RESEARCH REPORT
SWINE HEALTH

Title: Determination of the role of L83L, an uncharacterized ASFV protein that binds IL-1β during ASFV infection **– NPB #16-184**

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Industry Summary: African swine fever has many different genes. Very few of these genes have been studied and their function remains largely unknown. We took some of these unknown proteins and with bioinformatics predicted their potential functions. We then took predicted candidates and determined the cellular proteins that bind to these viral proteins. We found that one of the viral proteins binds with a cellular protein that is expressed in animals as a method for fighting viruses, this cellular protein is called Interleukin-1-Beta (IL-1β). Next we set out to delete this viral protein using molecular approaches. We were successful and were able to get viable virus that replicated well in cell cultures. Next we compared the ability of our mutant virus to cause disease in swine. When tested, our mutant showed disease characteristics similar to that of a parental virus. This tells us that this viral protein is not required for the virus to cause disease. Further studies incorporating this viral deletion into other attenuated strains could lead to a safer next-generation experimental ASFV vaccine.

Keywords: ASFV, Il1-B, L83L, ASF, Virus, swine

Scientific Abstract: African swine fever virus (ASFV) causes a contagious and frequently lethal disease of pigs that result in significant economic consequences to the swine industry. The ASFV genome encodes for more than 160 genes, but only a few of them have been studied and their functionality described. Here we report the characterization of open reading frame (ORF) L83L. A recombinant ASFV harboring a HA tagged version of the L83L protein was developed (ASFV-G-L83L-HA) and used to demonstrate that L83L is a transiently expressed early virus protein. A recombinant ASFV lacking the L83L gene (ASFV-G-ΔL83L) was developed from the highly virulent field isolate Georgia 2007 (ASFV-G) and was used to show that L83L is a non-essential gene for virus replication and pathogenicity. ASFV-G-ΔL83L had a similar replication ability in primary swine macrophage cell cultures when compared to its parental virus ASFV-G. Analysis of host-protein interactions for L83L using a yeast two-hybrid screen identified IL-1β as its host ligand. Experimental infection of domestic pigs showed that ASFV-G-ΔL83L is as virulent as the parental virus ASFV-G.

Introduction:

African swine fever (ASF) is a devastating hemorrhagic fever of pigs with mortality rates approaching 100 percent. ASF is endemic to Sub-Saharan Africa and maintains a life cycle in the wild through infection between soft ticks and feral pigs (wild pigs/bush pigs/warthogs). In Africa, ASFV causes major economic losses, threatens food security, and limits pig production in affected countries. The emergence of ASF virus in the Caucasus in 2007 demonstrated the constant threat of spread into ASFV free countries. Furthermore, from the initial introduction in Georgia, ASFV has now expanded to multiple countries in the region including Russia, where it has expanded widely within the country. Most recently in was diagnosed in the Ukraine and Poland. The risk of introduction from the Caucasus into Western Europe is high. The threat of introduction in the United States or neighboring countries has significantly increased since the introduction and recent spread throughout the Caucasus and Russia and Eastern Europe. An ASFV outbreak in the US would result in the immediate shut down of international markets for US pork and pork products and would have a devastating effect on the US economy. Importantly, there is currently no vaccine against ASFV. The only method of control is to detect and eliminate infected animals. In the Caucasus and Russia these methods have not been effective in controlling the current outbreak. Furthermore, the lack of alternative control methods has resulted in little cooperation from the public and pork producers who might sell animals at the first signs of disease and further spread the infection. An effective vaccine will both reduce the likelihood of introduction of ASFV into the US pork industry, and provide the industry with a functional method for control in case of introduction.

Fewer than half of the 189 predicted protein coding genes in ASFV have a predicted function, and fewer than half of these have been functionally characterized in vitro. Protein deletions in ASFV, that were previously determined to be a potential attenuated vaccine, were discovered before 1999 in the ASFV strains Malawi and E75. Recently we have tested these same gene knockouts in the current outbreak strain ASFV Georgia/2007. Although we have found similar results at low doses, at higher dose, applications of the vaccine candidates designed based on the work done prior to 1999 in other ASFV strains, the animals develop symptoms of ASF. This suggests that ASFV Georgia/2007 strain will potentially need additional relevant viral protein deletions to be an effective and safe vaccine. To date, to our knowledge no new protein targets have been successfully tested as potential vaccine targets since 1999.

Poxviruses are close relatives to ASFV, with Vaccinia Virus (VACV) being the most widely studied, however the sequence similarity between ASFV and Vaccinia is very low. In VACV two proteins A46R and A52R are antagonists for host IL-1. Also, in Vaccinia protein B15R mimics a soluble IL-1β receptor and decrease the effectiveness of IL-1β. This trend of blocking IL-1 by viral proteins can be seen in many different viruses. IL-1 is well characterized to have pro-inflammatory activity that is typically produced by monocytes and macrophages, while macrophages are primary cell type for ASFV infection, however IL-1 is not produced during viral infection. This trend of blocking IL-1 can be seen in many diverse viruses. However, to date no known mechanisms for blocking IL-1 signaling has been shown in ASFV. Importantly it is for the virus to block IL-1, while allowing the production of IL-1 during infection not only leads to viral attenuation, but also to improving immune response to potential vaccines. As an example, inactivation of B15R has shown an amplification of CD8+ memory T-cell responses and increased the duration of protective immunity after MVA infection. By using bioinformatics we determined that L83L was a likely candidate to bind IL-1β, and, importantly, we have confirmed this result by yeast-two hybrid screening.

In this study, we examined the role of a previously uncharacterized ASFV protein, encoded by ORF L83L, which is a highly conserved ORF among ASFV isolates. It was determined that L83L

is a non-essential gene which is transiently expressed as an early virus protein. Recombinant ASFV with a deletion in the L83L gene (ASFV-G-ΔL83L) was shown to have a similar ability to replicate in primary swine macrophage cell cultures as its parental virus ASFV-G. It was also shown that L83L specifically interacts with IL-1β, indicating a potential role for L83L in host immunomodulation. Nevertheless, infection of domestic pigs showed that ASFV-G-ΔL83L produces a disease similar to parental virus ASFV-G.

Objectives: i) Determine the role that L83L has for binding IL-1β during viral infection.
ii) Identify the exact binding site for IL-1β in L83L using a combination of computational structural biology and alanine scanning mutagenesis
(iii) Evaluate the role of L83L-IL-1β interaction in virus replication and pathogenesis by developing ASFV mutants unable to perform that interaction.

Materials & Methods:
Cell cultures and viruses: Primary swine macrophage cell cultures were prepared from defibrinated swine blood. Heparin-treated swine blood was incubated at 37°C for 1 hour to allow sedimentation of the erythrocyte fraction. Mononuclear leukocytes were separated by flotation over a Ficoll-Paque density gradient (specific gravity 1.079). The monocyte/macrophage cell fraction was cultured in plastic Primaria tissue culture flasks containing macrophage media, composed of RPMI 1640 Medium Technologies supplemented with 30% L929 supernatant and 20% fetal bovine serum for 48 hours at 37°C under 5% CO2. Adherent cells were detached from the plastic by 10 mM EDTA in phosphate buffered saline (PBS) and were then reseeded into Primaria T25, 6- or 96-well dishes at a density of $5x10^6$ cells per ml for use in assays 24 hours later.
Comparative growth curves between ASFV-G-ΔL83L and parental ASFV-G were performed in primary swine macrophage cell cultures. Macrophage cell monolayers were prepared in 24-well plates and infected at a MOI of 0.01 (based on HAD50 previously determined in primary swine macrophage cell cultures). After 1 hour of adsorption at 37°C under 5% CO2 the inoculum was removed and the cells were rinsed two times with PBS. The monolayers were then rinsed with macrophage media and incubated for 2, 24, 48, 72 and 96 hours at 37°C under 5% CO2. At appropriate times post-infection, the cells were frozen at ≤−70°C and then thawed lysates were used to determine titers by HAID50/ml in primary swine macrophage cell cultures. All samples were run simultaneously to avoid inter-assay variability.

Virus titration was performed on primary swine macrophage cell cultures in 96-well plates. Virus dilutions and cultures were performed using macrophage medium. Presence of virus was assessed by hemadsorption (HA) and virus titers were calculated by the Reed and Muench method.

Construction of the recombinant viruses: Recombinant ASFV-G-ΔL83L was generated by homologous recombination between the parental ASFV genome and a recombination transfer vector following infection and transfection of swine macrophage cell cultures (Neilan et al., 1997; Zsak et al., 1996). The recombinant transfer vector (p72mCherryΔL83L) contained flanking genomic regions with the left arm located between genomic positions 2916-3916 and the right arm located between genomic positions 4163-5163) and a reporter gene cassette containing the mCherry fluorescent protein (mCherry) gene under the control of the ASFV p72 late gene promoter. This construction created a 246-nucleotide deletion in the left variable region of ASFV-G, between nucleotide positions 3917-4162, by deleting the ORF sequence for L83L. Macrophage cell cultures were infected with ASFV-G and transfected with p72mCherryΔL83L. Recombinant ASFV-G-ΔL83L was purified to homogeneity by successive rounds of limiting dilution purification.

Recombinant transfer vectors p72mCherryΔL83L and p72mCherry-L83L-HA were obtained by DNA synthesis.

Development of the cDNA yeast two-hybrid library: A porcine primary macrophage cDNA expression library was constructed using monocytes/macrophages obtained from healthy swine. Macrophage cultures were prepared from defibrinated swine blood. Total RNA was extracted from adherent macrophage cells using an RNeasy Mini. Contaminant genomic DNA was removed by DNase treatment using TURBO DNA-free. The swine macrophage RNA was then used as a template to construct the library.

Library screening and mapping of IL-1 domain: The GAL4-based yeast two-hybrid system was used for this study. The 'bait' protein, ASFV Georgia L83L (nucleotide residues 4162-3917 of the ASFV Georgia genome), was expressed with an N-terminus fusion to the GAL4 Binding Domain. As 'prey', the previously described swine macrophage cDNA library containing proteins fused to the GAL4 Activation Domain (AD) was used. Mapping of the IL-1 binding site on the ASFV L83L protein was performed by a two-hybrid system using an alanine scanning methodology. A library containing 19 mutated forms of L83L was produced by DNA synthesis harboring each of the partially overlapping stretches of native amino acid sequences substituted by alanine residues.

Western blot analysis: Presence of L83L-HA protein was detected by *Western* blot analysis using an anti-HA monoclonal antibody in cell lysates of macrophage cell cultures infected with ASFV-G-L83L-HA. Infected cells were harvested using RIPA buffer and the NuPAGE LDS sample buffer system, and incubated at 70°C for 10 min. Samples were run under reducing conditions in precast Novex 4-12% bis-Tris acrylamide gels and transferred to polyvinylidene difluoride membranes. A goat anti-mouse IgG conjugated to horseradish peroxidase was used as secondary reagent. Western immunoblots were visualized using a Supersignal West Dura extended duration substrate according to manufacturer directions. Reactivity was detected with an Azure c300 Chemiluminescent Western Blot Imaging System and cSeries capture software 2014.

Next Generation Sequencing (NGS) of ASFV genomes: Macrophage cells were seeded as described and infected with ASFV; once the cytopathic effect was evident throughout the monolayer, DNA was extracted from infected cells as described previously (Krug et al., 2015). The extracted DNA was then used for full-length sequencing of the virus genome. Briefly, virus DNA was enzymatically sheared and assessed for fragmentation by measuring DNA size distribution. Adapters and library barcodes were added to the fragmented DNA by ligation. Using the Pippin Prep™ system, the appropriate size range of the adapter-ligated library was collected and normalized. The DNA library was then sequenced using a NextSeq following the manufacturer's protocol. Sequence analysis was performed using CLC Genomics Workbench.

Animal experiments: Animal experiments were performed under biosafety level 3 conditions in the animal facilities at PIADC following a protocol approved by the Institutional Animal Care and Use Committee. ASFV-G-ΔL83L was assessed for its virulence phenotype relative to the parental ASFV-G virus using 80-90 pound commercial breed swine. Five pigs were inoculated intramuscularly (IM) with either 10^2 or 10^3 HAD50 of ASFV-G-ΔL83L and compared with an additional two groups inoculated with similar doses of ASFV-G. Clinical signs (anorexia, depression, fever, purple skin discoloration, staggering gait, diarrhea and cough) and changes in body temperature were recorded daily throughout the experiment.

Results:

Objective 1: The role for L83L for virus replication was determined by deletion of L83L in ASFV-G. Next, we constructed a mutant virus using our virulent outbreak strain ASFV-G as a backbone. Construction of this virus was done by homologous recombination replacing L83L with RFP as depicted in Figure 1. Subsequent rounds of limiting dilutions/plaque purification

were performed to purify the virus. All this work was performed in primary swine macrophages to retain the ability of the resulting virus ASFV-G-ΔL83L to replicate in swine. In addition, using a similar approach we simultaneously made the mutant virus lacking the binding site where aa residues FTSE were substituted for alanines (ASFV-G-Δ13-L83L).

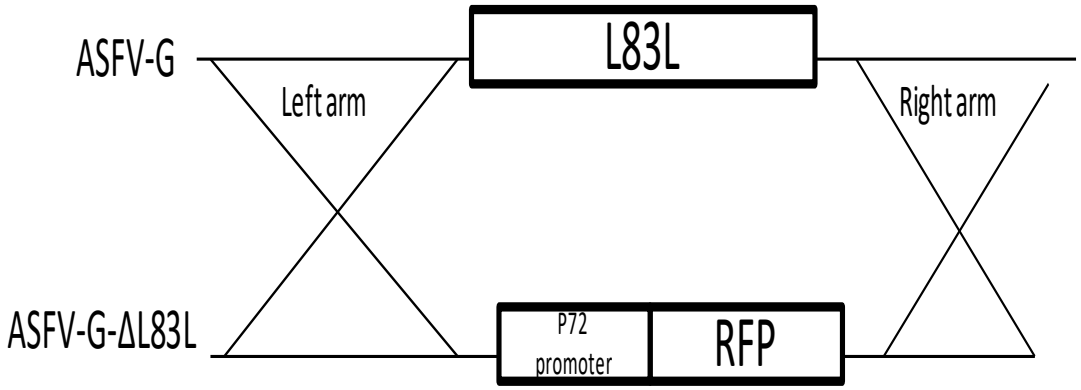

Figure 1: Construction of ASFV-G-ΔL83L

To evaluate the accuracy of the genetic modification and the integrity of the genome of the recombinant virus, full genome sequences of ASFV-G-ΔL83L and parental ASFV-G were obtained by NGS on an Illumina NextSeq® 500 and compared. The full-length genome comparison between ASFV-G-ΔL83L and parental ASFV-G revealed a deletion of 426 nucleotides corresponding with the introduced modification. Additionally, the consensus sequence of the ASFV-G-ΔL83L genome showed an insertion of 1294 nucleotides corresponding to the p72-mCherry cassette sequence. Besides the insertion cassette containing the reporter gene, no additional differences were observed between the ASFV-G-ΔL83L and ASFV-G genomes, confirming the ASFV-G-ΔL83L virus did not accumulate mutations during the process of homologous recombination and plaque purification. In addition, NGS confirmed the absence of any residual parental ASFV-G genome as contaminant of the ASFV-G-ΔL83L stock. ASFV-G-ΔL83L retained the ability to replicate in swine macrophages without any significant growth defect, as observed in Figure 2.

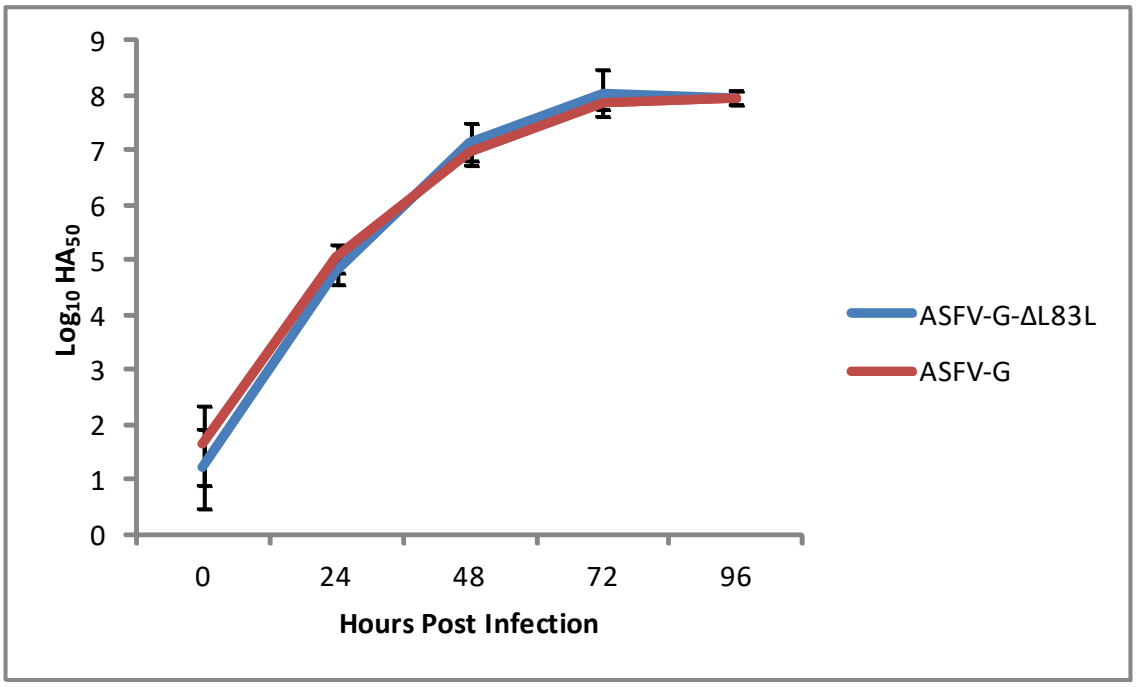

Figure 2: Growth Characteristics of ASFV-G-ΔL83L

Addition of recombinant human IL-1β (swine IL-1β is not commercially available) to cell cultures didn't affect virus growth in either virus. IL-1β serum samples from animals infected with both viruses showed no detectable difference in IL-1β. In conclusion for Objective 1 it was shown that disruption of L83L and IL-1β protein binding does not affect the ability of virus to replicate in swine macrophages.

Objective 2: L83L binding to IL-1β was determined by a yeast-two hybrid screen (Figure 3). Here the specificity of the L83L and IL-1β is shown. Only a positive protein-protein interaction will show growth on –ALTH plates. Non-selective –LT plates were included as a positive control for assay, and T7 and Lam were used as negative control for the assay.

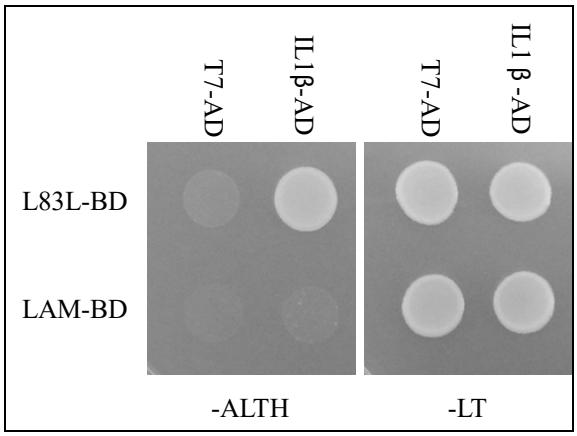

Figure 3: yeast-two hybrid screen for L83L protein binding to IL-1B

To determine the binding site in viral protein L83L, a series of 20 alanine mutants were constructed in the yeast two hybrid vector as fusions with the Gal4 Binding domain (BD). The strategy for this mutagenesis is depicted in Figure 4 where the indicated residues were substituted for alanine. As an example, in mutant 2-5 residues DTSL were substituted for AAAA. This was performed using site-directed mutagenesis. All mutants were fully sequenced to contain only the desired mutation.

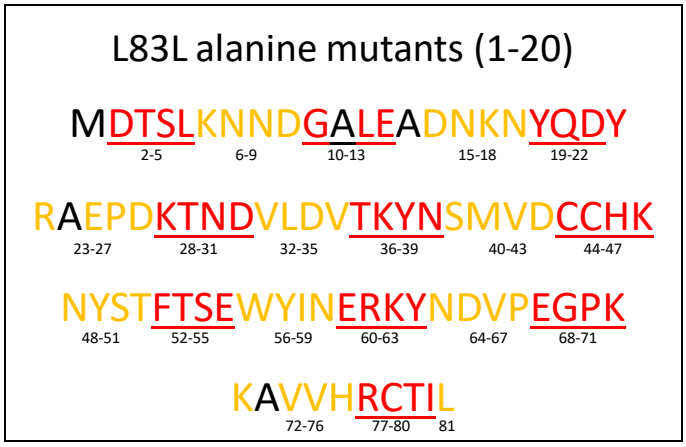

Figure 4: Alanine Mapping of L83L

All twenty of these alanine mutants were tested for their ability to maintain IL-1β binding. Only mutant 52-55 (FTSE) resulted in the lack of ability to bind IL-1β, and is identified as the binding site for IL-1β. Thus, the binding site for IL-1β in L83L are residues 52-55 FTSE.

Objective 3: Since L83L specifically binds IL-1β is was interesting to test if L83L may play a role immunomodulating the host immune response during the infection. In order to evaluate the effect of the deletion of the L83L gene on ASFV-G virulence, four groups of five 80-90 pound pigs were inoculated IM with either 10^2 or 10^3 HAD50 per animal as follows: one group was inoculated with 10^2 HAD50 of ASFV-G-ΔL83L, a second group was inoculated with 10^3 HAD50 of ASFV-G-ΔL83L, a third group was inoculated with 10^2 HAD50 of ASFV-G, and a forth group was inoculated with 10^3 HAD50 of ASFV-G. As expected, animals in both groups infected with ASFV-G exhibited increased body temperature (>104°F) by day 4 or 5 post-infection followed by the appearance of clinical signs associated with the disease including anorexia, depression, purple skin discoloration, staggering gait and diarrhea (Table 1). Signs of the disease aggravated progressively over time and animals either died or were euthanized in extremis by day 7 post-infection. Interestingly, animals receiving either 10^2 or 10^3 HAD50 of ASFV-G-ΔL83L presented with disease undistinguishable from those inoculated with ASFV-G. Both, time of presentation and severity of the clinical signs related with the disease in deletion mutant virus inoculated animals completely resemble those present in animals inoculated with the parental virus. Therefore, deletion of the L83L gene does not alter the virulence of the highly virulent ASFV-G isolate.

Table 1: Summary clinical signs post challenge.

			Fever		
Virus dose (HAD_{50})	**No. of survivors/total**	**Mean time to death (± SD)**	**No. of days to onset (± SD)**	**Duration No. of days (± SD)**	**Maximum daily temp, °F (± SD)**
ASFV-G-ΔL83L 10^2	0/4	7 (0)	5.25 (0.5)	1.75 (0.5)	105.28 ± 0.68
ASFV-G-ΔL83L 10^3	0/4	7 (0)	5. 50 (0.58)	1. 50 (0.58)	105.40 ± 0.54
ASFV-G 10^2	0/4	7.25 (0.5)	4.75 (0.5)	1. 50 (0.58)	105.20 ± 0.75
ASFV-G 10^3	0/4	7 (0)	4. 50 (0.58)	4.75 (1.7)	105.40 ± 0.54

Discussion:

Out of the more than 150 proteins encoded in the ASFV genome very few of them have been studied in detail. Understanding which viral proteins in ASFV are essential for the process of in vitro and in vivo virus replication as well as in virus virulence in swine is critical to enhance the possibilities of developing novel countermeasures to control the virus, therefore the disease. Identification of the function of virus genes, followed by their genetic manipulation, has enabled the development of potential ASFV live-attenuated vaccine candidates. Only a small number of genes have been successfully deleted using an infectious ASFV backbone (e.g: 9GL, UK, MGF, NL, CD2, Lectin), and another small number of genes determined to be essential for virus replication (e.g: EP152R, p30, p54, p72). This lack of information limits the current knowledge for most ASFV proteins only to the data of ORF functional genomics and predicted functions.

In this study we determined that L83L, a previously uncharacterized ASFV ORF, is actually a protein that is transiently expressed at early times during the infection of swine primary macrophages. It is also shown that L83L is a non-essential gene and its deletion from ASFV-G

genome does not significantly alter virus replication in swine macrophage cultures and, importantly, is not critical for ASFV virulence in swine, as the deleted virus ASFV-G-ΔL83L had similar pathogenesis as the parental ASFV-G.

In the field of ASFV, there still remains a great need for further experimental characterization of viral proteins and their possible role during infection in swine. Although it has been postulated that the virus modulates the host immune response to facilitate the progress of infection, the precise molecular mechanisms allowing ASFV to manipulate host cellular processes for its own survival are still not well understood. Very few host proteins are known to directly interact with ASFV proteins and further work is required to determine possible virus-host interactions that may be involved in determining the pathogenesis of ASFV in swine. In this study we identified that the viral protein L83L specifically binds the host gene IL-1β, a member of the IL-1 family of cytokines. IL-1β is a pro-inflammatory cytokine that is a key regulator of the inflammatory and immune responses which contributes to host defense against infection. Viruses have many mechanisms to evade the host immune response, and IL-1 family members have been shown to be a target for viral proteins to achieve this goal. Vaccinia Virus (VV), a virus with genomic and structural similarities to ASFV, has two separate mechanisms for blocking IL-1 pathways: 1) VV proteins A64R and A52R have been shown to interfere specifically with IL-1 signal transduction (Bowie et al., 2000). 2) VV protein B15R encodes for a soluble IL-1 receptor that binds only IL-1β. However, when B15R was deleted in VV, the virus was still lethal in a mouse model system, suggesting that the deletion of B15R alone was unable to attenuate the virus in mice, possibly due to a repetitive function of another viral protein, possibly A64R and A52R. Another viral mechanism to block IL-1β was previously shown in cowpox virus, where viral protein CrmA was shown to inhibit the IL-1β converting enzyme thus preventing cleavage of pro-IL-1β to active IL-1β (Ray et al., 1992). As we showed here, our preliminary studies do not indicate that deletion of L83L in the context of highly virulent Georgia2007 isolate affect virus replication or virulence in parentally infected swine. However, other studies in Vaccinia virus have shown that deletion of the protein that binds IL-1B, has been beneficial to reduce vaccine dose, perhaps incorporation of this deletion in an attenuated experimental vaccine, could reduce the dose required for vaccination. We have found in other studies that the line between attenuated and vaccine is very small, where some attenuated vaccines yield no protection, perhaps the addition of the L83L deletion could offer protection in these instances.