Proposal Example 2

Title and Hypothesis (1000 characters maximum):

Utilizing CRISPR Methods to Reduce Feline Immunodeficiency Virus Proviral Loads and Infectivity

RNA-directed gene editing (CRISPR) will reduce proviral loads and production of infectious virus in feline cells infected *in vitro*.

Specific Aims (2000 characters maximum):

RNA-directed gene editing or CRISPR (clustered, regularly interspaced, short palindromic repeats) is a molecular technique based upon a bacterial antiviral defense mechanism. CRISPR utilizes the bacterial protein, Cas9, coupled with a site-specific engineered guide RNA (gRNA) to locate and cleave the proviral genome target sequence. When implemented, the CRISPR/Cas9 editing system results in specific, double strand breaks in the targeted proviral DNA, resulting in the deletion of specific genetic loci. Subsequent host DNA repair occurs by standard cellular repair mechanisms, homologous recombination or non-homologous end joining (Charpentier and Doudna, 2013). A set of lentiviruses expressing anti-FIV gRNA/Cas9 (anti-FIV CRISPR reagent) has been previously engineered in Dr. Murphy's laboratory.

Specific Aim 1: Establish the optimal CRISPR treatment protocol.

Specific Aim 2: Determine if FIV infected feline cells treated with anti-FIV CRISPR reagent reduces
proviral loads and/or production of infectious virus relative to FIV infected/untreated cells.

Project Plan Significance (500 characters maximum):

This project is undertaken as a *proof a concept*; hypothesis-confirming results will provide an impetus for *in vivo* studies in FIV infected cats. If successful, this study will provide *in vitro* data to support an attempt to reduce the latent FIV proviral reservoir in chronically FIV-infected cats. FIV infected cats are an important animal model of HIV-1 pathogenesis in humans (Burkhard and Dean, 2003; Sparger, 2005). Knowledge gained from these studies could benefit both cats and humans alike.

Innovation (500 characters maximum):

Although the disease course is often prolonged, most cats infected with FIV eventually succumb to opportunistic infections and/or neoplastic disease as a result of virus-mediated destruction of the adaptive immune system. There are no current medical therapies that consistently and efficiently eliminate lentiviral reservoirs from infected latent cells (Archin et al., 2014; McDonnel et al., 2014). Gene editing technology provides a novel method to reduce or eliminate the archived proviral load.

Approach. Must include Rationale and Methods, Potential Problems and Alternatives, Experimental Rigor (statistics, validation of reagents, sample size, etc.). 8000 characters maximum.

Rationale and Methods:

FIV infection is a common and incurable infectious disease of domestic cats and invariably results in death once it progresses to the stage of feline acquired immunodeficiency syndrome (FAIDS) (Burkhard and Dean, 2003). FIV, and other lentiviruses like HIV, persist through time as a result of integration of the provirus into the host cell genome. We propose to use CRISPR/Cas9 technology as a strategy to specifically target the sequence of the integrated FIV provirus, resulting in sequence-specific excision of the viral genome from latently infected host cells.

Specific Aim 1: Establish the optimal CRISPR treatment protocol.

A control lentivirus expressing green fluorescent (GFP) will be utilized to establish the optimal parameters of infection. Various concentrations of primary feline peripheral blood mononuclear cells (PBMCs) will be infected with various titers of GFP expressing lentivirus in order to establish the correct multiplicity of infection (MOI). We will culture and observe the infected PBMCs using fluorescence microscopy to determine the MOI. Freshly isolated feline PBMCs will be obtained from SPF cats housed at the UC Davis Feline Nutrition Colony. An IACUC protocol is currently in place describing this process in detail. The control lentivirus expressing GFP is currently available in Dr. Murphy's laboratory. Feline PBMCs will be infected and cultured *in vitro* with FIV clade C lentivirus stocks in order to establish proviral integration and an acute infection, as described from previous protocols (Murphy et al., 2012). Viral infection status will be quantified through real-time PCR (McDonnel et al., 2012). Infectious viral stocks are archived and available in Dr. Murphy's laboratory.

Specific Aim 2: Determine if FIV infected feline cells treated with anti-FIV CRISPR reagent reduces
proviral loads and/or production of infectious virus relative to FIV infected/untreated cells.

Following infection, the FIV viral RNA gets integrated in the host DNA as an FIV proviral DNA. The proviral DNA is flanked by two direct repeat regions called long terminal repeats (LTRs) that will be the target of the CRISPR guide RNA. We will culture FIV infected feline PBMCs to reach peak proviral integration (SA 1). Treatment Group A cultures (~1 x 10e7 cells) will be subjected to the anti-FIV CRISPR treatment while treatment Group B cultures (~1 x 10e7 cells) will be used as controls and will not be treated with anti-FIV CRISPR. The dosage of the CRISPR treatment (viral titer/MOI) will be established using the control GFPexpressing lentivirus (SA 1). The CRISPR/Cas9 expressing lentivirus ("CRISPR treatment") will be applied at the appropriate MOI to the FIV infected PBMCs. We anticipate that this gene editing technology will make a site-specific double-stranded incision at both the 5' and 3' proviral LTR sequences. Cells will be cultured in vitro for 2, 3, 5 and 7 days. Approximately 1 x 10e6 cells will be removed at each time point and total DNA/RNA isolated (AllPrep, Qiagen). The proviral load and cell-associated viral RNA will be determined by real time PCR as described previously (McDonnel et al., 2012). Statistically significant reductions of FIV proviral loads in Group A relative to Group B will be supportive of the hypothesis. We will also evaluate the infectivity of FIV derived from CRISPR treated cells relative to virus derived from untreated cells. At the same time points described above, cell-free culture supernatants will be utilized to infect naive feline SPF PBMC cultures.

Infected PBMCs will be cultured for 5 additional days and the cell-associated DNA will be harvested by centrifugation using a Qiagen kit. Proviral copy number will be assessed using real time PCR. Statistically significant reductions of proviral DNA in PBMC cultures exposed to Group A supernatants relative to Group B will provide evidence supporting the study hypothesis.

Potential Problems and Alternatives:

Alternative FIV-permissible cell lines to primary PBMC include MCH-5 and/or CRFK (both currently available in the Murphy laboratory). In the event that the titer of currently available CRISPR stocks prove to be inadequate, high titer lentivirus (~1 x 10_9/ml) can be efficiently obtained from a supplier (Cyagen Biosciences).

Experimental Rigor:

All experiments will be performed in triplicate. Data will be presented as the mean of three or more values with a standard deviation. An analysis of variance (ANOVA) will be performed on each data set. Where global differences are identified, the Tukey-Kramer Multiple Comparisons Test will be used for pair-wise comparisons of the mean responses between treatment groups. A P value < 0.05 will be considered as statistically significant. Statistical comparisons will be performed with InStat software (GraphPad Software Inc., La Jolla, CA).