Understanding the Mechanism of CD4 T-Cell Inhibition by Ectromelia Virus

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Title: Understanding the Mechanism of CD4 T-cell Inhibition by Retrovirus

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Understanding The Mechanism of CD4 T-cell Inhibition by the B22 Protein of Ectromelia Virus

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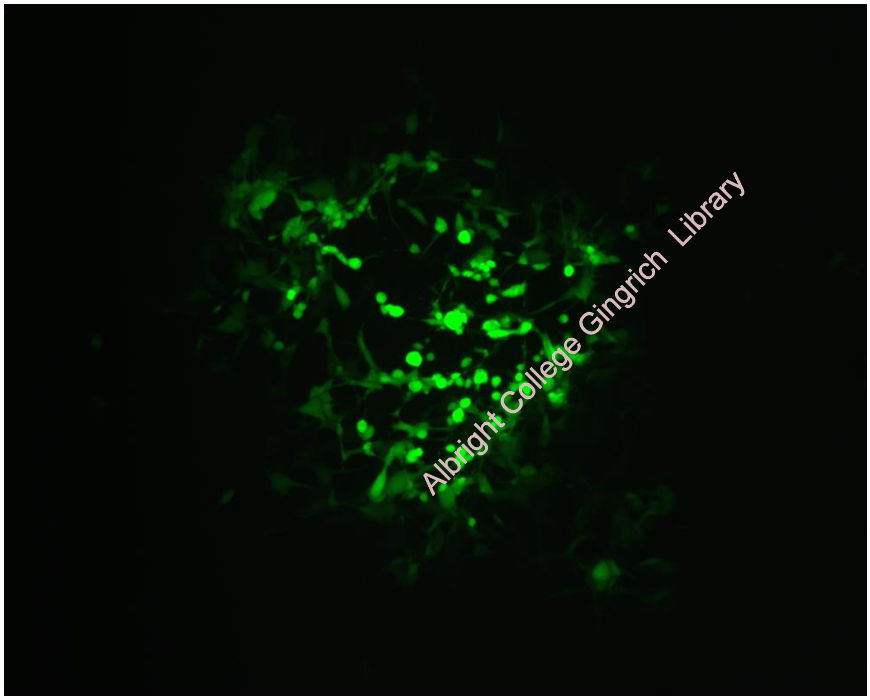

Abstract:

Viruses require entry into cells in order to replicate. Once inside of cells, the virus particles use host cell machinery to replicate. Host cells have developed ways of detecting viral infections and alerting the immune system to infections, but viruses have developed ways of preventing this activation of host immunity. Ectromelia virus (ECTV), the causative agent of mousepox, is a virus closely related to other poxviruses such as Variola virus (smallpox) and monkeypox virus. It was recently discovered that the B22 protein of monkeypox virus can cause inactivation of simian CD4 and CD8 T-cells and ECTV has a protein of similar gene sequence [1,2]. We previously observed that ECTV inactivates CD4 T-cells. The creation of an ECTV virus with a disruption in gene 174 would prevent the ECTV B22 protein from being formed during infection. The absence of the B22 protein appears to partially rescue the activation of CD4 T-cells during an ECTV infection. Further investigation of the actual mechanism of T-cell inhibition is needed.

Introduction:

Smallpox was at one time one of the most serious diseases to threaten humans. Thanks to the hard work of many individuals, smallpox was eradicated in 1977 [3]. The virus responsible for smallpox, Variola virus (VARV), is now securely stored in two laboratories, the Centers for Disease Control in Atlanta, Georgia and the Vector Institute in Koltsovo, Russia [3]. However, other related viruses can infect humans [4]. Monkeypox virus (MPXV) can do so, though it has yet to gain the ability to spread easily from person to person [4, 5]. On the other hand, Ectromelia virus (ECTV; also known as mousepox) is not capable of infecting humans. ECTV acts in mice as VARV and MPXV act in humans, because of this ECTV provides a safe model for the study of infectious human diseases [3, 6].

Viruses, including poxviruses, are not capable of replicating unless they enter a host cell. Once inside, they are able to take control of various aspects of the host cell machinery and create many copies of themselves. VARV, MPXV, and ECTV are all related viruses that are part of the same poxvirus sub-family [7]. In general, poxviruses infect a wide array of known species; orthopoxviruses are a genus of poxviruses that infect many types of mammals. Poxviruses are double stranded DNA viruses that code for about 200 genes in their 200 kilobase pair (kbp) genome [7].

The immune systems of mammals have developed ways of recognizing when cells have become infected by a virus. T-cells are one type of specialized immune cell capable of such recognition. Once a cell is infected, viral proteins are cut into small peptides and placed on molecules called major histocompatibility complexes (MHC) inside the cell [8]. The MHC:peptide complex then trafficks to the cell surface where the viral peptide awaits recognition by T-cells. Two main types of T-cells are capable of recognizing MHC:peptide complexes, CD4 and CD8 T-cells (**Figure1**). Once the T-cell recognizes the specific MHC:peptide complex it becomes activated. If it is a CD8 T-cell, it can potentially eliminate the infected cells. If it is a CD4 T-cell, it can secrete immunomodulatory molecules called cytokines that activate other immune cells and help to bring about an anti-viral response [8].

It was recently discovered that ECTV actively inhibits murine CD4 T-cells from recognizing and responding to infected cells (**Figure 2**). In a set of recently published papers on MPXV, it was observed that the virus also inhibits the activation of simian and human CD4 T-cells and CD8 T-cells [1]. Researchers were able to identify the protein, B22R, which is responsible for the inactivation of T-cells [1,2]. ECTV has an orthologous B22 protein with high sequence similarity, which is coded for by gene 174 [2]. These proteins belong to a protein

family called B22, and are by far the largest proteins in the poxvirus genome [1,2]. Proteins with similar sequences and structures tend to carry out similar functions in cells. Almost all orthopoxviruses have transmembrane glycoproteins of similar size and genome location [www.poxvirus.org]. It is highly unlikely that such large proteins would be conserved over the wide range of viruses without their function being of any importance.

We believe that the B22 protein in ECTV is partially, if not completely, responsible for murine CD4 T-cell inactivation *in vitro*. To test the possible function of B22 from ECTV, a mutant ECTV strain, Δ174, was created with a disruption in the B22 gene. The disrupted gene would prevent the expression of gene 174 and therefore prevent the presence of B22 in infected cells. The mutant virus was tested against the wild-type (w.t.) ECTV strain to compare CD4 T-cell function in the presence of both viruses. If the B22 protein is responsible for inactivation of CD4 T-cells, there should be more T-cells responding in the presence of the Δ174 virus than the w.t. ECTV.

Poxvirus research has many applications, not only related to a bioterrorism threat or a possible threat of a MPXV mutation allowing for easier spread from human to human. Poxviruses are also used as vectors for vaccine delivery [9], there are clinical trials using engineered poxviruses to treat cancer [10,11], and a rabbitpox protein is being used as an anti-inflammatory agent in cardiovascular disease patients in clinical trials [12]. Therefore, understanding the mechanisms of immune evasion can be used to gain a better understanding of the immune system as a whole. Poxvirus research has many human health benefits and ectromelia virus is a useful tool for examining the mechanisms of immune evasion techniques used by poxviruses [13].

Methods:

Cells and culture methods:

BSC1 and LIE^{d} cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Life Technologies) supplemented with 5% fetal bovine serum (FBS; Gemini BioProducts), and penicillin-streptomycin (Gemini BioProducts). NA79 CD4 T-cell hybridomas were cultured in Roswell Park Memorial Institute Media (RPMI, Life Technologies) supplemented with 10% FBS, penicillin-streptomycin, and 0.1% β-Mercaptoethanol (Life Technologies). All cell lines were maintained in 37°C incubators (5% CO_2) and split when cells reached approximately 80% confluency.

Viruses and infections:

The following viruses were used during the course of this work: wild-type ECTV (Moscow strain), ECTV (Moscow strain) expressing green fluorescent protein (GFP), and Δ174 ECTV (Moscow strain) expressing GFP. Infections were done in 500 μL of DMEM supplemented with 2% FBS for at least one hour prior to removal and replacement with normal growth media.

Creating the Δ174ECTV-GFP virus:

The left flanking region of gene 174 was amplified using a polymerase chain reaction (PCR) Primers used were:

- Forward- 5'-GCCTCGAGATGAATTTACAGAAATTATCTGG-3' with an Xhol cut site

- Reverse- 5’-GCGGATCCTAGCAATTATTTATTTCCAAAAATCC-3’ with a BamHI cut site

The amplified region of the virus was the upstream portion of the gene. The left flank PCR product as well as the GFP containing plasmid (P7.5 EGFP ECTV) were cut with the restriction enzymes Xho1 and BamHI (New England BioLabs). The restriction digests were performed according to the instructions provided by manufacturer. The portion to the left of the GFP gene in the plasmid was cut with these enzymes. The left flank was then inserted into the plasmid using T4 DNA Ligase (Life Technologies) according to manufacturer’s instructions. The plasmid was transformed into bacteria and isolated using a Quick Plasmid Miniprep kit (Life Technologies).

The right flanking region of gene 174was amplified using PCR. The primers used were:

- Forward- 5’-GCCTGCAGGATGGTAATCTGTTAGATATTGCAT-3’ with a PstI cut site
- Reverse-5’-GCGCGGCCGCTTAGTACCGATTATCCATAATTTCC-3’ with a Not1 cut site

The amplified DNA as well as the plasmid with the inserted left flank were cut with the restriction enzymes PstI and Not1 (New England BioLabs). The region to the right of the GFP gene in the plasmid was likewise digested. The right flank was inserted into the plasmid as described as above (**Figure 3**).

The plasmid containing the left and right flanking regions of gene 174 (from here referred to as the knock-out plasmid) was transformed into bacteria. The bacteria were grown in culture and the plasmid was isolated using a HiPure Plasmid Midiprep kit according to the instructions by Invitrogen. The isolated knock-out plasmid was transfected into BSC1 cells. Two hours later

the same cells were infected with w.t. ECTV. Because of the identical sequences (the flanking regions) in the virus and knock-out plasmid, homologous recombination occurred allowing for a large portion of gene 174 to be replaced with GFP. The GFP gene was used as a selection marker to identify mutant viruses. Green plaques were selected and the viruses were used to infect new BSC1 cells. This process was repeated nine times until stable green plaques were observed.

Confirmation of knock out virus:

PCR was used to confirm the knockout of gene 174. One set of primers was used that bound to the virus genome inside the flanking regions (**Figure 4a**). The PCR was run on both w.t. and Δ174 ECTV. Primers used:

- Forward: 5'-TCGCTGCCGTACAAGTATCT-3'
- Reverse: 5'-CAGTAGCAGAATAGCGCCACA-3'

A second PCR was done using primers that bind to the ECTV genome outside of the flanking regions (**Figure 4b**). Primers used:

- Forward: 5'- GCTACCGAAGACCAGCTATATG-3'
- Reverse: 5'-TCACTGCTACTTGATGACTGTG-3'

Flow cytometry:

Flow cytometry data was collected using a FACSCalibur instrument (BD Biosciences) equipped with both a red and blue laser. The collected data were analyzed using FCS Express 4 Flow Cytometry (De Novo Software; version 4.07). In experiments examining the spread of virus, $2.5x10^5$ cells were seeded into twelve-well plates. After the cells adhered to the surface, they were infected (MOI=0.01) with GFP-expressing ECTV or Δ174 ECTV as described above.

After 24, 48, or 72 hours, the cells were harvested using trypsin digestion. Virus spread in the culture was quantified by measuring the percentage of total cells that were positive for GFP expression.

To measure CD4 T-cell inactivation, $5x10^5$ LIE^d cells were placed in two wells of a 6-well plate. One million LIE^d cells were placed in the remaining 4 wells. One hour later, two of the wells containing $1x10^6$ LIE^d cells were infected with w.t ECTV-GFP (MOI=1). The remaining wells with $1x10^6$ LIE^d cells were infected with Δ174 ECTV-GFP (MOI=1). Three hours later 1μl of NA79 peptide (0.01mg/ml, final concentration, Gen Script) was added to one well of each infection condition. The remaining three wells received 1μl of dimethyl sulfoxide (DMSO) as a control. Two hours later that media was removed and the wells were rinsed twice with 1x PBS, R10 media was added to each well. Four hours later, $5x10^5$ NA79 hybridomas were added to each well. 1 μl of ST-246 (5μM, final concentration) was added to each well to prevent infection of hybridomas [14]. Cells were incubated 14 hours.

T-cell activation was measured using a Fluoreporter lacZ Flow Cytometry kit (Life Technologies) Cells were harvested using trypsin digestion. The cells were resuspended with 100μl staining media (4% FBS in 1x PSB) and incubated for 10 minutes in a 37°C water bath. Fluorescein di-β-D-galactopyranoside (FDG, 20 mM, final concentration) was diluted 1:10 in H_2O and incubated for 10 minutes in a 37°C water bath. 125 μl of the warmed FDG was added to the cell solutions to allow for FDG loading due to hypotonic shock. Upon the addition of FDG, the cells went back into the water bath for one minute. The FDG loading was stopped by adding 1mL of chilled staining media. Cells were placed on ice and stained with anti-MHC APC antibodies (BioLegends) to differentially label the LIE^d cells from the T-cell hybrids. Cells incubated on ice for 1.5 hours before being examined on the flow cytometer.

Results:

Confirmation of knockout virus:

To confirm the knockout of gene 174, two PCR-based tests were done. Both viruses produced a signal from primers at the junction between gene 42 and 43 as well as the junction between genes 43 and 44, which should be common to both viruses. However, as expected, only the w.t. virus produced a PCR product from primers that bound in the interior of gene 174 (**Figure 4c**). A second confirmatory PCR test was done using w.t. and Δ174 ECTV. In this case, primers were designed to bind within the flanking regions, which produced a band around 6kbp in the w.t. virus and a band below 2kbp in the Δ174 ECTV (**Figure 4d**). The w.t. virus encodes for the entire 174 gene, which is about 5.7 kbps. Most of gene 174 was replaced by the smaller GFP gene in the Δ174 ECTV, which creates a smaller PCR product when run on a gel.

Growth rate comparison:

The amount of virus infected cells were counted on days 1, 2, and 3 after infection and infected cells were counted using GFP expression. On day one 13.45±3.91% of cells were infected by w.t. ECTV and 11.65±4.92% were infected by Δ174 ECTV. On day two 59.84±2.89% and 55.70±7.68% of cells were infected by w.t. or Δ174 ECTV, respectively. On the final day 97.98±0.60% and 98.57±0.68% of cells were infected by w.t. or Δ174 ECTV, respectively (**Figure 5**).

Using both phase contrast and fluorescent microscopy, the plaques formed from w.t. and Δ174 ECTV were examined for a comparison of relative size. Plaques are holes formed in the monolayer of cells due to viral infection. As cells die, they pull up off the plastic and leave visible holes – or "plaques". The cell shape and size of viral plaques of both viruses are similar (**Figure 6a and c**). GFP expression in both viruses also looked identical (**Figure 6b and d**).

CD4 T-cell activation is restored in the presence of Δ174 ECTV*:*

The lacZ reporter assay enabled us to quantify the amount of CD4 T-cells activated in the absence and presence of ECTV. The LIE^d cells express MHC and can therefore present antigens, pieces of viral proteins, to CD4 T-cells. The antigen presenting cells are pulsed with NA79 peptides, which are loaded into the MHC and presented to T-cells. The presented peptide leads to the activation of T-cells. Activated T-cell hybridomas produce the enzyme β-Galactosidase. The next day the cells are treated with FDG. β-Galactosidase is capable of cleaving FDG into galactose and fluorescein, which emits green light when excited by a blue laser (**Figure 7**). Only the activated T-cells will contain fluorescein. The flow cytometer takes the solution of cells and shines a blue laser on them; the activated T-cells are counted due to their bright FDG signal.

A lacZ reporter system was used to identify activated CD4 T-cells. The positive control for maximum T-cell activation showed a large FDG peak between 10^2 and 10^3 as well as a small inactivated population of cells at 10^1 (**Figure 8**). There was a larger population of activated T-cells than non-activated cells. The stimulated cells infected with w.t. ECTV did not have a defined peak of activated CD4 T-cells between 10^2 and 10^3. There was a 47% reduction in T-cell activation while in the presence of the stimulating peptide. The Δ174 ECTV infected condition had similar activation to the positive control. There was a distinct peak between 10^2 and 10^3. The non-activated T-cells had a slight shift right in FDG expression, but the overall size and shape of the population was similar to that of the control (**Figure 8**).

Discussion:

The use of PCR allowed for the confirmation of the disruption of gene 174 in the mutant virus. The first set of primers used were complimentary with regions of the ECTV genome

internal to the flanking regions of gene 174. In the mutant virus, the part of the genome between the flanking regions should be replaced with the GFP gene, therefore, there would be nothing for the primers to bind to on the mutant virus. If the primers are not capable of binding, there will be no PCR product. We expected to see a PCR product for the w.t. virus, but not the Δ174 ECTV. The absence of a product in the Δ174 ECTV lane supports the creation of a disruption of gene 174.

A second set of primers were used to confirm the disruption of gene 174. The primers were complimentary with the ECTV genome within the flanking regions themselves. Both viruses would have sequences for the primers to bind to in their genome. The w.t. virus would be expected to have a larger PCR product. The primers would lead to the amplification of the entire gene 174, which is about 5.7 kbps. The deletion virus, while still having the identical flanking regions, would have a large portion of gene 174 replaced by the much smaller GFP gene. A smaller PCR product would be expected. Smaller DNA sequences travel more quickly through an agarose gel and result in bands lower on the gel. The lower band for the Δ174 ECTV PCR product confirmed the creation of a mutant virus.

Growth of the w.t. virus had to be compared to the Δ174 ECTV. The protein is not required for replication among cells in vitro; this was determined by the ease of infection of fibroblast cell lines. Deleting a gene could lead to the detection of the virus in the cell and apoptosis could result. To be sure that the gene disruption did not affect viral growth kinetics, the w.t. and Δ174 ECTV growth rates were compared. Both viruses infected the same amount of cells at all three time points and the relative appearance of plaques was indistinguishable. Thus, the qualitative and quantitative data lead to the conclusion that the B22 protein is not necessary for virus replication *in vitro*.

The positive control to assess T-cell activation used only peptide-treated cells without ECTV infection to find the maximum activation levels. The activated T-cells had a peak between 10^2 and 10^3; the non-activated cells had an FDG expression of 10^1. The w.t. ECTV condition showed very little activation; illustrating the w.t ECTV does inactivate murine CD4 T-cells. The Δ174 ECTV condition had an activation peak at the same location as the positive control. The deletion of the B22 protein gene resulted to the return of T-cell activation similar to that of conditions with no virus (**Figure 6**). In conclusion, the B22 protein is partially, if not completely, responsible for the inactivation of CD4 T-cells in mice.

The T-cell hybrids may behave differently than native T-cells. Hybridomas are immortalized by fusing a murine T-cell and a fibroblast cell, therefore they may not strictly behave as T-cells behave. Future experimentation could measure T-cell inhibition using ELIspot assays, which are another method of measuring T-cell stimulation. Antigen presenting cells (APCs) from mice would be infected or peptide-pulsed, similarly to those in the hyrid/lacZ assay. The day of the assay, mice would be sacrificed and CD4 T-cells would be purified from their spleen and incubated with the APCs. An ELIspot assay would then be carried out to detect interferon-gamma secretion. This *ex-vivo* approach would show activity of the w.t. and mutant viruses in an environment that more closely resembles what may be occurring *in vivo*.

VACV, the virus used for the smallpox vaccination, does not have a full B22 gene, but it does have a gene for the protein A35. The protein is known to disrupt the peptide loading of the MHC molecule and therefore diminishes CD4 T-cell activation [15]. ECTV also has the gene for the A35 protein [15]. It is possible that if the B22 protein is not solely responsible for the inactivation of T-cells, A35 could be working in conjunction with the B22 protein. To test if the

proteins both play a role in CD4 T-cell inactivation, a double knock-out virus could be created. A similar lacZ assay could be performed to test for T-cell activation in the absence of both proteins.

Further experimentation will make use of a plasmid that has been developed that contains gene 174 alone. B22 can be measured using a protein tag. Transfecting the plasmid into mammalian cells will allow for its expression in the absence of all other viral genes. If B22 is alone responsible for the inactivation, there should be a similar level of CD4 T-cell inactivation to viral infections. If similar inactivation occurs, this would suggest that B22 is necessary and sufficient for T-cell inactivation. Future work will include the use of B22 plasmid transfected cells during as lacZ assay.

Once we have more data on the inhibitory effects of B22, the exact mechanism of inhibition should be studied. B22 is believed to be expressed on the surface of infected cells [1] and does not appear to interfere with MHC peptide loading. B22 could be interacting with the co-stimulatory molecules on the CD4 T-cells which are required for the T-cell activation, or it could be interacting with the MHC molecule directly. To test for these interactions a co-immuno precipitation (CoIP) assay could be done to examine if B22 is physically interacting with a surface molecule on T-cells. In the CoIP assay, Antibodies, which are very specific to the molecules to which they bind, to the Myc-c tag at the end of the B22 protein will have a bead conjugated to it. The bead will increase the mass of the protein so that during centrifugation the protein:antibody:bead complex will pellet out of solution. Any protein that is strongly associated with the B22 protein will be pulled out of solution with the B22 protein:antibody:bead complex. The pellet can then be resuspended and run on a polyacrylamide gel. The gel will separate anything trapped in the pellet based on size. A second antibody that binds to CD4 or MHC-II could be used to treat the separated proteins. The antibody will have a tag that will allow the

visualization of where the antibody binds. If the CD4 antibody binds to the area where B22 is on the gel, it would imply that CD4 interacts with B22. If a MHC antibody binds, then the B22 protein interacts with the MHC molecule. Either of these interactions could cause the inhibitory interaction between the CD4 T-cells and virus-infected cells.

All tests to date carried out have been done *in vitro, in vivo* tests could illustrate the importance of the protein during an infection of the organism. MPXV with a B22 gene deletion was shown to have decreased virulence and mortality in infected monkeys when compared to the w.t. MPXV [2]. B22 could increase the ability of the ECTV to spread in murine tissues due to immune evasion during *in vivo* infections and increase the severity of the disease in mice. It would be very important to the understanding of the B22 protein to see the effect of its absence on the organismal scale. If B22 does work in ECTV as it does in MPXV, it would be conceivable that the deletion of the B22 gene would lead to a reduction in virulence and mortality in mice.

ECTV's B22 protein has a great sequence similarity to MPXV B22 protein [2], and upon initial investigation, appears to have a similar function. The poxviral B22 protein is believed to inhibit the activation of CD4 T-cells. The creation of the Δ174 ECTV allowed for the return of T-cell activation in the absence of the B22 protein. The exact mechanism of the inhibition remains unknown. A CoIP assay could indicate if the protein is acting on the MHC on the antigen presenting cells, or on the CD4 on T-cells. Both interactions could prevent the activation of T-cells *in vitro*. Understanding the mechanism of inhibition could elucidate novel mechanisms of the immune system. Furthermore, poxvirus proteins are currently being used in human drug trials [12], if the B22 protein does inactivate T-cells as it appears to, it could have possible use as an anti-inflammatory drug.

Figures:

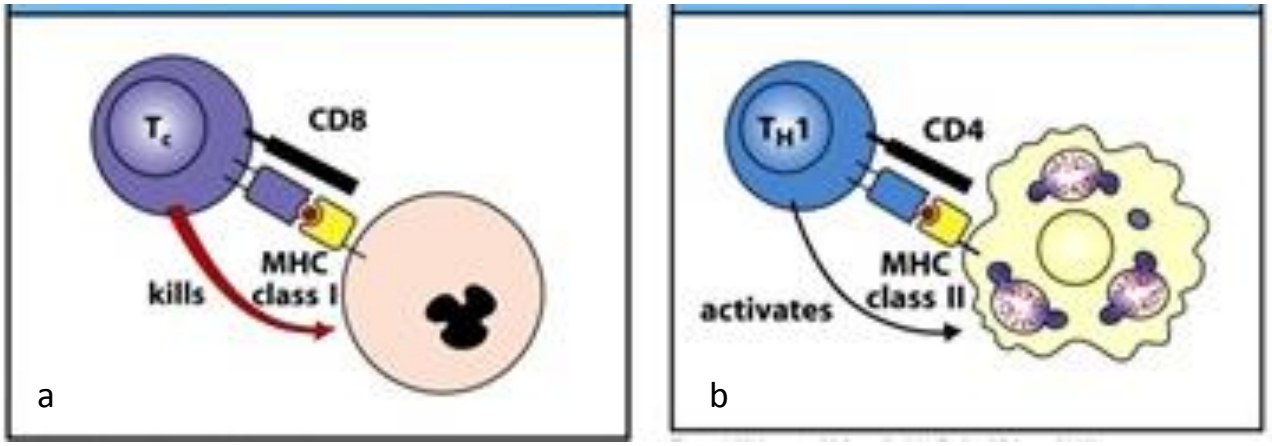

Figure 1: Activation of T-cells. CD8 T-cells are activated by peptides (red) which are presented to T-cells on class I MHC. Once activated T-cells can kill the infected cell (a). CD4 T-cells are activated by peptides presented to T-cells on class II MHC, once activated they can secrete molecules that bring about an immune response (b). Peptides from viruses or bacteria are placed on the MHC while still inside the cell. Once the peptide is in place, it moves to the surface of the cell where it can be recognized by the proper T-cell. The figure was taken from reference #8.

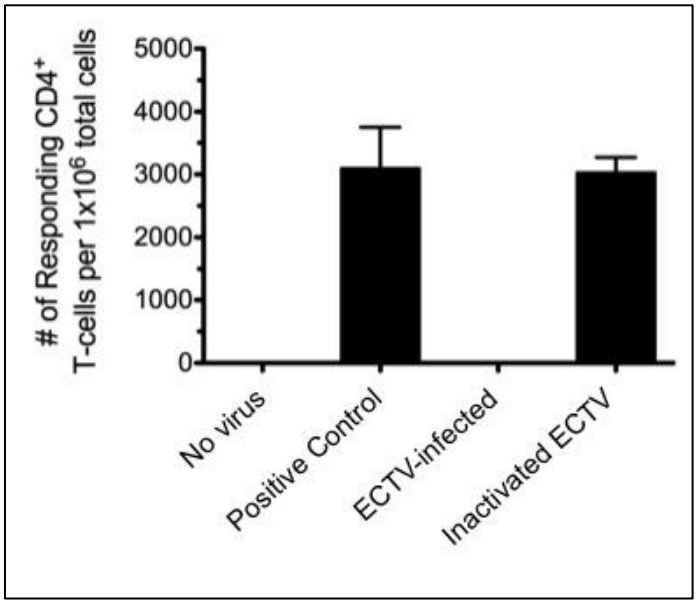

Figure 2: Live ECTV infection inhibits mouse CD4 T-cell responses. CD4 T-cells from mice were induced to produce IFN-gamma, which was detected using flow cytometry (*"Positive Control" column*). This treatment allowed for detection of the maximum possible CD4 T-cell response. Next, when live ECTV was included into the system under otherwise identical conditions, the CD4 T-cell response was no longer detectable (*"ECTV-infected" column*). Finally, if the ECTV was inactivated by ultraviolet radiation so that it was no longer a viable virus, the CD4 T-cell response was restored (*"Inactivated ECTV" column*). These data suggest that the inhibitory effect of the virus is an active process that requires live virus infection. Note: in the graph, the bars indicate the average value and the error bars represent the standard deviation of the data points.

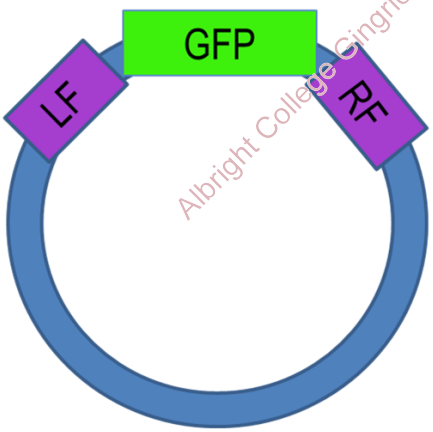

Figure 3: Knock out plasmid. The GFP gene has the left flanking region of gene 174 inserted to its left and the right flanking region of gene 174 to its right.

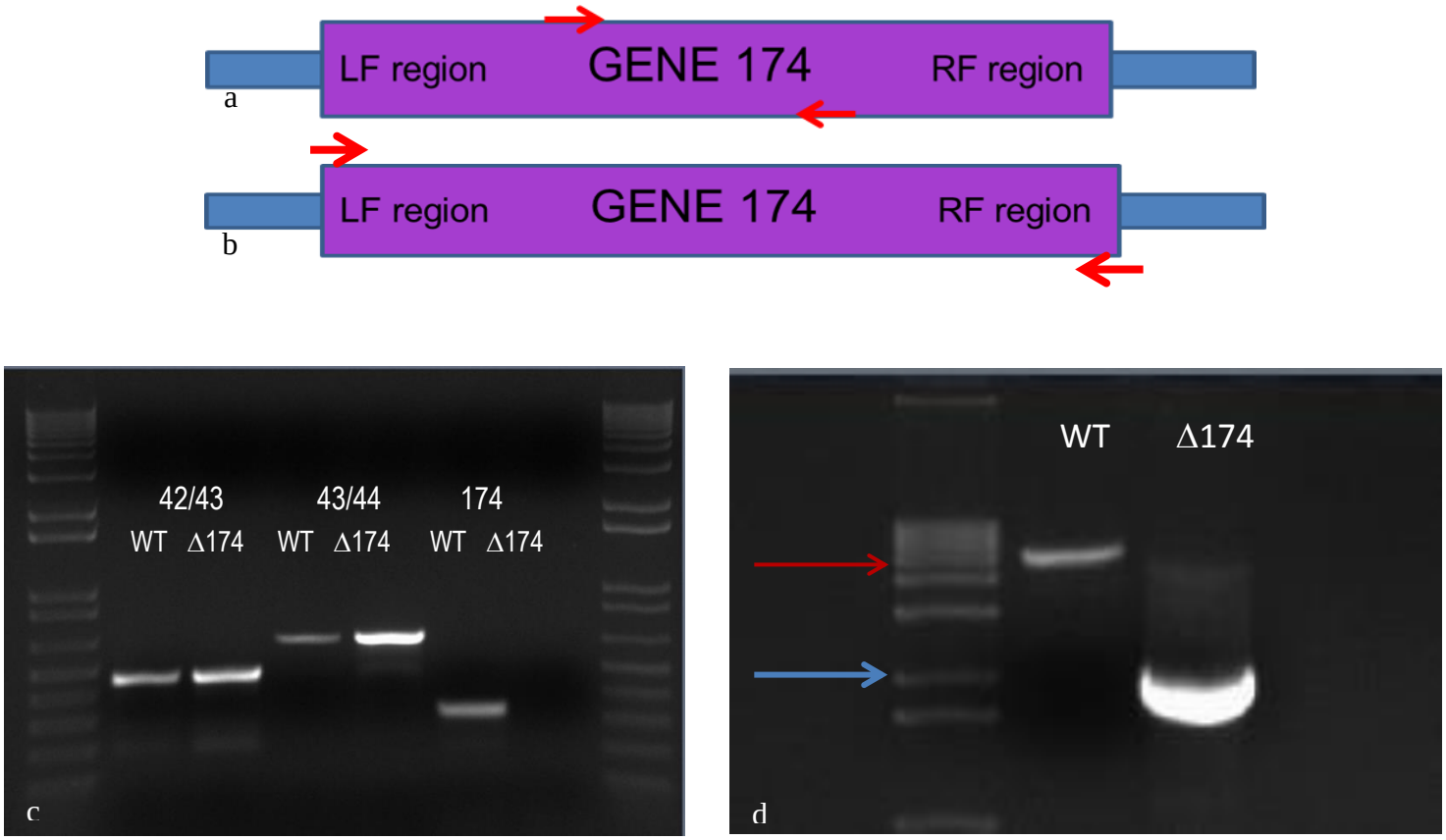

Figure 4: Confirmation of 174 disruption by PCR. PCR reactions were run using the proposed mutant virus with a gene 174 disruption and wild-type ECTV. The first set of reactions were carried out using primers that amplify the junction between genes 42 and 43. The second set of reactions were run using primers that amplify the junction between gene 43 and 44. The final set of reactions used primers that corresponded to regions of gene 174 that were replaced by GFP in the mutant virus (a,c). Identical PCR reactions were carried out using wild-type and Δ174 ECTV. PCR primers were used that bind at the termini of gene 174(b). The red arrow marks 6 kbp and the blue arrow marks 2 kbp. Gene 174 is ~5.7kbp, and the PCR product for wild-type is approximately the right size. The GFP gene that was used to replace the majority of the gene 174, in addition to its promoter and flanking regions, is ~2 kbp (d).

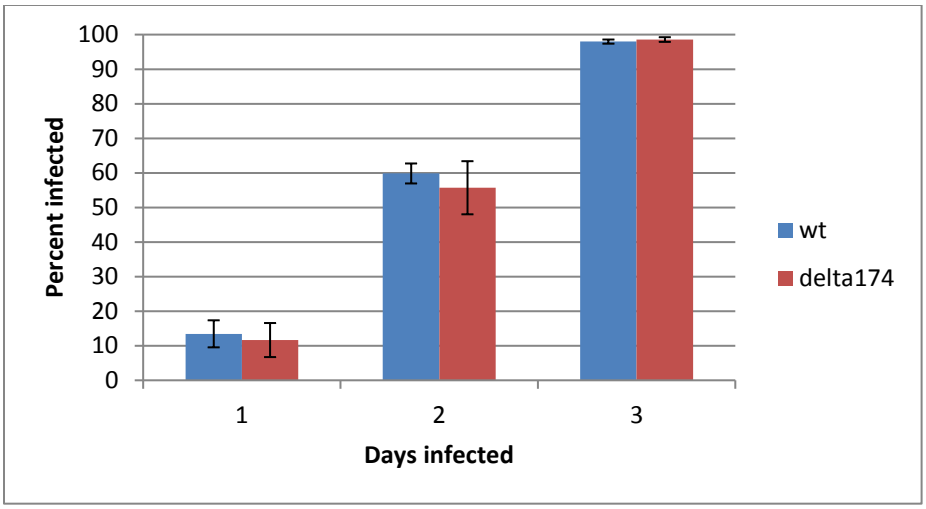

Figure 5: Growth Comparison . BSC1 cells were infected (MOI=0.01) with either w.t. ECTV-GFP orΔ174 ECTV. At 24, 48, or 72 hours, cells were harvested and GFP positive cells were counted as infected cells using flow cytometry. Note: in the graph, the bars indicate the average value and the error bars represent the standard deviation of the data points.

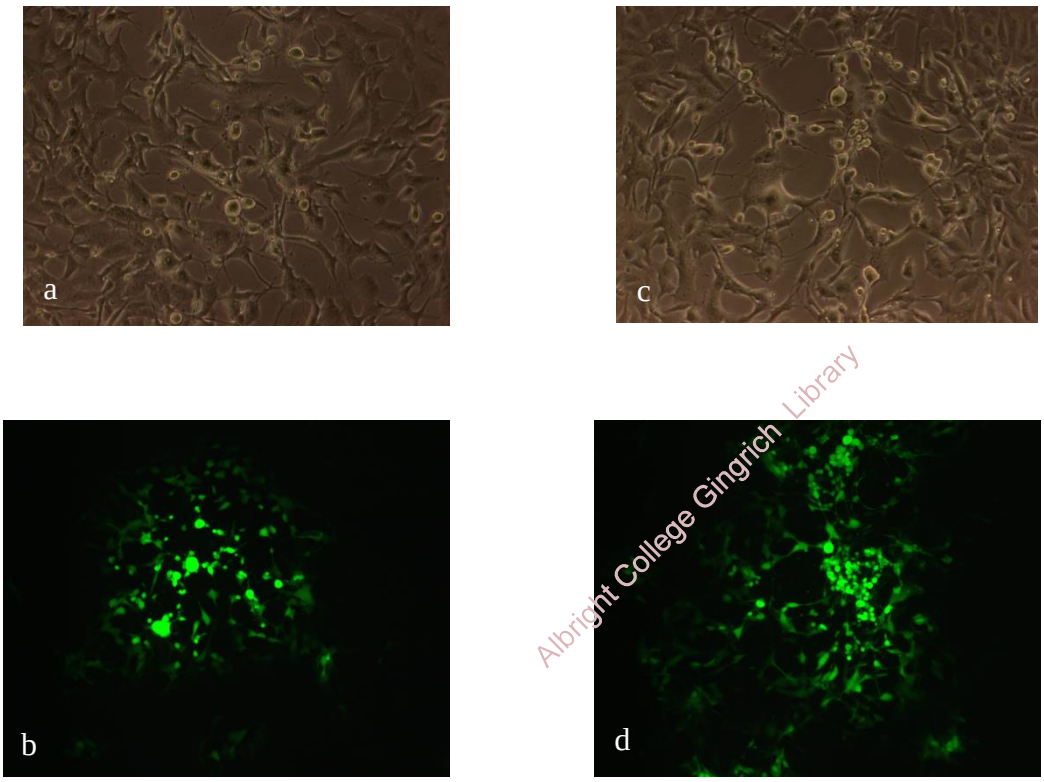

Figure 6: Plaque comparison. BSC1 cells were infected with w.t. ECTV-GFP (a and b) or Δ174 ECTV (c and d). A phase contrast microscope was used at 400x to take pictures of cells (a and c). A fluorescent microscope was used to visualize GFP expression in infected cells at 400x magnification (b and d).

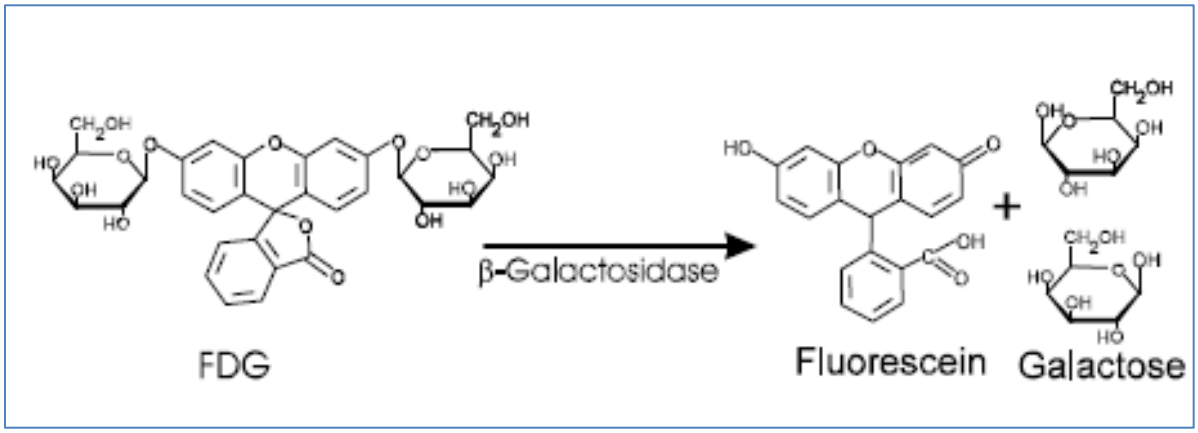

Figure 7: Structure and reaction of FDG [www.biotek.com]**.** Fluorescein di-β-D-galactopyranoside (FDG), when cleaved by the cellular enzyme β-Galactosidase, is converted to fluorescein and galactose. Fluorescein fluoresces green when excited by a blue laser. Only activated T-cells will produce β-Galactosidase and fluoresce.

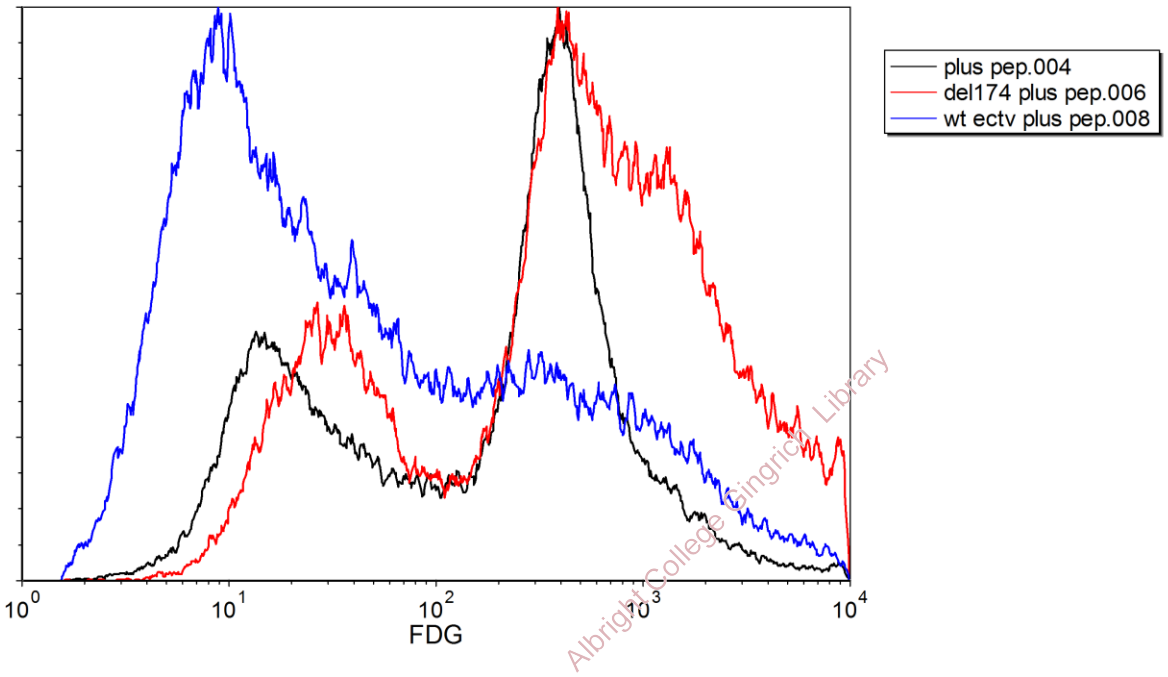

Figure 8:CD4 hybrid Activation Assay. Activation of T-cells was measured by plotting the amount of T-cells against the FDG expression. $5x10^5$ NA79 CD4 T-cell hybridomas were added to $1x10^6$ antigen presenting cells. The cells were stimulated with NA79 peptide. The plus peptide control (black line), were used to identify the maximum amount of T-cells capable of being activated in one assay. $5x10^5$ hybridomas were added to $1x10^6$ antigen presenting cells infected at an MOI=1 with w.t. ECTV (blue line) or Δ174 ECTV (red line) before stimulation with peptide. Flow cytometry was used to detect FDG expression.

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