Necroptosis: Role in Poxvirus Pathogenesis and Oncolytic Virotherapy

by

Sambhavi Subramanian

A Dissertation Presented in Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy

Approved April 2020 by the
Graduate Supervisory Committee:

Bertram Jacobs, Co-Chair
Jason Newbern, Co-Chair
Joseph Blattman
Kurt Gustin

ARIZONA STATE UNIVERSITY

May 2020

ABSTRACT

Necroptosis is a pro-inflammatory mechanism of programmed cell death. It has been implicated in many diseases such as inflammatory diseases, neurodegenerative diseases, cancer and during viral infections. The focus of this research work was to establish the relationship between poxvirus pathogenesis and necroptosis, and the translation implications of necroptosis in oncolytic virotherapy. Vaccinia virus (VACV) is the currently used vaccine for smallpox and it has also been developed as a vaccine vector for several pathogens. E3L is one of the key innate immune evasion genes of VACV and it encodes E3 protein composed of dsRNA binding domain in the C-terminus and Z-NA-binding domain (Z-NA BD) in the N terminus. Both domains are necessary for type 1 interferon resistance and pathogenesis. Recently, it has been shown that in *in vitro*, the N-terminus of E3 is necessary to inhibit necroptosis occurring through the host-encoded cellular proteins RIP3 and Z-NA-binding protein DAI interaction leading to phosphorylation of MLKL, the key executioner step in the pathway. The research work presented here clearly demonstrates that in a mouse model, the N-terminus of VACV E3 is necessary to inhibit necroptosis during pathogenesis in mice. Another poxvirus belonging to the same family as VACV is monkeypox virus (MPXV) and is an emerging human pathogen. MPXV contains a natural truncation in the N-terminus of its E3 homologue, F3. The results indicate that during MPXV infection in mice, pathogenesis was higher only in DAI knockout mice and not in MLKL knockout mice, suggesting that DAI is possibly activating other proteins not leading to necroptosis. The characterization of VACV as an oncolytic virus was carried out with a focus on future clinical trials. In

this study, a pan screening was conducted in various cancer cell lines as many cancers downregulate necroptotic proteins. The results reveal that the N-terminal deletion mutant of VACV selectively replicates in cancer cell lines with a deficient necroptotic pathway and thus, can be used as a potential treatment against specific tumors and evidently, provides abundant scope for future studies.

DEDICATION

To my incredible family and friends.

Thank you for your unwavering faith, encouragement, patience and love throughout this journey. This dream could not have been achieved without all of you.

To my grandparents.

Your love and prayers will always be a part of me. Grandpa, your last words will always inspire me to reach for the stars and beyond.

To my amazing parents.

My dad, who believes that knowledge is an indestructible wealth. Thank you for always motivating me and giving me the freedom to pursue my dreams.

My mom, who is a superwoman. Thank you, for being selfless, strong, supportive and patient.

Both of you are my pillars of strength.

To my uncle, aunt and cousin in AZ.

You are my home away from home. Uncle and Aunt, you are my role models. Thank you, for your support, intellectually and emotionally. Words cannot express my love and gratitude for all of you.

To my fabulous friends.

Thank you for being my support system, my sounding board when I needed it, for always being there and showering me with unconditional love! I could not have made it without you guys!

ACKNOWLEDGMENTS

I would like to express my sincere gratitude to my mentor, Dr. Bertram Jacobs for taking a chance on me and providing me with the opportunity to perform research in his lab. Thank you for your patience, guidance and support in giving me a chance to grow as a scientist. Your passion and enthusiasm for science is inspiring and above all, you have taught me the importance of being humane in the scientific world. To my graduate committee members, Dr. Jason Newbern, Dr. Joseph Blattman and Dr. Kurt Gustin, thank you for your time, advice and constructive feedback on the research projects.

I would like to thank the members of Jacobs lab for their support and friendship. Dr. Karen Kibler, thank you for training me in BSL3 and ABSL3 practices, helping with mice experiments and teaching me the importance of ethical practices in research. Dr. Jeffery Langland for providing insightful suggestions during lab meetings. Aradhana Kasimsetty, thank you for your hard work, dedication and contributions to this work. Julia Bergamaschi, thank you for your help with mice colonies and genotyping. Samantha Cotsmire, Mateusz Szczerba, Jackie Williams, James Bonner and Megan McCaughan, thank you for the scientific discussions, endless help, support and friendship. I would also like to thank the past members of the Jacobs lab who were instrumental in teaching me the ropes of virology and molecular biology when I joined the lab – Dr. Heather Koehler, Dr. Trung Huynh, Dr. Karen Denzler and Nobuko Fukushima.

I would like to acknowledge and thank the staff of ASU animal facility and to all the mice that made this research possible.

I would also like to thank School of Life Sciences for providing funding throughout my graduate program. I would like to express my gratitude to my teaching instructors – Dr. Valerie Stout, Dr. Maneesha Muralinath, Dr. Heather Bean, Gary Tahmahkera and Boyd Armer for their guidance and mentorship in helping me acquire teaching skills and instilling the joy of teaching in me.

I would like to thank School of Life Sciences, GPSA and Graduate College for providing me with travel funding to attend conferences that have helped shape my professional career.

TABLE OF CONTENTS

Page

LIST OF FIGURES viii

CHAPTER

1 INTRODUCTION 1

2 NECROPTOSIS INHIBITION OF VACCINIA VIRUS PATHOGENESIS IN MICE 14

Abstract 14

Introduction 16

Materials and Methods 20

Results 24

Discussion 29

Figures 32

3 MONKEYPOX INHIBITION OF NECROPTOSIS IN MICE 42

Abstract 42

Introduction 43

Materials and Methods 46

Results 49

Discussion 51

Figures 54

CHAPTER Page

4. CHARACTERIZATION OF NECROPTOSIS IN CANCER CELL LINES FOR ONCOLYTIC VACCINIA POTENTIAL 62

Abstract 62

Introduction 63

Materials and Methods 65

Results 69

Discussion 73

Figures 76

5. OVERALL DISCUSSION 86

REFERENCES 92

FIGURES

Figure Page

1. Programmed Cell Death Pathways 11

2. Canonical and Non-Canonical Necroptosis Pathways.....12

3. Programmed Cell Death in Human Diseases 13

4. Domains of E3 Protein 32

5. IFN Sensitivity Leads to Reduced Pathogenicity in a C57BL/6J Mouse Model33

6. Necroptosis Pathway is Important for IFN sensitivity of VACV-E3LΔ83.....34

7. VACV-E3LΔ83N Replicates and Spreads to the Brain at a Higher Titer in $DAI^{-/-}$, $RIP3^{-/-}$ and $MLKL^{-/-}$ Mice 35

8. pMLKL is Detected in Nasal Tissues During VACV-E3LΔ83 Infection in Mice 36

9. Schematic of E3 Z-NA-Binding Domain and Generated Mutants..... 37

10. Pathogenesis is Restored During VACV-E3L-P63A Infection in Mice Deficient in the Necroptosis Pathway 38

11. Intra nasal LD_{50} Study.....39

12. Intra cranial LD_{50} Study.....40

13. Proposed Model for Mechanism of Necroptosis Inhibition of VACV Pathogenessis in Mice 41

14. Domains of VACV E3 and MPXV F3.....54

15. Pathogenesis is Rescued in $DAI^{-/-}$and $MLKL^{-/-}$ During VACV-E3LΔ37N Infection 55

Figure Page

16. Individual Clinical Score during VACV-E3LΔ37N Infection 56

17. Pathogenesis is Rescued Only in DAI$^{-/-}$during MPXV infection. 57

18. Individual Clinical Score During MPXV Infection 58

19. MPXV Titers in Various Tissues.....59

20. MPXV is Neurovirulent in Mice 60

21. Proposed Model for Mechanism of MPXV Inhibition of Pathogenesis in Mice 61

22. Proposed Mechanism for Potential Use of N-terminal deletion Mutant of VACV as an Oncolytic Virus.....76

23. RIP3 and DAI Expression Levels in Human Cancer Cell Lines 77

24. RIP3 and DAI Expression Levels in Murine Cancer Cell Lines 78

25. Cell Viability Assay in Human Cancer Cell Lines 79

26. Cell Viability Assay in Human Cancer Cell Lines 80

27. Cell Viability Assay in Human Cancer Cell Lines 81

28. Cell Viability Assay in Murine Cancer Cell Lines 82

29. Cell Viability Assay after Viral Infection in B16 and E0771 cells 83

30. pMLKL Expression in E0771 Cells During VACV-E3LΔ83 Infection 84

31. Single Step Growth Curve in E0771 cells.....85

CHAPTER 1

INTRODUCTION

Infectious diseases caused by pathogens pose a serious concern for global human health and security. Among the diverse pathogens, viruses are amongst the most serious, often rapidly evolving pathogens that challenge economic and livelihood security in today's world. Viruses are obligate, intracellular pathogens that replicate using the hosts' cellular machinery. Hence, it is critical for viruses to develop strategies to evade the hosts' innate immune defense mechanism for effective replication and spread in the host system. When a virus infects a cell, the host cell senses the virus infection and responds by secreting interferon (IFN) leading to an innate immune response. The induction of IFN and other pro-inflammatory cytokines creates an anti-viral state in the cells and the virus is unable to replicate further.

IFNs are grouped into three types and the main cytokines produced in response to a viral infection are Type I IFNs (IFNα/β). The IFN signal transduction pathway consists of two signaling cascades: a virus-induced IFN production signal and an IFN receptor-mediated secondary signal to establish an antiviral state(1). During virus infection of the cell, detection of pathogen-associated molecular patterns (PAMPs) by host pattern recognition receptors (PRRs) leads to the IFN production signal. PAMPs are components or byproducts of microorganisms during their life cycle inside the host. These PAMPs can be recognized by four classes of pathogen recognition proteins (PRPs): toll-like receptors (TLRs), retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs),

nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), and cytoplasmic DNA sensors (2). Induction of type 1 IFN have shown to be regulated by TLRs and RLRs. Although each pathway induces type 1 IFN production through different adaptor and signaling molecules, both pathways converge onto three common signaling molecules: interferon regulatory factor 3 (IRF-3), IRF-7, and NF-κB. Following activation via the upstream recognition pathways, these signaling components bind the IFN-β promoter to produce IFN-β which acts upon the IFN-αβ receptor (IFNAR) in both autocrine and paracrine mechanisms(2, 3). This leads to the upregulation of IFN-stimulated genes (ISGs) and the IFN signaling cascade. The activation of this pathway leads to the upregulation of the genes under the control of the interferon stimulated response element promoter (ISRE), which includes interferon-induced effector molecules such as protein kinase R (PKR) and 2'-5' oligo adenylate synthetase (OAS). Upon virus infection, these "primed" cells inhibit viral replication and spread(4, 5).

Programmed cell death (PCD) is an orchestrated suicide mechanism activated in response to host cellular defense signals. This leads to cell death that prevents viral replication and spread, thus curbing the infection effectively(6-8). Programmed cell death pathways include apoptosis, pyroptosis and necroptosis and are differentiated based on morphological and immunological characteristics (Figure 1) (9-12).

Apoptosis is a caspase-dependent pathway that is induced by stimuli such as intracellular signals (intrinsic) or activation of death receptors (extrinsic). There are various intracellular inducers of the intrinsic pathway such as cytochrome c from mitochondria, SMAC/Diablo or Bax that are released in responses to variety of stresses.

The extrinsic pathway is initiated by the binding of extracellular death receptor ligands such as tumor necrosis factor (TNF), Fas ligand (FasL), and TNF-related apoptosis-inducing ligand (TRAIL) to their respective transmembrane receptors. Activation of the pathway includes the induction of caspases – 8 and -9 in response to cellular signaling. This leads to activation of caspases – 3 and -7 known as leading to cell death. Cell death is characterized by membrane blebbing, chromosomal fragmentation and the formation apoptotic bodies. This results in nonimmunogenic death as the apoptotic bodies retains the cellular content and are cleared by phagocytic cells(13, 14).

Pyroptosis is also a caspase- dependent cell death pathway. Signaling is initiated by activation of procaspase-1. This results in the formation of inflammasomes leading to activation of inflammatory cytokines such as IL-18 and IL-1β and thus, the pathway is considered highly immunogenic. The hallmark of pyroptosis is the loss of cell membrane integrity and the release of intracellular contents(13, 15).

Necroptosis is a recently identified pro-inflammatory mechanism of programmed cell death. This is a caspase-independent pathway involving serine/threonine kinase receptor interacting proteins (RIP) that regulate the signaling. Although, there are different pathways leading to necroptotic cell death, RHIM domain containing protein, RIP3, acts as the common point for the different adaptor proteins to dock leading to autophosphorylation and recruitment of Mixed Lineage Kinase Domain Like pseudo kinase (MLKL). Upon RIP3 activation, MLKL undergoes phosphorylation and trimerization leading to loss of membrane integrity and ultimately cell death(16-20).

The canonical pathway of necroptosis is initiated when RIP1 interacts with RIP3 leading to RIP1-RIP3 complex and is activated by Tumor Necrosis Factor (TNF)α. Non-canonical pathways include the interaction of one of the adaptor proteins, TIR domain-containing adaptor-inducing interferon-(TRIF) and DNA-dependent activator of Interferon (21) (21) with RIP3 leading to RIP3 phosphorylation and eventually MLKL phosphorylation leading to cell death (Figure 2) (16, 20, 22-24).

Necroptosis has been implicated during infections of pathogens such as bacteria and viruses serving as a key host defense mechanism. It is demonstrated that viruses have evolved mechanism to evade necroptosis. It has been identified that ICP 6 and ICP 10 encoded by herpes simplex viruses (HSV)1 and M45- encoded viral inhibitor of RIP activation (vIRA) found in HSV2 inhibit necroptosis (16, 25, 26). Another virus that has recently been implicated in evading necroptosis belongs to poxvirus family, vaccinia virus (VACV)(27).

Among the extensively studied genera of poxviruses is Orthopoxvirus genus that includes the causative agent of smallpox, variola virus, its prototypical member, vaccinia virus and the recently emerging pathogen, monkeypox virus. Smallpox caused by variola virus, was one of the deadliest diseases known to mankind that killed millions of people(28). Variolation was one of the first methods to control the spread of smallpox. The process involved administration of variola virus from smallpox scabs to people who never had the disease(29, 30). Although, fatality from variolation was lesser than smallpox itself, this practice was not deemed safe. In 1798, Edward Jenner introduced the concept of vaccination where cowpox was used as a vaccination agent against

smallpox(30-32). By the early 20th century, VACV was replaced as the vaccination agent for smallpox. A massive global eradication campaign was launched by the World Health Organization (WHO) in 1956 and by 1980, WHO declared the world to be smallpox free(30-34). Thus, VACV has played a pivotal role in the eradication of smallpox. Currently, in addition to being used as a smallpox vaccine, VACV is also used as vaccine vector for human disease

Poxviruses are a family of large brick-shaped, enveloped, double stranded DNA viruses. They replicate in the cytoplasm and encode most of the factors required for transcription and replication. Ineffective termination sequences during late transcription results in formation of double stranded (dsRNA), which acts as a pathogen associated molecular pattern (PAMP) through pattern recognition receptors (PRRs) (35, 36).

Vaccinia virus (VACV), a member of the Orthopoxvirus genus, primarily infects mammals. Due to its unique mechanism of replication, it has evolved to evade the host immune response with approximately one third of its genome dedicated to virulence factors(35, 37). VACV depends on its ability to circumvent the host's IFN system. One of the key innate immune evasion gene is E3L.The E3 protein has two main domains: a Z-DNA binding domain contained in the amino terminus, and a double-stranded RNA binding domain in the carboxy terminus(4, 38-41).

The C-terminus of E3 has been well characterized. It binds the dsRNA produced as a result of viral infections and sequesters it from PRRs. The domain is necessary for IFN resistance, host range and full pathogenesis in mice(4, 42). The N-terminus has been hard to characterize. *In vitro,* the domain is not necessary for IFN resistance and

replication. *In vivo*, the domain is necessary for pathogenesis and viral spread(4, 42). Intranasal infection of C57BL/6J mice with VACV-E3LΔ83N does not lead to pathogenesis. However, in IFNAR$^{-/-}$ mice, intranasal infection of C57BL/6J mice with VACV-E3LΔ83N leads to pathogenesis(41, 42). Recent study has demonstrated that the *in vitro*, N-terminus of VACV E3 is necessary to inhibit DAI- dependent necroptotic cell death(27). In Chapter 2, we demonstrate the role of necroptosis during VACV pathogenesis in mice.

Monkeypox virus (MPXV) is another member of the Orthopoxvirus genus that has garnered attention in the last few years due to the incidences of human transmission of the virus. MPXV infects a broad range of animal species(43-46) and can be transmitted to humans by means of direct contact with infected animals or human-human transmission through broken skin or respiratory droplets. Clinical manifestation of MPXV has been characterized by viral prodrome of fever, headache, chills and myalgias lasting 1-3 days. This is followed by rash eruption known as maculopapular exanthem progressing to vesicular and pustular stages and crusts form in 2-3 weeks. Although, the symptoms are similar to smallpox, MPXV infection causes lymphadenopathy that is a distinctive hallmark of the disease(47-50). MPXV is endemic in the West and Central areas of Africa, with the first human cases diagnosed in the Congo region of Africa in 1970(51, 52). Since 2005, Democratic Republic of Congo has reported over 1000 cases per year. Incidences outside of Africa although rare, have been documented in United States in 2003, Israel in 2018 and United Kingdom is 2018 and 2020 (43, 51-60). There are two strains of MPXV, a Central African strain and a West African strain. The Central

African strain is known to be more virulent with fatality of 10%. In comparison, West African strain causes milder disease(53, 61).

MPXV and VACV have significant genomic similarity. MPXV F3 protein is the homologue of VACV E3 protein. Although, the F3 protein has an intact C-terminus, the N-terminus has a natural truncation of 37 amino acids (61, 62). MPXV has a broad host range infecting wide range of cell types and hosts. Despite having a N-terminal truncation, MPXV is shown to phenotypically resemble a wild-type VACV (wtVACV) rather than VACV-E3LΔ37N(63). As previously mentioned, it has been shown that the N-terminal deletion mutant of VACV undergo DAI-dependent necroptotic cell death, *in vitro*, in L929 cells(27). Unpublished data in the lab indicate that MPXV has intermediate IFN sensitivity in L929 cells which is rescued in $DAI^{-/-}$ L929 cells. It is also observed that trimerization on MLKL which is thought to be the ultimate step of necroptosis is not seen during MPXV infection in L929 cells. In Chapter 3, we discuss the role of necroptosis during MPXV infection in mice.

Cell death is a common pathology in human diseases. Necroptosis has been implicated in various diseases such as ischemic brain injury, liver injury, neurodegenerative diseases such as multiple sclerosis and Alzheimer's disease, pathogen infections and in cancer (Figure 3) (64, 65).

The focus of Chapter 4 in this dissertation is to determine the translation outcome of using VACV as a potential oncolytic virus to target cancer. Necroptosis is shown to have dual effects, either promoting or reducing tumor depending on the cancer. It has

been identified that in some cancers, the markers for necroptosis is elevated. RIPK1 is commonly overexpressed in glioblastoma, pancreatic ductal adenocarcinoma and in lung tumor(66, 67). This upregulation has been correlated with poor prognosis. In most cancers, the key regulators of necroptosis are downregulated. This leads to cell proliferation and tumor growth. RIPK1 expression has been noted to be downregulated in head and neck squamous cell carcinoma (HNSCC)(68). One of the key regulators of necroptosis, RIPK3 is down-regulated in about two-thirds of cancers. In 85% of breast cancer patients, RIPK3 expression is reduced when compared to normal tissue. RIPK3 expression is also downregulated in melanoma, colorectal cancer and acute myeloid leukemia(69). These studies indicate that necroptotic regulators can serve as prognostic biomarkers in cancer identification and diagnostics.

Oncolytic virotherapy is one of the recent advances in cancer immunotherapy. It involves the use of viruses to selectively target cancerous cells and spare normal cells. Oncolytic viruses can be either viruses that naturally replicate in cancer cells and are non-pathogenic in humans or they can be viruses that are genetically manipulated for use in vaccine vectors(68, 70-72). The efficacy of oncolytic virus is dependent of many factors such as virus entry, replication and specific targeting(70, 73, 74). Mutations that cause cancer lead to dysfunction of the signaling systems responsible for cell death and proliferation. Thus, the viruses can be engineered to target these cellular pathways thereby inhibiting replication of cancerous cells. There are diverse range of viruses in clinical trials for the potential use as oncolytic viruses. These include type 1 herpes simplex virus (HSV), reoviruses, measles virus, vaccinia virus and oncolytic adenovirus

among other viruses(75-79). Different factors play a role in the selection of virus including but not limited to tumor tropism, viral genome, stability of virus, immunogenicity, size, pathogenicity and replication efficacy(77, 80).

In many cancers, the anti-viral response through IFN signaling has be shown to be dysregulated(74). An FDA – approved oncolytic virus talimogene laherparepvec (T-VEC), is a herpes simplex virus type 1 (HSV-1) that has been modified to target tumor cells(81, 82). The modification involved the deletion of gene encoding neurovirulence factor ICP34.5. This leads to viral replication in cancerous cells but in normal cells, the viral replication is inhibited by the interferon cells in the cells (83-85).

VACV is a 190kb double stranded DNA virus that replicates in the cytoplasm of cells. Due to its large genome size, recombination with up to 25kb of foreign DNA can be stably incorporated. The virus can replicate in a wide variety of cells and it's the chances of integration into host genome is low since it replicates in the cytoplasm(86, 87). VACV E3 protein has been widely studied for its important role of evading host immune defenses. E3 contains two domains – dsRNA binding at the C-terminus and Z-DNA binding domain at the N-terminus. Both domains are necessary for IFN resistance and pathogenesis. VACV with N-terminal deletion has been shown to have reduced pathogenesis in mice (5, 27, 42, 88-91).

Previous studies in the lab have shown that in vitro, N terminal deletion mutant VACV-E3LΔ54N selectively replicates in numerous cancer cell lines but not in the normal non-cancerous cells. In vivo studies in a SCID mouse xenograft model demonstrated that VACV-E3LΔ54N caused tumor regression in MDA-MB-435s,

melanoma model. VACV-E3LΔ54N spread and replicated to untreated tumors from treated tumor(63, 92). This was indicative of the potential of the virus to travel and destroy cancerous cells and thus, decreasing the occurrence of tumor resurgence. In Chapter 4, the correlation between necroptosis and the ability of VACV to replicate selectively in cancerous cells is discussed.

This goal of this dissertation is two-fold: to understand the role of necroptosis during pox virus pathogenesis and, as a translation focus to identify the potential of VACV as an oncolytic virus.

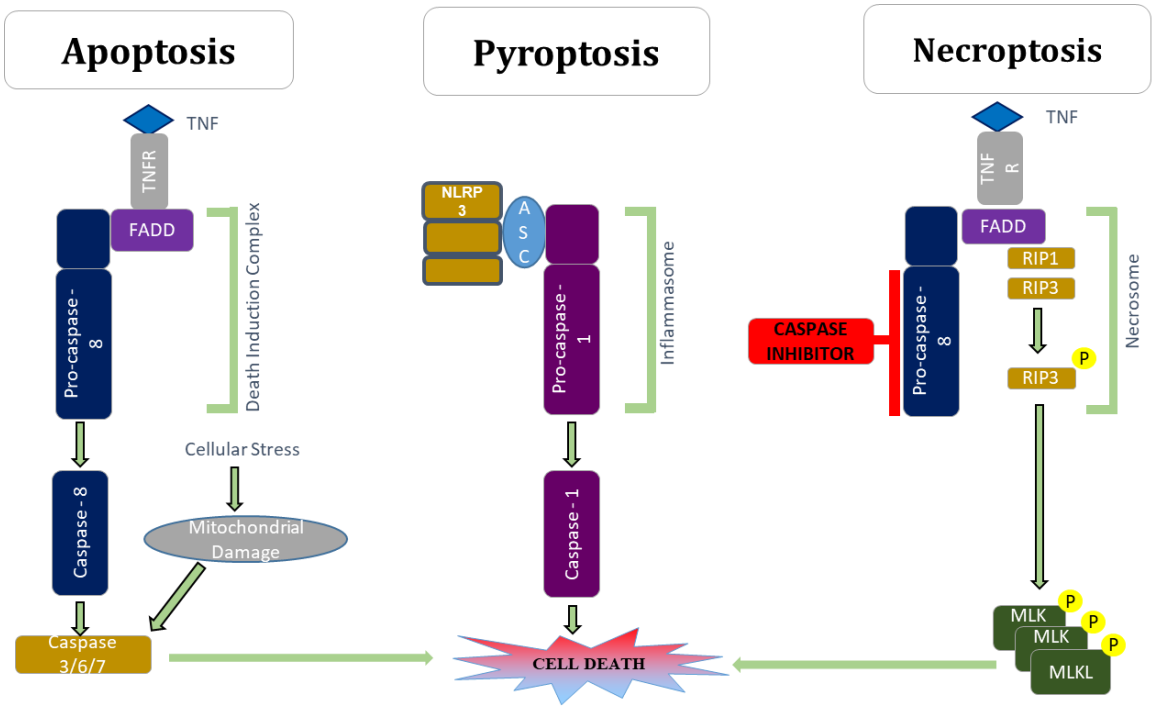

Figure 1: Programmed Cell Death Pathways. Apoptosis and Pyroptosis are caspase-dependent pathways and Necroptosis is a caspase – independent pathway. Apoptosis can be signaled through intrinsic and extrinsic pathways while pyroptosis signals through formation of inflammasome. Necroptosis results in the formation of necrosome leading to activation of MLKL and cell death.

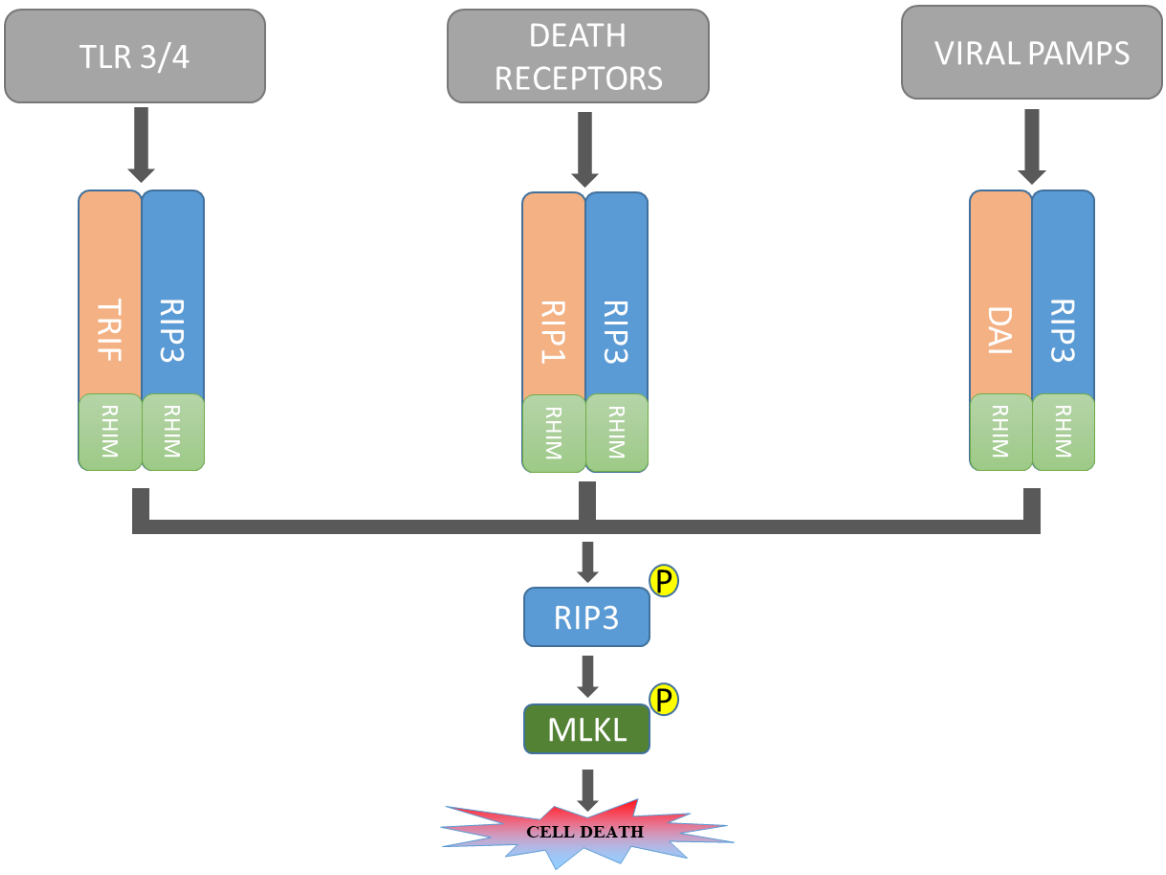

Figure 2: Canonical and Non-canonical Necroptosis Pathways. Canonical pathway occurs through activation of RIP1 adaptor proteins while the non-canonical pathways occurs through adaptor proteins DAI or TRIF leading to activation of RIP3 and phosphorylation of MLKL, resulting in cell death.

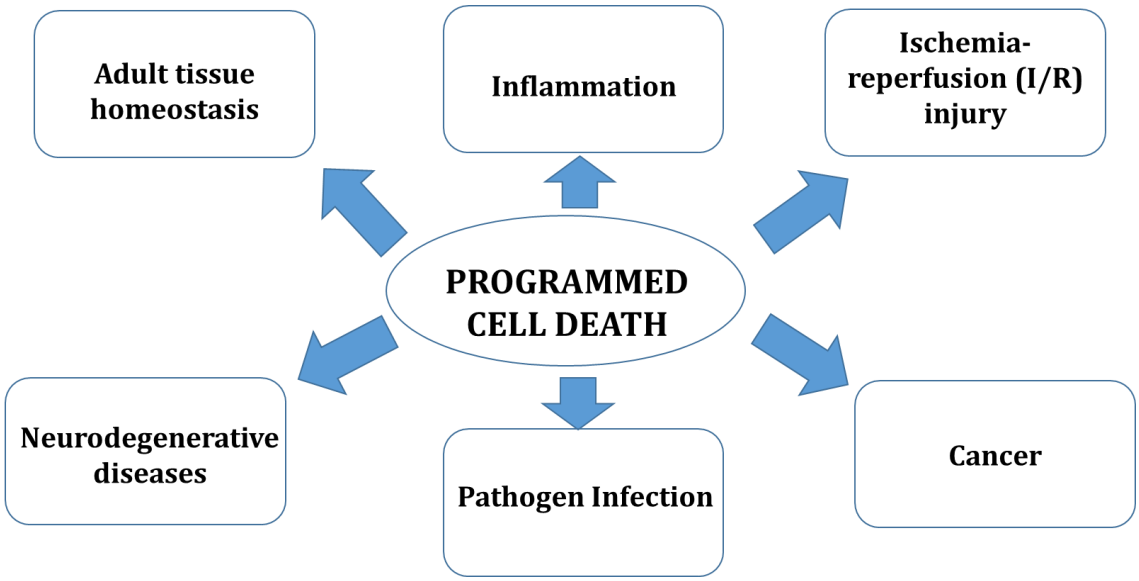

Figure 3: Programmed Cell Death in Human Diseases.

CHAPTER 2

NECROPTOSIS INHIBITION OF VACCINIA VIRUS PATHOGENESIS IN MICE

ABSTRACT

Vaccinia virus encodes the multifunctional protein, E3 that functions in evading the interferon induced antiviral response. The E3 protein contains two highly conserved functional domains. The well-characterized carboxy – terminus contains a double stranded RNA binding domain that aids in interferon (IFN) resistance. The less-well characterized amino terminus contains a Z-DNA binding domain. Deletion of N-terminus domain in E3 (VACV-E3LΔ83N) results in attenuation of the virus in mouse model and the pathogenesis is restored in an IFN knockout mouse model. Necroptosis is a pro-inflammatory, caspase independent mechanism of programmed cell death. In IFN treated L929 cells, VACV-E3LΔ83N induces necroptosis and death is dependent on host RIPK3 protein and on DAI, a sensor of virus infection. In this study, the role of IFN sensitivity of VACV-E3LΔ83N in necroptosis during pathogenesis in a mouse model was characterized. The results demonstrate that in a C57BL/6J model, IFN-treatment significantly reduced pathogenesis during Δ83N infection while there was no significant difference during wtVACV infection. Additionally, in a DAI or MLKL knockout model, there is no significant difference in pathogenesis between non-IFN treated and IFN-treated animals during Δ83N infection and the pathogenesis is higher than in C57BL/6J mice. Infection with point mutant of key predicted ZNA interacting residue (VACV_VACV-E3L-P63A) resulted in reduced pathogenesis in a C57BL/6J model

while this pathogenesis was restored in the DAI or MLKL knockout mouse model. In summary, this data suggests that virus-induced necroptosis is important for inhibiting vaccinia virus pathogenesis in mice.

INTRODUCTION

Type I IFNs (IFN-α/β) are potent, inducible cytokines that play a critical role in the innate immune system (1). Type I IFNs (IFN-α/β) are potent cytokines known primarily to inhibit virus replication. The IFN signal transduction pathway consists of two signaling cascades: a virus-induced IFN production signal and an IFN receptor-mediated secondary signal to establish an antiviral state. During virus infection of the cell, detection of pathogen-associated molecular patterns (PAMPs) by host pattern recognition receptors (PRRs) leads to the IFN production signal. PAMPs are components or byproducts of microorganisms during their life cycle inside the host. These PAMPs can be recognized by four classes of pathogen recognition proteins (PRPs): toll-like receptors (TLRs), retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), and cytoplasmic DNA sensors Induction of type 1 IFN have shown to be regulated by TLRs and RLRs . Although each pathway induces type 1 IFN production through different adaptor and signaling molecules, both pathways converge onto three common signaling molecules: interferon regulatory factor 3 (IRF-3), IRF-7, and NF-κB. Following activation via the upstream recognition pathways, these signaling components bind the IFN-β promoter to produce IFN-β which acts upon the IFN-αβ receptor (IFNAR) in both autocrine and paracrine mechanisms. This leads to the upregulation of IFN-stimulated genes (ISGs) and the IFN signaling cascade. The activation of this pathway leads to the upregulation of the genes under the control of the interferon stimulated response element promoter (ISRE), which

includes interferon-induced effector molecules such as protein kinase R (PKR) and 2'-5' oligo adenylate synthetase (OAS). Upon virus infection, these "primed" cells inhibit viral replication and spread(4, 5).

Vaccinia virus (VACV), a prototypical member of the Orthopoxvirus genus, is a dsDNA virus(35). VACV replicates entirely in the cytoplasm and encodes numerous innate immune evasion genes. About one-third of the genes encoded by VACV are responsible for host immune evasion mechanisms. One of the main characteristics of this virus is that it is IFN resistant and this is mapped to the E3L gene. The E3L protein has two main domains: a Z-DNA binding domain contained in the amino terminus, and a double-stranded RNA binding domain in the carboxy terminus. Both domains have been shown to be necessary for pathogenesis, PKR inhibition and IFN resistance(42, 86, 87, 93).

The function of the N-terminus has been enigmatic. In cell culture models, the N-terminus has been dispensable for IFN resistance and replication. In mouse models, this domain is necessary for pathogenesis and viral spread. In an intranasal infection of C57BL/6J mice model, recombinant viruses containing N-terminal deletions in E3L replicate efficiently in the nasal mucosa but fail to spread from the nasal cavity to the brain. Upon intracranial injection, these virus recombinants exhibit decreased virulence even when likely due to poor replication in neuronal tissue. In mice devoid of $IFN^{-/-}$ receptors, it was shown that the N-terminus of E3L was necessary to inhibit IFN response and VACVE3L Δ83N, a highly attenuated virus, regains its virulence, in both intranasal and intracranial infections to the same level as wtVACV. Additionally, during intranasal

infection of $PKR^{-/-}$ mice, the pathogenicity of VACVE3L Δ83N was not restored, suggesting that other IFN inducible pathways can inhibit the pathogenicity of VACVE3L Δ83N in the whole-animal model, in addition to PKR(4, 5, 41, 42).

Viral infections lead to the production of type 1 interferon in the infected cells that acts as the first line of defense creating an anti-viral state in the cells(94-96). Programmed cell death is one of the key anti-viral host defense mechanism to control viral spread. Although apoptosis is considered the primary cell death mechanism, alternative cell death pathways such as pyroptosis and necroptosis have gained significant interest in recent times(13).Viruses such as VACV encode anti-apoptotic genes and the activation of alternative death pathway becomes important during infection(4, 91).

VACV E3L gene is one of the key innate immune evasion gene(35, 97). Recently, it has been identified that N-terminus of VACV E3 is necessary to inhibit necroptosis, a programmed cell death pathway(27). Necroptosis is a recently identified pro-inflammatory mechanism of programmed cell death. It is a caspase-independent mechanism that leads to RHIM-dependent activation of RIP3 resulting in recruitment and phosphorylation of downstream pseudokinase known as mixed lineage kinase-like (MLKL). Activation of MLKL leads to loss in membrane integrity. The canonical pathway of necroptosis involves the RHIM - dependent interaction of RIP1 with RIP3 leading to conformation changes. Non – canonical activation of this pathway involves interaction of either DAI or TRIF leading to complex with RIP3 and activation of the executioner of necroptosis, phosphorylation and translocation of MLKL to the plasma membrane leading to cell membrane disruption(15-18, 20, 22, 23, 98, 99).

The Zα domain of VACV E3 protein is necessary for IFN resistance of wtVACV in murine L929 cells. The N-terminus of E3 competes with DAI to prevent DAI-dependent necroptotic cell death. IFN-treated L929 cells infected with VACV-E3LΔ83N led to RIPK3 activation and phosphorylation and aggregation of MLKL. This led to membrane blebbing and loss of plasma integrity of the cells. E3 and DAI belong to the same family of Zα binding domain containing family of proteins and can compete for Z-form nucleic acid produced during VACV infection(27).

In this study, the translation of the *in vitro* results to an *in vivo* mouse model was studied. The role of N-terminus of VACV E3 to inhibit necroptosis during pathogenesis in mice was studied.

MATERIALS AND METHODS

Mice

C57BL/6J, RIP3$^{-/-}$, DAI$^{-/-}$ and MLKL$^{-/-}$ were bred and housed at the Arizona State University animal facility. RIP3$^{-/-}$ were from Genentech, Inc. and rederived at Charles River, DAI$^{-/-}$ were received from Dr. Shizuo Akira, Osaka University and MLKL$^{-/-}$ were received from Dr. Jiahuai at State Key Laboratory of Cellular Stress Biology, School of Life Sciences, Xiamen University. Colonies were maintained pathogen-free. Four to six-week old mice were used for all the experiments. Mice of either sex was used in the experiments. All animal experiments were approved by the Arizona State University Institutional Animal Care and Use Committee (IACUC).

Viruses and Treatment

The Western Reserve (WR) strain of vaccinia virus (wtVACV), mutant N-terminal deletion of 83 amino acids of E3L (VACV-E3LΔ83N) and point mutants, VACV-E3LVACV-E3L-P63A and VACV-E3LVACV-E3L-E42A were generated as previously described(42, 100). Mouse interferon α (Calbiochem) was administered at 100 U/mouse along with the virus infection in the indicated experiments.(43, 51, 57)

Intranasal and intracranial infections of mice

Mice were anaesthetized using a cocktail containing 37.5 mg/ml ketamine, 7.5 mg/ml xylazine and 2.5 mg/ml acepromazine maleate. Intra-peritoneal injection of the anesthetic was administered at the dose of 1 µl/g of body weight of the mouse. For intranasal infections, anaesthetized mice were infected with 10µl of virus in dose range

10^3-10^8 pfu in 1 mM Tris pH 8.8 using a Pipetman loaded with a protein-loading tip. For intracranial infections, anesthetized mice were infected with 5 µl of virus in dose range 10^1-10^4 in 1 mM Tris pH 8.8 using a 30-gauge hypodermic needle and a 50 µl syringe. Mice were monitored for weight loss and clinical symptoms. Clinical symptoms that were evaluated include eye infection (left and right eye), breathing, hunching, ruffling of fur, sneezing and level of activity. Each clinical sign was scored on a scale of 0-3 with 3 representing severe symptomology. For intracranial infections, neurological symptoms were also monitored. Weight loss was also monitored with mice losing 10-20% of original weight receiving score of 1 and mice losing 20-30% of original weight receiving score of 2. Clinical score assigned was a total of weight loss and clinical symptoms score. Mice dropping below 30% of their initial weight or with a clinical score of 17 and above were euthanized.

Tissue Distribution

Mice were infected intranasally as described above with 10^6 pfu of wtVACV or 10^8 pfu of VACV-E3LΔ83N. At 6 days post infection, mice were sacrificed with isofluorane overdose and organs were harvested. Tissue samples were immediately snap frozen in liquid nitrogen. Each tissue was weighed, ground to a fine powder in liquid nitrogen using a mortar and pestle and resuspended in 10% wt/volume RPMI media supplemented with Anti- Anti (Thermo Fisher Scientific). Tissue homogenates underwent three cycles of freeze-thaws that consisted of freezing samples at -80°C and then slowly thawing the samples on ice, followed by a quick thaw at 37°C and sonication for 30s. Titers were determined by a standard plaque assay using BSC40 cell monolayers.

Western Immunoblot Analysis

Mice were infected intranasally as described above with 10^6 pfu of wtVACV or 10^8 pfu of VACV-E3LΔ83N. At 6 days post infection, mice were sacrificed with isoflurane overdose and organs were harvested. Tissue samples were immediately snap frozen in liquid nitrogen. Each tissue was weighed, ground to a fine powder in liquid nitrogen using a mortar and pestle and resuspended in 4 volumes of RIPA lysis buffer containing 1X Halt Protease and Phosphatase Inhibitor Cocktail (Pierce Thermo Scientific). The lysates were incubated on ice for 30minutes, centrifuged at 15,000g for 30 minutes and the resulting supernatant was saved in a new tube. Protein concentrations were determined by BCA analysis. The samples were boiled for 5 mins and equal protein was loaded on 10% SDS-PAGE gels. Proteins were transferred to nitrocellulose membrane, blocked in TBS-Tween containing 3% BSA for 1 hour at room temperature and probed with primary antibodies in TBS-T containing 3% BSA overnight at 4°C. Membrane was washed in TBS-T and probed with secondary antibody in TBS-T containing 3 % non-fat dry milk (Carnation) for 1 hour at room temperature followed by chemiluminescent development using SuperSignal West Dura Duration substrate (Pierce Biotechnology) and developed using X-ray film.

Statistics

Statistical analysis was done in GraphPad Prism by using a two-tailed, unpaired t test. ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$

RESULTS

VACV-E3LΔ83N is IFN sensitive in C57BL/6J mice. The WR strain of VACV is a mouse adapted, neurovirulent strain. E3L is a key innate immune evasion gene of VACV. The E3 protein has two domains, the C-terminus dsRNA binding domain and the N-terminus Z-DNA binding domain. Deletion of N-terminus domain in E3 results in attenuation of the virus in mouse models and pathogenesis of the N-terminal VACV mutant is restored in an IFNAR $^{-/-}$mouse model in intranasal and intracranial injection studies. We sought to ask if treatment with mouse interferon α along with virus at the time of infection would reduce the pathogenicity of VACV-E3LΔ83N. C57BL/6J mice were infected intranasally with either wtVACV or VACV-E3LΔ83N, with or without mouse interferon-α at the time of infection. Mice were monitored for weight loss and clinical symptoms. Mice infected with wtVACV had higher clinical score but there was no significant difference between non-interferon treated and interferon treated groups of mice. This was consistent with previous studies that have shown that wtVACV is IFN resistant in cells in culture and in mouse models. VACV-E3LΔ83N infection led to decreased pathogenesis in non-interferon treated groups and the treatment with interferon along with the virus significantly decreased the pathogenesis. These results are consistent with our previous studies that have shown that the N-terminus of E3 is critical to inhibit the IFN system (Figure 5).

IFN sensitivity of VACV-E3LΔ83N is dependent on the necroptosis pathway. Induction of necroptosis in IFN-treated mouse L929 cells and pathogenesis in C57BL/6J

mice has been shown to be correlated to the Z-DNA binding domain of E3. We assessed if the necroptosis pathway is important for the interferon sensitivity of VACV-E3LΔ83N. Mice deficient in the necroptosis pathway, RIP3$^{-/-}$, DAI$^{-/-}$ and MLKL$^{-/-}$ were infected intranasally with or without mouse interferon – α. Mice were monitored for weight loss and clinical symptoms. Pathogenesis of VACV-E3LΔ83N was restored in the RIP3$^{-/-}$, DAI$^{-/-}$ and MLKL$^{-/-}$ mice but there was no significant difference between the non-treated and treated IFN groups in each of the knockout mice. These results indicate that during VACV-E3LΔ83N infection, IFN sensitivity and the presence of an active necroptosis pathway is critical to inhibit pathogenesis in mice (Figure 6).

VACV-E3LΔ83N replicates and spreads to the brain at a higher titer in mice deficient in the necroptosis pathway. The WR strain of VACV is a mouse adapted, neurovirulent strain. In an intranasal infection, N-terminal deletion mutant of VACV, VACV-E3LΔ83N replicates in the nasal mucosa but spreads poorly to the brain as compared to wtVACV infection. However, in mice devoid of the interferon – α/β receptors, VACV-E3LΔ83N replicates and spreads to the brain at titers comparable to wtVACV levels. We sought to identify if the restoration in pathogenesis during VACV-E3LΔ83N infection in mice deficient in the necroptosis pathway also correlated with spread of virus to the brain. C57BL/6J, RIP3$^{-/-}$, DAI$^{-/-}$ and MLKL$^{-/-}$ mice were infected intranasally with 10^7 pfu of VACV-E3LΔ83N. Each cohort of mice was sacrificed at 6 days post infection, and the nasal turbinates' and brain were harvested. VACV-E3LΔ83N replicated to high titers in the sinus in all strains of mice. In C57BL/6J mice, spread to the brain was very low, whereas in RIP3$^{-/-}$, DAI$^{-/-}$ and MLKL$^{-/-}$ mice, the virus spread to a

significantly higher titer as compared to C57BL/6J. These results are consistent with the data that the induction of necroptosis is critical to inhibit pathogenesis in mice (Figure 7).

Necroptosis markers are detected during VACV-E3LΔ83N infection in C57BL/6J mice. To confirm that induction of necroptosis was responsible for inhibition of pathogenesis during VACV-E3LΔ83N, we tested for the presence of pMLKL and aggregated DAI and RIP3, which are hallmarks for programmed necrosis. C57BL/6J mice were either mock -, wtVACV or VACV-E3LΔ83N- infected intranasally at 10^6 pfu dose. At 4 days post infection, nasal turbinates, brain and lungs were harvested from each group of mice. Since, VACV-E3LΔ83N does not spread during intranasal infection, we probed for the presence of pMLKL in the nasal turbinates'. pMLKL was detected in the nasal turbinates' of mice infected with VACV-E3LΔ83N but was absent during mock or wtVACV infection. Aggregated DAI and aggregated RIP3 was present in the nasal turbinates', brain and lungs of mice infected with VACV-E3LΔ83N but not in mock- or wtVACV infection. These results confirm that N-terminus of E3 is key to preventing necroptosis induction and subsequently leading to pathogenesis in mice (Figure 8).

Inhibition of Z-DNA-binding correlates with induction of necroptosis in vivo. To confirm that the induction of necroptosis correlates with deficient Z-DNA binding ability of VACV, we utilized point mutants VACV_VACV-E3L-P63A that has a loss in Z-DNA binding and VACV_E2A that retains the Z-DNA binding ability. C57BL/6J, $RIP3^{-/-}$, $DAI^{-/-}$ and $MLKL^{-/-}$ mice were infected intranasally with either of the point mutants at 10^6 pfu dose and clinical scores were evaluated. In C57BL/6J mice, infection with VACV_VACV-E3L-P63A led to significant reduction in pathogenesis as compared

to mice infected with VACV_E2A. This result is consistent with our data that VACV-E3LΔ83N with a deletion in the Z-DNA binding domain leads to decreased virulence in mice. We also show that in mice deficient in the necroptosis pathway, RIP3$^{-/-}$, DAI$^{-/-}$ and MLKL$^{-/-}$, pathogenesis is restored during VACV_VACV-E3L-P63A which is represented by the significant increased in clinical score. Thus, the presence of an intact Zα – binding domain results in inhibiting necroptosis (Figure 10).

Pathogenesis of VACV-E3LΔ83N is not completely restored during intranasal and intracranial infections in mice. The amino terminus of E3 is necessary for full pathogenesis mice. In an intranasal infection in C57BL/6J mice, the LD_{50} for VACV-E3LΔ83N is 3 logs lower than wtVACV. We investigated if in mice deficient in the necroptosis pathway pathogenesis during VACV-E3LΔ83N leads to restoration of dose like wtVACV levels. Mice were infected with varying dose of wtVACV and VACV-E3LΔ83N in RIP3$^{-/-}$, DAI$^{-/-}$ and MLKL$^{-/-}$ mice and monitored for mortality for 14 days. We observed that although infection with high dose of VACV-E3LΔ83N led to increased pathogenesis, the pathogenesis was not restored to wtVACV levels during this route of infection. It has been shown that inability of VACV-E3LΔ83N to completely suppress PKR activation during infection leads to decreased pathogenesis in mice. Previously, we have shown that during intranasal infection, eif2α phosphorylation was detected in the nasal turbinates of mice infected intranasally with VACV-E3LΔ83N but was absent in mice in brains of mice infected intracranially with VACV-E3LΔ83N. In C57BL/6J mice, LD_{50} for VACV-E3LΔ83N is $1x10^5$ pfu as compared to $1x10^2$ for wtVACV (Figure 11). To test if the sequestration of PKR is important for increased

virulence in mice, we infected RIP3$^{-/-}$, DAI$^{-/-}$ and MLKL$^{-/-}$ mice intracranially with wtVACV and VACV-E3LΔ83N and observed for mortality for 14 days. We show that during an intracranial infection, there is a 3-log increase in pathogenesis in mice deficient in necroptosis during an intracranial VACV-E3LΔ83N infection with an LD_{50} of $1x10^2$ pfu. But the pathogenesis is not completely restored to wtVACV levels (Figure 12). We propose that the N-terminus of E3 is involved in more than one function during pathogenesis in mice.

DISCUSSION

In this study, the role of necroptosis during VACV pathogenesis in mice was investigated. VACV E3 protein is one of the key innate immune evasion proteins. It is believed to confer IFN resistance. While the C-terminal domain has been identified to be responsible for dsRNA binding and sequestration, the characterization of the conserved N-terminus has been enigmatic. The N-terminus is identified as the Z-NA binding domain belonging to the Zα family of proteins. Other notable members of this family include Zα domains of ADAR1 and DAI.

Previous studies have characterized that VACV encoding for a E3L protein lacking the Z-NA-BD (VACV E3LΔ83N) underwent DAI- dependent necroptotic cell death in IFN treated mouse L929 cells. wtVACV with an intact N-terminus is resistant to this programmed necroptosis *in vitro*. *In vivo* studies have demonstrated that the N-terminal deletion mutant of E3 (VACV E3LΔ83N) is apathogenic during intranasal infections in mice but the pathogenesis is rescued in mice deficient in the IFNα/β receptor ($IFNAR^{-/-}$) due to the IFN sensitivity of VACV E3LΔ83N.

In order to determine if the IFN sensitivity of the N-terminal deletion mutant of E3 of VACV leads to reduced pathogenesis in C57BL/6J mice, we sought to determine if treatment with IFN along with the virus reduced the pathogenicity of the virus. Our results demonstrate that during infection with wtVACV, IFN treatment did not significantly reduced pathogenesis. However, during VACV E3LΔ83N infection,

treatment with IFN significantly reduced pathogenesis. This correlates with the results that were previously seen in the IFNAR$^{-/-}$ mice with this virus. The results confirm that VACV E3LΔ83N is IFN sensitive in mice.

In vitro, the absence of an intact Z-NA BD led to DAI- dependent necroptosis suggesting that DAI competes with the Z-NA BD of E3 leading to programmed cell death. Our results in the mice model correlate with this finding. We observe that in mice deficient in the necroptosis pathway – DAI$^{-/-}$, RIP3$^{-/-}$ and MLKL$^{-/-}$, IFN sensitivity of VACV E3LΔ83N is abolished and the pathogenesis observed is higher than in C57Bl/6J mice. These results correlate with the *in vitro* observation the IFN sensitivity of VACV E3 is linked to the Z-NA BD domain of E3.

Previous studies have demonstrated that during IN infections, VACV E3LΔ83N replicates in the nasal tissue but does not spread to the brain. It was also observed that in IFNAR$^{-/-}$ mice, the virus significantly spread and replicated in the brain. In our study, we observed that viral spread to the brain is higher in the DAI$^{-/-}$, RIP3$^{-/-}$ and MLKL$^{-/-}$ mice as compared to C57Bl/6J mice during VACV E3LΔ83N infection. These results indicate that induction of necroptosis leads to restriction of viral replication and spread in mice.

In order to confirm the induction of necroptosis, we probed for the activation of MLKL phosphorylation. Our results indicate that MLKL phosphorylation is seen in the nasal tissue of mice infected with VACV E3LΔ83N and not with wtVACV. We have also observed aggregated RIP3 and aggregated DAI in the nose, brain and lungs of mice infected with VACV E3LΔ83N and not with wtVACV (unpublished data). These results

demonstrate that the N-terminus of VACV is necessary to inhibit necroptosis during pathogenesis in mice.

Z-NA-BD of E3 and DAI are both members of the Zα family of Z-NA-binding proteins. We evaluated the role of Z-NA-BD to inhibit necroptosis leading to pathogenesis. During intranasal infection with a point mutant VACV E3 VACV-E3L-P63A, we observe that pathogenesis is decreased in C57BL/6J mice but is restored in mice deficient in the necroptosis pathway – $DAI^{-/-}$, $RIP3^{-/-}$ and MLKL-/-. VACV E3 VACV-E3L-P63A mutation has a loss in Z-NA BD ability and was predicted to behave similar to VACV E3LΔ83N. Our results confirm that the loss in Z-NA -BD ability leads to necroptosis and reduced pathogenesis. During infection with a point mutant VACV E3 VACV-E3L-E42A, that retains the Z-NA-BD ability, we observed that the pathogenesis was significantly higher since necroptosis was inhibited. These results confirm that inhibition of DAI- dependent necroptosis correlated with ability of VACV E3 to bind to Z-DNA.

This research study has established importance of the role of N terminus of VACV E3 in evading the host IFN system during pathogenesis. The data presented here confirms that the N-terminus of VACV E3 is necessary to prevent the initiation of IFN-primed virally induced necroptosis in mice. This is a novel mechanism of action since it's a RHIM independent mechanism of viral inhibition of necroptosis that occurs independent of canonical activator, RIP1. This novel and innovative mechanism of necroptosis can be used as a tool in characterizing the mechanism of pathogen regulation and anti-viral defense.

FIGURES

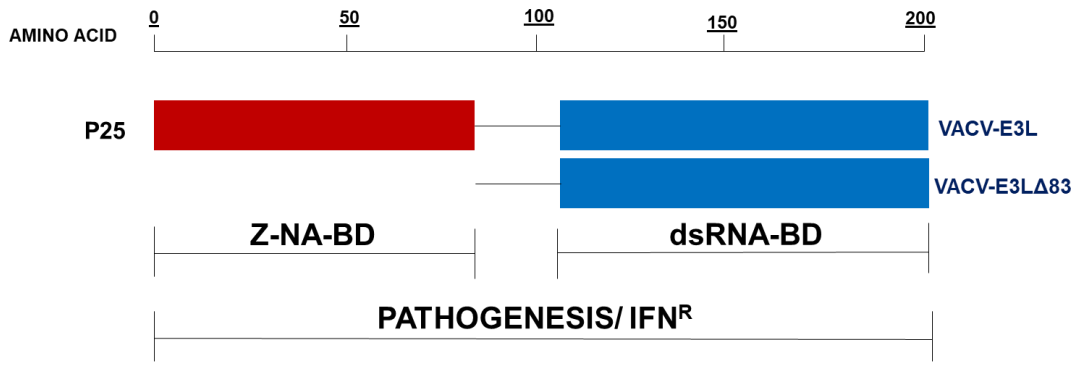

Figure 4: Domains of E3 protein. E3 is a 25 kDa protein containing two domains: a dsRNA binding domain on the carboxyl terminus and a Z-form nucleic acid binding domain in the amino terminus. Both domains have been shown to be responsible for IFN resistance and for full pathogenesis in mice. N-terminus is necessary to inhibit necroptosis. The two forms depicted here is the wtVACV containing full length of E3 and N-terminal deletion mutant VACV-E383N containing deletion of 83 amino acids in the N-terminus.

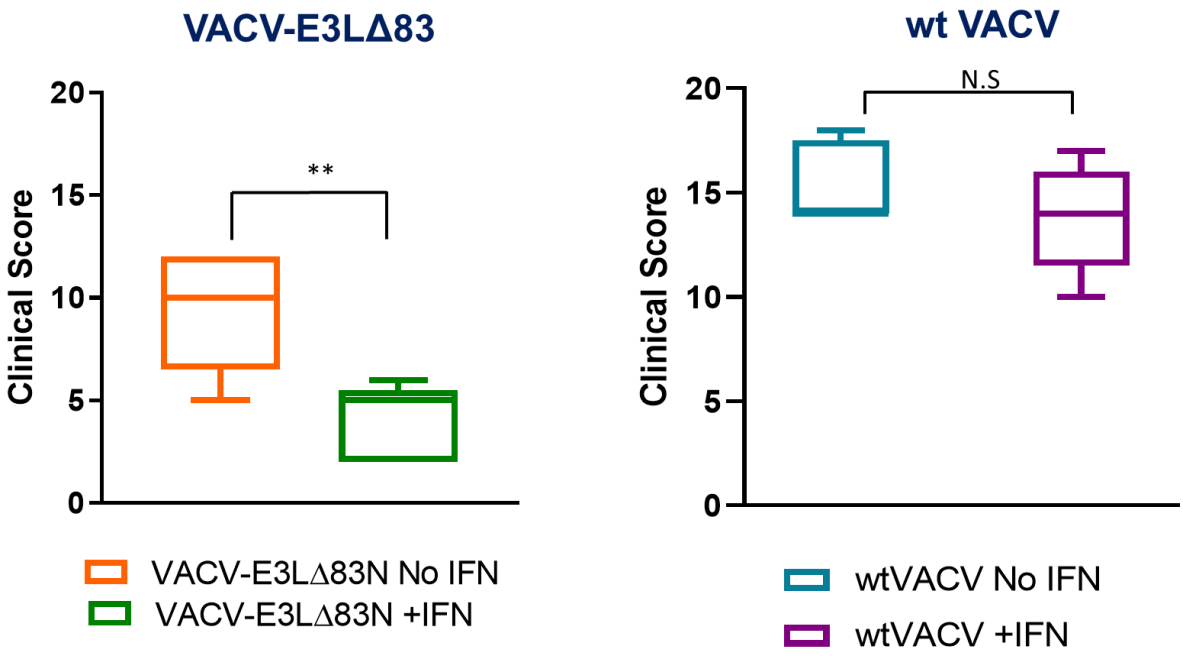

Figure 5: IFN sensitivity leads to reduced pathogenicity in a C57BL/6J mouse model. Groups of 5-10 C57BL/6J mice were infected with 10^6pfu of VACV-E383N and wtVACV, either with or without 100U of mouse IFNα. Mice were monitored for clinical symptoms and weight loss.

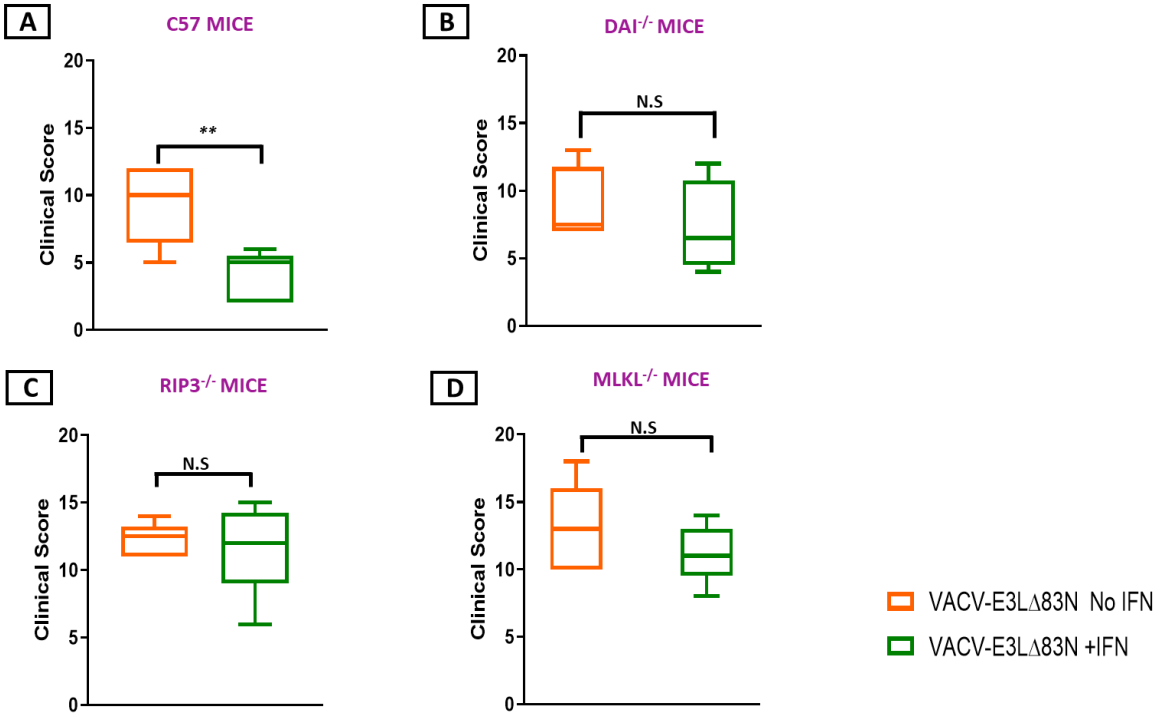

Figure 6: Necroptosis Pathway is Important for IFN sensitivity of VACV-E3LΔ83. Groups of 5-10 C57BL/6J, DAI$^{-/-}$, RIP3$^{-/-}$ and MLKL$^{-/-}$ mice were infected with 10^6pfu of VACV-E383N, either with or without 100U of mouse IFNα. Mice were monitored for clinical symptoms and weight loss**.**

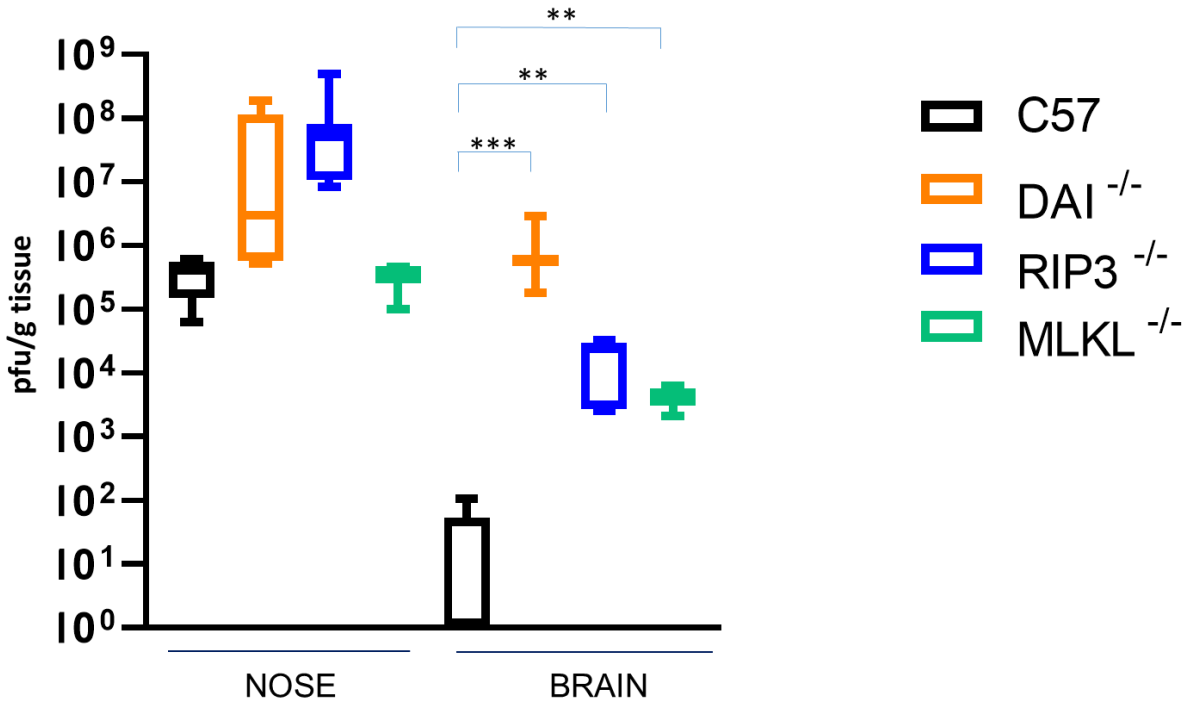

Figure 7: VACVE3Δ83N replicates and spreads to the brain at a higher titer in DAI$^{-/-}$, RIP3$^{-/-}$ and MLKL$^{-/-}$ mice. Groups of 5-10 C57BL/6J, DAI$^{-/-}$, RIP3$^{-/-}$ and MLKL$^{-/-}$ mice were intranasally infected with 10^8pfu of VACV-E383N. At 5dpi, nasal and brain tissues were harvested, frozen in liquid nitrogen and tittered in BSC40 to deduct viral replication and spread.

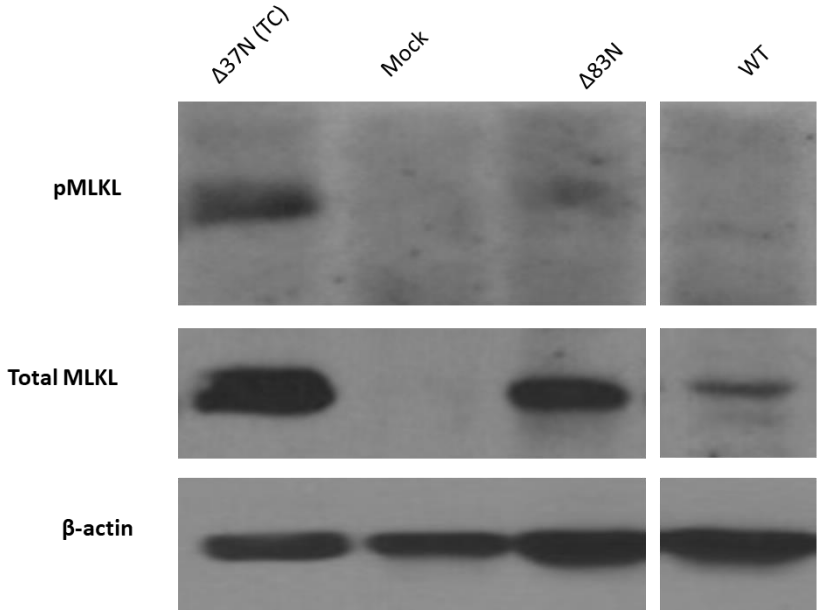

Figure 8: pMLKL is detected in nasal tissues during VACV-E3LΔ83 infection in mice. Groups of 5-10 C57BL/6J mice were infected intranasally with 10^8pfu of VACV-E383N or 10^6pfu of wtVACV. At 5dpi, nasal tissues were harvested, frozen in liquid nitrogen and assayed for pMLKL detection. Mouse L929 cells infected with VACV-E3 Δ37N were used as a positive control.

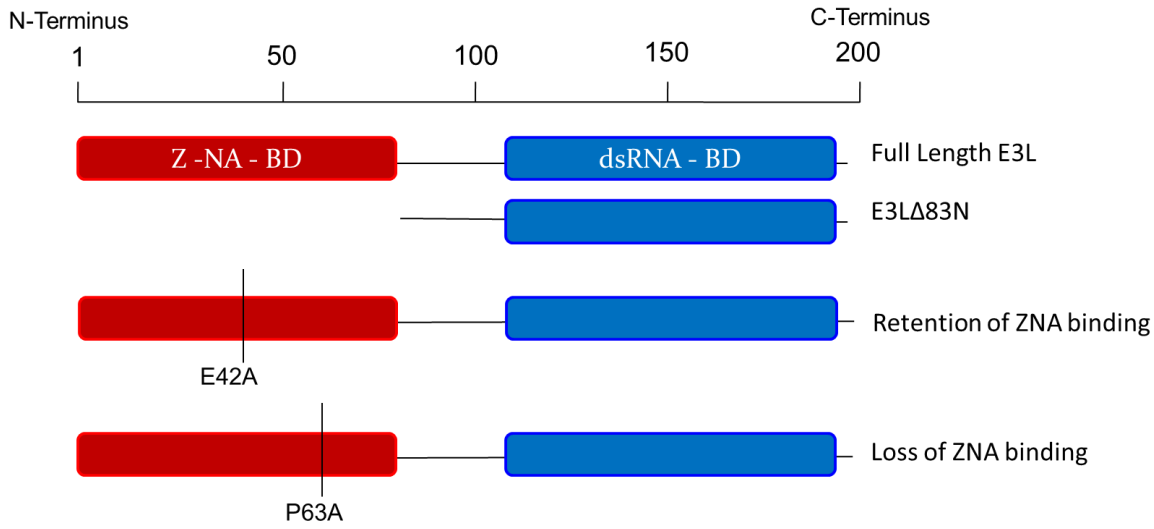

Figure 9: Schematic of E3 Z-NA-Binding Domain and Generated Mutants. E3 is a 25kDa protein. N-terminus contains Z-DNA binding domain. Point mutation in the N-terminus was generated to observe phenotype corresponding to loss of Z-NA binding (VACV-E3L_P63A). Control point mutant retaining the Z-NA binding ability was also generated (VACV-E3L_E42A).

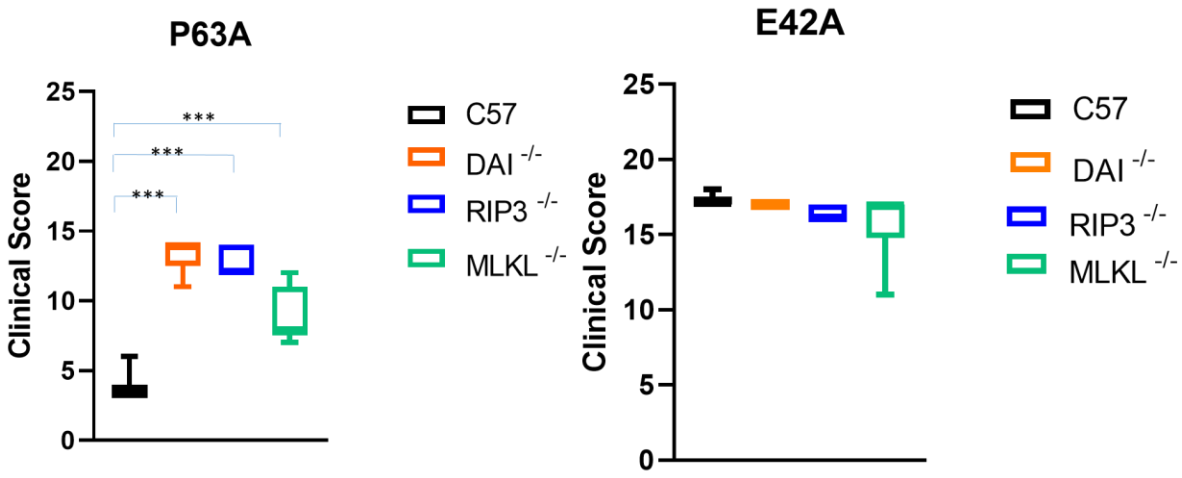

Figure 10: Pathogenesis is restored during VACV-E3L-P63A infection in mice deficient in the necroptosis pathway. Groups of 5-10 C57BL/6J, DAI$^{-/-}$, RIP3$^{-/-}$ and MLKL$^{-/-}$ mice were infected with 10^6pfu of VACV-E3L-P63A and VACV-E3L-E42A. Mice were monitored for clinical symptoms and weight loss**.**

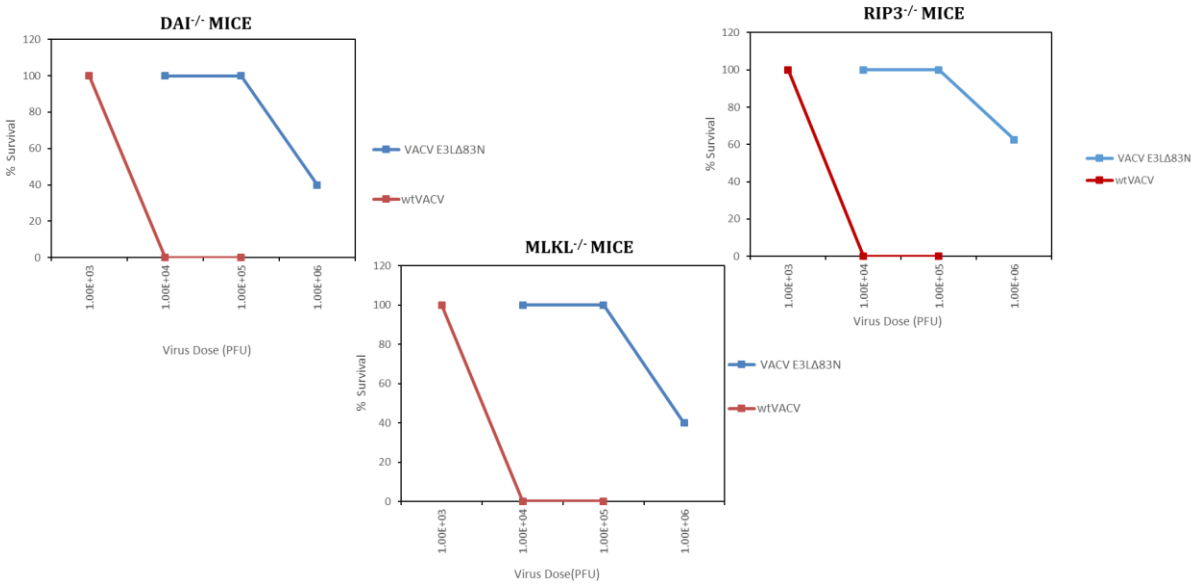

Figure 11: Intra nasal LD_{50} Study. Groups of 8-10, $DAI^{-/-}$, $RIP3^{-/-}$ and $MLKL^{-/-}$ mice were infected intranasally with varying increasing doses of wtVACV and VACV-E383N, Mice were monitored for clinical symptoms and weight loss**.**

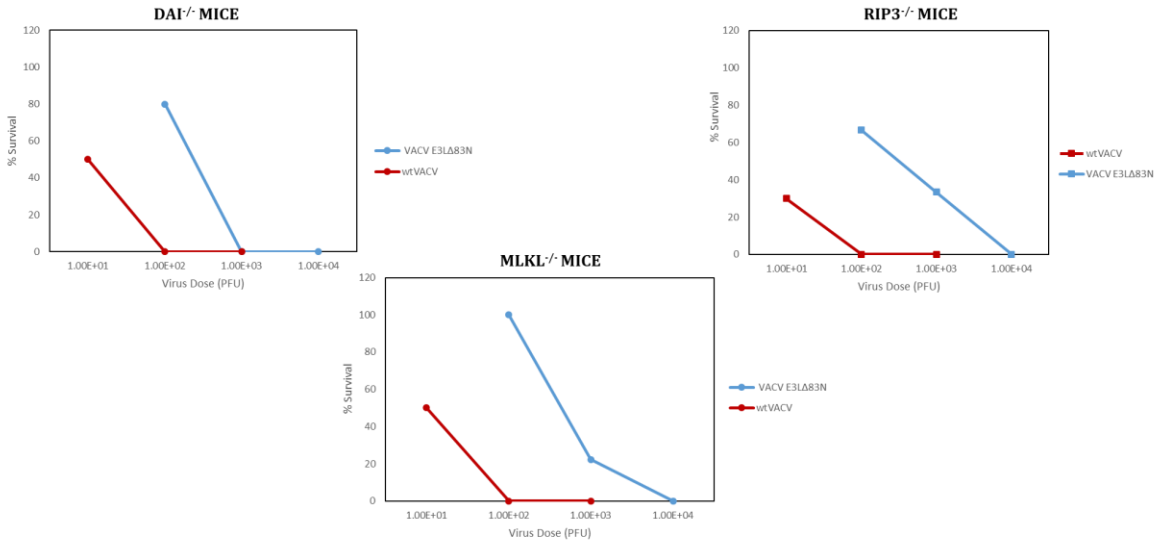

Figure 12: Intra cranial LD_{50} Study. Groups of 8-10, $DAI^{-/-}$, $RIP3^{-/-}$ and $MLKL^{-/-}$ mice were infected intracranially with varying increasing doses of wtVACV and VACV-E383N, Mice were monitored for clinical symptoms and weight loss.

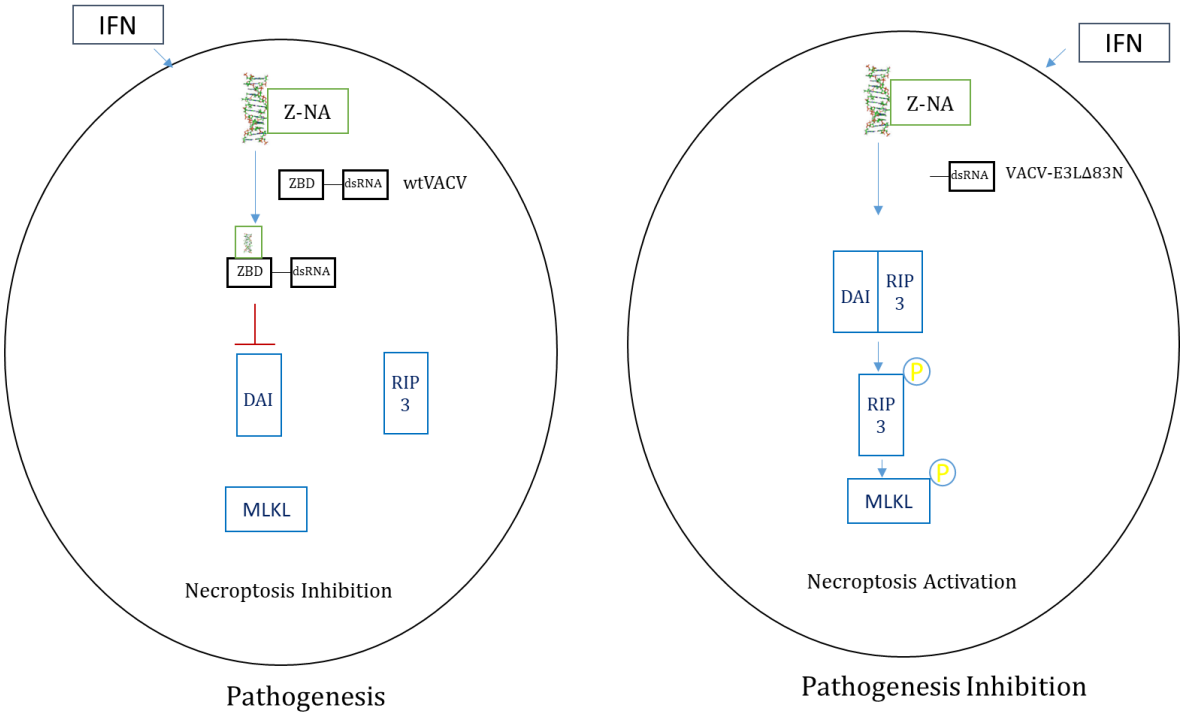

Fig 13: Proposed model for mechanism of necroptosis inhibition of VACV pathogenesis in mice.

CHAPTER 3

MONKEYPOX INHIBITION OF NECROPTOSIS IN MICE

ABSTRACT

Monkeypox is a recently re-emerging Orthopoxvirus infection of humans. Monkeypox caused by monkeypox virus (MPXV) is a member of the Orthopoxvirus genus, which includes variola virus and vaccinia virus. Vaccinia virus (VACV E3 is a homolog to monkeypox virus, F3. Monkeypox contains a natural truncation at the N-terminus of its F3 protein leading to the loss of first 37 amino acids. The N-terminus of E3 is critical to inhibit DAI-dependent necroptotic cell death during VACV infections in mice leading to increased pathogenesis. *In vitro*, MPXV shows interferon sensitivity that is DAI and RIP3 dependent but does not lead to MLKL trimerization and rapid cell death. In this study, the role of necroptosis during MPXV pathogenesis in mice was investigated. *In vivo* results demonstrate that during intra-nasal MPXV infection, pathogenesis was rescued in DAI ($DAI^{-/-}$) model but not in MLKL ($MLKL^{-/-}$) model, suggesting that DAI is inducing other proteins not leading to MLKL phosphorylation. identified that MPXV is neurovirulent showing increasing pathogenesis during an intracranial infection in mice. The data obtained in mouse model correlates with the *in vitro* data indicating that although the pathway is activated, MPXV inhibits necroptosis.

INTRODUCTION

Zoonotic infectious diseases are a cause for concern to the public health and global security(54). Monkeypox caused by monkeypox virus (MPXV) is a member of the Orthopoxvirus genus, which includes the deadly variola virus. Although it was first discovered in 1958 among monkeys, human monkeypox case was first reported in Democratic Republic of Congo (DRC) in 1970. MPXV is highly endemic to Central and West African countries. The Central African or Congo Basin clade is found to be more pathogenic than the West African clade(43, 51, 57).

In recent times, human monkeypox cases have been reported in humans in fifteen countries around the world. The clinical symptoms of monkeypox infection is similar to smallpox with the addition of lymphadenopathy that distinguishes it from smallpox. Although, the clinical manifestations of monkeypox are milder than smallpox, the fatality rates can range up to 10%(36, 53, 63). The current treatment for MPXV infection is the smallpox vaccine but there are potential side effects associated with the vaccination. With no current vaccine available for monkeypox infections and increasing incidence of the disease, it is imperative to understand the mechanism of pathogenesis of the virus(51, 57)

Among chordopoxviruses, E3L gene is highly conserved except for molluscum contagiosum virus and the avipoxvirus genus. For vaccinia virus, E3L expresses a protein consisting of a dsRNA binding domain on the carboxyl terminus and a Z-form nucleic acid binding domain on the amino terminus. The E3L gene encodes for two proteins, p25

and p20, that bind to dsRNA and sequester it away from many of the intracellular dsRNA PAMP. Although the E3L gene is highly conserved, leporipoxviruses myxoma virus (MYXV), MPXV and shape fibroma virus lack a full-length homologue. MPXV F3L gene is the homologue of E3L and it produces a protein truncated of the first 37 amino acids from the N-terminus known as p20 form. This is a result of leaky scanning and translation initiation at a second downstream start codon(61, 86, 101).

Both N-terminal and C-terminal domains of the VACV E3L protein are necessary for IFN resistance and pathogenesis in mice. *In vitro,* VACV-E3LΔ37N with a truncation of the first 37 amino acids is IFN sensitive in mouse L929 cells. *In vivo,* VACV-E3LΔ37N pathogenesis is reduced by 1,000-fold during an intracranial infection and 100-fold during an intranasal infection, as compared to wtVACV(27, 41, 42, 88).

It is also observed that the N-terminus of VACV E3 is necessary to inhibit programmed cell death, necroptosis. Necroptosis is a pro-inflammatory, anti-viral cell death pathway in which cells undergo a rapid and explosive death(15, 16). N-terminal deletion mutants of VACV undergoes DAI- dependent necroptotic cell death where DAI, an interferon stimulated gene containing a Za domain interacts with Z-form nucleic acids and a RHIM domain activating RIP3(27). This leads to downstream activation, trimerization and eventually, phosphorylation of MLKL. MLKL phosphorylation is the key executioner step of necroptosis leading to cell death(15, 20, 22, 23, 27, 99). *In vitro*, VACV-E3LΔ37N undergoes DAI-dependent necroptotic cell death leading to MLKL phosphorylation.

Unpublished data in the lab indicate that MPXV has intermediate IFN sensitivity in mouse L929 cells which is rescued in DAI$^{-/-}$ L929 cells. Although, MPXV replication is dependent on DAI and RIP3, MLKL trimerization is not seen in cells infected with MPXV. Although, MLKL phosphorylation is known to be the key executioner step of necroptosis, it is believed that MLKL trimerizes after being phosphorylated leading to cell death. Thus, although DAI is activated in MPXV infected cells, it does not lead to necroptotic cell death.

In this study, we investigated the effect of MPXV pathogenesis mice. The goal of the study was to determine if the results we observed in vitro also translated to a mouse model.

MATERIALS AND METHODS

Mice

C57BL/6J, DAI$^{-/-}$ and MLKL$^{-/-}$ were bred and housed at the Arizona State University animal facility. RIP3$^{-/-}$ were from Genentech, Inc. and rederived at Charles River, DAI$^{-/-}$ were received from Dr. Shizuo Akira, Osaka University and MLKL$^{-/-}$ were received from Dr. Jiahuai at State Key Laboratory of Cellular Stress Biology, School of Life Sciences, Xiamen University. Colonies were maintained pathogen-free. Four to six-week old mice were used for all the experiments. Mice of either sex was used in the experiments. All animal experiments were approved by the Arizona State University Institutional Animal Care and Use Committee (IACUC). All MPXV experiments were completed in a biosafety level 3 laboratory (BSL-3) in accordance with protocols approved by Arizona State University and the Center for Disease Control and Prevention (CDC).

Viruses and Treatment

The Western Reserve (WR) strain of vaccinia virus N-terminal deletion of 37 amino acids of E3L (VACV-E3LΔ37N) and Monkeypox (Zaire Strain 79). Zaire 79 is a Central African clade member of MPXV.

Intranasal and intracranial infections of mice

Mice were anaesthetized using a cocktail containing 37.5 mg/ml ketamine, 7.5 mg/ml xylazine and 2.5 mg/ml acepromazine maleate. Intra-peritoneal injection of the anesthetic was administered at the dose of 1 µl/g of body weight of the mouse. For intranasal infections, anaesthetized mice were infected with 10µl of virus in dose range 10^3-10^8 pfu in 1 mM Tris pH 8.8 using a Pipetman loaded with a protein-loading tip. For intracranial infections, anesthetized mice were infected with 5 µl of virus in dose range 10^1-10^4 in 1 mM Tris pH 8.8 using a 30-gauge hypodermic needle and a 50 µl syringe. Mice were monitored for weight loss and clinical symptoms. Clinical symptoms that were evaluated include eye infection (left and right eye), breathing, hunching, ruffling of fur, sneezing and level of activity. Each clinical sign was scored on a scale of 0-3 with 3 representing severe symptomology. For intracranial infections, neurological symptoms were also monitored. Weight loss was also monitored with mice losing 10-20% of original weight receiving score of 1 and mice losing 20-30% of original weight receiving score of 2. Clinical score assigned was a total of weight loss and clinical symptoms score. Mice dropping below 30% of their initial weight or with a clinical score of 17 and above were euthanized.

Tissue Distribution

Mice were infected intranasally as described above with 10^6 pfu of wtVACV or 10^8 pfu of VACV-E3LΔ83N. At 6 days post infection, mice were sacrificed with isoflurane overdose and organs were harvested. Tissue samples were immediately snap frozen in liquid nitrogen. Each tissue was weighed, ground to a fine powder in liquid

nitrogen using a mortar and pestle and resuspended in 10% wt/volume RPMI media supplemented with Anti- Anti (Thermo Fisher Scientific). Tissue homogenates underwent three cycles of freeze-thaws that consisted of freezing samples at -80°C and then slowly thawing the samples on ice, followed by a quick thaw at 37°C and sonication for 30s. Titers were determined by a standard plaque assay using BSC40 cell monolayers.

RESULTS

Pathogenesis is rescued in DAI$^{-/-}$and MLKL$^{-/-}$ during VACV-E3LΔ37N infection. The N-terminus of the E3L gene is necessary for pathogenesis of VACV in C57BL/6J mice. This has been demonstrated by reduction in pathogenesis during intranasal and intracranial infections in C57BL/6J mice. We hypothesized that the induction of necroptosis is critical to inhibition of pathogenesis in mice. C57BL/6J, DAI$^{-/-}$and MLKL$^{-/-}$ mice were infected intranasally with 10^7 pfu of virus and mice were monitored for clinical symptoms and weight loss. We demonstrate that VACV-E3LΔ37N is highly attenuated in C57BL/6J mice and is rescued in DAI$^{-/-}$and MLKL$^{-/-}$ mice (Figure 15 and 16). Thus, N-terminus of E3L gene is critical for inhibition of necroptosis leading to enhanced pathogenesis in mice.

Pathogenesis is rescued only in DAI$^{-/-}$during MPXV infection. The F3L gene of MPXV is believed to be a homolog of E3L gene of VACV. Although MPXV has an intact dsRNA binding C-terminus, it has a deletion of 37 amino acids in the Z-DNA binding N-terminal domain. We assessed if MPXV demonstrated pathogenesis like VACV-E3LΔ37N in mice deficient in necroptosis pathway. Our results demonstrate that MPXV as attenuated pathogenesis in C57BL/6J mice and this pathogenesis is rescued only in DAI$^{-/-}$ and not in MLKL$^{-/-}$ (Figure 17 and 18). This data is consistent with the results we observe in cell culture and suggest that during MPXV infection, DAI is possibly activating other proteins that does not lead to necroptosis.

MPXV does not spread to brain, lungs and spleen in DAI$^{-/-}$ during an intranasal infection. MPXV does not cause pathogenesis in C57BL/6J mice but in DAI$^{-/-}$ mice pathogenesis is restored with MPXV infection. To determine the mechanism of pathogenesis, viral spread to different tissues was assessed. C57BL/6J, DAI$^{-/-}$ and MLKL$^{-/-}$ were infected intranasally with MPXV and tissues were harvested. Virus was recovered from nasal tissues from all three different mice strains. However, we do not see a significant increase in viral spread to brain or lungs in the DAI$^{-/-}$ mice compared to the C57BL/6J. We also do not observe any viral spread to spleens in all three strains of mice (Figure 19).

MPXV (Zaire strain) is neurovirulent in mice. N-terminal deletion mutants of VACV demonstrate decreased neurovirulence in C57BL/6J mice**.** To assess the neurovirulence of MPXV, mice were infected intracranially and monitored for clinical symptoms and weight loss. We observed that MPXV is neurovirulent with LD_{50} of 10^1 pfu in C57BL/6J. LD_{50} in RIP3$^{-/-}$ and MLKL$^{-/-}$ were 10^2 pfu respectively. DAI$^{-/-}$ mice were the first group to infected intracranially. We did not predict for MPXV to be neurovirulent in mice and hence, the dose range was on the higher end. We observed that the virus was neurovirulent with only 20% survival in the lowest dose 10^3 pfu. It is also to be noted that the surviving mice did not show any symptom of infection and we believe that the mouse was not infected (Figure 20).

DISCUSSION

Monkeypox virus is an emerging human pathogen causing monkeypox disease. The treatment for monkeypox is the smallpox vaccine. The discontinuation of smallpox vaccination has led to increased susceptibility to monkeypox and is a global health security concern. Human monkeypox infections are believed to be transmitted with indirect or direct contact with live or dead animals. Although, the specific animal host reservoir of monkeypox virus remains unknown, it has been identified in range of animals including monkeys, dormice, squirrels (rope and tree) and rats. Thus, it is critical to understand the host – pathogen association and identify the mechanism of pathogenesis of the virus.

Monkeypox virus (MPXV) is a member of the genus Orthopoxvirus, which also includes vaccinia virus (VACV). Monkeypox expresses F3 protein that is an ortholog of VACV-E3 protein. However, F3 contains a deletion of the first 37 amino acids from the N-terminus. It is shown that VACV-E3 with 83 amino acid deletion in the N-terminus – VACV-E3Δ83N undergoes DAI-dependent necroptotic cell death leading to decreased pathogenesis in mice. In this study, we studied the role of necroptosis during monkeypox infection in mice.

Previously, we have demonstrated that the N-terminus of E3 is necessary to inhibit VACV pathogenesis. In order to compare the pathogenesis between VACV and MPXV infections, we utilized VACV-E3 with 37 amino acid deletion in the N-terminus -

VACV-E3Δ37N. During an intranasal infection with VACV-E3Δ37N, we observed that pathogenesis was low in C57BL/6Jmice, but this pathogenesis was restored in mice deficient in the necroptotic pathway. These results confirm our previous finding that N-terminus of E3 is critical to evade necroptosis in mice.

In an IN MPXV infection, we observed that pathogenesis was low in C57BL/6Jmice, but this pathogenesis was restored only in $DAI^{-/-}$ mice and not in $MLKL^{-/-}$ mice. This data correlates with our in vitro results, where in mouse L929 cells, MPXV has intermediate IFN sensitivity but the IFN sensitivity is rescued only $DAI^{-/-}$ L929 cells. In vitro, we also observed that MLKL phosphorylation is seen in MPXV-infected L929 cells but trimerization of MLKL is not observed. Recent studies have shown that TAM (Tyro3, Axl, and Mertk) kinases are involved in the oligomerization of MLKL that results in pore formation in the membrane and eventually cell death. We hypothesize that inhibition TAM kinases during MPXV infections leads to absence of MLKL trimerization and cell death. We also analyzed the spread of MPXV to different organs during IN MPXV infection in C57BL/6J mice and mice deficient in necroptotic pathway, namely $DAI^{-/-}$ and $MLKL^{-/-}$. Although we observed replication of the virus in all groups of mice, we did not observe a significant spread of the virus to the brain or lungs of $DAI^{-/-}$ as we predicted. MPXV has shown been shown to replicate more in the distal organs, we predict that the higher pathogenesis we see in $DAI^{-/-}$ could be due to the spread of virus to distal organs.

Neurovirulence of MPXV has not been studied. WR strain of VACV is a neurovirulent strain and the LD_{50} for VACV-E3Δ37N during an intra-cranial infection is

100 times that of wtVACV. Our results indicate that MPXV is highly neurovirulent in mice with LD_{50} in C57BL/6J being 10^1 pfu and 10^2 pfu in $RIP3^{-/-}$ and $MLKL^{-/-}$. Studies have shown that MPXV is not pathogenic in immunocompetent mice. Although, the IC route of infection is not a natural infection route, this could provide a model to study MPXV pathogenesis at a biological level and identify possible transmission mechanisms before validating the results in nonhuman primates and nonmurine small animal models.

Recently, it has been identified that the DAI-/- mice obtained from the lab of Shizuo Akira were of a mixed genetic background with C57BL/6J and 129 mixed background leading to the absence of subset of population NK1.1+ and Ly49H+ NK cells in the DAI-/- mice (102). This could possibly have impacted our viral replication and spread to the different organs during the intranasal MPXV infection. However, our neurovirulence study emphasizes that the pathogenesis we observed are consistent in all the necroptotic deficient mice. Future studies in this direction involve dissecting this genetic difference during MPXV pathogenesis.

This research study identifies the presence of an alternative independent mechanism that does not lead to necroptosis and provides an enhanced understanding of innate immune evasion by MPXV. Further elucidation of the probable pathways involved in the subversion of host's anti-viral immune response will be critical to the development of effective vaccine design against the most important re-emerging Orthopoxvirus for humans, MPXV.

FIGURES

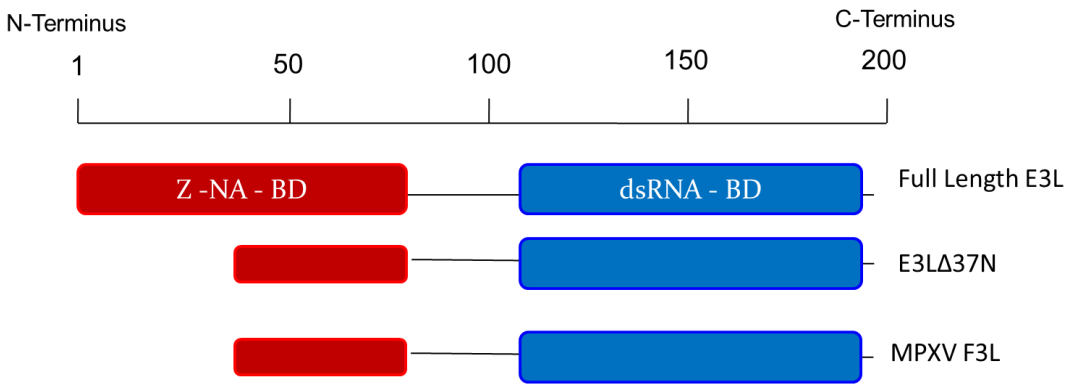

Figure 14: Domains of VACV E3 and MPXV F3. Depicted here are VACV E3 and MPXV F3 proteins. VACV E3 is a 25kDa protein containing N-terminal Z-NA binding domain and C-terminus containing dsRNA binding protein. MPXV F3 contains a natural 37 amino acid truncation at the N-terminus. wtVACV containing contains 37 amino acid deletion at the N-terminus VACV-E3Δ37N was used to compare the two homologues.

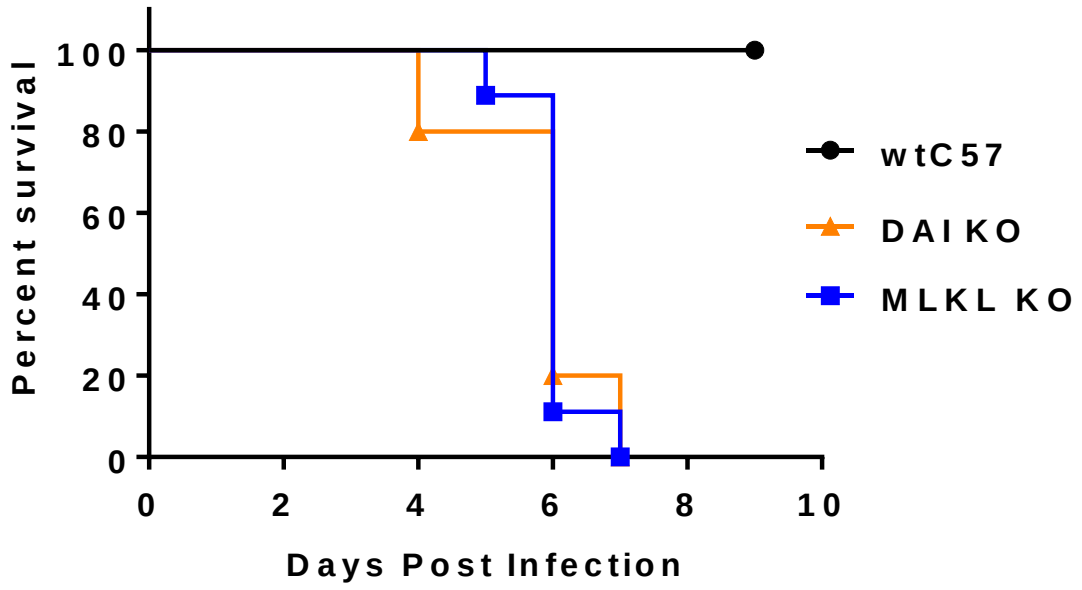

Figure 15: Pathogenesis is rescued in DAI$^{-/-}$and MLKL$^{-/-}$during VACV-E3LΔ37N infection. Groups of 5-10 C57BL/6J, DAI$^{-/-}$ and MLKL$^{-/-}$ mice were infected intranasally with 10^7pfu of VACV-E3LΔ37N. Mice were monitored for clinical symptoms and weight loss**.** Percent survival was plotted.

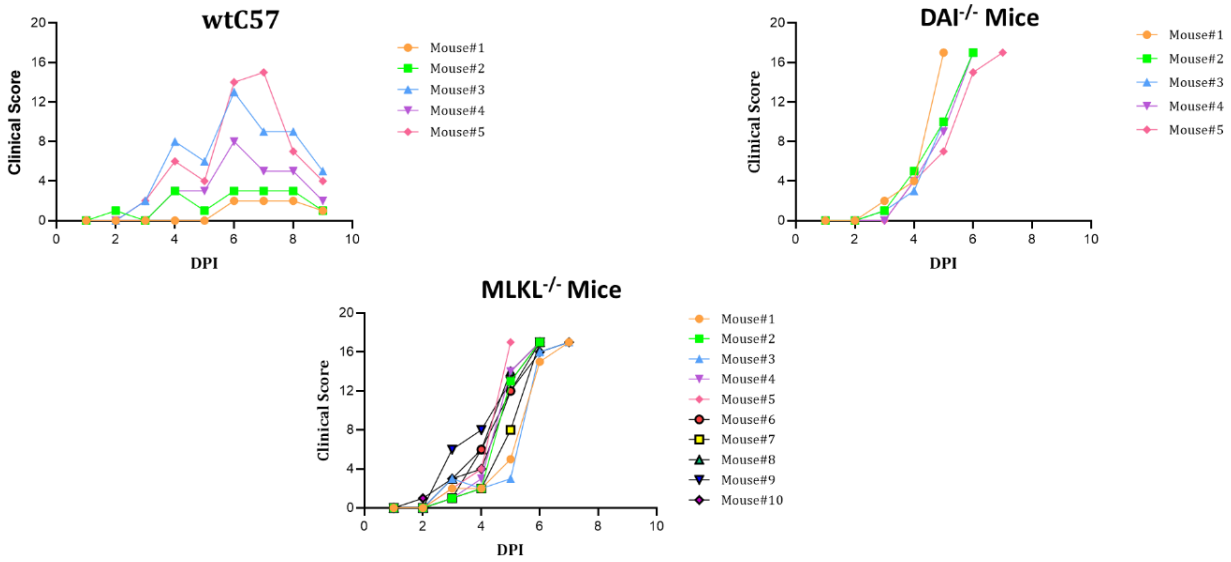

Figure 16: Individual Clinical Score during VACV-E3LΔ37N infection. Groups of 5-10 C57BL/6J, DAI$^{-/-}$ and MLKL$^{-/-}$ mice were infected intranasally with 10^7pfu of VACV-E3LΔ37N. Mice were monitored for clinical symptoms and weight loss. Shown here is the clinical score calculated for each individual mouse calculated as a combined score from clinical symptoms and weight loss.

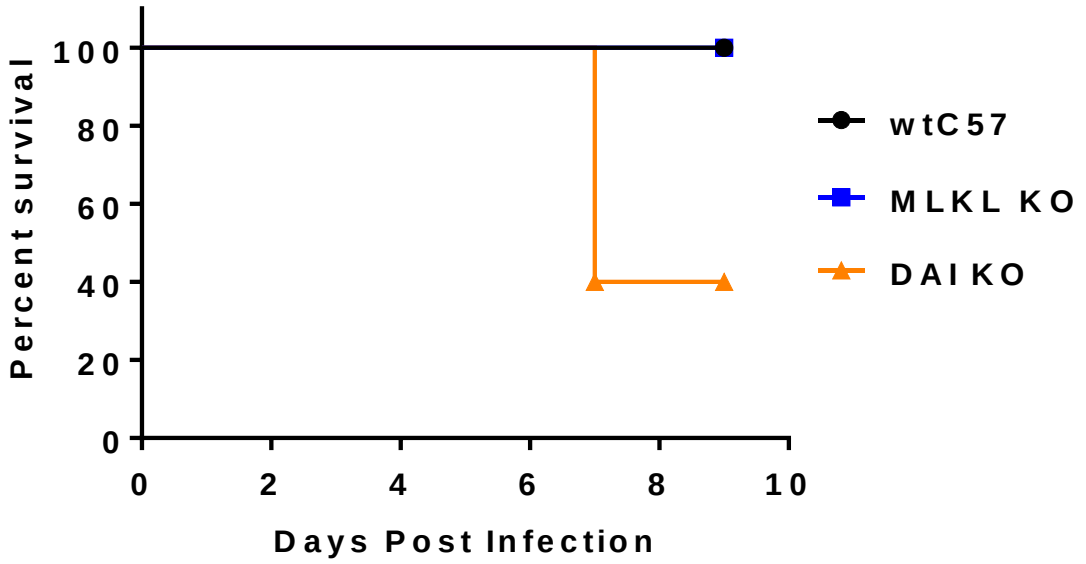

Figure 17: Pathogenesis is rescued only in DAI$^{-/-}$during MPXV infection. Groups of 5-10 C57BL/6J, DAI$^{-/-}$ and MLKL$^{-/-}$ mice were infected intransally with 10^7pfu of MPXV – Zaire 79 strain. Mice were monitored for clinical symptoms and weight loss**.**

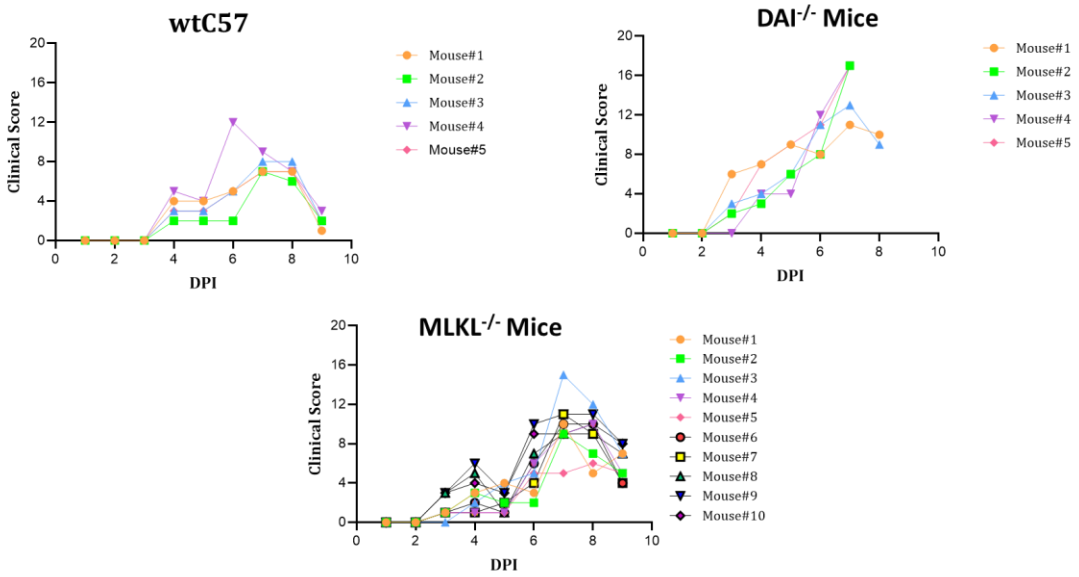

Figure 18: Individual Clinical Score during MPXV infection. Groups of 5-10 C57BL/6J, DAI$^{-/-}$ and MLKL$^{-/-}$ mice were infected intranasally with 10^7pfu of MPXV – Zaire 79 strain. Mice were monitored for clinical symptoms and weight loss. Shown here is the clinical score calculated for each individual mouse calculated as a combined score from clinical symptoms and weight loss.

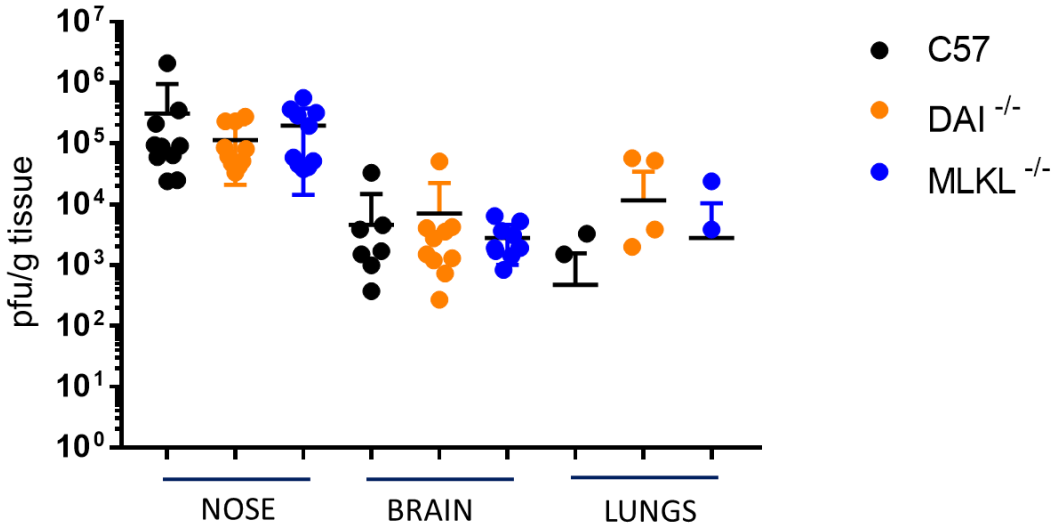

Figure 19: MPXV titers in various tissues. Groups of 8-10 C57BL/6J, DAI$^{-/-}$ and MLKL$^{-/-}$ mice were intranasally infected with 10^7pfu of MPCV – Zaire 79 strain. At 5dpi, nasal, brain and lung tissues were harvested, frozen in liquid nitrogen and tittered in BSC40 to deduct viral replication and spread.

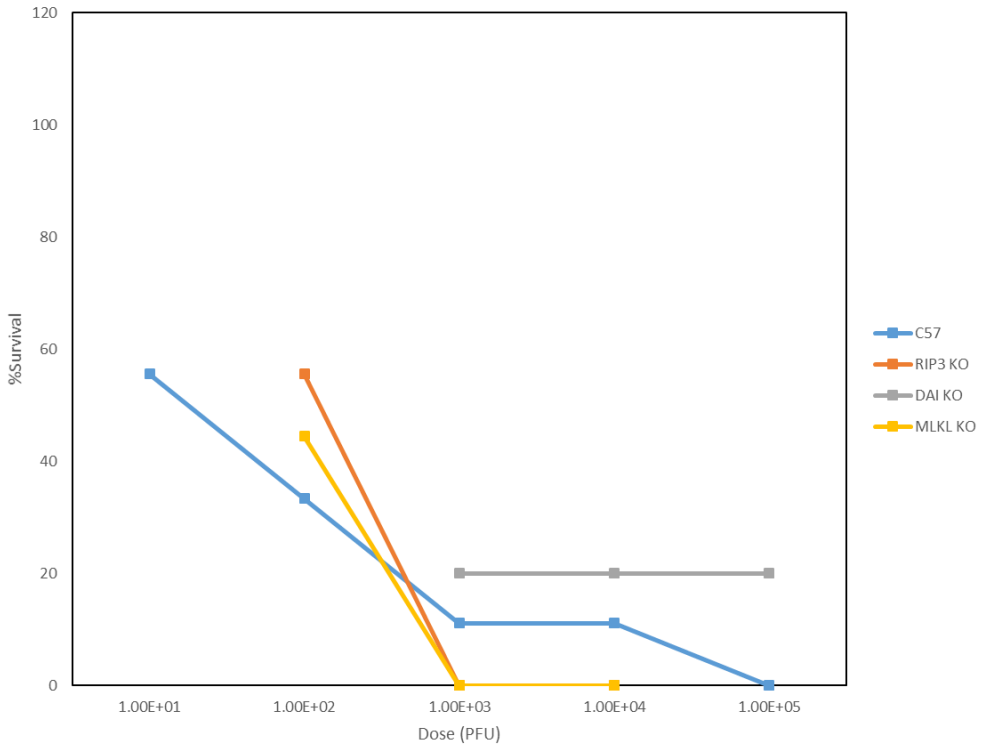

Figure 20: MPXV is neurovirulent in mice. Groups of 5-10 C57BL/6J, DAI$^{-/-}$, RIP3$^{-/-}$ and MLKL$^{-/-}$ mice were intracranially infected with varying increasing doses of MPXV-Zaire79 strain. Mice were monitored for clinical symptoms and weight loss. Percent survival was plotted.

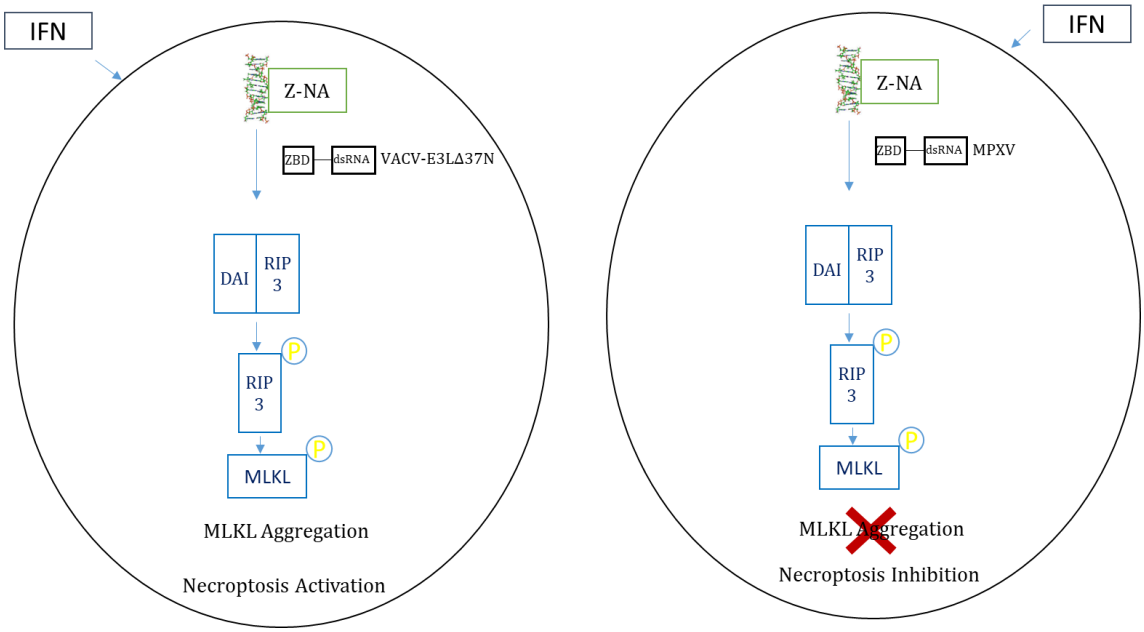

Fig 21: Proposed model for mechanism of MPXV inhibition of pathogenesis in mice.

CHAPTER 4

CHARACTERIZATION OF NECROPTOSIS IN CANCER CELL LINES FOR ONCOLYTIC VACCINIA POTENTIAL

ABSTRACT

Oncolytic virotherapy is emerging as a promising new therapeutic approach to target cancer. Cancer cells are known develop resistance to programmed cell death pathways. It has been identified that majority of cancer cell lines have a deficient necroptotic pathway. In this study, the potential use of vaccinia virus (VACV) mutants for oncolytic virotherapy was studied. Previous studies in the lab have shown that VACV with a nonfunctional E3 protein selectively replicates in necroptosis deficient cells but not in necroptosis competent cells, making them potential candidates for oncolytic virotherapy. In order to establish the efficacy and selectivity of this treatment, an accurate tumor model is required. Various cancer cell lines were screened for expression of necroptotic proteins and the functionality of the pathway was studied using cell viability assays. The absence of an active necroptotic pathway was correlated to the replication of the N-terminal deletion mutants of VACV. These results suggest the potential use of using VACV mutants as an oncolytic virus to target cancerous cells with a deficient necroptotic pathway while sparing the healthy cells with an active necroptotic pathway. Oncolytic VACV mutant viruses can also be used in combinatorial treatments to target highly resistant tumors.

INTRODUCTION

Oncolytic virotherapy is emerging as a promising new therapeutic approach to target cancer. It involves the use of viruses that can selectively replicate in and destroy cancerous cells while sparing normal cells(21, 70, 103). Genetic mutations in cells lead to dysregulation of cellular proteins that lead to uncontrolled growth of cells leading to tumors. One of the hallmarks of cancer cells is their ability to evade cell death. Therapeutics targeting the resistance of cancer cells to programmed cell death could pave way for potential advancement in the field of oncolytic virotherapy.

The three known programmed cell death pathways include apoptosis, pyroptosis and necroptosis(8, 13). Although, apoptosis has been well characterized, necroptosis has recently been shown to play an important role in anti-viral response. In a canonical pathway, necroptosis occurs when tumor necrosis factor (TNF) binds the tumor necrosis factor receptor (TNF-R) leading to a signal cascade through RHIM domains of the adaptor protein RIP3. RIP3 can interact with different adaptor proteins such as RIP1, DAI or TRIF to form a necrosome. These interactions cause autophosphorylation of RIP3 leading to MLKL phosphorylation and eventually cell death. MLKL is the key executioner step of necroptosis(16, 20, 98, 99, 104-107).

Vaccinia virus (VACV), a member of the Orthopoxvirus genus is a 190 kb double stranded DNA virus that replicates in the cytoplasm of infected cells. It has been used for vaccination against smallpox. One of the key innate immune evasion gene of VACV is

E3L. E3 protein has two domains, a double stranded RNA binding domain at the carboxyl- terminus that sequesters dsRNA during produced in cells during viral infection and a Z-DNA binding domain at the N-terminus(4, 35, 38, 40, 41, 91, 108). The N-terminus of VACV E3 has been shown to be necessary to inhibit DAI-dependent necroptotic cell death. DAI contains a Zα binding domain that competes with N-terminus of E3 during viral infection. Vaccinia virus containing mutation in the N-terminus activates DAI leading to necroptotic cell death in infected cells. VACV-E3LΔ83N was shown to be apathogenic in C57BL/6Jmice but pathogenic in $RIP3^{-/-}$ and $DAI^{-/-}$ mice. Thus, evasion of necroptosis is critical to VACV pathogenesis.

Majority of cancers are believed to have a silenced RIP3 expression due to genomic methylation near the RIPK3 transcription start site (TSS). The loss of RIP3 expression leads to greater resistance to programmed cell death pathway and to chemotherapeutic agents(66-69). Since N-terminal deletion mutant of VACV selectively replicate in the absence of an active necroptotic pathway, we propose to utilize this virus as a potential oncolytic virus to target cancer cells with a deficient necroptosis pathway. Our hypothesis is that the N-terminal deletion mutant would replicate in cells deficient in necroptosis pathway (cancer cells) and in normal, healthy cells this virus would not replicate due to an active pathway.

In this study, various cancer cell lines were screened to identify potential cancer lines to study the oncolytic potential of VACV.

MATERIALS AND METHODS

Cells and Viruses

Murine and Human cancer cell lines were used in the study. Murine cell lines included L929 cells obtained from ATCC and cultured in MEM+5%FBS medium, E0771 cells obtained from ATCC and cultured in RPMI 1640+10% FBS medium. JC cells obtained from ATCC and cultured in RPMI 1640+10% FBS medium. 4T1 cells cultured in RPMI 1640 +10% FBS and B16 cells cultured in DMEM+10% FBS. Human cancer cell lines included HT 29 cells cultured in McCoy's 5a medium supplemented with 10% FBS. HS578T cells were obtained from ATCC (ATCC HTB-125) and cultured in DMEM supplemented with 10% FBS and 0.01 mg/ml bovine insulin. T-47D cells were obtained from ATCC (ATCC HTB-133) and cultured in RPMI 1640 supplemented with 10% FBS. SKBR3 cells were obtained from ATCC (ATCC HTB-30) and cultured in McCoy's 5a medium supplemented with 10% FBS. U251, U87 and U118 cells were maintained in Eagle's Minimal Essential Medium (MEM) containing 5% FBS, while U343 was maintained in DMEM containing 5% FBS. Panc-1 cells were maintained in Dulbecco's Modified Eagle's Medium supplemented with 10% FBS. All cells were incubated at in the presence of 5% CO2. MDA-MB-435S cells were obtained from ATCC (ATCC HTB-133) and were maintained in Leibovitz's L-15 medium supplemented with 10% FBS, 2 mM L-glutamine and 0.01 mg/ml bovine insulin. These cells were grown in at 37°C with no CO2.

Vaccinia virus strain Western Reserve (WR), designated as VACV, was used as the parental viruses to generate VACV-E3LΔ83N, VACV-E3LΔ54N, as previously described and were used in the viral infection studies.

Treatment and Inhibitors of Cells

Cells used as a positive control for programmed necrosis were pre -treated with the pan-caspase inhibitor Z VAD-FMK (ZVD) (ApexBio) at 100μM for 1 hour and treated with mouse TNF-α (Sigma) at 20ng/ml for murine cells or human TNF-α for human cells following the one hour pretreatment. For human cells, SMAC mimetic (LCL161) was treated along with ZVD to inhibit apoptosis family of proteins. N-(6-(Isopropylsulfonyl) quinolin-4-yl)benzo[d]thiazol-5-amine (GSK872, GlaxoSmithKline) was used to inhibit RIP3 activity in cells.

Cell Viability Assay

Cell viability was determined using propidium iodide staining assay. For positive control, cells were treated with ZVD, Hoescht and Propidium Iodide and LCL161 (for human cells) . After 1-hour incubation, TNFα (human and mouse respectively) was added. For virus infections, cells were infected with virus diluted in MEM containing 2% FBS at an MOI of 5. Cell monolayers were infected with 100 μL of virus after aspirating the media off the dishes. Cells were incubated at 37C, 5% CO2 for 1 hour, with rocking every 10 minutes. Post infection, cells were over laid with appropriate medium containing Hoescht and Propidium Iodide. Cells were incubated for 4 hours for murine cell lines and 6 hours for human cell lines. Post-incubation, cells were imaged on an

EVOS™ FL Auto Imaging System (Invitrogen™) at 10X objective to evaluate the viability of cells.

Western Immunoblot Analysis

Cells were pre-treated with interferon (Universal IFN for human cells and mouse IFNα for murine cells) for 16 to 18 hrs. Interferon pre-treatment was 100u/ml for L929 cells and 1000U/ml for all the other cell lines that were screened. Cell lysates were prepared by scraping cells and centrifuging for 1,000 g for 10 minutes. Cell pellet was lysed in RIPA buffer (25mM Tris•HCl pH 7.6, 150mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) for 10 minutes. Proteins were reduced in Laemmli buffer containing DTT. Samples were boiled for 5 minutes, separated on a denaturing 10% SDS PAGE gel. Proteins were then transferred to PVDF membranes in transfer buffer (25 mM Tris, 192 mM Glycine, 20% methanol) for 60 minutes at 60 volts at 4C. Following transfer, the membrane was incubated in blocking buffer (140 mM NaCl, 3 mM KCl, 20 mM Tris [pH 7.8], 0.05% Tween 20, 3% nonfat milk) for 1 hour at room temperature. The blots were incubated in primary antibodies DAI or RIP3 as intended. Primary antibodies were diluted in blocking buffer and blots were exposed to antibodies. overnight. Secondary goat anti-rabbit IgG conjugated to horseradish peroxidase (1:5000, Santa Cruz) or anti-mouse IgG conjugated to horseradish peroxide were incubated at room temperature for 1 hour. Immunoreactive bands were visualized by chemiluminescent development using SuperSignal West Dura Duration substrate (Pierce Biotechnology).

Single-Step Growth Curve

B16 and E0771 cells were treated with or without mouse IFNα for 16-18 hours prior to infection. Cells were infected with a MOI of 5 of VACV and VACV-E3LΔ83N for 24 hours. At 1 hour and 24 hours, cells were harvested by scraping cells into media. The lysates underwent three freeze/thaws with 30min at -80C, 30 min on ice for each round of freeze/thaw. Virus was released by sonication and the resulting virus stocks were tittered in a monolayer of BSC40 and titers were calculated.

RESULTS

RIP3 expression vary among cancer cell lines. Many cancers downregulate RIP3 due to genomic methylation near its transcriptional start site. We screened different cancer cell lines to identify the levels of RIP3. The cancer cell lines that were screened included melanoma, glioblastoma, pancreatic cancer and breast cancer cell lines. Protein expression was determined using western blot analysis. We observed that RIP3 levels differed between the cell lines.

In murine cell lines, B16 – melanoma cell line and 4T1 - stage IV breast cancer line was shown to lack RIP3 expression in the presence and absence of mouse IFNα, when compared to RIP3 expressing L929 cells. The JC adenocarcinoma and E0771 breast adenocarcinoma were shown to express RIP3 in the presence and absence of mouse IFNα, when compared to RIP3 expressing L929 cells (Figure 24).

In human cancer cell lines, MDA-MB-435S melanoma, T-47D ductal carcinoma and HS578T ductal carcinoma were all shown to lack RIP3 expression. Conversely, the SKBR3 adenocarcinoma was shown to express RIP3. Panc-1, pancreatic carcinoma of ductal cell origin was shown to express RIP3. The glioblastoma cell lines that were screened, namely U87, U118, U251 and U343 were all shown to express RIP3 (Figure 23). HT29 cells expressing RIP3 were used as a positive control for this assay.

DAI expression is inducible in mouse cancer lines and is constitutive in human cancer cell lines. We screened the different cancer cell lines for DAI expression

since N-terminal deletion mutants of VACV undergo DAI- dependent necroptotic cell death and our goal is to identify the potential use of these viruses in oncolytic virotherapy.

All the human cell lines, SKBR3, MDA-MB-435S, T-47D, HS578T, Panc -1, U87, U87, U118, U251 and U343 have constitutive DAI expression (Figure 23). In murine cell lines, E0771, B16 and 4T1 have inducible DAI while treated with mouse IFNα while JC cell line has constitutive DAI (Figure 24). These results indicate that the N-terminal deletion mutant can selectively replicate in cells with deficient necroptosis pathway leading to potential as oncolytic virotherapy.

Necroptotic cell death is visualized with TNFα+ZVD treatment in cells with intact necroptosis pathway. We sought to determine the functionality of the necroptotic pathway with TNFα+ZVD treatment in cells. This assay identified the cells where the pathway is deficient signifying the potential cancer cell line candidates for VACV replication. In murine cell lines, L929 cell line was used as a positive control. JC and E0771 cell lines with intact RIP3 and DAI underwent necroptotic cell death as indicated by the uptake of propidium iodide stain signifying the loss of cell membrane integrity. B16 and 4T1 cell lines have loss in RIP3 expression as shown by western blots. These cell lines do not undergo necroptosis as indicated by healthy, viable cells stained with Hoechst dye (Figure 28). In human cell lines, HT 29 cells were used as the positive control. We observed that in SkBr3 cells, necroptotic cell death was observed. We did not observe necroptosis in MDA-MB-435S, T-47D and HS578T cells (Figure 26). These results correlate with the western blot observations that cell death is observed in the

presence of an intact necroptotic pathway. Our results in glioblastoma cells indicate that the presence or absence of MLKL should also be evaluated while identifying an intact necroptotic pathway. Although all four of the glioblastoma cells were expressing DAI and RIP3, cell death was observed only in U118 cell line indicating that the other lines namely U87, U251 and U353 were potentially not expressing MLKL leading to cell survival (Figure 27).

Cell viability of N-terminal deletion mutant of VACV correlates with loss of necroptotic pathway in cancer cell lines. Murine B16 and E0771 cell lines were chosen to characterize the replication competency of N-terminal deletion mutant of VACV. As previously noted, E0771 has an intact necroptotic pathway. The cells were infected with VACV E3LΔ54N and VACV E3LΔ83N at a MOI 5 and cell viability was assessed with propidium iodide staining. We observe that there is increased cell death during N-terminal deletion mutants' viral infection in these cells. In B16 cells, the loss of RIP3 expression, led to the inability of VACV E3LΔ54N and VACV E3LΔ83N to cause cell death in these cells. This was observed with the cells staying viable and there was no significant observation of propidium iodide uptake in these cells (Figure 29).

pMLKL is detected during VACV E3LΔ83N infection in E0771 cells. E0771 cells have an intact DAI and RIP3 system. We assessed the presence of pMLKL as a marker during infection with VACV E3LΔ83N in E0771 cells. Our results indicate that during infection with wtVACV, pMLKL was not detected in these cells. However, during infection with VACV E3LΔ83N, we detected pMLKL in these cells. The positive control

used in the experiment was treatment of E0771 cells with TNFα+zVAD that leads to pMLKL expression (Figure 30).

Type 1 IFN sensitivity correlates with presence of an active necroptotic pathway. Type 1 IFN system is involved in anti-viral responses. During viral infections, production of IFN primes the cells for programmed cell death. We assessed the IFN sensitivity of N-terminal deletion mutant VACV E3LΔ54N and VACV E3LΔ83N in E0771 cells by determining the growth kinetics during single step growth curve. Our results indicate that wtVACV replicates to high titers with or without mouse IFNα. During VACV E3LΔ83N replication, there is a 3-log reduction in titers in the presence of mouse IFNα and replication was inhibited during VACV E3LΔ54N infection in the presence of mouse IFNα. These results indicate that the inhibition of necroptosis correlate with VACV replication in E0771 cell line (Figure 31).

DISCUSSION

The potential use of N-terminal deletion mutants of VACV as an oncolytic virus is likely to be dependent on the necroptosis pathway in the cancerous cells. We screened various cancer cell lines to determine the presence or absence of the necroptosis pathway. These included melanoma, breast cancer, glioblastoma and pancreatic cancer cell lines. Both murine and human cell lines were screened to identify potential lines that can be served as mouse model system to test the efficacy of the potential oncolytic viruses.

It has been published that most cancer cell lines have a deficient RIP3 expression(69). We sought to identify the cell lines for their RIP3 expression. In human cell lines, SKBR3, Panc-1 and all the screened glioblastoma cells expressed RIP3 whereas T-47D, HS578T and MDA-MB-435S were all RIP3 negative. In murine cell lines, JC and E0771 expressed RIP3 while BALB/C 4T1 and C57BL/6J B16 were both RIP3 negative. These results indicate the potential cell lines where the N-terminal deletion mutant of VACV would replicate.

Since, VACV E3Δ83N and VACV E3Δ54N undergo DAI – dependent necroptotic cell death, we also screened the cell lines for DAI expression. We observed that all human cancer cell lines expressed constitutive DAI expression suggesting that the presence of type 1 interferon was not necessary for activation in these cells. In murine cell lines, only JC cells expressed constitutive DAI expression while B16, E0771 and 4T1 expressed IFN inducible DAI expression.

In order to test the functionality of the necroptosis pathway, we performed cell viability assays. We used the classical inducer of necroptotic cell death TNFα and ZVD to screen for cells undergoing necroptotic cell death. As hypothesized, cells with active necroptotic pathway underwent necroptotic cell death as evidenced by the uptake of propidium iodide stain.

In order to test the replication of N-terminal deletion mutant of VACV, we chose two murine cell lines B16 and E0771. We infected cells with VACV E3Δ83N and VACV E3Δ54N and observed for cell death. AT 4hpi, we observe that B16 cells that were shown to be RIP3 deficient were viable while the E0771 cells having an intact necroptotic pathway had significant increase in cell death. These results indicate that N-terminal deletion mutant of VACV could be used as a potential oncolytic virus in cells without an active necroptotic pathway. The results also correlate with replication data, where we observed that in E0771 cells, presence of moue IFNα reduced the replication of N-terminal deletion mutant of VACV.

Previous studies in the lab that *in vitro*, VACV E3Δ54N selectively replicated in certain cancer cell lines and also demonstrated that in a xenograft model of cancer using MDA-MB-435s cells, VACV E3Δ54N significantly reduced tumor volume in mice. At that time, the correlation to necroptosis was not identified. The discovery of necroptosis as a pathway to inhibit replication has advanced our understanding of the biological significance of programmed cell death pathways during VACV replication and spread.

In the current study, potential cancer cell lines where replication of N-terminal VACV mutants correlated with loss in necroptotic pathway have been identified. Future work in this direction includes characterizing the effects of viral treatment in a syngeneic mouse model to determine the response of a functioning immune system to VACV treatment. These studies will provide significant insight into the functioning of the biological and immune response when tumors are treated with viruses

FIGURES

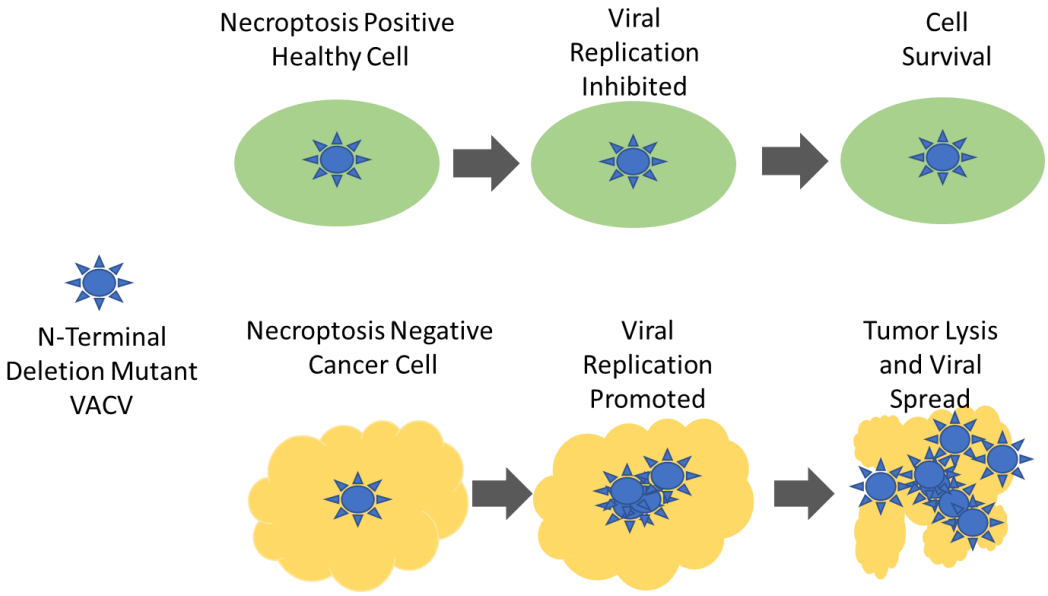

Figure 22: Proposed Mechanism for Potential Use of N-terminal deletion Mutant of VACV as an Oncolytic Virus

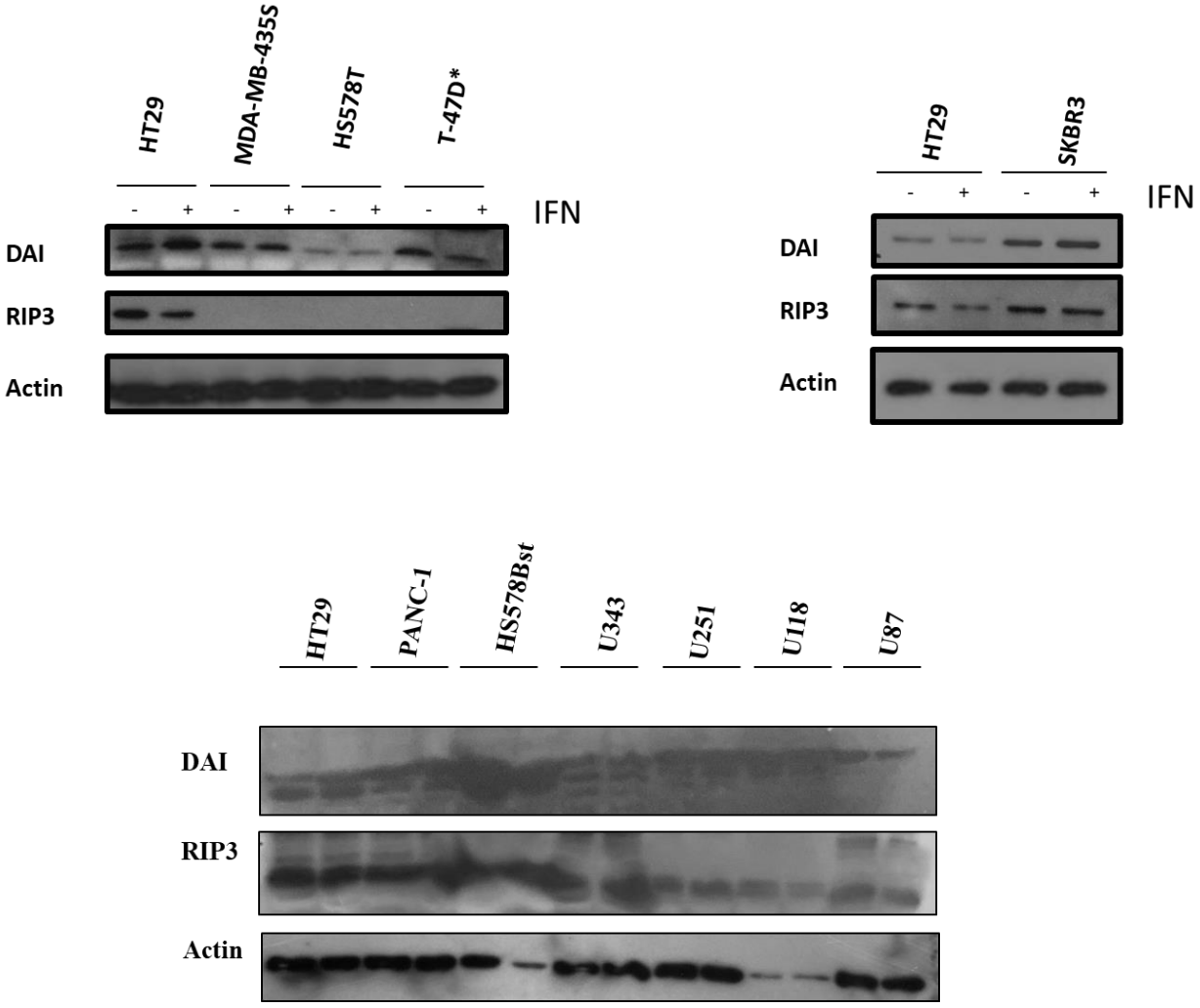

Figure 23: RIP3 and DAI Expression Levels in Human Cancer Cell Lines. Protein expression levels were determined for the given human cancer cell lines. Cells either had no pre-treatment or had treatment with 1000U/ml of universal IFN for 16-18 hours prior to harvest. Harvested cells were lysed and probed for protein expression.

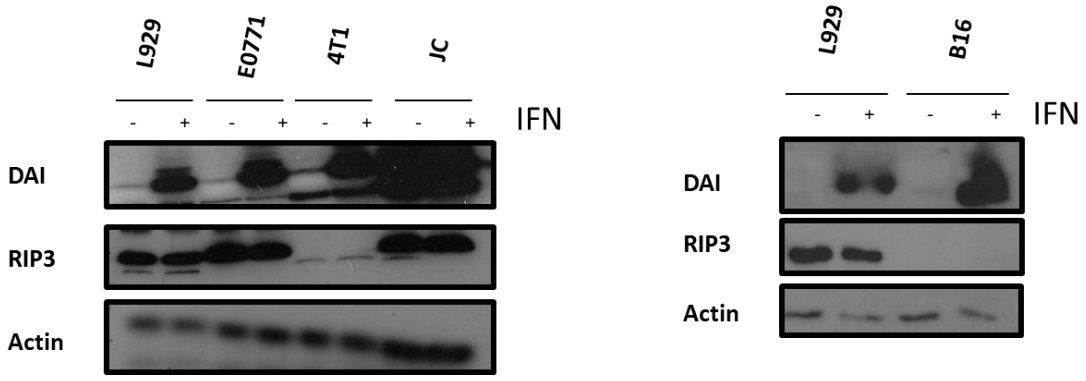

Figure 24: RIP3 and DAI Expression Levels in Murine Cancer Cell Lines. Protein expression levels were determined for the given murine cancer cell lines. Cells either had no pre-treatment or had treatment with 100U/ml of mouse IFNα for 16-18 hours prior to harvest. Harvested cells were lysed and probed for protein expression.

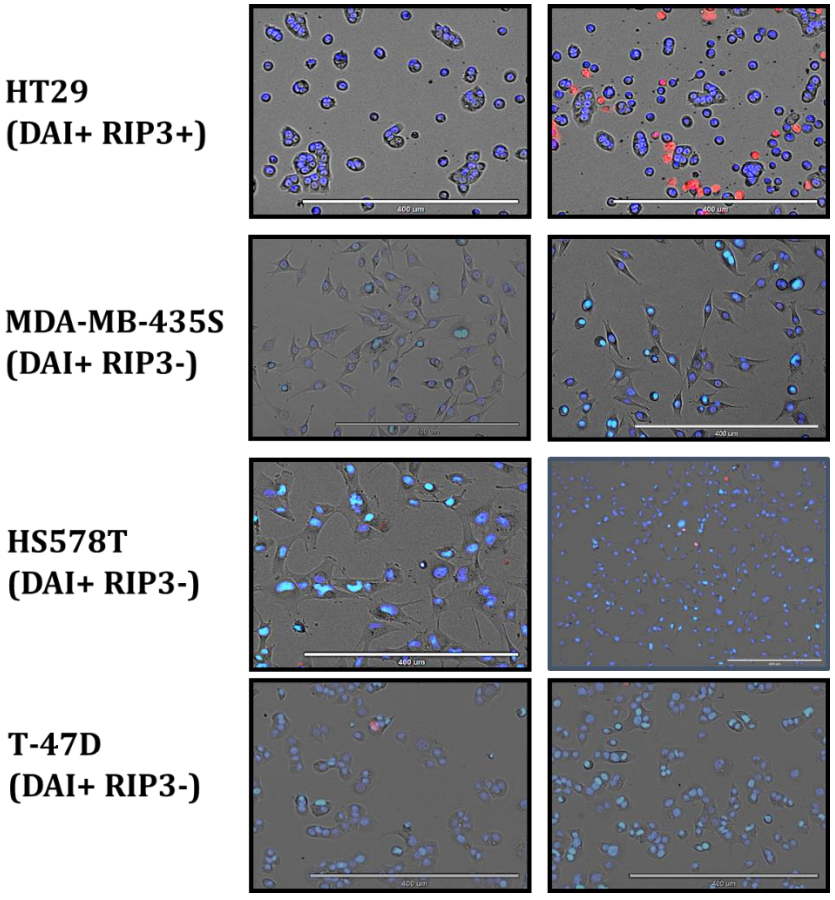

Figure 25: Cell Viability Assay in Human Cancer Cell Lines. Fluorescent microscopy of human melanoma post treatment with TNFα, zVAD-fmk and SMAC mimetic. Blue (Hoescht) represents nuclear staining and Red (Propidium Iodide) indicates cell death.

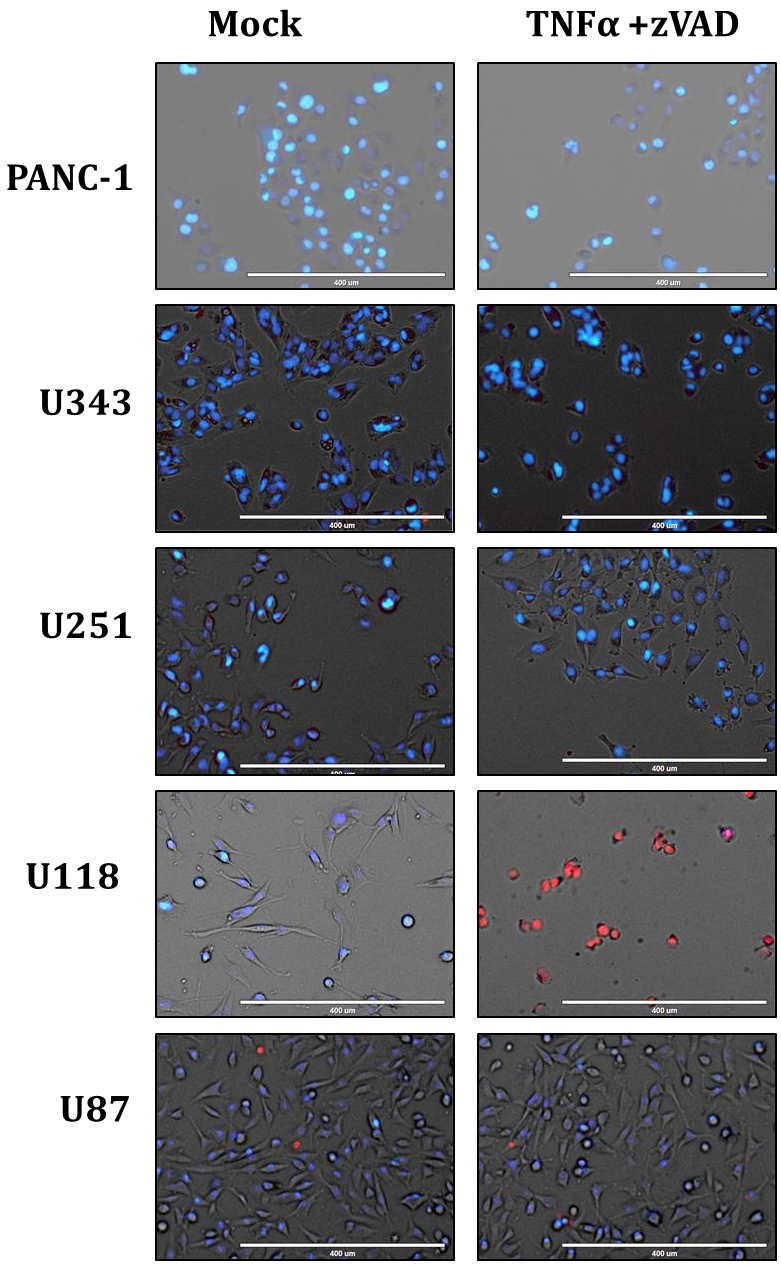

Figure 26: Cell Viability Assay in Human Cancer Cell Lines. Fluorescent microscopy of human melanoma post treatment with TNFα, zVAD-fmk and SMAC mimetic. Blue (Hoescht) represents nuclear staining and Red (Propidium Iodide) indicates cell death.

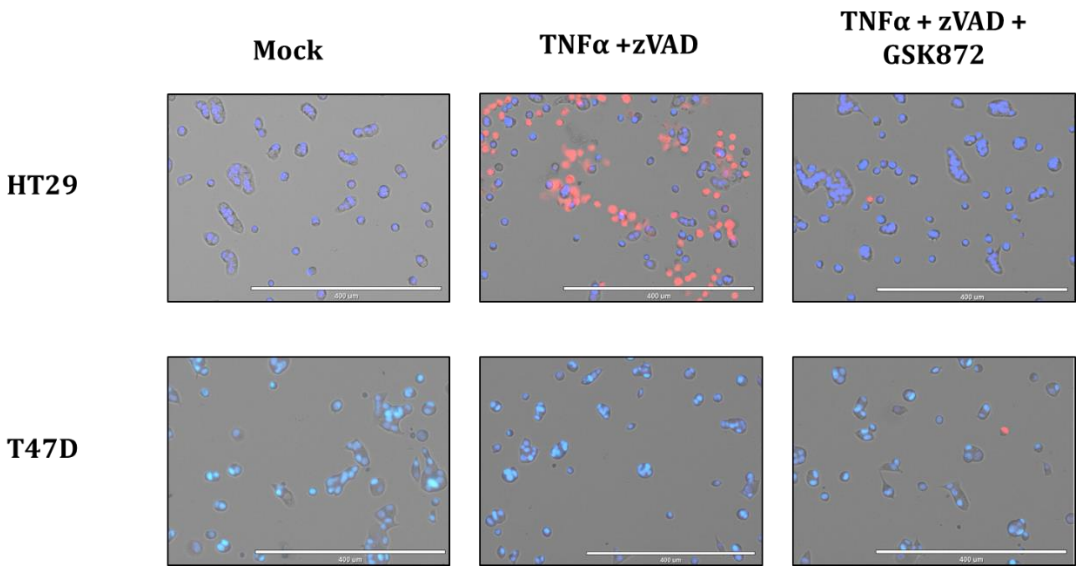

Figure 27: Cell Viability Assay in Human Cancer Cell Lines. Fluorescent microscopy representative human cancers post treatment with TNFα, zVAD-fmk and SMAC mimetic, LCL161, +/- RIP3 inhibitor GSK872. Blue (Hoescht) represents nuclear staining and Red (Propidium Iodide) indicates cell death.

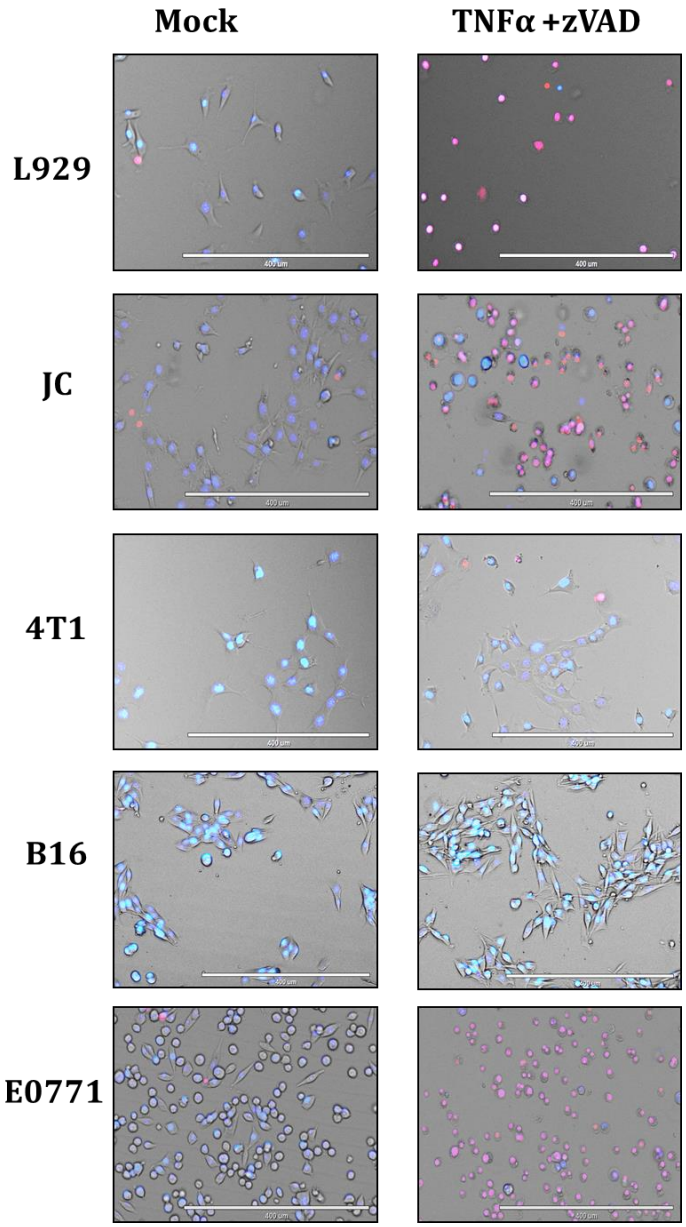

Figure 28: Cell Viability Assay in Murine Cancer Cell Lines. Fluorescent microscopy of murine cancer cells post treatment with TNFα, zVAD-fmk and SMAC mimetic. Blue (Hoescht) represents nuclear staining and Red (Propidium Iodide) indicates cell death.

`

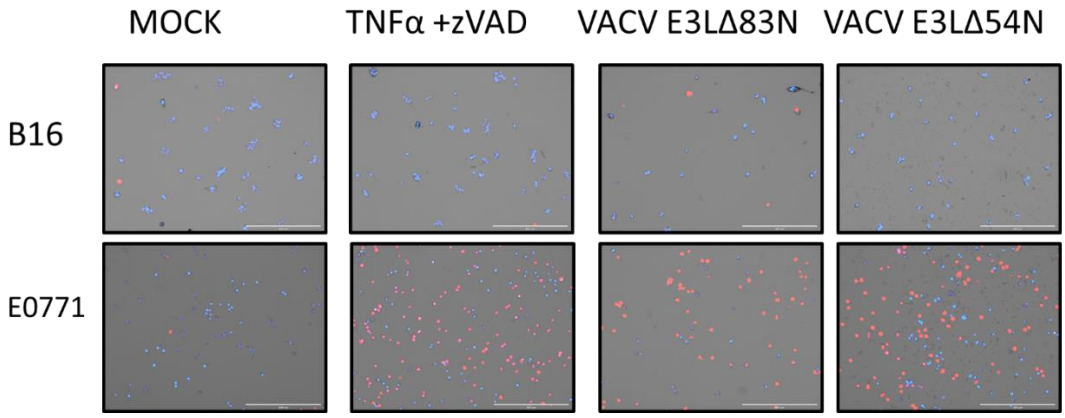

Figure 29: Cell Viability Assay after Viral Infection in B16 and E0771 cells. Fluorescent microscopy of B16 and E0771 cells. Cells were either mock-treated, treated with TNFα and zVAD-fmk or with N-terminal deletion mutatnt of VACV – VACV-E3LΔ83N or VACV-E3LΔ37N . Blue (Hoescht) represents nuclear staining and Red (Propidium Iodide) indicates cell death.

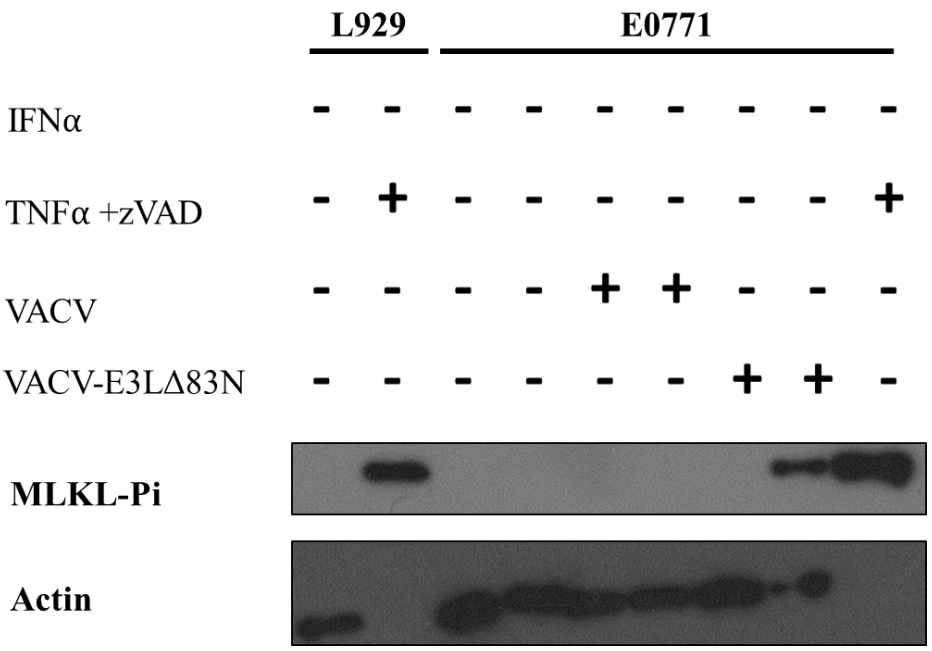

Figure 30: pMLKL Expression in E0771 Cells During VACV-E3LΔ83 Infection.
Cells treated/infected with mouse universal IFNα, TNFα/zVAD and VACV as indicated.

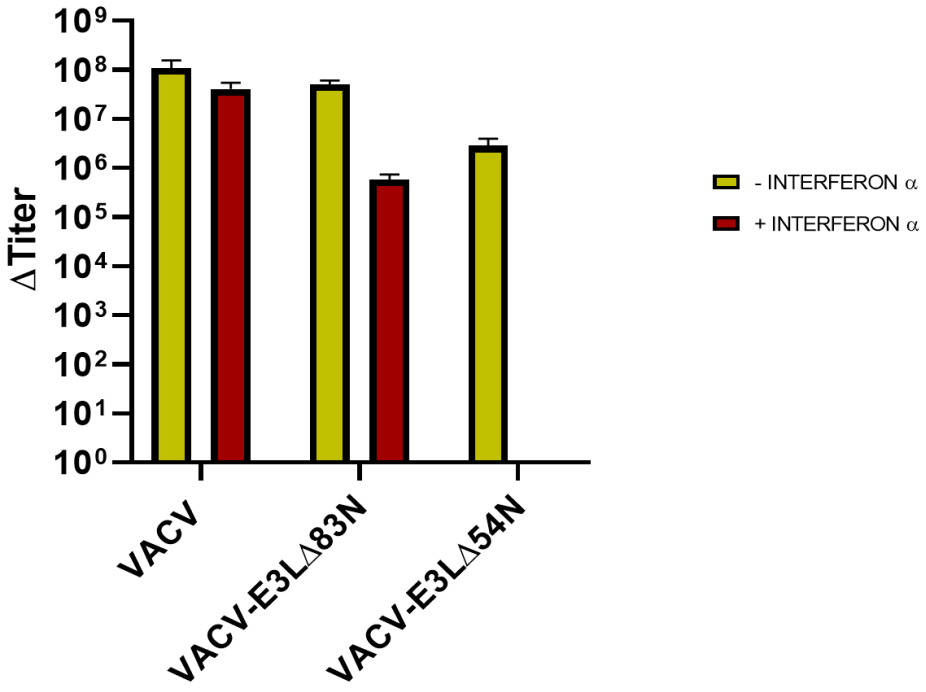

Figure 31: Single Step Growth Curve in E0771 cells. The cells were infected with wtVACV, VACV-E3LΔ83N and VACV-E3LΔ54N at a MOI of 5. Viruses were harvested at 0 and 24 hpi and tittered by plaque assay in BSC40 cells.

CHAPTER 5

OVERALL DISCUSSION

The E3L gene of vaccinia virus (VACV) encodes a protein that contains two domains: dsRNA binging domain in the C-terminus and Z-NA binding domain in the N-terminus. Both the domains are necessary for IFN resistance and pathogenesis in mice. Recently, it has been identified that the N-terminus of E3 is necessary to inhibit DAI-dependent necroptotic cell death in cells in culture. In this investigation, the role of necroptosis in poxvirus pathogeneses and its potential outcome in oncolytic virotherapy was dissected.

N-terminal deletion mutant of VACV (VACV E3Δ83N) is not pathogenic in C57BL/6J mice but in mice devoid of IFN receptors ($IFNAR^{-/-}$), the virus becomes pathogenic. We demonstrate that the results correlate when we treat C57BL/6J mice with mouse IFNα along with the virus during an intranasal infection in mice. Our results indicate that the pathogenesis, measured as a result of clinical symptoms and weight loss, was significantly lower in mice treated with mouse IFNα along with VACV E3Δ83N compared to mice that were not treated with mouse IFNα. We also observed that during wtVACV infection, treatment with mouse IFNα did not significantly alter the pathogenesis score. These results indicated that *in vivo*, VACV E3Δ83N is IFN sensitive.

In order to determine if the IFN sensitivity of VACV E3Δ83N observed in mice is due to the necroptotic pathway, we utilized mice deficient in the necroptotic pathway. Our results demonstrate the in $RIP3^{-/-}$, $DAI^{-/-}$ and $MLKL^{-/-}$, the IFN sensitivity of VACV

E3Δ83N is abolished and pathogenesis is these mice are higher than that observed in C57BL/6J mice. We were also able to show that in C57BL/6J mice infected intranasally with VACV E3Δ83N, phosphorylation of MLKL is observed in nasal tissue while this phenomenon is not observed during wtVACV infection. These observations confirm that the N-terminus is necessary to inhibit DAI-dependent necroptotic pathway and thus, leading to pathogenesis in mice.

These results led us to further investigate the effect of a potential Z-NA-binding domain in E3 on programmed necrosis. We utilized point mutants of VACV that either retained the Z-NA binding ability (VACV_VACV-E3L-E42A) or lost the Z-NA binding ability (VACV_VACV-E3L-P63A) to study pathogenesis and compared the results to our previous study. We demonstrate that during VACV_VACV-E3L-P63A intranasal infection, C57BL/6Jmice have reduced pathogenesis and it is rescued in mice deficient in the necroptosis pathway ($DAI^{-/-}$, $RIP3^{-/-}$ and $MLKL^{-/-}$). During VACV_VACV-E3L-E42A infection, there is no significant difference between the groups and the pathogenesis is higher than that observed during VACV_VACV-E3L-P63A infection. These correlate with our predictions and confirm that the Z-NA binding domain of E3 has a significant effect on the inhibition of programmed necrosis for VACV and play a pivotal role during pathogenesis in mice.

Our model for VACV pathogenesis in mice is that Zα domain of N-terminus of E3 is replaceable by Zα domain of DAI. Hence, during wtVACV infection, E3 competes with DAI and prevents DAI sensing leading to inhibition of necroptosis. This results in viral replication and spread leading to pathogenesis observed in mice. During VACV

E3Δ83N infection, the loss of N-terminus leads to sensing by DAI leading to necroptosis and resulting in decreased viral replication and pathogenesis in mice.

Monkeypox virus (MPXV) is another member of the Orthopoxvirus genus that has recently emerged as a human pathogen. MPXV contains a natural truncation in the N-terminal Zα domain of its E3 homologue, F3. Since our results with VACV from the same genus indicate that Z-nucleic acid-binding function of the N terminus of E3 is important for inhibition of induction of necroptosis, we sought to understand its role during pathogenesis of MPXV in mice.

In order to compare MPXV pathogenesis to VACV, we utilized VACV with N-terminal truncation of 37 amino acids (VACV-E3LΔ37N). Our results indicate that during intranasal infection of C57Bl/6 mice with VACV-E3LΔ37N, pathogenesis was low, and this was restored in both $DAI^{-/-}$ and $MLKL^{-/-}$ mice, suggesting that the results are consistent with our observations with VACV-E3LΔ83N. Due to similar mutation in the N-terminal domain of MPXV, we predicted similar results. However, during MPXV infection, although the pathogenesis was lower in C57BL/6J mice, it was restored only in $DAI^{-/-}$ not in $MLKL^{-/-}$ mice. These results also correlate with *in vitro* data where in L929 cells, MPXV has intermediate sensitivity which was rescued in $DAI^{-/-}$ L929 cells and there was no MLKL trimerization that occurred in MPXV infected cells. VACV-E3LΔ83N replicates and spreads to the brain at a significantly higher titer in mice deficient in the necroptotic pathway. Data from MPXV pathogenesis study indicate that there was no significant difference in spread to brain and lungs during MPXV infection in $DAI^{-/-}$ mice compared to C57BL/6Jmice. We predict that the replication and spread could

have been higher to the distal organs leading to increased pathogenesis. These results collectively suggest during MPXV infections, DAI is activating other proteins that do not lead to necroptotic cell death.

Programmed cell death has been implicated in human diseases. In Chapter 4, we aimed to study the translational outcome of VACV undergoing necroptosis. Recently, necroptosis has been implicated in many cancers. About two-thirds of cancer are known to have a downregulated necroptotic pathway. Previous studies in the lab have identified that VACV-E3LΔ54N selectively replicates in cancerous cells and not in normal non-cancerous cells. In vivo, in a xenograft model of cancer with MDA-MB-435s tumors, regression of tumor was observed at a dose of $1x10^7$ pfu. The regression was observed in both treated and untreated tumors suggesting that the virus was able to translocate and replicate to the untreated side. In a syngeneic BALB/c model with 4T1 tumors, although complete tumor regression was not observed, the rate of tumor growth was decreased. These results indicated the potential of using N-terminal deletion mutant as an oncolytic agent.

The discovery of necroptosis inhibiting replication of N-terminal deletion mutant of VACV and the occurrence of downregulated necroptotic protein markers in many cancers, led to the hypothesis that N-terminal deletion mutant of VACV is a potential oncolytic agent in cancers with deficient necroptotic pathway. The screening was performed on various cancer cell lines such as melanoma, glioblastoma, pancreatic and breast cancer cell lines for the necroptotic markers. In the absence of an active necroptotic pathway, cancer cell lines undergo the classical TNFα+ZVAD death and the

N-terminal deletion mutants – VACV E3Δ83N and VACV E3Δ54N replicated in these cell lines. There is a vast scope for expansion of this research to further screening of various cancer cell types to identify necroptotic markers for developing personalized diagnostics in the field of oncolytic virotherapy.

This work establishes a relationship between necroptosis and poxvirus pathogenesis. VACV E3 does not contain an identifiable RHIM domain and thus, is believed to undergo a novel mechanism of inhibiting necroptosis. Data presented in this dissertation demonstrate that the N-terminus of VACV E3 is critical to inhibit necroptosis and induce pathogenesis in mice. However, VACV EΔ83N does not rescue pathogenesis to wtVACV levels in mice deficient in necroptosis pathway. This leads to the question if there are other IFN-inducible pathways that are playing a role during pathogenesis. It has been shown that during vaccinia infections, PKR activation and necroptosis are distinct mechanisms that the virus must evade. Previous research has shown that virulence of VACV-E3LΔ83N was not rescued in a PKR knock-out mouse model. However, it has also been demonstrated that during VACV-E3LΔ83N pathogenesis, eif2α phosphorylation is seen during intranasal infection that is absent during an intracranial infection. Presumably, role of inhibition of PKR and necroptosis by using VACV mutants that fail to inhibit PKR in pathogenesis will provide greater significance to understand the use of programmed necrosis as an innate host defense mechanism. Further research in understanding of the role of the Z-NA-BD in E3 can provide insight into the pathogenesis of monkeypox virus, as it contains a natural truncation in the N-terminal Zα domain of its E3 homologue, F3. The duality of MPXV in being pathogenic despite missing this

essential region of the E3 innate immune evasion protein is unique to MPXV. The results demonstrated in this work identify that a DAI-dependent, RIPK3- dependent inhibition of virus replication that is independent of necroptotic cell death occurs during MPXV infection in mice. Thus, it is imperative to identify the innate immune evasion mechanisms that occur during Orthopoxvirus infections to provide a better understanding of the most important re-emerging Orthopoxvirus disease in humans, MPXV.

To conclude, this research work also establishes the potential of using N-terminal deletion mutants as an oncolytic virus to target specific cancer cell lines. It is critical to target tumors with a combinatorial treatment as tumors consist of non-heterogenous population of cells. Further in the field of cancer research, chemotherapeutic agents are known to be effective against cells that have an upregulated RIP3 expression, Hence, when combined with VACV mutant that can selectively replicate in cells with deficient RIP3, this would be a highly efficient and effective oncolytic treatment. The further elucidation of mechanism of Z-NA-BD of Orthopoxviruses will be critical to development of effective VACV based vaccines and viral oncolytic therapies in medical research.

REFERENCES

1. Basler CF, GarcÍA-Sastre A. VIRUSES AND THE TYPE I INTERFERON ANTIVIRAL SYSTEM: INDUCTION AND EVASION. International Reviews of Immunology. 2002;21(4-5):305-37.

2. Kawai T, Akira S. The roles of TLRs, RLRs and NLRs in pathogen recognition. Int Immunol. 2009;21(4):317-37.

3. McCormick KD, Ghosh A, Trivedi S, Wang L, Coyne CB, Ferris RL, et al. Innate immune signaling through differential RIPK1 expression promote tumor progression in head and neck squamous cell carcinoma. Carcinogenesis. 2016;37(5):522-9.

4. Chang HW, Watson JC, Jacobs BL. The E3L gene of vaccinia virus encodes an inhibitor of the interferon-induced, double-stranded RNA-dependent protein kinase. Proceedings of the National Academy of Sciences of the United States of America. 1992;89(11):4825-9.

5. Langland JO, Jacobs BL. Inhibition of PKR by vaccinia virus: role of the N- and C-terminal domains of E3L. Virology. 2004;324(2):419-29.

6. Kaiser WJ, Upton JW, Mocarski ES. Viral modulation of programmed necrosis. Current Opinion in Virology. 2013;3(3):296-306.

7. Labbé K, Saleh M. Cell death in the host response to infection. Cell Death & Differentiation. 2008;15(9):1339-49.

8. Lamkanfi M, Dixit VM. Manipulation of host cell death pathways during microbial infections. Cell host & microbe. 2010;8(1):44-54.

9. Upton JW, Chan FK-M. Staying Alive: Cell Death in Antiviral Immunity. Molecular cell. 2014;54:273-80.

10. Jorgensen I, Rayamajhi M, Miao EA. Programmed cell death as a defence against infection. Nature Reviews Immunology. 2017;17(3):151-64.

11. Duprez L, Wirawan E, Berghe TV, Vandenabeele P. Major cell death pathways at a glance. Microbes and Infection. 2009;11(13):1050-62.

12. Elmore S. Apoptosis: A Review of Programmed Cell Death. Toxicologic Pathology. 2007;35(4):495-516.

13. Duprez L, Wirawan E, Vanden Berghe T, Vandenabeele P. Major cell death pathways at a glance. Microbes and infection. 2009;11(13):1050-62.

14. Nagata S, Tanaka M. Programmed cell death and the immune system. Nature Reviews Immunology. 2017;17(5):333-40.

15. Galluzzi L, Vitale I, Aaronson SA, Abrams JM, Adam D, Agostinis P, et al. Molecular mechanisms of cell death: recommendations of the Nomenclature Committee on Cell Death 2018. Cell Death and Differentiation. 2018;25(3):486-541.

16. Mocarski ES, Guo H, Kaiser WJ. Necroptosis: The Trojan horse in cell autonomous antiviral host defense. Virology. 2015;479-480:160-6.

17. Robinson N, McComb S, Mulligan R, Dudani R, Krishnan L, Sad S. Type I interferon induces necroptosis in macrophages during infection with Salmonella enterica serovar Typhimurium. Nature Immunology. 2012;13(10):954-62.

18. Hildebrand JM, Tanzer MC, Lucet IS, Young SN, Spall SK, Sharma P, et al. Activation of the pseudokinase MLKL unleashes the four-helix bundle domain to induce membrane localization and necroptotic cell death. Proceedings of the National Academy of Sciences of the United States of America. 2014;111(42):15072.

19. Kaiser WJ, Sridharan H, Huang C, Mandal P, Upton JW, Gough PJ, et al. Toll-like receptor 3-mediated necrosis via TRIF, RIP3, and MLKL. The Journal of biological chemistry. 2013;288(43):31268-79.

20. Moriwaki K, Chan F-M. RIP3: a molecular switch for necrosis and inflammation. Genes & Development. 2013;27(15):1640-9.

21. Guo ZS, Lu B, Guo Z, Giehl E, Feist M, Dai E, et al. Vaccinia virus-mediated cancer immunotherapy: cancer vaccines and oncolytics. Journal for ImmunoTherapy of Cancer. 2019;7(1):6.

22. Omoto S, Guo H, Talekar GR, Roback L, Kaiser WJ, Mocarski ES. Suppression of RIP3-dependent necroptosis by human cytomegalovirus. Journal of biological chemistry. 2015;290(18):11635-48.

23. Upton JW, Kaiser WJ, Mocarski ES. DAI/ZBP1/DLM-1 complexes with RIP3 to mediate virus-induced programmed necrosis that is targeted by murine cytomegalovirus vIRA. Cell host & microbe. 2012;11(3):290-7.

24. Kuriakose T, Man SM, Malireddi RKS, Karki R, Kesavardhana S, Place DE, et al. ZBP1/DAI is an innate sensor of influenza virus triggering the NLRP3 inflammasome and programmed cell death pathways. Sci Immunol. 2016;1(2):aag2045.

25. Guo H, Omoto S, Harris PA, Finger JN, Bertin J, Gough PJ, et al. Herpes simplex virus suppresses necroptosis in human cells. Cell host & microbe. 2015;17(2):243-51.

26. Mocarski ES, Upton JW, Kaiser WJ. Viral infection and the evolution of caspase 8-regulated apoptotic and necrotic death pathways. Nature reviews Immunology. 2011;12(2):79-88.

27. Koehler H, Cotsmire S, Langland J, Kibler KV, Kalman D, Upton JW, et al. Inhibition of DAI-dependent necroptosis by the Z-DNA binding domain of the vaccinia virus innate immune evasion protein, E3. Proceedings of the National Academy of Sciences. 2017;114(43):11506.

28. Fenner F, Henderson DA, Anita I, Jezele Z, Ladnyi ID.

29. Behbehani AM. The smallpox story: life and death of an old disease. Microbiological reviews. 1983;47(4):455-509.

30. Breman JG, Henderson DA. Diagnosis and Management of Smallpox. New England Journal of Medicine. 2002;346(17):1300-8.

31. Breman JG, Arita I, Fenner F. Preventing the return of smallpox. The New England journal of medicine. 2003;348(5):463-6.

32. Greenberg RN, Kennedy JS. ACAM2000: a newly licensed cell culture-based live vaccinia smallpox vaccine. Expert Opinion on Investigational Drugs. 2008;17(4):555-64.

33. Jacobs BL, Langland JO, Kibler KV, Denzler KL, White SD, Holechek SA, et al. Vaccinia virus vaccines: past, present and future. Antiviral research. 2009;84(1):1-13.

34. The global eradication of smallpox. Final Report of the Global Commission for the Certification of Smallpox Eradication. History of International Public Health. 1980;4:122 pp.

35. Buller RM, Palumbo GJ. Poxvirus pathogenesis. Microbiological reviews. 1991;55(1):80-122.

36. Stanford MM, McFadden G, Karupiah G, Chaudhri G. Immunopathogenesis of poxvirus infections: forecasting the impending storm. Immunology and cell biology. 2007;85(2):93-102.

37. Liu S-W, Katsafanas GC, Liu R, Wyatt LS, Moss B. Poxvirus Decapping Enzymes Enhance Virulence by Preventing the Accumulation of dsRNA and the Induction of Innate Antiviral Responses. Cell Host & Microbe. 2015;17(3):320-31.

38. García MA, Meurs EF, Esteban M. The dsRNA protein kinase PKR: virus and cell control. Biochimie. 2007;89(6-7):799-811.

39. Herbert A, Alfken J, Kim YG, Mian IS, Nishikura K, Rich A. A Z-DNA binding domain present in the human editing enzyme, double-stranded RNA adenosine deaminase. Proceedings of the National Academy of Sciences of the United States of America. 1997;94(16):8421-6.

40. Rich A. A role for Z-DNA binding in vaccinia virus pathogenesis. PNAS : Proceedings of the National Academy of Sciences of the United States of America. 2003;100(12):6974-9.

41. White SD, Jacobs BL. The Amino Terminus of the Vaccinia Virus E3 Protein Is Necessary To Inhibit the Interferon Response. Journal of Virology. 2012;86(10):5895-904.

42. Brandt TA, Jacobs BL. Both carboxy- and amino-terminal domains of the vaccinia virus interferon resistance gene, E3L, are required for pathogenesis in a mouse model. Journal of virology. 2001;75(2):850-6.

43. Fine PE, Jezek Z, Grab B, Dixon H. The transmission potential of monkeypox virus in human populations. International journal of epidemiology. 1988;17(3):643-50.

44. Guarner J, Johnson BJ, Paddock CD, Shieh W-J, Goldsmith CS, Reynolds MG, et al. Monkeypox transmission and pathogenesis in prairie dogs. Emerg Infect Dis. 2004;10(3):426-31.

45. Khodakevich L, Jezek Z, Kinzanzka K. ISOLATION OF MONKEYPOX VIRUS FROM WILD SQUIRREL INFECTED IN NATURE. The Lancet. 1986;327(8472):98-9.

46. Zaucha GM, Jahrling PB, Geisbert TW, Swearengen JR, Hensley L. The Pathology of Experimental Aerosolized Monkeypox Virus Infection in

Cynomolgus Monkeys (Macaca fascicularis). Laboratory Investigation. 2001;81(12):1581-600.

47. Di Giulio DB, Eckburg PB. Human monkeypox: an emerging zoonosis. The Lancet Infectious Diseases. 2004;4(1):15-25.

48. Huhn GD, Bauer AM, Yorita K, Graham MB, Sejvar J, Likos A, et al. Clinical Characteristics of Human Monkeypox, and Risk Factors for Severe Disease. Clinical Infectious Diseases. 2005;41(12):1742-51.

49. Ježek Z, Szczeniowski M, Paluku KM, Mutombo M. Human Monkeypox: Clinical Features of 282 Patients. The Journal of Infectious Diseases. 1987;156(2):293-8.

50. Reynolds MG, Yorita KL, Kuehnert MJ, Davidson WB, Huhn GD, Holman RC, et al. Clinical Manifestations of Human Monkeypox Influenced by Route of Infection. The Journal of Infectious Diseases. 2006;194(6):773-80.

51. Parker S, Nuara A, Buller RML, Schultz DA. Human monkeypox: an emerging zoonotic disease. Future Microbiology. 2007;2(1):17-34.

52. Durski KN, McCollum AM, Nakazawa Y, Petersen BW, Reynolds MG, Briand S, et al. Emergence of Monkeypox - West and Central Africa, 1970-2017. MMWR Morb Mortal Wkly Rep. 2018;67(10):306-10.

53. Huhn GD, Bauer AM, Yorita K, Graham MB, Sejvar J, Likos A, et al. Clinical characteristics of human monkeypox, and risk factors for severe disease. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America. 2005;41(12):1742-51.

54. Khodakevich L, Jezek Z, Messinger D. Monkeypox virus: ecology and public health significance. Bulletin of the World Health Organization. 1988;66(6):747-52.

55. Reed KDMD, Melski JWMD, Graham MBMD, Regnery RLP, Sotir MJPMPH, Wegner MVMDMPH, et al. The Detection of Monkeypox in Humans in the Western Hemisphere. The New England Journal of Medicine. 2004;350(4):342-50.

56. Ng OT, Lee V, Marimuthu K, Vasoo S, Chan G, Lin RTP, et al. A case of imported Monkeypox in Singapore. The Lancet Infectious Diseases. 2019;19(11):1166.

57. Reed KD, Melski JW, Graham MB, Regnery RL, Sotir MJ, Wegner MV, et al. The detection of monkeypox in humans in the Western Hemisphere. The New England journal of medicine. 2004;350(4):342-50.

58. Rezza G. Emergence of human monkeypox in west Africa. The Lancet Infectious Diseases. 2019;19(8):797-9.

59. Vaughan A, Aarons E, Astbury J, Balasegaram S, Beadsworth M, Beck CR, et al. Two cases of monkeypox imported to the United Kingdom, September 2018. Euro Surveill. 2018;23(38):1800509.

60. Noam E, Hagit A, Elad M, Yuval S, Yonit W-W, Nir P, et al. Diagnosis of Imported Monkeypox, Israel, 2018. Emerging Infectious Disease journal. 2019;25(5):980.

61. Chen N, Li G, Liszewski MK, Atkinson JP, Jahrling PB, Feng Z, et al. Virulence differences between monkeypox virus isolates from West Africa and the Congo basin. Virology. 2005;340(1):46-63.

62. Arndt WD, White SD, Johnson BP, Huynh T, Liao J, Harrington H, et al. Monkeypox virus induces the synthesis of less dsRNA than vaccinia virus, and is more resistant to the anti-poxvirus drug, IBT, than vaccinia virus. Virology. 2016;497:125-35.

63. Arndt WD. Characterization of the E3L amino-terminus in poxvirus replication and tumor regression [Ph.D.]. Ann Arbor: Arizona State University; 2010.

64. Fuchs Y, Steller H. Programmed Cell Death in Animal Development and Disease. Cell. 2011;147(4):742-58.

65. Lockshin RA, Zakeri Z. Cell death in health and disease. J Cell Mol Med. 2007;11(6):1214-24.

66. Gong Y, Fan Z, Luo G, Yang C, Huang Q, Fan K, et al. The role of necroptosis in cancer biology and therapy. Molecular Cancer. 2019;18(1):100.

67. Najafov A, Chen H, Yuan J. Necroptosis and Cancer. Trends Cancer. 2017;3(4):294-301.

68. Su Z, Yang Z, Xie L, DeWitt JP, Chen Y. Cancer therapy in the necroptosis era. Cell Death & Differentiation. 2016;23(5):748-56.

69. Koo G-b, Morgan MJ, Lee D-g, Kim W-j, Yoon J-h, Koo JS, et al. Methylation-dependent loss of RIP3 expression in cancer represses programmed necrosis in response to chemotherapeutics. Cell Research. 2015;25(6):707-25.

70. Chan WM, McFadden G. Oncolytic Poxviruses. Annual Review of Virology. 2014;1(1):191-214.

71. Dunn GP, Bruce AT, Sheehan KCF, Shankaran V, Uppaluri R, Bui JD, et al. A critical function for type I interferons in cancer immunoediting. Nature immunology. 2005;6(7):722-9.

72. Varghese S, Rabkin SD. Oncolytic herpes simplex virus vectors for cancer virotherapy. Cancer gene therapy. 2002;9(12):967-78.

73. Bos JL. ras oncogenes in human cancer: a review. Cancer research. 1989;49(17):4682-9.

74. Thorne SH, Bartlett DL, Kirn DH. The use of oncolytic vaccinia viruses in the treatment of cancer: a new role for an old ally? Current gene therapy. 2005;5(4):429-43.

75. Chaurasiya S, Chen NG, Fong Y. Oncolytic viruses and immunity. Current Opinion in Immunology. 2018;51:83-90.

76. Kimata H, Imai T, Kikumori T, Teshigahara O, Nagasaka T, Goshima F, et al. Pilot Study of Oncolytic Viral Therapy Using Mutant Herpes Simplex Virus (HF10) Against Recurrent Metastatic Breast Cancer. Annals of Surgical Oncology. 2006;13(8):1078-84.

77. Lawler SE, Speranza M-C, Cho C-F, Chiocca EA. Oncolytic Viruses in Cancer Treatment: A Review. JAMA Oncology. 2017;3(6):841-9.

78. Russell SJ, Peng K-W, Bell JC. Oncolytic virotherapy. Nature Biotechnology. 2012;30(7):658-70.

79. Zheng M, Huang J, Tong A, Yang H. Oncolytic Viruses for Cancer Therapy: Barriers and Recent Advances. Molecular Therapy - Oncolytics. 2019;15:234-47.

80. Harrington K, Freeman DJ, Kelly B, Harper J, Soria J-C. Optimizing oncolytic virotherapy in cancer treatment. Nature Reviews Drug Discovery. 2019;18(9):689-706.

81. Andtbacka RHI, Kaufman HL, Collichio F, Amatruda T, Senzer N, Chesney J, et al. Talimogene Laherparepvec Improves Durable Response Rate in Patients With Advanced Melanoma. Journal of Clinical Oncology. 2015;33(25):2780-8.

82. Kohlhapp FJ, Kaufman HL. Molecular Pathways: Mechanism of Action for Talimogene Laherparepvec, a New Oncolytic Virus Immunotherapy. Clinical Cancer Research. 2016;22(5):1048.

83. Conry RM, Westbrook B, McKee S, Norwood TG. Talimogene laherparepvec: First in class oncolytic virotherapy. Hum Vaccin Immunother. 2018;14(4):839-46.

84. Harrington KJ, Puzanov I, Hecht JR, Hodi FS, Szabo Z, Murugappan S, et al. Clinical development of talimogene laherparepvec (T-VEC): a modified herpes simplex virus type-1–derived oncolytic immunotherapy. Expert Review of Anticancer Therapy. 2015;15(12):1389-403.

85. Raman SS, Hecht JR, Chan E. Talimogene laherparepvec: review of its mechanism of action and clinical efficacy and safety. Immunotherapy. 2019;11(8):705-23.

86. Arndt W, Mitnik C, Denzler KL, White S, Waters R, Jacobs BL, et al. In Vitro Characterization of a Nineteenth-Century Therapy for Smallpox. Plos One. 2012;7(3):9.

87. Beattie E, Kauffman EB, Martinez H, Perkus ME, Jacobs BL, Paoletti E, et al. Host-range restriction of vaccinia virus E3L-specific deletion mutants. Virus genes. 1996;12(1):89-94.

88. Brandt T, Heck MC, Vijaysri S, Jentarra GM, Cameron JM, Jacobs BL. The N-terminal domain of the vaccinia virus E3L-protein is required for neurovirulence, but not induction of a protective immune response. Virology. 2005;333(2):263-70.

89. Denzler KL, Babas T, Rippeon A, Huynh T, Fukushima N, Rhodes L, et al. Attenuated NYCBH vaccinia virus deleted for the E3L gene confers partial protection against lethal monkeypox virus disease in cynomolgus macaques. Vaccine. 2011;29(52):9684-90.

90. Jacobs BL, Langland JO. When two strands are better than one: the mediators and modulators of the cellular responses to double-stranded RNA. Virology. 1996;219(2):339-49.

91. Kibler KV, Shors T, Perkins KB, Zeman CC, Banaszak MP, Biesterfeldt J, et al. Double-stranded RNA is a trigger for apoptosis in vaccinia virus-infected cells. Journal of virology. 1997;71(3):1992-2003.

92. Mitnik CR. Poxvirus interferon -resistance genes: Their evolutionary past and role in anti-cancer therapy [Ph.D.]. Ann Arbor: Arizona State University; 2004.

93. Watson JC, Chang HW, Jacobs BL. Characterization of a vaccinia virus-encoded double-stranded RNA-binding protein that may be involved in inhibition of the double-stranded RNA-dependent protein kinase. Virology. 1991;185(1):206-16.

94. Samuel CE. Antiviral actions of interferons. Clinical microbiology reviews. 2001;14(4):778-809, table of contents.

95. Ivashkiv LB, Donlin LT. Regulation of type I interferon responses. Nature reviews Immunology. 2014;14(1):36-49.

96. Perry AK, Chen G, Zheng D, Tang H, Cheng G. The host type I interferon response to viral and bacterial infections. Cell Research. 2005;15(6):407-22.

97. Seet BT, Johnston JB, Brunetti CR, Barrett JW, et al. Poxviruses and immune evasion. Annual Review of Immunology. 2003;21:377-423.

98. Upton JW, Kaiser WJ, Mocarski ES. Virus inhibition of RIP3-dependent necrosis. Cell host & microbe. 2010;7(4):302-13.

99. Wu XN, Yang ZH, Wang XK, Zhang Y, Wan H, Song Y, et al. Distinct roles of RIP1-RIP3 hetero- and RIP3-RIP3 homo-interaction in mediating necroptosis. Cell death and differentiation. 2014;21(11):1709-20.

100. Makarova O, Kamberov E, Margolis B. Generation of deletion and point mutations with one primer in a single cloning step. BioTechniques. 2000;29(5):970-2.

101. Arndt WD, Cotsmire S, Trainor K, Harrington H, Hauns K, Kibler KV, et al. Evasion of the Innate Immune Type I Interferon System by Monkeypox Virus. Journal of virology. 2015;89(20):10489-99.

102. Upton JW, Kaiser WJ, Mocarski ES. DAI/ZBP1/DLM-1 Complexes with RIP3 to Mediate Virus-Induced Programmed Necrosis that Is Targeted by Murine Cytomegalovirus vIRA. Cell Host & Microbe. 2019;26(4):564.

103. Kirn DH, Wang Y, Liang W, Contag CH, Thorne SH. Enhancing poxvirus oncolytic effects through increased spread and immune evasion. Cancer research. 2008;68(7):2071-5.

104. Holler N, Zaru R, Micheau O, Thome M, Attinger A, Valitutti S, et al. Fas triggers an alternative, caspase-8-independent cell death pathway using the kinase RIP as effector molecule. Nature immunology. 2000;1(6):489-95.

105. Kaiser WJ, Upton JW, Mocarski ES. Receptor-interacting protein homotypic interaction motif-dependent control of NF-kappa B activation via the DNA-dependent activator of IFN regulatory factors. Journal of immunology (Baltimore, Md : 1950). 2008;181(9):6427-34.

106. Wang H, Sun L, Su L, Rizo J, Liu L, Li-Feng W, et al. Mixed Lineage Kinase Domain-like Protein MLKL Causes Necrotic Membrane Disruption upon Phosphorylation by RIP3. Molecular cell. 2014;54:133-46.

107. Zhang D-W, Shao J, Lin J, Zhang N, Lu B-J, Lin S-C, et al. RIP3, an energy metabolism regulator that switches TNF-induced cell death from apoptosis to necrosis. Science (New York, NY). 2009;325(5938):332-6.

108. Kahmann JD, Wecking DA, Putter V, Lowenhaupt K, Kim Y-G, Schmieder P, et al. The solution structure of the N-terminal domain of E3L shows a tyrosine conformation that may explain its reduced affinity to Z-DNA in vitro. Proceedings of the National Academy of Sciences of the United States of America. 2004;101(9):2712-7.