Single-Cell Analysis of RNA Expression following Virus Infection

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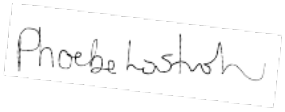

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Abstract

Epstein-Barr virus (EBV) and Kaposi's sarcoma herpesvirus (KSHV) are human gammaherpesviruses. Gammaherpesviruses are known to be host-specific and cause lifelong infection. Macrophages are infected by gammaherpesviruses, but this process is poorly defined; it is unknown if they undergo lytic replication, latency, or some combination of these. Prior studies in the van Dyk lab utilizing MHV68, a murine model for gammaherpesviruses, shows efficient viral entry in both macrophage-like cells and permissive control fibroblasts. Permissive control fibroblasts are fully susceptible to infection done by MHV68. However, macrophage-like cells show a defect in subsequent viral replication and viral gene expression. Based on prior lab findings using bulk cell analyses, we wanted to know if viral gene expression is uniformly low/absent in infected macrophage-like cells or if there is a small population of cells with robust viral gene expression.

We hypothesized that viral gene expression was contributed by a small percentage of macrophage-like cells when compared to permissive control fibroblast cells, in which most cells show abundant viral gene expression. To analyze viral gene expression, we used single-cell analysis of viral RNA expression through flow cytometric detection of RNA hybridization. We analyzed RNA expression in J774 macrophage-like cells versus 3T12 permissive fibroblasts through a RNA PrimeFlow. Through this RNA assay, we used RNA probes specific to cellular beta-actin (degraded in active virus replication), TMERs (viral ncRNAs that are abundantly expressed in lytic and latent infection), and ORF18 (viral late RNA only expressed in active lytic infection). We found that viral gene expression in J774 macrophages was low and that there was not a population of cells that showed abundant viral expression. The decrease in ORF18 signal was more pronounced in J774 macrophages than TMER signal, consistent with either a defect in

viral replication/late gene expression or with latent infection in J774 macrophages. Caveats to our interpretation are the need for additional optimization of our infection and RNA PrimeFlow protocols since overall viral gene expression appeared to be low in both cell types. Nonetheless, this experiment demonstrated how single-cell analysis can shed greater light on bulk analysis, particularly in cases of heterogeneous responses within cell populations. Our data allows us to potentially develop vaccines that can prevent latent infection done by the gammaherpesvirus.

Introduction

Gammaherpesviruses (Epstein-Barr virus and Kaposi's sarcoma herpesvirus)

The gammaherpesvirus family is a large double-stranded DNA known for causing inflammatory diseases and cancer, especially in immunocompromised people (Diebel et al., 2015). Epstein Barr virus (EBV) and Kaposi's sarcoma-associated herpesvirus (KSHV) are human gammaherpesviruses known to develop life-long infections within their hosts via latency in lymphocytes (Diebel et al., 2015). During latency, EBV and KSHV can express a variety of proteins and noncoding RNAs that can affect the host's cell cycle, increase cell proliferation, and inhibit apoptosis (Cesarman, 2013). EBV is known to cause several human malignancies, primarily B cell lymphomas, carcinomas of the stomach and nasopharynx, NK (natural killer) and T cell lymphomas, and leiomyosarcomas (Morales-Sánchez and Fuentes-Panana, 2018). EBV uses a combination of lytic and latent infections to persist within its host. Like EBV, KSHV also uses lytic and latent infections to establish infections in its host. KSHV is known for causing Kaposi's sarcoma and primary effusion lymphoma. An overview of lytic and latent infections is needed to understand how EBV and KSHV can infect their hosts.

Latent and Lytic Infections

Lytic infections occur when a virus infects a host cell, replicates within the host, and eventually bursts through the cell membrane. Latent infections are when a virus will stay within the host without causing symptoms, through this kind of infection there will be no active viral production that occurs. However, with latent infections, the virus can be reactivated to perform lytic replication, and the host can begin to have viral symptoms.

Lytic infection for herpesviruses and their gene expression are divided into three different stages, immediate-early (IE), early (E), and late (L) (Gruffat et al., 2016). The immediate-early genes can be transcribed in the absence of *de novo* protein synthesis (Gruffat et al., 2016). The immediate-early genes are then used to code for viral regulatory proteins that are involved in the transcriptional control of the early genes. The viral early genes are then used for replication of the viral genome, expression of late viral genes, and the accumulation of early and late viral mRNAs (Gruffat et al., 2016). After viral DNA replication, the late viral genes begin to be transcribed, which eventually leads to the production and release of infectious viral particles. We know a great deal about how immediate-early and early genes are regulated; however, the mechanism behind late viral genes is less understood. The Sun lab identified several viral proteins that were necessary for late viral gene expression (Gruffat et al., 2016). There are five viral genes for the mouse model of gammaherpesvirus, MHV68 – ORF18, ORF24, ORF30, ORF31, and ORF34 – whose deletion led to loss of late viral gene expression but had no effect on early viral gene expression or viral DNA replication (Figure 1).

While these five viral genes are important for viral gene expression, there are non-coding RNAs (ncRNAs) that are necessary for lytic replication. Both lytic and latent infections require a certain subset of viral genes to work. One non-coding RNA that is present during viral infection is tRNA-miRNA-encoded RNAs (TMERs). TMERs are dispensable for lytic replication and latency, but they are required for the pathogenesis and infection of immunocompromised individuals (Knox et al., 2021). Viral ncRNAs, like TMER, are essential for pathogenesis. We now know that lytic infection is involved in upregulating promoter activity in a variety of hosts and viral pol III-dependent transcripts (Knox et al., 2021). Depending on the cell type and the environment, these gammaherpesviruses can result in active lytic replication or latent infection.

Immediate-Early Viral Genes	Early Viral Genes	Late Viral Genes
K3	ORF6	ORF18**
Rta	TMER*	ORF24
M8		ORF30
ORF73		ORF31
		ORF34
		M7
* - Involved in lytic replication and latency ** - Involved in lytic replication only		

Figure 1: Viral genes that are present in the different stages of viral infection.

Viral Replication and Gene Expression in Fibroblasts and Macrophage-Like Cells

3T12 fibroblasts and J774 macrophage-like cells are good candidates for understanding more about gammaherpesvirus infections, specifically using MHV68, a mouse model for gammaherpesviruses. These two cell types are good candidates because 3T12 fibroblasts have been studied greatly when it comes to infection done by MHV68. 3T12 fibroblasts serve as a good reference control when it comes to determining if a cell is undergoing lytic infection versus latency. J774 cells were another good candidate because we don't have a good baseline of whether they undergo lytic replication or latency. Macrophages are also an important part of our immune system, so it's beneficial to understand how viruses can infect the cell. Gaining a better understanding of J774 cells can also help us develop ways to prevent latency and gammaherpesvirus infections.

Prior experiments done in the van Dyk lab have indicated that 3T12 fibroblasts are fully permissive to active lytic replication of the virus, they are very well-characterized, and serve as a reference control to the J774 cells. However, while macrophages can be infected by gammaherpesviruses, the process is poorly defined. From the van Dyk lab, we know that post-translational modifications and viral protein expression at the single-cell level have shown a

complete defect in most macrophage cells with a small percentage of those cells being positive for these protein markers. Prior research from the van Dyk lab has indicated that there may be a division of how cells are infected based on prior bulk cell analyses, but we would like to know what this looks like on a single-cell level. Figure 2 depicts the viral genome copies in 3T12 fibroblasts and J774 macrophages, 24 hours post-infection, 48 hours post-infection, and 72 hours post-infection. As expected, the 3T12 fibroblasts have a normal amount of viral genome copies compared to a typical infection level indicating that there is no disruption in viral replication (Figure 2). In J774 cells (blue line), there is a delay in the number of viral genome copies that are made, indicating that there is a relative defect in J774 viral replication. Figure 2 depicts the overall viral gene expression in 3T12 fibroblasts and J774 cells. As mentioned earlier, there are different waves of virus gene expression: immediate early, early, and late. ORF73 is an immediate early gene, ORF6 is an early gene, and M7 is a late gene. For J774 cells, there is less expression of ORF73, ORF4, and M7 when compared to 3T12 fibroblasts (Figure 3). This difference in viral gene expression was more pronounced in ORF6 and M7, indicating for J774 cells there is a greater defect in viral RNAs which are needed at later stages of infection. This defect in viral replication and viral gene expression in J774 cells led to our research question.

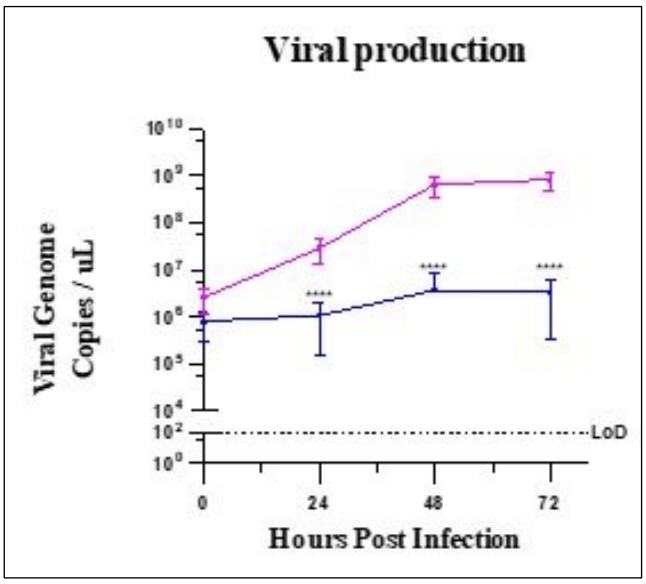

Figure 2: 24-hour and 48 hours post-infection (hpi) RNA expression graph depicting viral genome copies in 3T12s (pink line) and J774s (blue line), 24 hpi, 48 hpi, and 72 hpi. Unpublished data from Linda van Dyk's lab

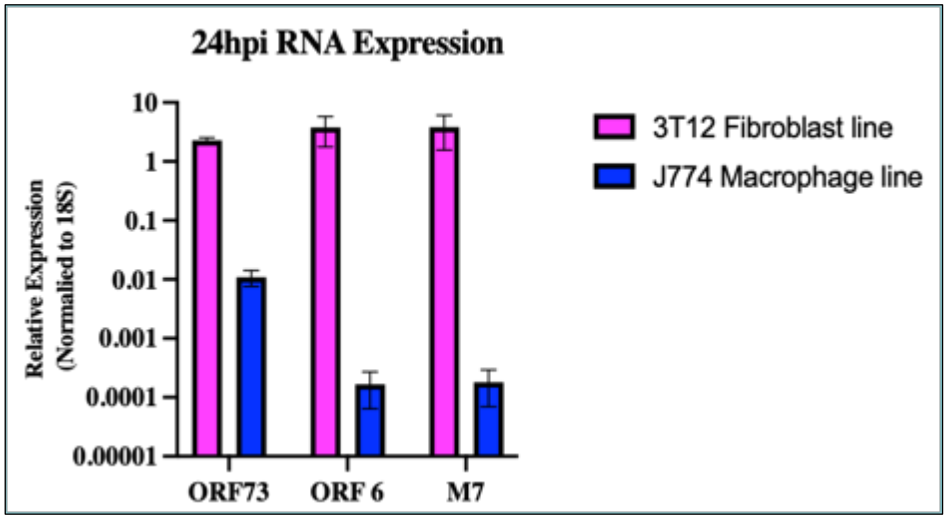

Figure 3: Relative expression of viral genes ORF73 (immediate early), ORF6 (early), and M7 (late) in 3T12s and J774s. Unpublished data from Linda van Dyk's lab.

RNA PrimeFlow

While we know a great deal about what happens when 3T12 fibroblasts are infected with MHV68, our understanding of whether J774 macrophages undergo lytic replication, latency, or some combination is limited. This is important because it allows us to better understand how latent infection of gammaherpesvirus can work, allowing us to develop vaccines that prevent latency. Our primary research goal was to determine if viral gene expression is uniformly low or absent in infected J774 cells by examining gene expression at the single-cell level. To gain an understanding of the overall viral gene expression and what kind of viral replication J774 cells undergo, we needed to use single-cell analysis. We employed RNA PrimeFlow to understand this viral gene expression in J774 macrophages. RNA PrimeFlow is a combination of flow cytometry and fluorescent in-situ hybridization (Figure 4). PrimeFlow is a scientific method that allows us to detect the expression of viral non-coding and messenger RNAs during lytic infection on a single cell level (Oko et al., 2019). To utilize RNA PrimeFlow, we needed to identify and use three different PrimeFlow target genes. We employed one specific viral ncRNA TMER because TMERs are abundantly expressed in all stages of infection, both lytic and latent. TMERs give us a good baseline to know if our infection was successful; all cells should express TMERs if there was successful viral replication. ORF18 is a viral late RNA that is only expressed in active lytic replication. This viral gene allows us to understand if cells are undergoing lytic replication or latency. If ORF18 is present and abundantly expressed, then we know that J774 cells are undergoing lytic replication, however, if there is a defect in ORF18 expression then the J774 cells may be undergoing latency. The cellular beta-actin is degraded during active virus replication. So, like TMERs, cellular beta-actin can be used as a baseline to determine if our viral infection was successful. However, our data analysis did not include cellular beta-actin. The primary aim of this project was to conduct RNA PrimeFlow analysis of TMERs, ORF18, and

cellular beta-actin 24 hours post-infection to understand J774 viral gene expression at the single-cell level.

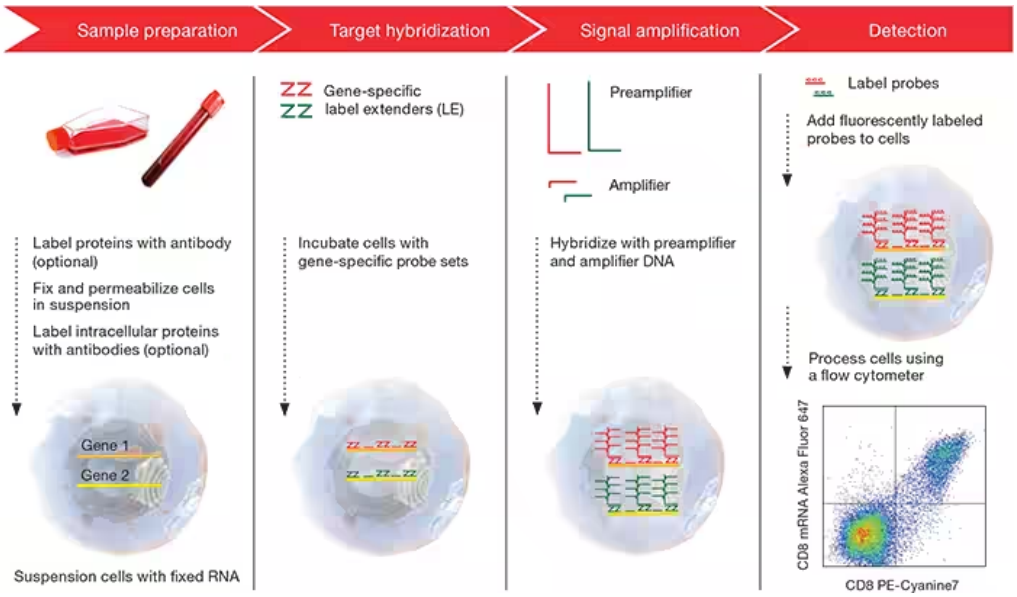

Figure 4: RNA PrimeFlow schematic from ThermoFisher Scientific.

Results

TMER expression is low in J774 macrophages when compared to 3T12 fibroblasts

To determine how infection with the murine gammaherpesvirus MHV68 impacts the percentage of cells that express TMER and ORF18 or co-express the two viral genes, we first conducted an RNA PrimeFlow that incubated 3T12 fibroblasts and J774 cells with both viral genes ORF18 and TMER. TMERs are abundantly expressed in all stages of infection, both lytic and latent. By analyzing TMER expression we can make sure that the infection was successful, which allows us to have an accurate analysis of ORF18 expression. For the WT 3T12 fibroblasts, there was 2.5 times more expression of TMER than the WT J774 macrophages (Figure 5). Although the percentage of J774 macrophages that TMER was low, both cell lines indicated that there was a successful infection, however, for both cell lines, there was low viral expression.

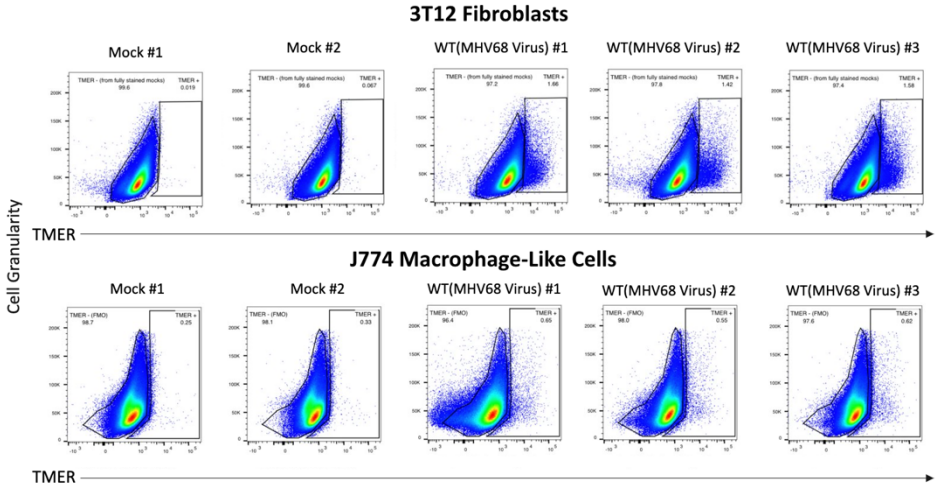

Figure 5. TMER expression is low in J774 cells when compared to 3T12 cells. 3T12 cells and J774 cells were incubated with viral ncRNA, TMER and were processed by flow cytometry. These graphs are showing the cells that expressed exclusively TMER and the cells that did not express TMER. These graphs show one mock infection trial and one wild-type (WT) infection for both 3T12 and J774 cells, there was a total of two mock infections and three WT infections for each cell line.

ORF18 expression is low in J774 macrophages when compared to 3T12 fibroblasts

To determine how an infection with MHV68 impacts ORF18 expression, we first conducted an RNA PrimeFlow that incubated 3T12 fibroblasts and J774 cells with both ORF18 and TMER. ORF18 is abundantly expressed only in active lytic infection. The WT 3T12 fibroblasts had 8.5 times more ORF18 expression than the WT J774 macrophages. This low expression of ORF18 in J774 macrophages is consistent with prior data in the van Dyk lab which indicated that there is a defect in viral replication and late viral gene expression in J774 cells (Figure 6).

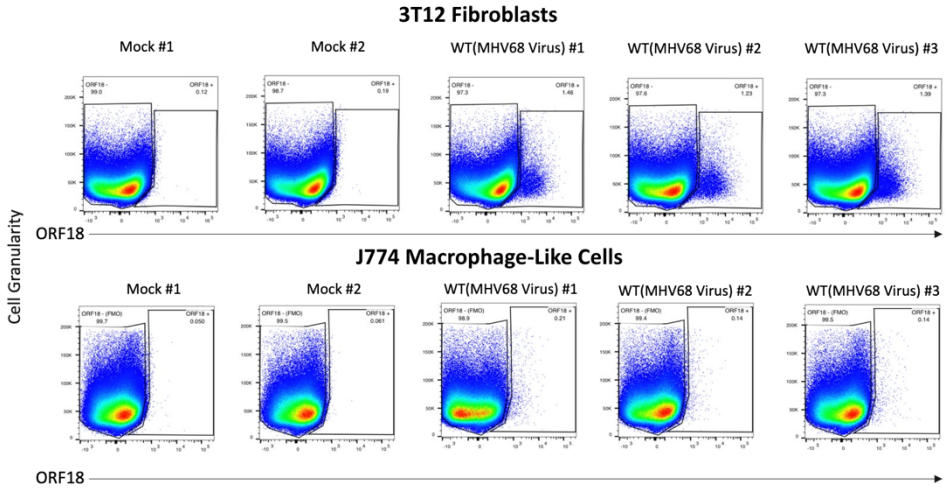

Figure 6. ORF18 expression is low in J774 cells when compared to 3T12 cells. 3T12 cells and J774 cells were incubated with viral late RNA gene, ORF18 and were processed by flow cytometry. For both cell types, the mock infections had no ORF18 expression. These graphs show just one mock infection and one wild-type (WT) infection, but there was a total of two mock infections and three WT infections.

Co-expression of TMER and ORF18 was low in J774 cells when compared to 3T12 fibroblasts

To determine the percentage of cells that were expressing only TMER, only ORF18, or both ORF18 and TMER, we conducted an RNA PrimeFlow that incubated 3T12 fibroblasts and J774 cells with both ORF18 and TMER. As expected, the mock infections for both 3T12 fibroblasts and J774 cells, had almost no cells that expressed TMER and ORF18. About 99.9-100% of cells had no TMER or ORF18 expression (Figure 7). For the WT 3T12 fibroblasts, an

average of 0.33% of cells expressed only ORF18 and only an average of 0.039% of WT J774 macrophages expressed only ORF18. The WT 3T12 fibroblasts had an average of 0.97% of cells that expressed only TMER, while the WT J774 macrophages had an average of 0.34% of cells that expressed only TMER. The WT 3T12 fibroblasts had an average of 0.48% of cells that co-expressed TMER and ORF18, however, the WT J774 macrophages had an average of 0.064% of cells that co-expressed TMER and ORF18. (Figure 7). As expected, the overall viral gene expression in J774 cells was lower compared to 3T12 fibroblasts.

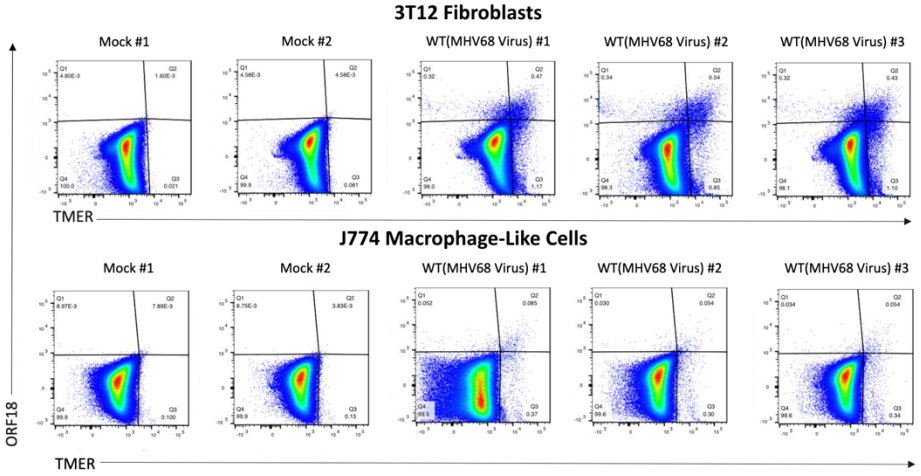

Figure 7. Co-expression of TMER and ORF18 was low in J774 cells when compared to 3T12 cells. 3T12 cells and J774 cells were incubated with ORF18 and TMER via RNA PrimeFlow and processed through flow cytometry. These graphs show which cells expressed only TMER, only ORF18, or expressed both ORF18 and TMER. Quadrant one indicates the cells that expressed only ORF18, quadrant two indicates the cells that expressed both ORF18 and TMER, quadrant three indicates the cells that only express TMER, and quadrant four indicates the cells that express neither ORF18 or TMER.

The percentage of cells that were ORF18+, TMER+, and TMER+ORF18+ is lower in J774 cells

Overall, J774 macrophages had a lower percentage of cells that expressed ORF18 and TMER when compared to 3T12. This indicates that the J774 cells, when infected, had a latent infection rather than a lytic one. The mock J774 infections and the WT J774 macrophages had a similar percentage of cells with an ORF18 signal, indicating that the J774 cells did not have a

lytic infection. TMER expression in J774 macrophages was also low, but there was a noticeable difference between the mock infections for J774 macrophages and WT J774s. 3T12 fibroblasts still had a noticeable expression of TMERs and ORF18. The percentage of cells that co-expressed the viral genes was higher in 3T12 fibroblasts when compared to J774 macrophages. Approximately, 0.48% of 3T12 fibroblasts co-expressed TMER and ORF18, while 0.0643% of J774 macrophages co-expressed TMER and ORF18 (Figure 9). However, for both cell lines, overall viral gene expression was low when compared to past experiments.

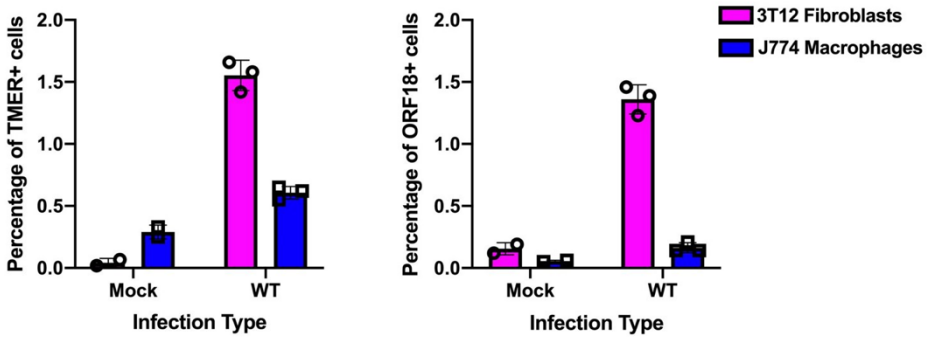

Figure 8. The percentage of cells that were ORF18+ and TMER+ were low in J774 cells. The WT 3T12 cells and WT J774 cells were incubated with ORF18 and TMER via RNA PrimeFlow and the mock infections for both cell lines were incubated with no viral RNA genes. Each bar represents the average percentage of cells that were positive for TMER or ORF18. Each symbol represents one of the mock or WT infections, there was a total of two mock infections and three WT infections for each cell line. The error bar represents the estimated error for the percentage of cells positive for TMER or ORF18.

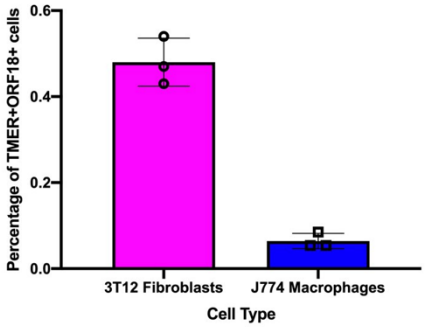

Figure 9. The percentage of cells that were TMER+ORF18+ was low in J774 cells. These bar graphs represent the WT 3T12 cells and WT J774 cells that were incubated with ORF18 and TMER via RNA PrimeFlow. Each bar represents the average percentage of cells that were positive for TMER and ORF18. Each symbol represents one of the WT infections, there was a total of three WT infections for each cell line. The error bar represents the estimated error for the percentage of cells positive for both TMER and ORF18.

Discussion

To determine if viral gene expression was uniformly low or absent in J774 macrophage-like cells we utilized RNA PrimeFlow to understand what percentage of cells expressed the viral genes, TMER and ORF18. We also wanted to understand what kind of infection J774 macrophages undergo: lytic replication, latency, or a combination. We employed two different target genes (TMERs, and ORF18) to analyze viral gene expression in J774 cells. We found that a low percentage of J774 macrophages had TMER and ORF18 gene expression when compared to 3T12 fibroblasts. As expected, 3T12 fibroblasts had cells that expressed ORF18 and TMER. While J774 cells did have TMER expression, a low percentage of cells had ORF18 expression. However, there are some caveats to the experiment that we performed. For example, from prior experiments done in the van Dyk lab, we know that viral gene expression is typically higher than what we found.

Despite the low detection, we are still able to draw some conclusions from our data. There was a low percentage of cells that had TMER expression in both 3T12 fibroblasts and J774 macrophages. TMER reflects viral infection, so our infection rate was lower than expected. However, 3T12 fibroblasts still had a higher percentage of cells that expressed only TMER, reflecting lytic infection of the fibroblasts. Prior experiments done in the van Dyk lab indicate that 3T12 fibroblasts typically have a higher percentage of cells that express TMER. In fibroblasts around 79.8% of cells expressed TMER (Oko, 2019). The 3T12 fibroblasts for my experiment had a low percentage of cells that expressed TMER because there may have been some errors in the protocol. Permeabilization is an important part of RNA PrimeFlow and when this step is not done correctly the viral gene probes are not able to enter the cells properly, there

will be a low percentage of cells that actively express TMER. This experimental error may have also caused the J774 macrophages to have a low percentage of cells that express TMER as well. However, despite having a low percentage of cells that express TMER, the 3T12 fibroblasts still had a higher percentage of cells that expressed TMER when compared to J774 macrophages. Since TMERs are abundantly expressed in both lytic and latent infections it was expected that both cell types would express this viral gene. Although both cell lines had low TMER expression, this TMER expression allows us to be confident that our cells were infected with MHV68.

The percentage of cells that had ORF18 expression in 3T12 fibroblasts was low just like TMER expression. Past experiments in the van Dyk lab have found about 61% of 3T12 fibroblasts have ORF18 expression (Oko, 2019). However, we do see again that 3T12 fibroblasts had a higher percentage of cells that expressed ORF18 when compared to the J774 macrophages. This low percentage of cells that expressed the viral gene may have been due to an error in the permeabilization of cells during RNA PrimeFlow. We can still draw some conclusions from the percentage of cells that expressed ORF18. 3T12 fibroblasts undergo lytic replication, so as expected there is ORF18 expression. However, with the J774 macrophages less than 0.10% of cells expressed ORF18. Since ORF18 is only expressed during lytic infections, the J774 macrophages may be undergoing latency rather than lytic replication. While the J774 macrophages are being infected with MHV68, the virus appears to be undergoing latency. However, since the percentage of cells that expressed ORF18 was so low, this conclusion is still tentative. It would be beneficial to conduct this experiment again and look at what other viral genes J774 macrophages can express. This will allow us to be more certain in our conclusion that J774 macrophages undergo latency. When we looked at the percentage of cells that were

TMER+ORF18+, the 3T12 fibroblasts still had a higher percentage of cells that co-expressed the two viral genes compared to the J774 macrophages. By looking at the co-expression of TMER and ORF18 we can gain a better idea of whether J774 macrophages are undergoing latency. Again, the J774 macrophages had a low percentage of cells that were TMER+ORF18+. This indicates that the J774 macrophages could be undergoing latency. However, due to the low percentage of cells that expressed TMER and ORF18 the experiment needs to undergo optimizations before we can be certain in our conclusions of viral infection in J774 macrophages.

As mentioned earlier, the viral gene expression was low in both 3T12 fibroblasts and J774 cells. Prior experiments had a higher percentage of viral gene expression. To improve this area of the experiment and have more certainty in our results we would need to optimize some parts of the experiment. We would primarily want to optimize our infection protocol and RNA PrimeFlow protocol. Although we did have a successful virus infection, based on the viral gene expression, not all the cells were able to be infected. To get the best possible results, we want to optimize this protocol, so that we know that all the cells are being infected with MHV68. We also want to optimize the RNA PrimeFlow protocol. This protocol is long and tedious which can lead to some errors along the way. One important step of the RNA PrimeFlow protocol is the permeabilization step. The permeabilization step allows us to make sure the target gene probes, TMER and ORF18, can enter the cell. If this step does not work successfully, we can potentially end up with low viral gene expression because not all the target gene probes can enter the cell. We would like to gain more of an understanding of what is happening in J774 cells, so we do plan on changing some parts of the experiment. We plan to analyze the cells at different time points post-infection because depending on the viral gene it can take longer for some genes to be expressed, especially late viral genes. We would also include additional viral genes and add a

latently infected cell line as a counterpart to the 3T12 fibroblasts as a control. To better understand the per-cell gene expression we would need to conduct more analysis of the data we have gathered. This would allow us to determine which J774 macrophages are undergoing either latency or lytic replication. These optimizations and additional experimental changes will allow us to be more certain in J774 cells undergoing latency and they will allow us to know for certain that viral gene expression is uniformly low.

This experiment allowed us to gain a better understanding of whether J774 macrophages undergo latency or lytic replication. Although our results are tentative, we can make a baseline conclusion that macrophage-like cells undergo latency. The overall goal of the van Dyk lab is to gain a better understanding of late viral gene expression of the gammaherpesvirus and develop ways to prevent latency and create a vaccine. However, to prevent latency and create a vaccine to protect against gammaherpesviruses it would be beneficial to conduct several more experiments that will draw broader and more accurate conclusions. This experiment allowed us to gain better insight into how MHV68 can infect J774 macrophages. Through this experiment, we were able to learn more about macrophages and gain a better understanding of latency. This experiment can potentially offer us ways to prevent latent infection in individuals infected with gammaherpesviruses, such as EBV and KSHV, and potentially develop vaccines that can protect against gammaherpesviruses.

Methods

Culturing 3T12 Fibroblast Cells and J774 Macrophage-Like Cells

The 3T12 fibroblast cells and J774 macrophage-like cells were obtained from mice in Dr. Linda van Dyk's lab. The J774 cells and 3T12 fibroblasts were washed with sterile phosphate buffered saline (PBS, pH 7.4). Trypsin was then used to remove the cells from the bottom of the flask. For the J774 cells, the trypsin was quenched with 10% cDMEM; the 3T12 fibroblasts were quenched with 5% cDMEM. The cells were then counted using a Bio-Rad automatic cell counter and Trypan blue. The recorded cell count was then used to determine the exact volume of cells needed to create a $5*5^5$ cells/mL solution (J774 cells) and a $2*10^5$ cells/mL solution (3T12 fibroblasts). 2 mL of the solution was added into three 6-well plates for J774 cells and three 6-well plates for the 3T12 fibroblasts. The plates were then incubated overnight at 37°C.

Infection with MHV68

To infect the 3T12 and J774 cells, the supernatant was removed from each well and each well was rinsed with PBS. Trypsin was then added to each well and incubated. Once they were done incubating the cells were then quenched with 10% cDMEM for J774 cells and 5% cDMEM for 3T12 fibroblasts. The cells were then counted via a Bio-Rad automatic cell counter using Trypan blue. This count was then used to determine how much virus was needed and how many cells we would be infecting. The multiplicity of infection for each cell line was one. There were $8.83*10^5$ J774 cells in each well and $5.28*10^5$ 3T12 fibroblasts in each well. An inoculum was made by adding WT MHV68 virus to 10% cDMEM or 5% cDMEM. The inoculum was added to each well and placed into an incubator. They were then rocked every 15 minutes for one hour

and the inoculum was then removed after the hour and replaced with 10% cDMEM or 5% cDMEM. The well plates were incubated for 24 hours at 37°C.

RNA PrimeFlow

24 hours post-infection (hpi) RNA PrimeFlow was conducted on the 3T12 and J774 cells. The cells were first harvested using trypsin and 5% or 10% cDMEM. The supernatant from each cell was collected into a 1.5 mL Eppendorf tube. The cells were spun down at ~400 x g for 10 minutes at 4°C. Sample 4 from the 3T12 fibroblasts and from the J774 cells was used for the Live/Dead stain. Sample 4 was split into two different Eppendorf tubes and one tube was resuspended in 4% PFA. The two different Eppendorf tubes were mixed back into the original sample 4 Eppendorf tube. The Live/Dead Aqua Stain was made using a 1:100 dilution and the cells were resuspended in this solution. The cells were then fixed and permeabilized using fixation buffer 1X, 1X permeabilization buffer, and 1X fixation buffer 2. The samples were then hybridized with the following probes: Actin Type 1, ORF18 Type 6, and TMER Type 4. After probe hybridization, the cells were then incubated overnight at 4°C. The second day of RNA PrimeFlow involved signal amplification using pre-warmed PreAmp Mix, Amp Mix, and Label Probe Diluent. After signal amplification, the cells were stored at 4°C in the dark. Before running on the flow cytometer, the cells were filtered through 70 uM filter into titer tubes.

Data Analysis

Data analysis of the flow cytometry was done using FlowJo. Before gating the mock infections and WT infections the raw data was first gated. First, the generous cells were gated, this included about 99% of cells being gated. From the generous cells the single cells were gated via side scatter (SSC) then the single cells were gated via forward side scatter (FSC). From these

three different populations the live cells were gated, which included about 50% of cells. Once these gates were applied to all the populations the mock and infections were gated. To gate the infections properly, the mock infection population was gated and applied to the infections. Once the mock infection gate was applied to the infections, the cells that were outside of the mock infection population were gated and were gated as the infected population. No statistical analysis was conducted.

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