Proposal

Sandor Haas-Neill: *Extracellular vesicles with rabies viral glycoprotein may deliver anti-HSV1&2 drugs to neurons*

Introduction: Herpes simplex virus (HSV), existing in two serotypes (HSV-1 and HSV-2), is a double stranded DNA virus that can cause lesions on human mucosa in its lytic phase, and persists in a latent phase inside neural ganglia (1; 2). Infections are life-long and, beyond the general unpleasantness to patients of occasional outbreaks of lesions (when the virus periodically emerges from latency), HSV can lead to complications such as additional sexually transmitted infections caused by open HSV sores; obstructive urethral swelling; in rare cases, meningitis; and infection of a newborn, which can result in blindness, deafness, and even brain damage (3; 4; 5). Although these complications are infrequent in the population, HSV is prevalent enough that they constitute a significant burden globally (6).

Extracellular vesicles (EV) are lipid bilayer capsules that can contain various cargo types including DNA, mRNA, miRNA, and protein (7). Due to their ability to transfer signalling molecules and functional cargo to cells and because they do not elicit an immune response, EV have gained a lot of attention as potential vehicles for drug delivery (8; 9; 10). Alvarez-Erviti *et al*., 2011 (10) have demonstrated that by engineering dendritic cell vesicles to express a rabies viral glycoprotein (RVG) fused to a protein normally expressed on the vesicle membrane - Lysosome-associated membrane glycoprotein 2 (LAMP2B) – the vesicles are able to deliver siRNA directly to the brains of mice. Another implication of this is that, like the rabies virus and like HSV, EV expressing the LAMP2B-RVG fusion protein are able to access peripheral and central neurons efficiently (11).

Hypothesis: I hypothesize that extracellular vesicles expressing the LAMP2B-RVG fusion protein and containing anti-HSV compounds will target neurons and combat latent HSV infection in vitro and in vivo.

Aim 1: Assess which cargo for the LAMP2B-RVG-EV most effectively targets latent infection of neurons in vitro. EV will be loaded via electroporation with acyclovir (a drug used to treat lytic HSV); with siRNA to HSV's latency associated transcript LAT; with a nuclease targeted to HSV genes as a positive control; with dsDNA that codes for inhibitory miRNAs on an HSV viral promoter. I will also use a negative control of LAMP2B-RVG-EV loaded with nothing. To determine which of these cargoes may have the strongest impact on the infection, I will assess the state of the virus following these treatments to HSV latently infected dendritic cells with qRT-PCR of viral RNA, and measuring late viral protein.

Aim 2: Track the in vivo movement of viral particles as well as LAMP2B-RVG-EV following different injection methods to assess the similarity of the distributions in a live system. First, I will track the movement of HSV-1 (lip abrasion) and HSV-2 (vaginal) following entry into a mouse using a bioluminescence assay. Secondly, also using a bioluminescence assay, I will track the path taken by LAMP2B-RVG-EV following entry to a mouse via lip abrasion, vagina, intraperitoneal injection, and intravenous injection. Finally, I will compare the movements of the HSV virus as well as the EV to make an assessment of how appropriate the overlap is for potential treatment of HSV.

Aim 3: Test the degree to which the in vivo treatment of HSV-infected Guinea Pigs with LAMP2B-RVG-EV can abolish reactivation of the virus. I will measure HSV viral RNA in the spinal fluid of guinea pigs. I will also count the number of lesions that result both from reactivated HSV that has been treated in the latent phase with LAMP2B-RVG-EV containing the positive control from Aim 1 (targeted nuclease) and from another of the cargoes with the best

potential to destroy latent HSV. This aim will put into perspective the potential for LAMP2B-RVG-EV to be employed in a clinical setting to cure life-long HSV infections.

This study will seek to determine two things. The first is the extent to which current HSV drugs and simple treatments can be effective if delivered straight to the neurons that are latently infected. The second is whether EV expressing rabies viral glycoprotein can be as effective as adeno-associated virus for delivering targeted nucleases to neurons, while being immunologically inert.

Background: HSV-1 and HSV-2 share 83% of the coding regions of their genomes but behave differently; most prevalently, HSV-2 infects only genital tissues, where HSV-1 *can* infect genital tissues but more commonly infects orofacial mucosal tissues (12). 84 polypeptides are coded for by these shared coding regions and about half of the 84 are needed by HSV-1 for replication (13; 14). Around its DNA, the virus possesses an icosapentahedral capsid; a layer of protein with no definite shape, called a tegument; and a viral envelope (1).

As noted, HSV-1 is the cause of facial mucosal lesions. Increasingly, it is also the cause of genital lesions (140 million people between 15-49, in 2012) and is thought to infect an estimated 67% of the world's population under the age of 50 (3.7 billion people, 2012)(15). Globally, HSV-2 infects an estimated 11% of individuals between the ages of 15-49 (417 million people infected) and is the cause of the majority of genital lesions (16).

HSV is highly contagious. It spreads through skin contact and usually at sites of frequent viral shedding such as between mucosal surfaces or where there are breaks in the skin (lesions) because the virus has difficulty passing through keratinized debris on healthy skin (17; 2). Upon gaining entry to the body, HSV first infects epithelial tissue before moving into sensory nerve endings and then, by travelling across synapses, into neural ganglia, where latent infection is established (2).

HSV entry and latency: Several glycoproteins present on the viral envelope are necessary for entry into the cell. Glycoprotein B (gB) achieves initial heparin sulphate binding on the cell surface to bring the virus into proximity with the cell; glycoprotein C (gC) can also bind heparin sulphate but is not required for viral entry (18). The next required step is for glycoprotein D (gD) to bind one of three high affinity receptors: herpes entry mediator A (HveA), expressed in monocyte-dendritic lineage and lymphoid tissues (which may contribute to the affinity for neurons); nectins, with nectin-1α (also known as HveC) being of particular interest because it is also expressed on sensory neurons; and 3-O-sulphotransferase sulphated heparin sulphates (19; 20; 2; 21; 18). Glycoprotein H (gH) and glycoprotein L (gL) are also necessary for viral entry into the cells: they form a fusion complex with one another, as well as with gB, to fuse the host cell plasma membrane with the viral envelope (22).

Following entry of the tegument and capsid into the cell, "immediate early" genes are expressed. These include major viral transcription factor (ICP4), which is needed for other events preceding replication (known from knockdown results in 1 generation of viral infection) (23). The trans-inducing factor (VP16) is a transcription factor and a component of the tegument that is already translated; it complexes with two more transcription factors - host octamer binding protein transcription factor (Oct-1) and host cell factor-1 (HCF) - to express immediate early genes as fast as possible (24; 25). Immediate early gene expression is required for the expression of "early" genes, which code for DNA synthetic machinery proteins (26). Viral DNA

replication, along with both immediate early and early proteins is necessary for efficient expression of the "late" genes, which encode components of the new viron structures (26). It is likely that the transcriptional environment of different host cells has an impact on the HSV genome expression, meaning that HSV gene transcription may be somewhat repressed in neurons, helping the virus to establish latency (26).

During latency, HSV does not replicate itself or destroy its host cells; the viral genome exists in heterochromatin and the latency associated transcript (LAT) is most predominantly expressed (27; 28). That being said, 'lytic' gene expression is still detectable via qRT-PCR during the latent phase of the viral lifecycle (29). LAT DNA is 8.3 kb and produces a 2.0 and a 1.5 kb transcript via alternative splicing, which contains multiple open reading frames, but translated products have not been detected in vivo (30; 14). The transcript also possesses a 6.3 kb exon which is spliced into multiple miRNAs which, *in vitro*, limit immediate early gene expression (31). Generally, the LAT functions to keep the viral infection latent, and to reduce the rate of reintroduction of the lytic phase (32; 33). Since the virus does not emerge from cells during this phase, it has little interaction with the immune system.

HSV interaction with the immune system: Re-emergence of the lytic phase of the viral lifecycle is poorly understood; however, ex vivo reactivation of HSV can be achieved via inhibition of CD8 as well as γ-interferon (IFN-γ)(34; 35). It is also known that a local immune response is present in HSV infected neural ganglia in the form of elevation of cytokines derived from lymphoid tissue (36). This response can be stymied with the introduction of the anti-HSV drug, acyclovir, to the environment (37). This evidence suggests that reaction suppression and the initial control of the infection are mediated by CD8 expressing T cells and cytokines (IFN-γ) from lymphocytes (2).

When coming out of latency, viral particles travel down the axon to prepare to infect the epithelium (38). Antibodies have been shown to block transmission of virus back from dorsal neurons to keratinocytes in a two chamber ex-vivo model (39).

HSV is also equipped with several mechanisms of immune evasion, the most simple of them being its ability to infect certain immunocompetent T cells (40). Also among these are the fact that its UL41 gene codes for vhs protein, which is antagonistic to the antiviral effect of INF-alpha, and that its ICP47 protein, encoded by the US12 gene, can inhibit antigen processing and presentation in both the HLA class I and class II pathways (41; 42).

Current treatments and challenges of vaccine development: Drugs currently on the market for HSV treatment - acyclovir, famciclovir, and valacyclovir - are all HSV DNA polymerase inhibitors (43; 44; 45). Logically, then, they target the lytic phase of the HSV lifecycle and can be used to reduce symptoms of HSV such as lesions but are unlikely to have much impact on the dormant phase.

Latency also seems to be the dominant factor as to why HSV vaccines have not been developed for clinical use. Even in individuals with both cell-mediated and humoral immunity the virus can still re-emerge from latency, and it is difficult to prevent latency from being established in an uninfected animal – although some report to have done it (upon entry of the virus into the body)(46; 47; 48). Koelle and Corey, 2003 (2) have also suggested that although some approaches appear to work in animal models, it is doubtful that any of these same

methods in humans can achieve "sterilizing" immunity (completely preventing infection of a cell).

Extracellular vesicles and other drug delivery vehicles: There is clearly a need for a delivery mechanism capable of accessing neurons to target latent HSV. Some adeno-associated viruses (AAV) have displayed some neurotropism; an example of one that was used for the delivery of HSV gene targeting engineered nucleases is AAV8 (49). AAV8 was successful in its delivery to neurons, and the targeted nuclease was successful in cleaving a number of the latent HSV-1s UL19 and UL30 genes (49). One challenge associated with using viruses as cargo delivery vectors, however, is that they elicit an immune response: AAV injected into mouse muscle initiated the development of an antibody response to the capsid (50). Another problem with using viruses for transgene cargo delivery specifically is reduced delivery efficiency in subsequent treatments (51). EV do not share either of these problems; they can be produced to be immunologically inert, and they can be administered to deliver cargo many times without the efficiency of delivery being affected (10). EV tropism, however is not always as easy to explain as viral tropism, but it can be made to be.

By generating EV expressing the LAMP2B-RVG fusion protein Alvarez-Erviti *et al*., 2011 (10) produced EV that have acquired the natural neurotropism of the rabies virus due to the affinity of the rabies viral glycoprotein for nicotinic acetylcholine receptors expressed on neurons of the central and peripheral nervous system (52). Although rabies most likely does not reflect the exact behaviour of HSV travel through the nervous system, its ability to confer tropism to EV with a single protein is powerful and saves a lot of trouble that could be endured by trying to implement gB, gD, gH, and gL on the surface of an EV in a conformation that makes them functional. These RVG expressing EV are potentially an extremely effective tool for HSV research and treatment, as I hope to explore.

Background Summary: HSV-1 is a virus that infects 67% of people under 50 globally, and HSV-2 infects 11% of these same people globally (15; 16). Although the rate of serious complications is rare, the sheer volume of the infected results is a considerable disease burden in terms of suffering and economic costs (6). These HSV viruses are easy enough to treat and vaccinate against during their destructive lytic phase, but when they are latent inside neuronal ganglia with its genome in heterochromatin it can evade CD8 expressing T cells, IFN-γ cytokines, and viral DNA polymerase inhibitors are of limited use (43; 46; 47; 48). The infection lasts for life due to the latent phase, and periodically symptoms and morbidity can re-emerge. To adequately tackle this massive global issue, we need to focus on what can be done to access the virus during its latent phase in order to excise it from the body once and for all.

Extracellular vesicles have great potential as drug carriers because they are immunologically inert and because they do not lose efficiency as delivery vehicles for genes after the initial administration, as viral carriers do (10). By expressing rabies viral glycoprotein as a membrane surface fusion protein with the transmembrane protein LAMP2B, Alvarez-Erviti *et al*., 2011 (10) successfully created a population of EV with strong neurotropism. These EV have great potential for the treatment of HSV, as they can access the peripheral and central nervous system where the latent infection resides.

Methods: For this experimental design, I will use HSV-1 McKrae and HSV-2 333 strains because they are equally highly virulent in adult mice (53). Extracellular cellular vesicles used

will be designed using the method described in Alvarez-Arviti *et al*., 2011 (10): in brief, dendritic cells possessing granulocyte/macrophage-colony stimulating factor are selected from a bone marrow isolate from inbred C57BL/6 mice. Four days prior to exosome purification, one population of dendritic cells will be transfected with plasmids containing the gene construct for LAMP2B-RVG; a second population of cells that are not transfected will also be used. Four days following transfection, the vesicles are isolated and can be loaded via electroporation. For my purposes the BALB/c mouse bone marrow and dendritic cells will be used, however, as this will be the mouse strain employed. Likewise, to achieve the other aims, I will use vesicles from Lund human mesencephalic (LUHMES) cells for Aim 1 and hairless strain guinea pig bone marrow derived dendritic cells for Aim 3.

EV cargo anti-HSV plasmid construct was designed with knowledge that the promoters of both immediate-early and early HSV genes contain a TATA box or initiator element, or both, and differ greatly from the promoters of late HSV genes, which promoters necessarily possess both an initiator element and TATA box and do not possess cis acting elements upstream of the transcription start site (54). A gC promoter - consisting of a TATA box inside a 15 bp sequence (GGGTATAAATTCCGG), is sufficient to initiate gC transcription (55) - will be positioned in front of the 8.3 kb LAT DNA on a plasmid, which will have its own promoter deleted. This will mean that when the virus would normally emerge from latency, and transcribe late genes, it will also transcribe this exogenous LAT gene, resulting in upregulation of the miRNAs that target and repress other late genes. This has been designed in such that the virus will continue its latent phase when it would otherwise have become lytic once again.

Animal and Cell Models: LUHMES cells will be used as a model in Aim 1 due to their ability to support HSV-1 latency *in vitro* without the need for constant drug treatment required by other model cell lines (56). BALB/c mice will be used for Aim 2 due to the rapid establishment of latency by HSV-1 within 24 hours of first infection (57). This will allow easy and rapid visualization of the path taken by the virus through the body, as the virus will reach neural ganglia quickly. Hairless strain guinea pigs will be used in Aim 3 because HSV in guinea pigs will spontaneously reactivate, as it does in humans (where it does not do so in mice)(58; 53). Furthermore, in a hairless guinea pig, lesions can be easily seen without the animal having to undergo uncomfortable denudation (59).

Laboratory Methods: EV will be isolated using the method of differential centrifugation described in (60): in brief, cells will be grown in plain media for 48 hours before the media is collected and centrifuged at 1300xg, 12000xg. Then vesicles will be pelleted at 100,000xg and resuspended in 100 µL of phosphate-buffered saline (PBS). Cargo will be loaded into EV using the optimized electroporation protocol described in Pomatto *et al*., 2019 (61), involving 750 volts and 10 pulses. Each population of EV concentration and amount will be determined in 2 ways: via Nanosight count and using a Bradford assay for protein concentration.

The Nanosight passes a laser through a 1:10 diluted sample of EV. When the light refracts on the surface of the vesicle, the computer retraces the particle's position in the fluid. Based on the knowledge that they are spherical, the Nanosight can estimate the size of each vesicle and can count each one to provide an estimate of concentration. An injection rate of 80 will be used on a Nanosight LM14C, model type: NS14C.

The Bradford assay will use a known standard of bovine serum albumin (BSA) serially diluted to generate a standard curve. Vesicles are diluted 1:10, and are lysed in 1XRIPA buffer by freezing and thawing 5 times before samples are added to the clear bottom plate alongside BSA. A protein dye is added to the sample and a spectrophotometer measures the absorbance of each well at 750 nm. Protein concentration measured by the Bradford assay will be combined with concentration data from the Nanosight to generate a value of protein per vesicle.

Transfection will be done with "TRANS-HI DNA Transfection Reagent" using the manufacturer's instructions. In brief: cells are seeded 24 hours prior to transfection at a density of $1x10^5$ cells per mL, such that the cells are no more than 80% confluent and no less than 70% confluent at the time of transfection. 0.5 μg of DNA plasmid (containing the LAMP2B-RVG fusion protein gene and a selectable marker) will be used to treat cells along with 1.5 μL of TRANS-HI on a 24 well plate. Transformed cells will be grown on a larger scale and used for the generation of EV.

Aim 1: I will assess which cargo for the LAMP2B-RVG-EV most effectively targets latent infection of neurons in vitro. To determine what cargo type might be best to treat HSV in a clinical setting where EV can easily access the latent infection in neurons, LAMP2B-RVG-EV groups will be loaded via electroporation with one of acyclovir, nothing, the plasmid construct described earlier in the methods, siRNA complementary to the LAT transcript, and the targeted nuclease described in Aubert *et al*., 2016 (49). LUHMES cells infected with HSV will be treated with different groups of cargos and measured for their viral RNA and gD protein levels to determine which cargo type has the largest impact on the reactivation of latent virus following treatment in latency.

LUHMES cells, upon infection with HSV-1 and simultaneous treatment with acyclovir, stably support a latent infection in 48 hours, making them different to most other cell lines in which *in vitro* the virus remains lytic unless constantly incubated with an anti-HSV drug such as acyclovir (56). Treating latently HSV-1 infected LUHMES cells with 200 ug LAMP2B-RVG-EV will follow 24 hours of starving infected cells in plain media (to achieve alignment of cell cycle between all cells in the population). All treatments will be repeated in 12-hour intervals. 48 hours after the initial treatment, cells will be treated with a phosphoinositide 3-kinase (PI3K) inhibitor, which should induce reactivation (56).

After another 48-hour period in complete media, I will measure the impact of the treatments on cell culture by employing quantitative, reverse-transcriptase polymerase chain reaction (qRT-PCR) to measure the transcripts used for detection of HSV-1 in vaginal secretions described in Burrows *et al*., 2002 (62): using the HSVpolF (5-GCTCGAGTGCGAAAAAACGTTC-3) and HSVpolR (5-TGCGGTTGATAAACGCGCAGT-3) primer set, which amplifies to a 140-bp product. In brief: HSVpol mRNA is reverse transcribed with a reverse transcriptase before the resulting template DNA can be used for quantitative PCR. The first step of quantitative PCR is the same as that of normal PCR, where DNA is amplified in cycles by having primers anneal to a known sequence and another known sequence downstream to make a copy of the DNA between the two primers. In quantitative PCR, however, there is a fluorescent dye that binds to DNA present in the sample during the amplification, and there is a fluorescence reader that measures the fluorescence of each well in real time. Each sample will reach a certain threshold of fluorescence at a cycle number corresponding to the original amount of mRNA of interest in the

sample – termed its Ct. Cts are then compared to provide a quantitative estimate of the original amount of mRNA in the sample. Less HSVpol mRNA relative to control in a sample will indicate that the particular LAMP2B-RVG-EV cargo was able to either destroy latent HSV, or prevent it from re-entering its lytic phase.

ELISA, described in Voller *et al*., 1978 (63), will also be performed using a custom kit with anti-HSV-gD fixed in the wells (indirect capture). In brief: the samples will be pipetted into different wells containing the fixed antibody. The HSV protein gD, if present, will bind to the antibody. A second, free, primary antibody will be added to the well and will bind a different part of the gD protein. Finally, a secondary antibody with a fluorescent reporter will bind to the 'free' primary antibody, and the signal can be read if gD is present. The greater the fluorescent signal, the higher the concentration of gD in the sample. This will detect and compare the amount of recombinant gD viral envelope protein (product of a late gene) that has been expressed in the cell lysates and media. Less gD protein relative to control in a sample will indicate that a particular LAMP2B-RVG-EV cargo was able to destroy latent HSV or prevent it from re-entering its lytic phase.

Cell lysis will be done before assessing the change of mRNA and gD protein levels inside the cell following treatment with LAMP2B-RVG-EV. Some cells will have lysed due to the virus, however, so this media will also need to be collected - its EV pelleted and discarded (as in this caseIare not interested in them) - and pooled with the cell lysates of the corresponding vesicle treatments. Cells will then be washed twice in 4 mL of PBS and will be placed on ice. 700 mL of 1XRIPA buffer containing protease inhibitor will be added to the plates and shaken every 2 minutes over a 10 minute period. Cells will then be removed from the plate with a scraper and stored in a -80˚C freezer to further aid in lysis before analysis.

Aim 2: Track the in vivo movement of viral particles as well as LAMP2B-RVG-EV following different injection methods to assess the similarity of the distributions in a live system. To determine the appropriateness of LAMP2B-RVG-EV localization for the treatment of HSV in nuclear ganglia, three separate groups of BALB/c mice will be treated with one of HSV-1 expressing the *Renilla* and firefly luciferase genes; luciferase expressed in LAMP2B-RVG-EV; or the control: EV isolated for non-transfected dendritic cells. While HSV-1 will enter the mouse via lip and vaginal abrasions, the LAMP2B-RVG-EV will be injected intraperitoneally, injected intravenously, or applied to lip and vaginal abrasions. To determine the best method for delivery of LAMP2B-RVG-EV cargo to HSV-1 infected cells, fluorescence images taken between all the vesicle groups will be overlaid and assessed for best match to the HSV-1 groups.

The *in vivo* EV bioluminescence assay will be performed in a method similar to that described in Grange *et al.,* 2014 (64) using 12 BALB/c mice. Prior to treatment, I will label both populations of EV with 1 µM of the "Vybrant Cell Tracer 'DiI'" during an ultracentrifugation (not the same as the initial isolation), followed by two ultracentrifugation wash steps in PBS. Two mice in each of four subgroups of three mice will be injected with 200 µg LAMP2B-RVG-EV; the third mouse in each subgroup will be injected with the labelled normal dendritic cell EV. EV will be introduced to one subgroup intravenously, to one subgroup intraperitoneally, to one subgroup through application of EV to lip abrasions, and to one subgroup through application of EV to vaginal abrasions. After application of EV, the mice will be anesthetized with 2.5% isoflurane. Images will be taken after 15 minutes, 5 hours, and 24 hours on an IVIS Lumina II

imaging system (64). The illumination settings used will be a binding factor of 4, a 2 second exposure time, and an f/stop of 2. There will be 12 fields of view.

The HSV *in vivo* bioluminescence will be done with 6 BALB/c mice using the recombinant *Renilla* and firefly luciferase strain described in Luker *et al*., 2002 (65). This luciferase expressing HSV-1, (2 mice for each entry point) and a control group of just PBS (1 mouse for each entry point) will be applied topically to lip and vaginal abrasions (as these are the major entry points in a natural setting) and fluorescence readings will be taken following 15 minutes, 5 hours and 24 hours and following the method described in Luker *et al*., 2002 (65). Once again, the illumination settings used will be a binding factor of 4, a 2 second exposure time, and an f/stop of 2. There will be 12 fields of view. The data from these two bioluminescence experiments will be overlaid and compared to determine the best route of entry of LAMP2B-RVG-EV for the potential treatment of HSV-1.

Aim 3: Test the degree to which the in vivo treatment of HSV infected guinea pigs with LAMP2B-RVG-EV can abolish reactivation of the virus. To determine the capacity of LAMP2B-RVG-EV to deliver therapeutic cargo in a clinical setting for the treatment of HSV-1&2, three tests will be performed against one control group. Eight hairless female guinea pigs will be infected with HSV-1 McKrae strain via lip abrasion, while another group of 8 will be treated with HSV-2 333 strain via vaginal abrasion. Two days following this treatment, the number of lesions will be counted and recorded, as this is the day when the initial outbreak should be strongest (59). These two guinea pig populations will then be subdivided into four groups of two guinea pigs each. One set of two guinea pigs from each subgroup will remain as control and so will be treated with PBS. A second set of two guinea pigs from each subgroup will be treated with targeted nuclease loaded vesicles (positive control) will be injected intravenously. A third set of two guinea pigs from each subgroup will be injected with the best cargo inside an EV for treating HSV determined in Aim 1. A fourth set of two guinea pigs from each subgroup will be injected with AAV8 carrying the same targeted nuclease (a second positive control). This treatment will be repeated once a week (same day same time) for three weeks. At the end of the treatment phase –four weeks to allow the virus to re-emerge from latency - lesions will be counted and guinea pigs will be sacrificed to take spinal fluid measurements of viral HSV RNA using the same probes as were used in aim 1. A student's t-test will be used to determine the significance of the lesion counts. This will determine the efficacy of EV in comparison to AAV8 for the delivery of cargo to latent HSV *in vivo*.

Feasibility: The first experiment is a standard cell treatment with EV, which I have done many times. The other two aims utilize experiments that have been done previously, namely, bioluminescence detection of HSV and EV, and treatment of a live model with a cargo-carrying vector. There are many studies to draw from when it comes to optimizing the protocols of bioluminescence of viruses and EV for our purposes. Aubert *et al*., 2016 (49) showed that a nuclease targeted to HSV DNA in latent neurons is able to mutate it after being delivered by adenovirus; with this in mind, the only real variable in the third experiment becomes the efficacy of the LAMP2B-RVG-EV itself. There is also a near-guarantee of seeing lesions following re-emergence of the virus in experiment 3: it is known that 4 to 6 weeks following the initial HSV infection of the guinea pig is when the virus tends to reactivate and lesions re-emerge (59).

The theory is realistic because LAMP2B-RVG-EV have already been shown to have the ability to access the brains of mice after intravenous injection, which demonstrates excellent neurotropism compared to EV without RVG, suggesting that the vesicles may use the same mechanism as rabies to reach the brain – traveling through neurons. With this in mind, it becomes quite obvious how this vesicle system could be used to access with high efficiency latent HSV hiding inside neurons.

I will have access to all the equipment and facilities necessary to address my aims: an ultracentrifuge, an electroporator, a qRT-PCR machine, cell culture facilities, and animal facilities. A nanosight is available to me, which is owned and maintained by the Hoar lab on the main McMaster campus. An IVIS Lumina II is available through the central animal facility at McMaster University, which will be sufficient to carry out the bioluminescence experiments. I also have access to Living Image 4.0 software, which is needed to analyze the images. Herpes simplex virus is a containment level 2 pathogen, which is appropriate for our lab.

Expected Outcomes and Caveats: It is expected that the LAMP2B-RVG-EV will easily be able to enter the LUHMES cells and deliver their payloads. Acyclovir will most likely not have an effect on viral RNA or gD production following latency, as it inhibits viral DNA polymerase, already relatively inactive during latency. The targeted nuclease should cause a significant drop in both gD protein production and viral RNA measurements compared to control, as it will cut the DNA right out of the latently infected cell and is known to be capable of addressing the problem of latency (49). siRNA targeting the LAT transcript may have an interesting or unexpected effect, but previously when LAT has been knocked down with an AAV, it surprisingly resulted only in HSV-1 struggling to reactivate (66). Finally, the plasmid construct could potentially prevent reactivation of HSV-1, while not actually removing any of the latent viral DNA. It could be that upregulation of LAT under the inhibition of PI3K condition is not enough to keep the virus in latency, in which case one might expect to see only a slight depression in RNA and gD levels compared to control.

Although bat-associated rabies travels through the blood to the blood brain barrier and crosses it (2; 67; 68), HSV and wild type rabies invade the neural periphery and then travel anterograde along the axons of neurons, crossing synapses to reach the brain. Therefore, it would make sense to see light move from peripheral neurons to more dense regions of ganglia in the *in vivo* bioluminescence assay. It remains to be seen if LAMP2B-RVG-EV are also capable of this, as Alvarez-Erviti *et al*., 2011 (10) merely introduced the vesicles into mice intravenously and successfully detected their siRNA cargo in the brain. EV are known to be able to cross the blood brain barrier (69), and although this bioluminescence experiment can potentially elucidate the path of travel of LAMP2B-RVG-EV and HSV, there is a possibility that the results will simply show large signals in the brain.

Another caveat of the bioluminescence assay is that it may give the false impression of a higher percentage overlap than exists between HSV and LAMP2B-RVG-EV, as neighbouring neurons inhabited by one or the other exclusively could appear as one light-mass.

There is another possibility that both LAMP2B-RVG-EV and HSV-1 expressing luciferase will not exist in a high enough concentration to generate a signal in BALB/c mice that have had abrased lips swabbed with EV and HSV containing PBS. This could potentially be remedied by increasing the concentration of vesicle or LAMP2B-RVG-EV, although there is a possibility this

still will not generate sufficient bioluminescence. If this turns out to be the case, 200 μg of virus and LAMP2B-RVG-EV can be injected into the mouse lip and vaginal mucosa to increase the concentration to a visible level. This would be used only as a last resort to show the resulting distribution of EV/HSV, however, as it poorly mimics the reality of HSV infection or, in the case of LAMP2B-RVG-EV, the reality of how they might be implemented as an ointment drug treatment should the results of this study be otherwise promising.

In Aim 3, as a positive control for the test of the efficacy of delivery of the LAMP2B-RVG-EV, the targeted nuclease can be expected to work as well as it does when delivered by AAV8 in Aubert *et al*., 2016 (49). Therefore, one could expect there to be fewer lesions at the end of 4 weeks after the initial EV treatment and less viral RNA in spinal fluid than in the empty vector treatment. The plasmid construct has the same potential and caveats as in Aim 1, in that if LAT upregulation on a late gene promoter is enough to stymie reactivation, I would expect to see less spinal viral RNA and fewer lesions in both the HSV-1&2 infected guinea pigs.

Aspects of the targeted nuclease that render it difficult to translate into a human treatment are its off-target effects. That is, guided nucleases have not been developed that are accurate enough to cause a DNA cleavage at the intended site alone. Imperfect complementarity is tolerated by the complex formed by cas9 and its guide RNAs, which results in DNA cleavage far away from the intended site. This can increase the recipient's risk of cancer, among other things. Guided nucleases are being used in this study solely as positive controls for assessing the ability of LAMP2B-RVG-EV to affect its side of the treatment, and would be considered for use in humans only once better targeted nucleases have been developed.

Impact: This research will achieve three core ends, but it has the potential to lead to some useful collateral developments, as well. For example, the possibility of LAMP2B-RVG-EV being topically applied to lip or vaginal mucosa is a particularly exciting prospect for drug development. The delivery system is most likely to engage the same peripheral neurons as HSV through this entry route, and if it proves effective for treatment of the virus, the system could potentially be translated to the clinic in the form of a topical ointment. This is much less invasive than intravenous and intraperitoneal injection, and is therefore ideal as a mechanism of treatment. Additionally, the second aim of this research could potentially generate evidence that the ability of rabies to move in an anterograde fashion up the axons of peripheral neurons and through synapses to the brain can be conferred to an EV simply by expressing one protein on its surface. Such a finding would be interesting and novel and could have several indirect applications to human health and disease.

While the collateral findings of this research might be interesting and useful, its three core goals could point to serious advancement in the treatment of HSV-1&HSV-2. It will show whether or not LAMP2B-RVG-EV can be made to be as efficient as AAV in their delivery of anti-HSV cargo to neurons; it will identify which potential cargo may have the greatest impact on reactivation of the latent virus; and it will determine the appropriateness of the LAMP2B-RVG-EV system as a mimic to the *in vivo* pathing of HSV. The virus that infects greater than half the world's population has been allowed to coexist with our species for too long: its time has come. If I can prove that LAMP2B-RVG-EV are capable of delivering cargo to neurons,I will be one step closer to a safe, immune-inert delivery system that can access the virus where it hides and that, with the development of a safe and effective cargo, will contribute to end the plague of HSV.

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