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Building a T-Cell Granulysin Expression System to Investigate Tuberculosis Infection

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Building a T-Cell Granulysin Expression System to Investigate Tuberculosis Infection

A Major Qualifying Project
Submitted to the Faculty of
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Degree in Bachelor of Science
In
Biology and Biotechnology
By
Edith Sawyer

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Project Advisors: Lou Roberts, PhD; Samuel Behar, MD PhD
Abstract

Tuberculosis is a bacterial infection caused by *Mycobacterium tuberculosis*, commonly presents as an infection of the alveolar sacs and can be difficult to treat. Granulysin, a lytic enzyme employed by CD8 cells during infection, has been shown to degrade *M. tuberculosis*. This project utilizes cloning, transfection, flow cytometry, and qPCR to build lentivirus and gamma-retrovirus expressing three isoforms of granulysin. These viruses can be used to infect CD8 cells and express human granulysin to better study granulysin’s interaction with *Mycobacterium tuberculosis*. Gaining a more complete understanding of these interactions can help identify future targets to help develop a new vaccine against tuberculosis.

Introduction

The World Health Organization (WHO) estimated over 10.4 million cases of tuberculosis existed worldwide in 2015 [1]. Tuberculosis (TB), a bacterial infection caused by *Mycobacterium tuberculosis*, is most commonly observed as bacteria infecting alveolar sacs of the lungs. Symptomatic cases typically result in coughing, chest pain, and trouble breathing [2]. While most infections are transmitted through aerosolized droplets, *M. tuberculosis* can also be transmitted through open wounds or ingestion, resulting in infections outside the lung [3]. Tuberculosis infection is observed in and documented as two stages, identified as active and latent. Latent infections are asymptomatic cases which are not transmissible among people, while active infections can be transmitted in many ways, most frequently through airborne particles [3].

Upon infection by *M. tuberculosis*, macrophages near the site of infection are often the first host cells to come into contact with the pathogen. These macrophages phagocytose the bacteria in the beginning steps of the humoral immune response [3]. The endocytic vacuole containing the phagocytosed pathogen typically fuses with a lysosome, whose lytic enzymes kill the pathogen. While some pathogens have developed methods of preventing this fusion from occurring in order to prolong survival, it remains unclear the exact mechanism *M. tuberculosis* uses to avoid degradation within the macrophage [3]. The ability of *M. tuberculosis* to avoid destruction by hiding within phagosomes is a major reason why treating such infections can be difficult.

When considering the active cases of tuberculosis documented in 2016, the proportion of fatal cases differs dramatically in various countries. For example, 20% of cases in poorer African countries result in fatality, while only 5% of cases in more developed countries are fatal [1]. Currently, antibiotics are the most widely used method of treatment for tuberculosis infection, despite the fact that there are very few known antibiotics which are ultimately successful in killing *M. tuberculosis*. The two most commonly used and effective antibiotics are isoniazid and rifampin; these are often prescribed in conjunction with other antibiotics including pyrazinamide, doxycycline, and ethambutol in the case of active infections [2]. Though a limited number of effective antibiotics against TB have been identified, treatment regimens vary vastly on a case-by-case basis depending on disease intensity and resistance of each strain. Typically, regimens
begin with daily doses of isoniazid, rifampin, pyrazinamide, and ethambutol for eight weeks to combat the diseases most aggressive phase and continue with additional doses of isoniazid and rifampin for 18 weeks [4]. A significant cause of mortality in developing countries is the emergence of antibiotic-resistant *M. tuberculosis* strains. Attempts to develop a vaccine for tuberculosis prevention have not been resoundingly successful to date, though an effective vaccine offers a promising alternative to antibiotics and could significantly decrease the burden of disease worldwide [1].

Research conducted by the Behar lab at the University of Massachusetts Medical School in the Department of Microbiology and Physiological Systems focuses primarily on understanding the immunological response of the body to a tuberculosis infection. More specifically, the lab aims to identify the role of CD8 cytotoxic T-cells during *M. tuberculosis* infection. The goal is to identify particular mechanisms of action of the immune system and steps of infection that may be targeted to inform the design and development of vaccines against *M. tuberculosis*.

During an infection, the human body launches an immune response consisting of many distinct components, including two main types of white blood cells: B-cell and T-cell lymphocytes. Each of these components serve different purposes in fighting infection. B-cells help fight against infection by secreting antibodies specific against antigens. CD8 T-cells act to lyse host cells that are infected with virus or bacteria in order to prevent the pathogen from replicating and infecting other cells. CD8 T-cells do this by binding to and recognizing antigens on the infected cell surface, composed of digested antigen presented on the surface of macrophages. This interaction signals the T-cell to release cytotoxic granules into the intracellular space, which contain molecules called perforin and granzymes [5]. Perforin works by poking holes in the microbial cell wall or membrane of an infected cell so that granzymes can enter the cell. Granzymes are serine proteases that induce apoptosis by generally cleaving protein peptide bonds within the target cell [6]. An example of one of these granzyme serine proteases is an enzyme called granulysin.

Granulysin is a lytic enzyme encoded by a gene found on Chromosome II. The granulysin gene is 3.9 kb long and contains six distinct exons. This gene is transcribed, spliced, and translated into three different inactive protein isoforms which are each approximately 15 kDa, with most difference among isoforms found within the second exon. This 15 kDa precursor protein is produced constitutively and has a shorter half-life compared to its 9 kDa counterpart although the exact half-lives for each are unknown. Upon stimulation, the precursor is then post-translationally processed through proteolytic cleavage on both the N- and C-termini into one of three active 9 kDa protein isoforms [7]. These three isoforms are referred to in most literature as isoforms 519, 520, and 522; their lengths are 390, 420, and 460 base pairs, respectively. Through the remainder of this report these isoforms will be referred to as isoforms 1, 2, and 3. Figure 1 visually summarizes the process involved in expressing and processing the granulysin isoforms.
Differences in the functions of the 15 kDa and 9 kDa isoforms remain unclear, though many are hypothesized. Research suggests that while the 15 kDa isoform is expressed and secreted constitutively from the CD8 cell, it does not survive long within the cell before it must be post-translationally processed [7]. Otherwise, it is quickly degraded. The 15 kDa protein is only converted to a 9 kDa protein in the case of a proinflammatory response to help mediate both intracellular and extracellular infections, including *M. tuberculosis* [8]. Little is known about the differences between the three 9 kDa isoforms other than that they are formed by alternative splicing, and that the variations between isoforms exist mostly within the gene’s second exon. Additionally, isoform 2 is widely regarded to be the most prevalent in humans [7].

Previous research has demonstrated granulysin’s ability to degrade *M. tuberculosis* when directly applied to the bacteria [9]. However, the *in vivo* interactions between *M. tuberculosis* and active granulysin are not widely understood. As a result, this project aims to better understand the role of granulysin during a TB infection. The purpose of this project is to develop retroviral vectors expressing each of the three mRNA isoforms of granulysin. Two vectors are used for each isoform, resulting in six final constructs. One viral vector is lentivirus using the LeGO iG2 plasmid and the second is gamma retrovirus using the pMIGII plasmid. Figure 2 shows an overview of each viral system used to drive expression of the granulysin isoforms.

Each mRNA isoform is expressed in two separate viral vector systems because each system offers a separate set of advantages for future expression or implementation. Lentiviruses, unlike gamma retroviruses, are able to infect non-dividing cells, offering a possible advantage to increase expression in T-cells. However, while gamma retroviruses allow for preferential insertion sites in target genomes, lentiviruses randomly insert their genetic information into their
target. Additionally, lentiviral vectors are shown to have lower transduction efficiency than gamma retroviruses [10].

Figure 2: Overview of plasmid system

Figure 2 depicts key components of both viral vector systems including restriction enzyme cut sites, markers, promoters, and envelope/packaging plasmids. The gamma retroviral plasmid pMIGII uses a constitutive 5’ LTR promoter which allows for constant expression of granulysin isoforms located between EcoRI and BamHI cut sites. The 5’ LTR and the 3’ LTR, located between the GFP marker and ampicillin resistance gene, mark the viral coding region of the plasmid. The ampicillin resistance gene allows for bacterial colony selection but does not influence virus production. pMIGII was transfected with Ecopak packaging plasmid and pVSVG envelope plasmid. The broad tropism associated with pVSVG allows for the virus created by the system to non-specifically infect many different types of cells.

The lentivirus LeGO iG2 uses a constitutive SFFV promoter derived from the spleen focus-forming virus which also allows for constitutive expression of granulysin isoforms. The SFFV promoter and the 5’ truncated LTR located between the GFP and ampicillin resistance genes mark the beginning and end of the gene sequence encoding plasmid production. LeGO iG2 was transfected with psPAX2 packaging plasmid and pMD2G envelope plasmid which is also broadly tropic, similar to pVSVG, and is a widely used lentiviral envelope plasmid.

The purpose of this project is to develop retroviral vectors expressing each of the three mRNA isoforms of granulysin. These three isoforms were cloned into lentiviral and gamma-retroviral vectors and later transfected into mammalian cells lines where granulysin expression was confirmed and quantified.
Methods

Cloning of the Granulysin Isoforms

Synthesizing Granulysin and Oligos

Complete sequences for each of three total granulysin isoforms were synthesized by Genscript (Piscataway, NJ) in packaging plasmids with specified cut sites as indicated in Figure 2. Primers used for PCR reactions were ordered from IDT (Coralville, IA). Primers were reconstituted to 100 μM with DNase-free water and stored at -20 °C. Table 1 details the name, function, and sequence of each primer oligo used for granulysin isolation.

<table>
<thead>
<tr>
<th>Oligo Name</th>
<th>Function</th>
<th>Sequence (5'→3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward-1,3</td>
<td>Forward primer to amplify granulysin isoforms 1 and 3 from packaging plasmid for LeGO and pMIG ligation</td>
<td>AATAATGGATCCATGGCTACCTGGGCCCCTCTCTG</td>
</tr>
<tr>
<td>Forward-2</td>
<td>Forward primer to amplify granulysin isoform 2 from packaging plasmid for LeGO and pMIG ligation</td>
<td>AATAATGGATCCATGGAAGGTCTGGTCTTCTCTCT</td>
</tr>
<tr>
<td>Reverse-LeGO</td>
<td>Reverse primer to amplify granulysin isoforms 1, 2 and 3 from packaging plasmid for LeGO ligation</td>
<td>AATAATCCTGCAGGTCAGAGGGGACCTGT</td>
</tr>
<tr>
<td>Reverse-pMIG</td>
<td>Reverse primer to amplify granulysin isoforms 1, 2 and 3 from packaging plasmid for pMIG ligation</td>
<td>AATAATCCTCGAGTCAGAGGGGACCTGTAGAAGG</td>
</tr>
</tbody>
</table>

*Table 1: Name, function, and sequence of each primer oligo used for granulysin isolation*

Granulysin Amplification and Isolation by PCR

Polymerase Chain Reaction (PCR) using the ThermoFisher Phusion Hot Start Flex Polymerase kit and 100 μg of template DNA was used to amplify and isolate all three granulysin isoforms from the packaging plasmids in which they resided. Primers that were designed and ordered to match each isoform also contained restriction enzyme sites which would be later used for ligation purposes. The PCR protocol used was the following: initial denature at 98 °C for 30 sec followed by 98 °C for 10 sec, 59 °C for 30 sec, and 72 °C for 30 sec repeated 33 times, with a final extension of 72 °C for 10 minutes.
Gel Electrophoresis

Subsequent to PCR to isolate and amplify the three granulysin isoform inserts, a 1.2% agarose gel was run in order to determine if the three insert sizes matched the expected size for each isoform. Table 2 details the expected sizes for each isoform.

<table>
<thead>
<tr>
<th>Isoform #</th>
<th>Expected Size (base pairs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>390</td>
</tr>
<tr>
<td>2</td>
<td>420</td>
</tr>
<tr>
<td>3</td>
<td>522</td>
</tr>
</tbody>
</table>

*Table 2: Expected sizes for each granulysin isoform*

Restriction Digest

Double restriction digests were run on PCR reaction products. LeGO was cut by SbfI and BamHI restriction enzymes and pMIG was cut by EcoRI and XhoI restriction enzymes, each in CutSmart reaction buffer. The protocol for the restriction digest was followed exactly from the NEB restriction digest protocol. Reactions were incubated for one hour at 37 °C.

Ligation

Each of the three granulysin isoforms were ligated with both LeGO lentivirus or pMIG gamma retrovirus backbones resulting in six unique ligation reactions. Reactions were performed by exactly following the NEB T4 ligation protocol. The final volume for each reaction was 20 µL and reactions were placed in a thermocycler and run at 16 °C overnight. The ligation reaction was heat inactivated at 65 °C for 15 minutes.

Transformation

Eight samples (six experimental and two negative controls) were then transformed using the NEB High Efficiency Transformation Protocol along with pUC19 positive control. 10 µL of each sample and 2 µL of the positive control were used for the transformations. Transformation plates were incubated on LB + ampicillin plates at 37 °C overnight.

Colony PCR

Eight colonies were picked from each experimental plate and inoculated in LB + ampicillin medium to grow liquid cultures overnight for DNA isolation. Each colony was also used to provide template plasmid for PCR amplification. ThermoFisher Taq polymerase and Hot Start Flex Master Mix kit were used along with 10.0 µM forward and reverse primers used for granulysin isolation. The PCR protocol used was the following: initial denature at 98 °C for 30
sec followed by 94 °C for 10 sec, 59 °C for 30 sec, and 72 °C for 15 sec repeated 33 times with a final extension of 72 °C for 10 minutes.

DNA Isolation

DNA was isolated using the QIAprep Spin Miniprep Kit from colonies harboring vectors containing granulysin as determined from colony PCR and diagnostic gel electrophoresis results. The protocol was followed exactly from the QIAprep Kit instructions. DNA concentration and purity were determined using Nanodrop.

Sequence Confirmation

Purified plasmid DNA from selected colonies were sent to Etonbio along with primers used for granulysin isolation and following their instructions for sequencing. Sequence results were aligned and cross-examined in Benchling with known granulysin isoform sequences. Plasmids with sequences which aligned with expected sequences were used moving forward for transfections into mammalian cell lines.

Transfecting Cloned Plasmid into Mammalian Cell Lines

Cell Culture Techniques and Protocols

Cell culture medium used was 10% Fetal Bovine Serum (FBS) complete RPMI (cRPMI) sterile filtered through a 0.22-micron filter. The mixture for cRPMI was as follows:

RPMI Medium – 425 mL  
FBS – 50 mL  
HEPES Buffer – 5 mL  
Non-Essential Amino Acids (NEAA) – 5 mL  
Penicillin/Streptomycin Antibiotic Mixture (Penstrep) – 5 mL  
Sodium Pyruvate – 5 mL  
L-Glutamine – 5 mL

All mammalian cell culture procedures and methods were performed in a sterile hood and all cell lines were grown in a 37 °C incubator with 5% CO₂ and humidified air.

Transfection Protocols

Viral plasmids for granulysin isoforms 1, 2 and 3 were used to transiently transfect 293T embryonic kidney cells. Supernatant, containing virus, from 293T cells transfected by isoforms expressed by gamma-retroviral plasmids was then used to transfect stable cell lines of GP+E86 fibroblasts. Supernatant from 293T cells transfected by isoforms expressed by lentiviral plasmids was saved for future titering purposes. Both transient and stable transfections were performed by
exactly following the protocol provided in *Generation of T-cell receptor retrogenic mice* steps 19-27 [11].

**Flow Cytometry, Cell Sorting and Titering**

**MACSQuant Flow Cytometry**

After transient (293T) and stable (GP+E86) transfections, cell lines expressing granulysin isoforms and non-transfected controls were grown to confluence. The cells were harvested by trypsinization, washed in RPMI, and counted in cRPMI with FBS. 100 μL of solution from each sample were taken and cells were resuspended in 100 μL of MACS buffer (2% calf serum 98% PBS) while the remaining cells were washed again using RPMI. Cells resuspended in MACS buffer were screened using MACSQuant Analyzer flow cytometry to identify GFP⁺ fluorescence of each population.

Cell populations were gated using forward scatter (FSC) vs side scatter (SSC) to identify the cell population within solution that most closely matched the expected size of living cells. This population was then gated using FSC vs FSC in order to identify single cells in the population because cells that clump together are more likely to be dead or abnormal. This final cell population was analyzed using SSC vs FITC (GFP) to visualize how many of the living, single cells in each sample were positive for GFP fluorescence.

**GFP⁺ Cell Sorting**

After successful transfections into GP+E86 cells were confirmed by flow cytometry, the remaining cells from each sample were resuspended in 1% FBS in DPBS (with no calcium, magnesium, or phenol red) running medium at a concentration of 1 million cells per mL. Additional collection medium of 20% FBS in RPMI and cRPMI with gentamycin and fungizone was prepared. The running medium cell suspension and collection medium were taken to the University of Massachusetts Medical School cell sorting service to sort and collect the top 50% of GFP⁺ cells. These cells were cultured and grown as previously indicated to confluence.
Titering

In a 6-well plate, 2 million stable transfected GP+E86 cells expressing isoform 2 or 3 were plated in 2 mL of cRPMI and incubated at 37 °C for 24 hours to produce concentrated virus. Virus used to titer isoform 1 was retrieved from medium removed and frozen during harvests one and six of lentiviral transfection. Experimental plate set-up for all isoforms are depicted in Figure 3. Dilutions used to determine viral titer for isoforms 2 and 3 were made by exactly following the titering protocol in *Generation of T-cell receptor retrogenic mice* steps 31-35 [11]. The same titering protocol used for isoforms 2 and 3 was also used for isoform 1 except isoform 1 virus was not diluted.

Transfected titer plates were left to incubate for 72 hours and analyzed using flow cytometry for GFP⁺ cells. Final titer was determined by identifying the dilution for each isoform that produced a percentage of GFP⁺ cells closest to 50% and using the formula: percent positive x 0.01 x dilution factor x 10⁴ to determine titer for each isoform.

![Titering experimental set-up](image)

*Figure 3: Experimental plate set-up for titering*
qPCR mRNA Quantification

GP+E86 cells were lysed and RNA was isolated using the TRIzol Reagent RNA isolation protocol (Invitrogen). Isolated RNA was then transcribed into cDNA using by following the Invitrogen Superscript III kit protocol. Random hexamers were used as primers to transcribe cDNA non-specifically.

qPCR analysis was used to quantify the expression of granulysin in GP+E86 in conjunction with a β-Actin control and a non-transfected GP+E86 control. The β-Actin control was used to normalize the production of granulysin within each sample. The qPCR reactions were set up using the following reaction:

10 μL – ThermoFisher Fast SYBR green master mix
1 μL - Paired 20 μM forward and reverse primer mixture
8 μL - RNAse free water
1 μL - 1:10 dilution cDNA

Granulysin primers used for qPCR analysis were non-specific to various granulysin isoforms. Forward and reverse primer sequences used in the reaction are shown in Table 3.

<table>
<thead>
<tr>
<th>Oligo name</th>
<th>Function</th>
<th>Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>qPCR- Forward</td>
<td>Forward primer to amplify all isoforms of granulysin for qPCR analysis</td>
<td>GTGAAGAGACCCCACCCGAATAG</td>
</tr>
<tr>
<td>qPCR- Reverse</td>
<td>Reverse primer to amplify all isoforms of granulysin for qPCR analysis</td>
<td>TAGGACGCGGGAGGTACG</td>
</tr>
</tbody>
</table>

*Table 3: Name, function, and sequence of granulysin primer oligos used for qPCR analysis*

Finally, the qPCR reaction was set-up in a 96-well plate as depicted in Figure 4.
The qPCR reaction was run under the following thermocycling conditions:
  Activation - 95° C for 20 sec
  Denature - 95° C for 3 sec
  Anneal/Extend - 60° C for 30 sec
  Repeat denature and anneal/extend cycles 40 x

Average Ct values were determined for each triplicate reaction and ΔCt values were determined by subtracting the average β-Actin Ct values from the average granulysin Ct values for each isoform. Relative expression was determined compared to β-Actin and non-transfected granulysin controls by using the equation: $2^{-\Delta\text{Ct}}$. 
Results

DNA Cloning

Gel electrophoresis results from granulysin isolation and amplification shown in Figure 5 indicate that all three isoforms were amplified to their expected sizes. The no template control (NTC) was used as a negative control in order to ensure there was no contamination in the reactions. These sequences and backbone plasmids (LeGO and pMIGII) were digested using restriction enzymes to create compatible overhangs between backbones and inserts. Granulysin and backbone plasmids were then ligated and transformed into DH5α E. coli cells. Eight colonies were picked from each plate and were tested using colony PCR to determine if they harbored the vector containing the expected granulysin isoforms. Figure 6 shows results of colony PCR. DNA from granulysin-positive colonies was sent to be sequenced to confirm proper granulysin insertion. Sequencing results were analyzed in Benchling (Figure 7) and confirmed granulysin incorporation in the following plasmids: isoform 1 in LeGO backbone, isoform 2 in pMIG II backbone, and isoform 3 in pMIG II backbone.
Transfections

293T cells were transiently transfected using each of the confirmed retroviral plasmids, three transfections in total. Flow cytometry was used to determine the transfection efficiency in these cells by quantifying GFP fluorescence in the cell populations. Figure 8 panel A and B show the gating process used to confirm fluorescence in 293T cells. Panel C depicts non-transfected wildtype control and panel D depicts the transfected isoform 2 cell line. The cell line transfected with vector shows much higher fluorescence than non-transfected wildtype. Flow cytometry on 293T cells transfected with isoforms 1 and 3 yielded the same results.

Figure 7: Example of sequencing results showing sample match to expected granulysin sequence

Figure 8: Forward scatter vs side scatter (A) and forward scatter vs forward scatter (B) of 293T cells used to gate sample populations. FITC fluorescence vs side scatter used to determine transfection efficiency in wildtype 293T population (C) and 293T population transfected with granulysin isoform 2 (D) shows higher GFP+ fluorescence in transfected cell lines.
After transient transfections into 293T cells were confirmed by flow cytometry, isoforms 2 and 3 were transfected into GP+E86 stable cell lines. Transfections into GP+E86 fibroblasts were also confirmed by flow cytometry as shown in Figure 9. Increased fluorescence of isoforms 2 and 3 compared to the wildtype confirmed successful transfection into fibroblasts.

Figure 9: Normalized FITC fluorescence across wildtype (orange) and transfected (blue & red) GP+E86 cell lines also shown increased fluorescence in transfected cell lines.

After transfection was confirmed in GP+E86 cell lines and cells were sorted to isolate the top 50% most GFP+, qPCR was used to quantify granulysin mRNA expression in isolated. Figure 10 shows granulysin expression normalized to an external B-actin control and normalized to a non-transfected control. Wildtype, isoform 2 and isoform 3 samples had equivalent B-actin expression with average Ct values of 23.8, 24.6, and 23.7 respectively. Relative granulysin expression compared to B-actin was calculated and displayed in Figure 10. Low granulysin expression in wildtype control and high granulysin expression in transfected samples confirms the results obtained by flow cytometry, namely that cells transfected with granulysin isoforms express granulysin mRNA.
Finally, titering experiments were performed to determine the viral load produced by the transfected cell lines. Titer was derived by using flow cytometry to determine the number of GFP+ cells per well. Analysis showed isoforms 2 and 3 produced significantly higher viral loads than Isoform 1. Titer values are shown in Table 4.

![Figure 10: qPCR analysis of cDNA from lysed GP+E86 cells show six-fold more granulysin production in transfected cells than wildtype relative to consistent β-actin control.](image)

<table>
<thead>
<tr>
<th>ISOFORM #</th>
<th>DILUTION</th>
<th>%GFP+</th>
<th>TITER</th>
</tr>
</thead>
<tbody>
<tr>
<td>ISOFORM 1</td>
<td>No dilution</td>
<td>34</td>
<td>0.03x10&lt;sup&gt;5&lt;/sup&gt;</td>
</tr>
<tr>
<td>ISOFORM 2</td>
<td>1:33</td>
<td>35</td>
<td>1.16x10&lt;sup&gt;5&lt;/sup&gt;</td>
</tr>
<tr>
<td>ISOFORM 3</td>
<td>1:10</td>
<td>49</td>
<td>0.49x10&lt;sup&gt;5&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

*Table 4: Titer analysis of granulysin isoforms*
Discussion

The objectives of this project were to design and build retroviral plasmids containing three granulysin isoforms, transfect mammalian cell lines with granulysin-expressing retroviruses, and quantify expression in transfected cell lines. Overall, project objectives were met.

Attempts to clone granulysin isoforms into retroviral backbones were successful, resulting in sequence-confirmed plasmids containing each isoform. The key limiting factor throughout the cloning process was time. Lack of available time led to progressing with only one or two unique isoforms cloned into either lentiviral or gamma-retroviral backbones. Moving forward, successfully cloning the remaining retroviral plasmids would allow for a more wholistic analysis of the lenti and gamma-retroviral systems, as well as all three granulysin isoforms in comparison to one another.

Transient transfections of isoforms 1, 2 and 3 into 293T cells and stable transfections of isoforms 2 and 3 into GP+E86 cells where shown to be successful using flow cytometry. Fluorescence along with qPCR data confirmed successful transfections (via GFP) and expression of granulysin mRNA (via qPCR) in GP+E86 cells transfected by isoform 2 and 3 gamma-retroviruses.

Titering data reveals significantly lower viral yield from isoform 1 compared to isoforms 2 and 3. This could be the result of using medium from the first harvest of the lentiviral transfections for the titering protocol. Repeating titering experiments using medium from later harvests could result in higher titers as there was likely more viral production during harvests 2-5 of lentiviral transfection. Alternatively, lower titers could be the result of the lentiviral plasmid itself. Literature regarding lentiviral gene vectors suggests that lentiviruses tend to produce lower titers than gamma-retroviruses [10]. Additionally, re-sorting GP+E86 stable cell lines to isolate top 10% GFP+ cells could also help increase viral titers in isoforms 2 and 3 if necessary for future transfections into T-cells. However, because sorting the top 50% of GFP+ cells yielded approximately 700,000 cells per isoform, sorting the top 10% of GFP+ cells would likely yield fewer cells.

The intent of the Behar lab is to continue moving forward with isoforms 2 and 3 confirmed in pMIG gamma-retroviral plasmids. These isoforms will be transfected into T-cells, and granulysin protein production and activity will be confirmed. SDS PAGE gels with immunoblot will confirm protein-level granulysin expression, and ELISA assays will confirm enzyme activity. Isoform non-specific anti-granulysin antibodies are available for purchase through Invitrogen, Biolegand, and several other companies. The eventual goal of the Behar lab is to use retroviral plasmids containing human granulysin to create a retrogenic mouse model that can be used to study interactions between granulysin and Mycobacterium tuberculosis over the course of infection. The successful completion of this projects work has been a necessary and important step toward realizing the goal of this research.
References


Additional Bibliography


