes (Grusby et al., 1990). Lipase activity produced by cytotoxic T lymphocytes (CTLs) was tested and confirmed to have the same substrate preference for tributyrate which lead Grusby et al. to believe that the secretion was pancreatic lipase. To confirm their results, CTLs were then grown in IL-4 which showed a marked increase in the production of pancreatic lipase. Therefore, it was concluded that IL-4 could control the transcriptional activity of genes such as pancreatic lipase suggesting a role for lipase in T-cell mediated cytotoxicity. The CTL produced lipase was sequenced to determine exact structure and revealed the presence of 12 exons, rather than 13, suggesting a lipase other than pancreatic lipase as seen in Figure 2 (Kaplan et al., 1996).

The CTL lipase was later identified as pancreatic lipase related protein 2 (Lowe 2002). Though pancreatic lipase related protein 2 has a few minor changes in the lid domain and broader substrate preferences compared to pancreatic lipase, the same catalytic triad is present (Lowe 2002). It is thought that these minor differences in function and substrate preferences could be due to differing colipase dependency and/or slight variations in protein structure (Lowe 2000). However, current modeling of both structures suggests the same active site with no difference in the position of the residues or the residues present (Lowe 2000).
Figure 2. The exon sizes for murine CTL lipase, pancreatic lipase (PL), and pancreatic lipase related protein 1 (PLRP1). Although PL and PLRP1 have almost the same number of base pairs per exon and total number of exons, CTL lipase seems to have base pairs from exons 1 and 2 in PL joined together creating one large exon 1 (Image adapted from Kaplan et al., 1996).
Recently, IL-4 induction of pancreatic lipase related protein 2 (Pnliprp2) in CTLs has been found to increase their ability to kill tumor cells (Alves et al., 2009). Two approaches were used to study the cytotoxic effects of pnliprp2; one used cytotoxic lymphocytes isolated from BALB/c and C57BL/6 mice, while the other focused on recombinant Pnliprp2. The experiment revealed that pancreatic triglyceride lipase (Pnlip) and Pnliprp2 were toxic to tumor cells in the presence of the appropriate substrates. However, without the presence of these triglyceride substrates, the lipases indirectly targeted the tumor cells as mediated by triglyceride biproducts. Furthermore, a lipid-dependent form of CTL cytotoxicity was noticed in the absence of perforin suggesting a new mechanism by which CTLs could destroy tumor cells. However, only one lot of IL-4 induced CTLs was able to mediate lipid-dependent cytotoxicity, which could be due to a random phenomenon or contamination. Therefore, the results found by Alves et al. support previous studies on the toxicity of pancreatic lipases produced by cytotoxic lymphocytes but are not conclusive.
**Lipase assays**

Several commercial kits are available to test the enzymatic activity of lipases, including colorimetric and ELISA (enzyme-linked immunosorbent assay) based tests. The QuantiChrom Lipase Assay Kit by BioAssay Systems is a colorimetric assay which can be used to detect the enzymatic activity of lipase in serum, plasma, and other samples (BioAssay Systems datasheet). The assay is derived from the “BALB-DTNB Method” which uses dimercaptopropanol tributyrate (BALB) as a substrate to be cleaved by lipase (Furukawa et al., 1982). After the cleavage of the ester bonds, the SH groups react with the chromogenic agent, 5-5’ dithiobis(2-nitrobenzoic acid), to form a yellow product (Kurooka and Kitamura, 1978). The final color intensity, measured by a microplate reader set to a wavelength of 412nm, is directly proportional to the amount of enzymatic activity in the sample (BioAssay Systems datasheet). The “BALB-DTNB Method” is recommended over other assays because it is sensitive enough to measure low serum levels and demonstrates a linear relationship between enzyme concentration and the enzyme reaction time (Furukawa et al., 1982).
Research Objective

The purpose of this study was to investigate the activity of pancreatic lipase in disease-resistant BALB/c mice and susceptible C57BL/6 mice at timepoints which were previously analyzed in the DNA microarray. A colorimetric assay was used to detect the enzymatic activity of lipase in the spleen homogenates of mice at 3 and 7 days after infection, and in naïve mice. Furthermore, the sequences of pancreatic lipase and pancreatic lipase related proteins 1 and 2 were analyzed to determine basic sequence similarities.
MATERIALS AND METHODS

Virus

The LP-BM5 isolate of MuLV was generated from the mixture of 10^6 MuLV-infected SC-1 mouse fibroblast cells with 10^6 uninfected cells SC-1 cells in 35 ml of complete media. The medium was exchanged on days 1, 3, and 5, and cell free plus cell-associated virus was harvested on day 6. The titer of the 2010 virus was 3.2x10^3 plaque forming units per milliliter as measured by the XC plaque assay (Sonia Bakkour). Supernatants were stored in aliquots at -80 °C until use.
**Animals**

Six to eight week old female C57BL/6 and BALB/c mice were purchased from Taconic (New York) and housed in Mount Holyoke College Animal Facility. Infected animals were injected intraperitoneally with 1mL of LP-BM5 and sacrificed by carbon dioxide euthanasia after 3 and 7 days. Spleen and pancreas were collected from four naïve and four MuLV infected animals of each strain for both timepoints. Due to the proximity of the pancreas to the spleen, careful inspection of each spleen was necessary to prevent potential contamination.
**Tissue homogenization**

Spleen and pancreas were each weighed using a Mettler Toledo balance and suspended in 2 ml of cold phosphate buffered saline (PBS) with a pH of 7.4. A Polytron PT3100 was used to completely homogenize the tissues in 15mL round bottom tubes. Cold PBS was used to clean the probe between animals with the same treatment and cold 75% ethanol was used between infected and naïve animals of each strain to clean the probe. Samples were centrifuged for 5 minutes at 14,000 rpm. The supernatant was transferred to a new test tube and stored at -80ºC for one to two weeks before being used. According to the assay manufacturers, lysates should be used within one month, if not used immediately.
**Protein concentrations**

Nanodrop spectrophotometry

Two microliters of each sample were loaded onto the Nanodrop ND-1000 spectrophotometer and the absorbance values were recorded using Protein 480 nm wavelength reading before and after freezing. The head was cleaned initially with bleach, and with distilled water between each sample. Protein concentrations of samples were then normalized to approximately 14 mg/ml for each spleen and pancreas lysate.

Bradford Assay

A Coomassie Plus (Bradford) Protein Assay kit (Thermo Scientific) with bovine serum albumin (BSA) was used to create a standard curve. Spleen and pancreas lysates were diluted 1:100 in cold PBS and 10 µl was added to 500 µl of room temperature (25 °C) Coomassie Reagent. After fifteen minutes, the absorbance was measured on the Beckman DU640 spectrophotometer at a fixed wavelength of 595 nm. The protein concentration of each sample was determined by inserting the absorbance into the straight line equation generated from the BSA standard curve. Sample concentrations were tested before freezing and after thawing for accuracy.
**Lipase assay**

A commercial kit (QuantiChrom Lipase Assay Kit by BioAssay Systems) was used to measure the lipase activity in tissue lysates. Working reagent was prepared in advance for the total number of wells to be used based on the per well amount needed, according to the protocol. Each sample was run in triplicate wells with the pancreas samples used as a control to verify the assay was working properly. The working reagent was time-sensitive, and was used within an hour of constitution. A calibrator with an intensity known to be equivalent to 735 U/L and distilled water were also added to the plate as controls. The working reagent was pipetted into each well containing 10 µl of lysate. A microplate reader set to a wavelength of 412 nm was used to read the optical density of the wells at 10 and 20 minutes after addition of samples. Lipase activity was calculated using the formula provided on the product sheet:

\[
\frac{(\text{OD}_{20 \text{ minutes}} - \text{OD}_{10 \text{ minutes}})}{\text{OD}_{\text{calibrator at 20 minutes}} - \text{OD}_{\text{water at 20 minutes}}} \times 735 \ (\text{U/L})
\]

The lipase activity was measured in units/liter. The BioAssay datasheet defined one unit of enzyme as catalyzing the cleavage of 1 µmole substrate per minute, at an assay pH of 8.5.
Data analysis of lipase activity

The lipase activity for individual mice in a condition was calculated by averaging the lipase activity found in the three wells. The average of the condition was calculated by averaging the mean lipase activity of the three wells for each animal. Standard deviations were calculated for the averages using the deviation between the averages. A Student’s T-test was performed to analyze statistical significance between the conditions.

SPSS version 16.0 was used to perform a full factorial univariant analysis of variance (ANOVA) for all samples. In addition, the means and standard deviations were calculated to determine statistical significance.
**Sequence analysis**

BLAST (Basic Local Alignment Search Tool) was used to find sequence alignments in GenBank similar to the pancreatic lipase (Pnlip) transcript identified in the microarray data (NM_02925). BLAST was also used to search for other sequences similar to CTL lipase. Sequence similarities were compared with other murine derived transcripts by comparing the max identity, max score, query coverage, and E value. The max identity reveals the highest percentage of identical bases found within the match, while the max score tells how well the sequence matches the query (GenBank Overview, 2010). For a nucleotide sequence, a perfect match would have a max score exactly twice the number of nucleotides. The query coverage, however, shows the percentage of the query sequence that matches the subject sequence (GenBank Overview, 2010). Finally, the E value shows the number of sequences you would expect to find in a database similar to the query sequence (GenBank Overview, 2010). A significant E value for nucleotides, which indicates a very strong match is $1 \times 10^{-23}$ (GenBank Overview, 2010).

The transcript sequences for similar sequences, pancreatic lipase related proteins 1 and 2, stored by GenBank, were then used to determine the C+G content of each. The largest open reading frame of each was compared using ORF Reader. Sequences were then aligned using ClustalX and a consensus sequence between Pnlip and CTL lipase (M30687.1 from Kaplan et al., 2006) was constructed in SeaView. If extra fragments were at either end of the alignment,
they were removed and the sequence was realigned. BLAST2 analysis was then used to illustrate the similarity between the sequences of CTL lipase and Pnlip using a dot matrix plot.
RESULTS

Spleen weights

The weight of the spleen from each animal was measured and progression in spleen weight change over time in the two strains was compared. BALB/c spleens in naïve animals weighed less than BL/6 animals, but upon infection showed an increase in weight (Figure 3). The standard deviations per condition suggest a low biological variability in the mice from these conditions (Table 1). Each individual BL/6 mouse, however, decreased from the initial spleen weight after infection and formed a mean plateau around 80mg (Figure 4). ANOVA analysis revealed a significant interaction between the time after infection (naïve, 3D, or 7D) and the strain of mouse (BALB/c or BL/6) on the weight of the spleen, F (2,17) = 3.61, p < 0.05. Therefore, the effect of time after infection on spleen weight was different for BALB/c and BL/6 mice.
Table 1. The individual spleen weights (mg) for each animal per condition (n=4 per condition).

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<th>Individual Spleen Weights (mg)</th>
<th>Average ± SD</th>
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<td>BALB/c</td>
<td></td>
<td></td>
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<td>Naïve</td>
<td>88.3  73.8  84.0  84.0</td>
<td>82.5 ± 6.2</td>
</tr>
<tr>
<td>3D</td>
<td>88.8  93.4  106.8  98.9</td>
<td>97.0 ± 7.7</td>
</tr>
<tr>
<td>7D</td>
<td>139.8 103.2 88.9 95.0</td>
<td>106.7 ± 22.8</td>
</tr>
<tr>
<td>BL/6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Naïve</td>
<td>82.0  -    90.8 105.6</td>
<td>92.8 ± 11.9</td>
</tr>
<tr>
<td>3D</td>
<td>92.3  70.7  65.8 87.0</td>
<td>79.0 ± 13.0</td>
</tr>
<tr>
<td>7D</td>
<td>62.6  92.3  89.7 75.2</td>
<td>80.0 ± 14.0</td>
</tr>
</tbody>
</table>
Figure 3. The mean of spleen weights measured in naïve, 3 day and 7 day infected BALB/c and BL/6 mice (n=4 per condition).
Figure 4. Spleen weights (mg) of individuals for naïve, and infected animals at various timepoints.
**Protein concentrations**

Protein concentration of spleen and pancreas lysates was initially measured using a Nanodrop spectrophotometer. However, this technique was considered unreliable after inconsistency in measurements. Therefore, a Bradford assay was then used to determine the protein concentrations in lysate samples of spleen and pancreas both before and after freezing. The standard curve for bovine serum albumin before freezing had an $R^2$ value of 0.996 while the $R^2$ value for after freezing was 0.990 (Figures 5 and 6). The lysate absorbance values were inserted in the corresponding straight line equation and the protein concentrations were calculated both before and after freezing. Almost all concentrations increased after freezing, suggesting a potential experimental error (Table 2). Furthermore, measurements could not be taken immediately before freezing 7 day samples, so the values were excluded. For these reasons, we elected not to use the protein concentration in our calculation and to instead show lipase activities per liter of spleen lysate. Also, this was the recommendation of the lipase kit manufacturer (personal communication).
Figure 5. The BSA standard curve measured at a fixed wavelength of 595 nm to be used for comparison to lysate samples before freezing at -80°C.
Figure 6. The BSA standard curve measured at a fixed wavelength of 595 nm to be used for comparison to lysate concentrations after freezing at -80°C.
Table 2. The average protein concentrations (mg/ml) for each condition before and after freezing (n=4).

<table>
<thead>
<tr>
<th>Average Protein Concentrations (mg/ml)</th>
<th>Before</th>
<th>After</th>
</tr>
</thead>
<tbody>
<tr>
<td>BALB/c Naïve</td>
<td>12.0 ± 3.3</td>
<td>17.4 ± 6.3</td>
</tr>
<tr>
<td>BALB/c 3D</td>
<td>17.3 ± 9.0</td>
<td>23.8 ± 9.3</td>
</tr>
<tr>
<td>BALB/c 7D</td>
<td>-</td>
<td>15.2 ± 6.3</td>
</tr>
<tr>
<td>BL/6 Naïve</td>
<td>12.0 ± 2.0</td>
<td>11.1 ± 2.9</td>
</tr>
<tr>
<td>BL/6 3D</td>
<td>8.0 ± 5.7</td>
<td>14.0 ± 4.3</td>
</tr>
<tr>
<td>BL/6 7D</td>
<td>-</td>
<td>8.2 ± 4.5</td>
</tr>
</tbody>
</table>
**Lipase activity**

Lipase activity was measured in the spleen of four BALB/c and four BL/6 mice per condition (Table 3). Each condition in the spleen showed a large amount of biological variability between individual mice in lipase activity (Figure 7). If the means of the condition are considered, a peak of lipase activity occurs by day 3 and decreases by day 7, which still remains more elevated than baseline levels (Figure 8). BALB/c mice show the same trend, but have a higher level of lipase activity at each timepoint tested. ANOVA results suggest that lipase activity was dependent on the effects of strain not the treatment condition, $F (1,18) = 4.50, p < 0.05$. 
Table 3. The lipase activity (U/L) in individual animals for each condition (n=4 per condition).

<table>
<thead>
<tr>
<th></th>
<th>Individual Lipase Activity (U/L)</th>
<th>Average ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>BALB/c Naïve</td>
<td>131.9 21.2 0.0 125.6</td>
<td>69.7 ± 69.0</td>
</tr>
<tr>
<td>BALB/c 3D</td>
<td>248.1 54.2 143.0 192.4</td>
<td>159.4 ± 82.2</td>
</tr>
<tr>
<td>BALB/c 7D</td>
<td>216.7 41.6 125.0 74.0</td>
<td>114.3 ± 76.4</td>
</tr>
<tr>
<td>BL/6 Naïve</td>
<td>76.2 0.0 11.8 71.5</td>
<td>39.9 ± 39.6</td>
</tr>
<tr>
<td>BL/6 3D</td>
<td>16.5 90.3 78.5 158.0</td>
<td>85.8 ± 58.0</td>
</tr>
<tr>
<td>BL/6 7D</td>
<td>18.1 108.4 67.5 17.3</td>
<td>52.8 ± 43.9</td>
</tr>
</tbody>
</table>
Figure 7. Lipase activity (U/L) as measured in spleen lysates for individual naïve and infected mice at various timepoints.
Figure 8. Mean lipase activity in BALB/c and BL/6 mice at naïve, 3 day, and 7 day timepoints (n=4 per condition).
Sequence analysis

The accession number from the microarray data for pancreatic lipase (NM026925) was searched using nucleotide BLAST for mouse genomic and transcript data to determine other sequences which produced significant alignments (Table 4). The top hit received for transcripts, other than the exact sequence, was pancreatic lipase related protein 1 (Pnliprp1) which had a max identity of 69% and an E Value of 9E-154. The max score was less than twice the length of the nucleotide sequence, suggesting that the sequence was not a great match to the query. However, the query coverage demonstrated that 89% of the query sequence matches the subject sequence. The second highest hit was with pancreatic lipase related protein 2 (Pnliprp2) which also had a max identity of 69% but a weaker E Value of 3E-134. Furthermore, both the max score and query sequence had lower values suggesting that Pnlip is closer in nucleotide sequence to Pnliprp1 than Pnliprp2. A consensus sequence for Pnlip and pnliprp2 was constructed using Seaview, which demonstrated a large number of ambiguous residues confirming the BLAST results.

To further examine the potential differences between pnliprp2 and CTL lipase, the accession number (M30687.1) published by Grusby et al. in 1996 was compared to other sequences through BLAST. The results identified the transcript as being murine cytotoxic T lymphocyte lipase from a C57BL/6 mouse, also identified as Pnliprp2. The Pnliprp2 sequence was 40 base pairs longer than that of Pnlip, with a higher C+G content (50.5%) compared to Pnlip (48.5%). In
addition, results from ORF Finder, an NCBI tool used to find open reading frames, revealed that Pnliprp2 had 11 open reading frames, while Pnlip had 9. The most likely protein product, chosen by selecting the longest open reading frame, was 482 amino acids long for Pnliprp2 and only 465 amino acids long for Pnlip. Sequences were then aligned and compared using CLUSTALX which showed that approximately 85% of the amino acid residues are highly conserved between these two proteins. BLAST 2 was then used to generate a dot matrix to compare the regions of dissimilarity. The dot matrix displayed four deletions in the sequence and three insertions in the sequence when comparing the breaks in the line to known patterns (Figure 9).
Table 4. Sequences which produced significant alignments when using nucleotide BLAST to search for similar sequences to the pancreatic lipase transcript found in the microarray results from Tepsuporn et al.

<table>
<thead>
<tr>
<th>Accession number</th>
<th>Max identity (%)</th>
<th>Max score</th>
<th>Query coverage (%)</th>
<th>E value</th>
<th>Number of gaps</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pnlip NM_026925.3</td>
<td>100</td>
<td>2733</td>
<td>100</td>
<td>0.0</td>
<td>0</td>
</tr>
<tr>
<td>Pnliprp1 NM_018874.2</td>
<td>69</td>
<td>551</td>
<td>89</td>
<td>9E-154</td>
<td>21</td>
</tr>
<tr>
<td>Pnliprp2 NM_011128.1</td>
<td>69</td>
<td>486</td>
<td>87</td>
<td>3E-134</td>
<td>41</td>
</tr>
</tbody>
</table>
Figure 9. Dot matrix of CTL lipase (Pnliprp2) compared to pancreatic lipase (Pnlip) as generated by BLAST (bl2seq) which demonstrates regions of sequence dissimilarities.
DISCUSSION

In a DNA microarray experiment, the gene expression of several pancreatic enzymes were determined to be differentially expressed between BALB/c disease resistant and C57BL/6 susceptible mice at three days post-infection (Tepsuporn et al., 2008). Pancreatic lipase, colipase, and pancreatic lipase related protein 1 were all found to be differentially expressed at this timepoint in the spleen and considered to be resistance-associated, with higher levels of expression seen in the BALB/c mice. Tepsuporn et al. concluded that the pancreatic enzymes were produced by cells resident to the spleen and could play a potential role in the immune response by remodeling lymphatic and blood vessels or by inducing protease-activated receptors. Although some serine proteases have been recognized as mediators of leukocyte activation following shock, pancreatic lipase has not yet been considered outside of its role in the digestion of triglycerides within the small intestine (Waldo et al., 2003). To further investigate this novel discovery, I was interested in comparing the lipase activity in both strains of mice at 3 and 7 days after MuLV infection and before infection.
**Spleen weights**

Spleens were collected and weighed to determine whether BL/6 or BALB/c mice were showing signs of splenomegaly at three and seven days post infection with MuLV. Figure 1 shows an increase in the spleen weight for BALB/c mice after infection while the spleen weight of BL/6 mice decreases after infection. These results suggest that the BALB/c spleen is immediately infiltrated with cells, cytokines, or other factors involved in the innate immune response which effectively activate the necessary response needed to eliminate the virus.

Studies which focus later in the immune response to LP-BM5 in BALB/cBy and BL/6 mice, five to ten weeks after initial infection, noted that the susceptible strain had a significantly increased spleen weight of 225 mg, while the resistant strain remained at a normal weight of 100mg (Makino et al., 1990). Meanwhile, results from other investigations into earlier timepoints (naïve, 3 days, and 7 days) by Anna Bender and Lara Coulter have shown that both BALB/c and BL/6 mice increase in their spleen weights with time after infection (MHC Thesis 2001 and MHC Thesis 2003). The standard deviations in both studies showed large biological variability among individuals and used one to four replicates per condition. Therefore, additional biological replicates should be considered at these earlier timepoints before a conclusion can be reached about the trend of spleen weight in BALB/c and BL/6 mice.
Protein concentrations

To evaluate the yield of protein in each tissue lysate, the protein concentration for each spleen was measured using Nandodrop spectrophotometry. The reliability of the technique was questioned after repeated samples from one lysate measured many different concentrations. Therefore, the technique was replaced with a more specific protein concentration assay which compared the absorbance of a range of control samples of a known protein standard to my unknown samples.

A Bradford assay using bovine serum albumin was used to create a standard curve of concentrations between 0 and 750µg/ml. After inserting the absorbance values of the spleen lysates into the straight line equation, the concentrations were calculated and found to vary considerably within each condition. There was no noticeable trend between the protein concentrations measured from BALB/c or BL/6 mice before or after freezing. However, it was discovered that the protein concentration of lysates tested before freezing were often considerably lower than the concentrations measured after thawing. These results would suggest that some form of evaporation or protein lysis occurred between freezing and thawing to make the samples more concentrated.

Evaporation is unlikely because each microcentrifuge tube was securely capped and stored in Wheaton boxes at -80°C for two weeks until use. Unfortunately, these results suggest a potential error in the Bradford assay, although each standard curve had an R² value of greater than 0.90. Potential errors could have
been in the dilution of the lysate samples or in the purity of the PBS diluent.

Because of these potential errors, I elected to display the total enzymatic activity per liter of lysate without controlling for protein concentration per lysate.
**Lipase activity in spleens**

The QuantiChrom Lipase Assay kit was used to quantify the enzymatic activity of pancreatic lipase in the spleen and pancreatic tissue lysates of BALB/c and BL/6 mice at 3 and 7 days post infection and in naïve mice. My results suggest that while the BALB/c and BL/6 mice both show the same trend after infection, the BL/6 animals never express as much lipase activity as the disease resistant BALB/c animals (Figure 5). However, the large biological variability found within both strains in each treatment means that the data were not statistically significant and suggests that the trend may not be sufficient to explain how BALB/c mice are able to recover from the infection. Furthermore, the data revealed a significant effect of strain on the amount of lipase activity rather than an effect of the treatment (p<0.05).

Although the lipase assay I used comes from a commercial kit, there are many potential sources of error and other complications which cannot be overlooked. The company markets the kit as a way to measure the activity of pancreatic lipase based on cleavage of a substrate and the formation of a yellow product with an intensity proportional to the enzymatic activity. As noted on the information sheet provided by the company, there are two main lipases secreted by the pancreas: pancreatic lipase and pancreatic lipase related protein 2. The substrate supplied in the kit, a tributyrate, is able to be cleaved by both pancreatic lipase and pancreatic lipase related protein 2 but not pancreatic lipase related protein 1. During the assay development the same substrate was tested using
isoelectric profiling and molecular weight analysis in samples of human pancreatic juices which ultimately confirmed the presence of more than one lipase being measured (Kurooka and Kitamura 1978). These results were compared to other lipolytic enzymes and it was concluded that the enzymes were both pancreatic lipases and not another type of lipase, such as lipoprotein lipases and carboxylesterases (Kurooka and Kitamura 1978). Therefore, while the assay is sensitive enough to determine the difference between other enzymes and the pancreatic lipases, the assay can detect at least two different pancreatic lipases because of the substrate used.

Other complications with the technique include the kinematic nature of the experiment. The absorbance of each well was measured at ten and twenty minutes after the addition of the substrate and these values were used in the equation to determine the lipase activity of the sample. Although many of the wells showed an increase in absorbance values from ten to twenty minutes, a significant number showed the opposite, yielding a negative lipase activity value. Technical support within BioAssay Systems was unable to provide a definitive explanation as to how or why this would happen but mentioned a few variables which could affect the readings. Their first recommendation was to eliminate all bubbles from each well before reading the sample by centrifuging or gently tapping down the 96 well plate. Due to the detergent-like nature of the working reagent, bubbles form easily. All large bubbles were removed before the plate was read but a few small bubbles remained in some wells. It was stated that if the wells were still providing
negative values after any bubbles were disrupted, the next step would be to ensure that the sample is diluted enough to fall on the lipase curve provided by the company. The highest reading recommended by the company was an optical density (OD) of 2. Therefore, all samples with absorbance readings higher than two during the ten minute interval were diluted. With these modifications in mind, all of the samples were run again on the same 96 well plate to ensure consistency.

Though there were many potential errors and complications when using the lipase kit, the trend derived from the colorimetric lipase assay was similar to that found on the DNA microarray and when using real-time PCR to analyze the mRNA levels of pancreatic lipase, as determined by Nikita Kolhatkar (MHC Thesis, 2009). Rather than the 108 fold difference found using the microarray data (Tepsuporn et al., 2008), mRNA of pancreatic lipase suggested that there was a 1.67 fold difference between the strains at 3 days post infection with a significant amount of variability between individual animals. The results from these two different techniques conclude that although pancreatic lipase was thought to have a potential role in the immune response to LP-BM5 infection, it is unlikely that it plays an important protective role for the recovery or resistance to disease of BALB/c mice.
**Microarray data compared to lipase activity**

The microarray data revealed a 108 fold difference in expression of pancreatic lipase between BALB/c and BL/6 mice at three days post infection. However, this was an indirect comparison. The direct comparison made was between the MuLV and mock infections for each strain. The mock virus is a cell-free supernatant from uninfected SC-1 cells grown under the same conditions as the MuLV-infected culture. This was originally thought to be a placebo which would expose the animal to an intraperitoneal injection but without resulting in an immune response. However, recent experiments on genes differentially expressed in the microarray data, such as pancreatic lipase, have shown large increases in gene expression in mock infected animals (Nikita Kolhatkar, MHC Thesis 2009 and personal communication). These results suggest that the mock infection is stimulating the immune response in ways that cannot be explained. Earlier investigations into supernatant taken from SC-1 mouse fibroblast cells has not provided any evidence of potential chemicals or cell extracts which could be present in supernatant of these cells and be immune stimulators. Rather than comparing MuLV infected animals to animals undergoing an unexplainable immune response, naïve animals were chosen as the baseline. Unfortunately, using naïve animals excludes the intraperitoneal injection given to the treated condition suggesting that some of the differential response noticed could be due to a response stimulated by an injection. Future studies using another placebo
injection should be considered, such as PBS or culture media, to provide the same response to injection without an immune response to foreign proteins.
Sequence analysis of lipases

Basic sequence analysis revealed that the highest percentage of identical nucleotides between pancreatic lipase and pancreatic lipase related proteins 2 is 69%. This suggests that although the sequences of nucleotides are similar to each other, there are significant differences in the sequence. The BLAST2 dot matrix showed small changes in the nucleotide sequences with roughly four deletions and three insertions. Meanwhile, a CLUSTALX alignment and comparison of the protein sequences suggested that approximately 85% of the amino acids were highly conserved between the two proteins. Furthermore, a BLAST search and structural comparison of CTL lipase supported that it was the same transcript as pancreatic lipase related protein 2. Therefore, primer sets should be designed specific to pancreatic lipase related protein 2 (NM_011128.1) to further evaluate expression differences of this enzyme which is now thought to mediate tumor cell death (Alves et al., 2009).
**Future work**

Although expression of pancreatic lipase does not appear to provide a protective advantage to the BALB/c mice after MuLV infection, other pancreatic digestive enzymes have recently become implicated in mechanisms for other immune responses (Alves et al., 2009). Studying these newly recognized proteases and their receptors by more highly specific assays could be beneficial in understanding the early response within BALB/c and BL/6 mice infected with MuLV.

Proteases are a class of enzymes active in many different biological processes including protein degradation, cell death, and tissue differentiation (Dale and Vergnolle, 2008). In addition to these functions, specific types of enzymes such as serine proteases are able to cleave protease-activated receptors to activate signal transduction processes which in turn regulate the inflammatory response (Dale and Vergnolle, 2008).

Although there are different protease-activated receptors, which are currently differentiated dependent on the resulting cascade effects, it would be interesting to investigate the known proteases and their receptors in the MAIDS model system to investigate whether the two strains of mice are using the same receptors to activate the innate immune response. It would also be interesting to consider the protease-activated receptors individually because it has been suggested that some pathogen proteases are able to disarm the receptors,
providing an escape mechanism for the pathogen by preventing the innate immune response from being properly initiated (Dale and Vergnolle, 2008).

Enzymes associated with pancreatic function are currently being found to be important molecules in the innate immune response associated with shock and the destruction of tumor cells (Waldo et al., 2003). A study that examined the ability of individual organs to produce inflammatory responses associated with shock, noted that the pancreas was the only organ that was able to activate naïve leukocytes without the addition of exogenous enzymes (Waldo et al., 2003). They concluded that the pancreas can stimulate cell activation and kill naïve neutrophils needed for the innate immune response due to digestive enzymes including trypsin, lipase, and chymotrypsin. Therefore it would be interesting to compare serum levels of other lipases and digestive enzymes being produced by the pancreas to the number of circulating neutrophils within the first three days after infection by MuLV in both BALB/c and BL/6 mice.

Two lipases, pancreatic lipase and pancreatic lipase related protein 2, found in the pancreas, and IL-4, induced CD8 T cells (CTLs) to generate products found to be toxic to tumor cells in the presence of appropriate lipid substrates (Alves et al., 2009). It was concluded that for the effects to be valid in vivo, the lipid substrate must be present in the serum. The authors suggest that this is possible due to the incorporation of the substrates in very low density lipoproteins. Future studies should focus on other aspects of the immune response due to the potential of other overlapping interactions.
In the context of future experiments with the MAIDs model system, it would be interesting to determine if the small amount of lipase activity present was produced from CTLs or other cells within the spleen. Before isolating the CTLs from the animals, it would be pertinent to compare the presence of IL-4 in each condition. If there is no difference in the amount of IL-4 present, it would suggest that the animals have the same ability to induce lipase production by CTLs. However, if there is more IL-4 found in BALB/c animals it would be worthwhile to consider the effect of this cytokine on CTLs.

It has also been discovered that pancreatic lesions with associated inflammatory cell infiltration, along with acinar cell and pancreatic ductal destruction, are found in BL/6 mice at four weeks after infection with LP-BM5 MuLV (Wantanabe et al., 2003). The most predominant cell types observed infiltrating and destroying the pancreas were CD4⁺ T cells and Mac-1⁺ cells (Wantanabe et al., 2003). Mac-¹⁺ cells, such as macrophages, natural killer cells, granulocytes, and activated lymphocytes, express cell surface glycoproteins (CD11b/CD18) that play a role in cellular adhesion (Schleiffenbaum et al., 1989). Surprisingly, pancreatic destruction by these cell types did not have any significant effect on the serum levels of lipase, amylase, and glucose in BL/6 mice (Wantanabe et al., 2003). It would be interesting to compare these results, which used the BL/6 background, to disease-resistant BALB/c mice in order to investigate potential pancreatic destruction at this later stage after MuLV infection.
Conclusion

To conclude, results of lipase activity in spleen suggest that the strain, rather than the treatment with MuLV, had the main effect on the lipase activity. Although the trend resembles that seen in the microarray data, with the BALB/c mice expressing more than the BL/6, the biological variability within each condition suggests that the results are not statistically significant. However, the spleen weights were significantly different between strains and conditions, suggesting that more cells are infiltrating the spleen upon infection in BALB/c mice.

Future work with pancreatic enzymes such as CTL lipase, also confirmed to be pancreatic lipase related protein 2 by sequence analysis, and their receptors, could provide valuable information on whether the innate immune system in the disease-resistant BALB/c resistant strain is activated earlier and in a unique way to give the selective advantage for protective immunity in the future. Other future studies should consider the effect of CTL lipase on the activation and mortality of neutrophils in the early innate response, or the destruction of the pancreas by CD4\(^+\) T cells in resistant strains during the adaptive immune response.


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