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THE ROLE OF THE COMPLEMENT SYSTEM IN MOUSEPOX INFECTION

by

Elizabeth Andrea Moulton

A dissertation presented to the
Graduate School of Arts and Sciences
of Washington University in
partial fulfillment of the
requirements for the degree
of Doctor of Philosophy

May 2011

Saint Louis, Missouri

ABSTRACT OF THE DISSERTATION

The Role of the Complement System in Mousepox Infection

by

Elizabeth Andrea Moulton

Doctor of Philosophy in Biology and Biomedical Sciences

Molecular Microbiology and Microbial Pathogenesis

Washington University in St. Louis, 2011

Professor John P. Atkinson, Chairperson

Poxviruses subvert the host immune response by producing immunomodulatory virulence factors, including a complement regulatory protein. Ectromelia virus provides a mouse model for human smallpox infection where the virus and the host's immune response have also co-evolved. Using this model, we investigated the role of the complement system in a poxvirus infection. Ectromelia virus inoculated via multiple routes caused increased mortality by 7 to 10 days post-infection in C57BL/6 mice that lack C3, the central component of the complement cascade. In C3^{-/-} mice, ectromelia virus disseminated earlier to target organs and generated higher peak titers compared to the congenic controls. Specifically, increased hepatic inflammation and necrosis correlated with these higher tissue titers and likely contributed to the morbidity in the C3^{-/-} mice. *In vitro*, the complement system in naïve C57BL/6 mouse sera neutralized ectromelia virus, primarily through the recognition of the virion by natural antibody and activation of the classical and alternative pathways. Sera deficient in classical or alternative pathway components or antibody had

reduced ability to neutralize viral particles, which likely contributed to increased viral dissemination and disease severity *in vivo*. The increased mortality of C4^{-/-} or Factor B^{-/-} mice also indicates that these two pathways of complement activation are required for survival. The importance of complement in the immune response to poxviruses accounts for why the virus encodes a potent complement regulatory protein, Ectromelia Inhibitor of Complement Enzymes (EMICE). Infected cells produce EMICE within 6-8 hours of infection. Recombinant EMICE produced in *E. coli* has cofactor activity against C3 and C4, regulates mouse complement, and protects ectromelia virus *in vitro*. In summary, the complement system acts in the first few minutes, hours, and days to control this poxviral infection, and the virus counters this activity through production of its regulatory protein EMICE.

ACKNOWLEDGMENTS

I wish to thank my mentor, John Atkinson, for his support professionally and personally during our entire time working together. I am inspired by his immeasurable curiosity and unending dedication that enable him to balance his research with his clinical responsibilities. Despite his many obligations, he always made time for me and was willing to assist with whatever was needed, which includes offering to weigh mice over the holidays.

When I started, I did not realize that I would have the benefit of a second mentor, Mark Buller. I thank him for his wisdom and guidance, which extends far beyond this project. This research could never have happened without his encouragement and expertise.

The EMICE studies were performed in collaboration with Paula Bertram. In addition to her supportive friendship since we began in the lab together, she produced the recombinant PICEs, characterized their regulatory activity *in vitro*, and performed the EMICE Western blots. This work was also aided by Kathy Liszewski's expertise on complement regulation.

I have benefited immeasurably from the generous assistance of the skilled members of the Buller laboratory. Ed Hembrador, who is always willing to help with anything, generated the viral stocks and performed viral infectivity assays. Scott Parker established the quantitative PCR protocol, in addition to thoughtful discussions. Christina Oberle and Erin Touchette provided assistance and instruction in the mouse studies. Nanhai Chen generated the Δ EMICE virus. Finally, Jill Schriewer helped generate the rescue +EMICE virus, and all of the work I performed in the Buller laboratory benefited from her vast knowledge and talent.

The histopathological analysis was enhanced by the expertise of Grant Kolar and John Sagartz, and samples were prepared by the Digestive Diseases Research Core Center.

I am grateful to all the other members of the Atkinson laboratory who all contributed in their own way to this work. Specifically, Marilyn Leung and Xiaobo Wu aided with the numerous complement-deficient mice used in this work. Madonna Bogacki helped me navigate through the journal submission process. Claudia Kemper's support and encouragement extended far beyond science. In addition to sharing a bay with me, Amy Caudy and Celia Fang were superb companions and confidants on this journey through science.

I would like to thank the members of my thesis committee for their guidance of this project. It owes much of its success to them. I particularly appreciate Michael Diamond's critical reading of my manuscripts and advice throughout my training.

This work was also aided greatly by Erin Mehlhop, who helped us acquire and maintain the complement-deficient mice; Ram Akilesh, who shared his knowledge of quantitative PCR and other techniques; and Chung Lee, who kindly provided the IL-18 binding protein.

I am grateful to the investigators whose generous gift of the complement-deficient mice made most of this work possible. The MSTP training grant, Pulmonary and Critical Care training grant, DBBS, and Rheumatology division supported me during this work.

Finally, I thank my friends and family for going through this with me. Some of the times have been challenging, but we have made it after all.

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Chapter 1

Introduction

Clinical Significance

Poxviruses remain a threat to the human population despite the eradication decades ago of naturally circulating variola virus (VARV), the causative agent of smallpox. Smallpox, with its up to 30% mortality rate, could devastate the large unvaccinated population if released accidentally or by bioterrorists [1]. Closely related monkeypox virus (MPXV) has also emerged as a human pathogen, and certain strains of MPXV cause a disease that is clinically indistinguishable from smallpox [2]. A less virulent strain of MPXV has already caused one outbreak in the United States [3]. The current smallpox vaccine can cause life threatening complications [4]. Antiviral options are limited and ineffective at treating smallpox or vaccine related disease. A better understanding of poxviral pathogenesis is required to devise novel therapeutic options for smallpox and other emerging poxviral infections. Furthermore, the study of this family of infectious agents has provided insights into viral pathogenesis and revealed novel facets of the host's immune response.

Poxviruses' Infection and Replication

Within the poxviridae family of large (~200 kb) double-stranded DNA viruses, the orthopoxvirus genera includes VARV, MPXV, vaccinia virus (VACV), and mousepox' (ectromelia virus, ECTV). In general, the highly conserved center of the genome houses the genes essential for replication; whereas, the more variable ends of the genome contain genes important for virus-host interactions. This large genomic capacity enables the orthopoxviruses to encode multiple proteins devoted to modulating the host's immune system, in addition to the many proteins required for their cytoplasmic replication cycle.

To initiate infection, the virus binds to the host cell via an unidentified receptor, and a membrane fusion event releases the viral core into the cytoplasm (Figure 1-1) [5]. The complete transcriptional system packaged in the virion synthesizes the initial mRNAs to produce the enzymes required for DNA replication and transcription and anti-immune defense molecules, including a complement regulatory protein. DNA replication is followed by transcription and translation of the intermediate and late mRNAs. A single genome is packaged and matures into intracellular mature virus (IMV). IMV is either released from the cell during lysis or enveloped by a Golgi membrane to form intracellular enveloped virus (IEV). The IEV rides the microtubules to the plasma membrane where it acquires an actin tail and the outer membrane surrounding the virion fuses with the plasma membrane to release extracellular enveloped virion (EEV). Some of these virions remain associated with the cell: the cell-associated enveloped virus (CEV). Incomplete understanding of the function of each of the multiple infectious forms (IMV, EEV, and CEV) complicates the study of orthopoxvirus infection [6-8].

Ectromelia Infection

ECTV infects mice in the wild. While it replicates in most types of mammalian cells in tissue culture, its host-range is narrowly restricted to rodents [5]. The natural route of infection is thought to be via skin abrasions, which is most commonly mimicked in the laboratory by inoculating the footpad [9,10]. Following replication at the site of inoculation, the virus migrates to the draining lymph node, and replication there generates a primary viremia that seeds the liver, spleen, and other organs (Figure 1-2). Replication in these organs generates a

secondary viremia that leads to foci of infection in distal organs including the skin. The primary lesion develops at the inoculation site after approximately a week. Several days later, certain susceptible mouse strains develop the characteristic cutaneous pox lesions for which these viruses are named [10]. The footpad or subcutaneous route of inoculation enables the study of early events during the course of poxvirus infection (Figure 1-2, days 0-3), stages at which the innate immune system predominates in the host's response [11].

Because of the array of inbred and genetically manipulated mouse strains available, the ECTV model offers much promise for analyzing the role of individual components of the immune response to poxvirus infection in a natural host. Inbred mouse strains with differing degrees of susceptibility to ECTV provide a basis for determining the host factors that influence susceptibility to poxviruses [11].

ECTV infection is 100% lethal in the susceptible strains BALB/c, DBA/2, and A/J [12]. In the A/J strain, the infection and necrosis of the spleen and liver is so severe that the mice die 7–8 days post-infection before the primary lesion develops [13]. In contrast, the resistant strain, C57BL/6, has an asymptomatic infection that is cleared from the footpad by day 18 post-infection [11,12]. Direct comparisons of the immune response in sensitive and resistant strains have associated resistance with induction of a strong type 1 cytokine response (including interferon- γ (IFN- γ), tumor necrosis factor- α (TNF- α), and interleukin-2) and a robust cytotoxic lymphocyte (CTL) response [12].

Crossing C57BL/6 mice with susceptible DBA/2 mice and then selecting those animals with decreased susceptibility identified several resistance to *mousepox* (*mp*) loci. The *mp-1* locus on chromosome 6 mapped to the natural killer (NK) gene complex, which is

consistent with NK cells' role in protection [14,15]. Another allele, *mmp-2*, mapped near complement component C5. In preliminary experiments, passive transfer of C5 into a DBA/2 mice promotes lymphocyte recruitment to the spleen with histology similar to that observed in the resistant strain [16].

Resistant strains of mice carrying specific genetic deficiencies have helped define the requirements for a protective immune response, including both the timely clearance of infection and survival (Table 1-1). Similar to the observations from comparing sensitive and resistant mouse strains, antiviral cytokines and CTL responses are crucial. For example, lack of IFN- γ [17-20], perforin [19-22], or CD8⁺ T cells [23,24] in a normally resistant background results in 100% mortality even with low inoculums. Mice deficient in macrophages, toll-like receptor 9 [25], or NK cells [14,26] also have increased mortality during the acute infection. Mice lacking either granzyme A or B have a mild increase in mortality, but a lack of both results in 100% mortality with a mean time to death slightly greater than that of the perforin-deficient mice [19,27]. Along the same line, mice deficient in either TNF receptor, p75 or p55, have increased morbidity (p75 > p55); however, the lack of both receptors results in a more severe phenotype with very early morbidity at two days post-infection [28]. Infection of mice that cannot produce inducible nitric oxide synthase 2 is uniformly lethal at higher doses despite the fact that the mice generate normal IFN- γ and CTL responses [29]. In contrast, mice lacking MHC-II, B cells, CD40, or CD40 ligand (CD154) survive the acute infection but eventually succumb approximately 2 weeks to 2 months post-infection [19,20,23]. Depletion of CD4⁺ T cells did not increase mortality; however, it impaired viral clearance at 21 days post-infection, particularly from the skin [24].

Complement

The complement system is an ancient system of host defense with the central molecules established more than one billion years ago based on their presence in cnidarian coral [30]. In mammals, the system consists of around 30 serum and cell-associated proteins that interact to initiate or regulate a proteolytic cascade, similar to the clotting cascade, that mediates the powerful effector mechanisms of complement [31]. Hepatocytes synthesize most of the soluble proteins of the complement system; however, other cell types, such as epithelial cells, endothelial cells, monocytes, and other leukocytes also produce many components [32,33]. The complement system is central to the innate immune system and enhances the activity of the humoral arm of the adaptive response [34].

Three distinct pathways activate the complement cascade: classical, lectin, and alternative (Figure 1-3) [31]. C1q, as part of the C1 complex, traditionally binds to the Fc portion of IgM and IgG antibody to trigger the classical pathway. C1 also directly binds a variety of alternate ligands: viral glycoproteins, bacteria, and host proteins including, C-reactive protein and SIGN-R1, which interact with microbes [35-37]. Mannan-binding lectin (MBL) or ficolins recognize repetitive carbohydrate motifs on the surface of pathogens and infected cells and activate complement similar to C1q [38,39]. Spontaneous turnover of C3 is believed to initiate the alternative pathway on foreign surfaces that lack complement regulatory proteins [35,40]. Recent reports demonstrate that properdin binds to pathogens and directs assembly of the alternative pathway C3 convertase, in addition to its traditional role stabilizing this convertase [41]. The three pathways converge at the step of C3 cleavage and share a common terminal pathway leading to formation of the membrane attack

complex (MAC). Regardless of the initiating pathway, C3b produced by the C3 convertase deposits on surfaces and initiates a positive feedback loop by forming additional C3 convertases through the alternative pathway.

This proteolytic amplification cascade attacks invading microorganisms and diseased host cells through opsonization and lysis [35,40]. Due to its destructive potential, the host tightly regulates this system by expressing soluble and membrane-bound complement regulatory proteins [42]. Multiple human diseases have been linked to deficiencies in either enhanced activation or impaired regulation of the complement system [31]. Insufficient regulation results in excessive activation, leading to undesirable inflammation and tissue damage. A functional deficit in complement regulatory proteins causes paroxysmal nocturnal hemoglobinuria and predisposes to atypical hemolytic uremic syndrome, glomerulonephritis, and age-related macular degeneration [43-46].

Human deficiencies in complement activation fall into two categories. Deficiency of a component early in the pathway (C1-C4) predisposes to infections with pyogenic bacteria as well as autoimmune diseases; whereas, deficiency of a terminal pathway component (C5-C9) is most commonly associated with increased susceptibility to bacterial infections, especially *Neisseria* [47]. Evidence derived from both patients and animal models has implicated complement as an essential part of host defense against multiple virus families [48-54].

Complement in Viral Infection

Complement system activation can exert multiple antiviral effects (Figure 1-3) [35,55,56]. Opsonization of the virion may block attachment or promote destruction by phagocytosis. The MAC disrupts the membrane integrity of the virion or virally infected cells. The anaphylatoxin cleavage products, C3a and C5a, attract and activate proinflammatory and immune effector cells, as well as enhance epithelial resistance to viral infection [57]. Finally, complement activation induces and instructs the adaptive response and serves, along with Fc receptors, as an effector arm of antibody [56,58-60].

Complement activation increases neutralization of virus by natural antibody, which is predominantly of the IgM isotype [60-63]. Natural IgM can effectively trigger the classical pathway, and this synergy between two arms of the innate immune system helps protect the host until the development of specific antibodies [60,64]. Natural antibodies are low affinity, polyreactive, and present in sera at relatively low titers [64,65]. Not only does natural antibody protect the host by preventing the dissemination of invading pathogens through the bloodstream, but it also helps induce the immune response by targeting antigen to the spleen [64,66]. These functions improve survival in mouse models of vesicular stomatitis virus infection and sepsis (cecal ligation and puncture) [66,67]. Natural antibody is mainly produced by B-1 cells, which reside predominantly in the peritoneum and pleura [64]. In the well-studied model of influenza infection, both the natural IgM present before infection from B-1 cells and the IgM produced in response to the infection by B-2 cells are required for survival [68]. This natural IgM requires complement activity to neutralize influenza *in vitro* [62].

Optimal activation of antibody and T cell responses depends on the workings of the complement system [56,58-60]. Early T cell-independent antibody responses to vesicular stomatitis virus require C3 and C4 [63]. Complement deficiency reduces the activation of CD4⁺ T cells and the subsequent ability to induce T cell-dependent antibody responses to influenza and West Nile viruses [50,69]. Proper CD8⁺ T cell responses to these two viruses and lymphocytic choriomeningitis virus are also complement-dependent [50,69,70]. Many leukocytes synthesize complement proteins, and the C3 produced by wild-type myeloid cells transplanted into C3-deficient mice enables normal induction of protective antibody responses to peripheral herpes simplex virus infection [33,71]. This indicates that complement's impact on the adaptive immune response may result from local production and activation or may only require a low concentration (compared to serum levels) of complement proteins such as in tissue sites and body secretions.

To evade complement's activities, viruses use multiple strategies to hinder complement activation [40,55,56,72]. Viruses incorporate the host's complement regulatory proteins into their envelopes or upregulate these proteins on the infected cell's surface [73-77]. Some virally infected cells avoid classical pathway activation by shedding or internalizing antibodies that bind their surface [78]. More commonly, viruses produce their own complement regulatory proteins to protect themselves. Some of these proteins are unique to the virus [79,80], while others have structural and functional homology to the host's own regulators [49,81]. The multiple strategies that viruses have developed to evade complement provide further evidence of this system's importance in antiviral defense.

Viral Neutralization by Complement

Complement-mediated neutralization occurs when activation exceeds the viruses' ability to protect themselves. *In vitro* experiments have demonstrated neutralization in the absence of specific antiviral antibody for multiple viruses: parainfluenza virus [75], influenza virus [62], simian virus 5 and mumps virus [82], West Nile virus [51], human immunodeficiency virus [83], vesicular stomatitis virus [84], and vaccinia virus [76,85,86]. Several mechanisms mediate neutralization *in vitro*. For example, alternative pathway-initiated C3b deposition on two closely related paramyxoviruses results in the aggregation of simian virus 5, while mumps virus is lysed by engagement of MAC [82]. Even low concentrations of antiviral antibodies added to naïve sera increase neutralization and greatly expand the group of viruses sensitive to complement. Along the same lines, removing or blocking the viruses' complement defense strategies enhances neutralizing activity. Natural antibody can initiate neutralization of herpes simplex virus that lacks glycoprotein C, a complement regulatory protein that lacks homology to the host regulators [87].

Poxviral Inhibitors of Complement Enzymes

Complement regulatory proteins use three mechanisms to control complement activation: decay accelerating activity (DAA) dissociates the subunits that comprise the C3 and C5 convertases (Figure 1-4A), cofactor activity (CA) irreversibly inactivates the convertases when C3b or C4b are proteolytically cleaved by factor I (FI) (Figure 1-4B), and irreversible binding of inhibitors blocks MAC insertion and formation [35]. Most cells protect their membranes by expressing complement regulatory proteins, including DAF (decay accelerating factor;

CD55) and MCP (membrane cofactor protein; CD46), which regulate the C3 convertases using either DAA or CA, respectively (Figure 1-4). These proteins economically target the C3 and C5 convertases, as this is the step where the three initiating pathways converge (Figure 1-3).

The poxvirus inhibitors of complement enzymes (PICEs) have approximately 35% homology to host regulatory proteins [88]. The PICEs are composed of 4 complement control protein repeats (CCP), like their human homologues MCP and DAF. Historically, the most thoroughly studied proteins in this family are vaccinia complement control protein (VCP) and inflammation modulatory protein (IMP) from cowpox (CPXV) [89,90]. Recent studies by our laboratory and others have defined many of the regulatory properties of smallpox inhibitor of complement enzymes (SPICE) and monkeypox inhibitor of complement enzymes (MOPICE) [91,92].

VCP: VCP was the first secreted microbial protein identified as having a role in immunomodulation [90]. Using a combination of DAA and CA, VCP inhibits the classical (CA & DAA) and alternative (CA only) pathways [86,88,93,94]. VCP also prevents complement's enhancement of antibody-dependent neutralization of IMVs by both the classical and alternative pathways [86]. Function-blocking antibodies to VCP restore complement's ability to enhance neutralization [85,86]. The ability to bind and regulate C3b requires all four CCPs [95,96]. VCP attaches to the cell surface through a covalent linkage between its unpaired N-terminal cysteine and the viral protein A56 [97]. This mechanism may mediate VCP's inclusion in EEVs, since A56 is also present [98]. A fraction of VCP (~5-30%) released from cells infected *in vitro* forms a disulfide linked dimer, via that same N-

terminal cysteine, that possesses enhanced regulatory activity [99]. The multiple heparin binding sites present may influence the function and localization of VCP *in vivo* [96,100-104].

IMP: Culture supernatants from CPXV infected cells protect sensitized erythrocytes from complement activation and hemolysis in a dose dependent manner [105]. This protein has been studied more *in vivo* than *in vitro* (see below)[106].

SPICE: SPICE resembles VCP in that it has CA against both pathways, has DAA for the classical pathway C3 and C5 convertases, binds heparin, and a proportion of it exists as a dimer [91,92,107]. SPICE differs from VCP by only 11 amino acids (Figure 1-5), but these changes enhance SPICE's DAA by ~5-10 fold and CA by more than 100 fold for the human complement system [91,92,107,108].

MOPICE: Despite lacking most of CCP-4, MOPICE retains functional activity and dimerizes through an unpaired cysteine in the residual CCP-4 (Figure 1-5) [99]. It has ~1/100 of SPICE's CA, similar to VCP. Unlike SPICE and VCP, MOPICE lacks DAA for either pathway, which implicates CCP-4 as being essential for DAA [91,92].

EMICE: The ECTV genome demonstrated a homologue to SPICE and VCP, ectromelia inhibitor of complement enzymes (EMICE) [109]. As illustrated in (Figure 1-5), EMICE differs from SPICE by only 26 amino acids. Interestingly, 40% of the amino acid differences between EMICE and SPICE as well as a contiguous two amino acid deletion occur in CCP-1, while SPICE and VCP have an identical CCP-1. The remainder of EMICE, CCPs 2-4, resembles VCP more than SPICE, with 7 and 15 amino acid differences, respectively.

Poxviruses and Complement *in vivo*

The initial experiment that examined the role of a PICE *in vivo* used VACV that lacked VCP (Δ VCP-VACV). The lesions produced by Δ VCP-VACV upon injection into the back of rabbits or C4-deficient guinea pigs were smaller than those induced by wild-type VACV [86]. In a mouse model, similar experiments compared the IMP-deficient virus (Δ IMP-CPXV) to its parent virus following footpad inoculation. In contrast to the earlier results, Δ IMP-CPXV resulted in greater inflammation (swelling, induration, and mononuclear infiltrates) and tissue damage (hemorrhage) [105]. One interpretation of these data is that, in the case of Δ VCP-VACV, the increased complement activation was able to control the infection earlier resulting in less tissue destruction; in Δ IMP-CPXV infection, the additional complement activation, either directly or indirectly (via the increased inflammatory infiltrate), damaged the tissue. VCP and IMP likely use similar mechanisms to prevent complement activation and reduce inflammation, thereby protecting the virus (illustrated by Δ VCP-VACV) and its local environment (illustrated by Δ IMP-CPXV).

CPXV infection of complement-deficient mice suggests a role for complement in the host response. C5-deficient mice infected in the footpad with wild-type CPXV showed significantly greater induration, hemorrhage, and ulceration compared to the congenic C5-sufficient strain [89,105]. One of two studies also measured greater edema in the C5-deficient strain [89]. In the second study, equivalent edema occurred in both strains, and not only with the wild-type CPXV but also with Δ IMP-CPXV [105]. These results indicate that IMP affects the level of edema independent of C5 activity. Similarly, C3^{-/-} mice developed greater inflammation than the control mice following infection with wild-type CPXV [110].

In contrast, if infected with Δ IMP-CPXV, the $C3^{-/-}$ mice had slightly less inflammation than the wild-type mice. As prior experiments showed, Δ IMP-CPXV induced greater inflammation than CPXV in wild-type mice, but the two viruses induced comparable responses in the $C3^{-/-}$ mice.

Finally, the mapping of one of the genetic resistance alleles to ECTV to the region of the chromosome that contains C5 suggests that the complement system may have a role in protecting C57BL/6 mice [15,16]. The C5-deficient susceptible mouse strain, DBA/2, did not efficiently recruit lymphocytes to the site of infection. Preliminary data described in one paper indicated that passive transfer of C5 into this strain changed lymphocyte targeting so that it resembles that seen in the resistant strain, C57BL/6 [16].

In subsequent chapters, the impact of the complement system on ECTV infection will be investigated *in vivo* using complement-deficient mice. The interaction between ECTV particles and mouse complement will be investigated *in vitro*. Finally, the viral complement regulator, EMICE, will be characterized.

Figures

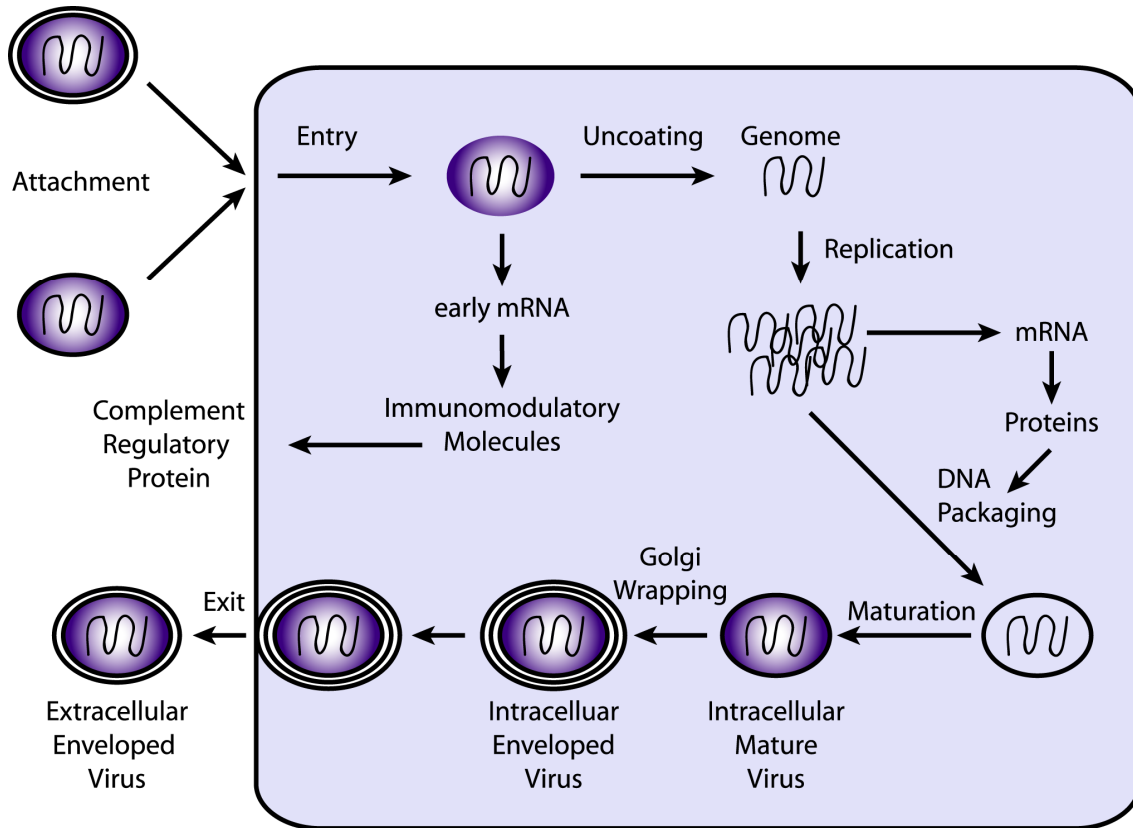


Figure 1-1: Poxvirus replication cycle

To initiate infection, the virus binds to the host cell via an unidentified receptor, and a membrane fusion event releases the viral core into the cytoplasm [5]. The complete transcriptional system packaged in the virion synthesizes the early mRNAs to produce immune defense molecules and the enzymes required for DNA replication and transcription. DNA replication is followed by transcription and translation of the intermediate and late mRNAs. A single genome is packaged and matures into the IMV. IMV is either released from the cell during lysis or enveloped by a Golgi membrane to form IEV. The IEV rides

the microtubules to the plasma membrane where it acquires an actin tail and the outer membrane surrounding the virion fuses with the plasma membrane to release EEV. Some of these virions remain associated with the cell (cell-associated enveloped virus, CEV. Incomplete understanding of the function of each of the multiple infectious forms (IMV, EEV, and CEV) complicates the study of orthopoxvirus infection [6,7].

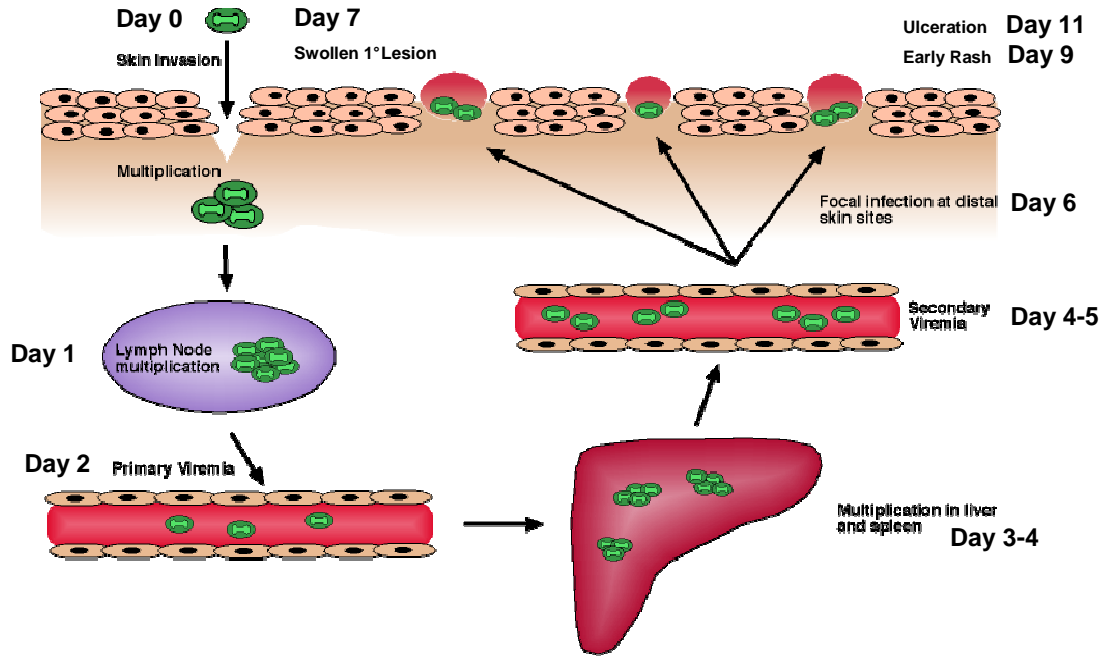


Figure 1-2: Ectromelia infection in a susceptible mouse strain

Following entry through a break in the skin, the virus replicates and disseminates to the draining lymph node. Replication at the initial site and in the draining lymph node generates the primary viremia that infects the spleen and liver. Virus released from these target organs causes a secondary viremia, which seeds distal sites like the skin. The primary lesion develops at the entry site after approximately a week. Several days later, certain susceptible mouse strains develop the characteristic cutaneous pox lesions for which these viruses are named [10]. Figure modified from Chung Lee.

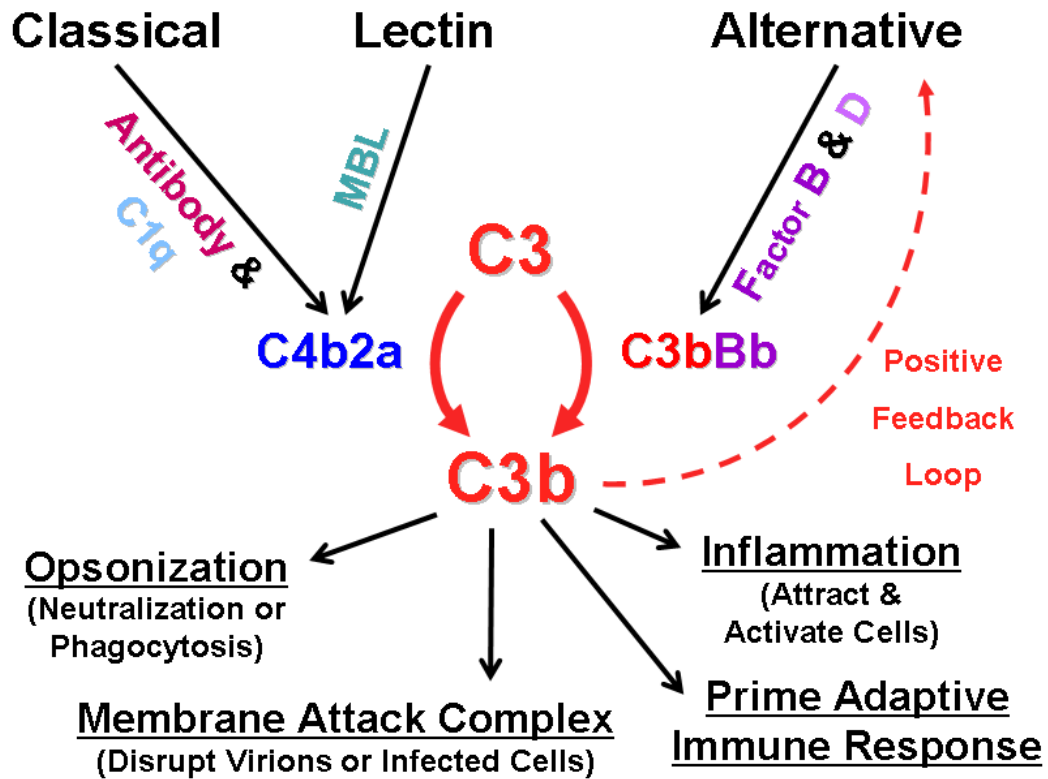
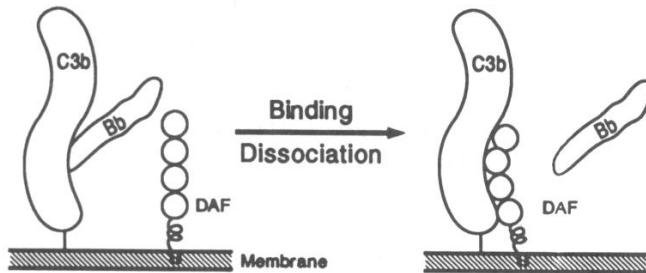


Figure 1-3: Complement activation pathways

The classical, lectin, and alternative pathways each initiate the complement cascade by forming a C3 convertase that cleaves C3 to C3b. The binding of C1q to antibody triggers the classical pathway. The carbohydrate motifs on pathogens activate the lectin pathway through MBL. The classical and lectin pathways form the C4 containing convertase, C4b2a. C3 deposited by either of these two pathways or from spontaneous activation of C3 initiates the alternative pathway. Upon binding to C3b, factor B (FB) is cleaved to Bb by factor D (FD), which forms the alternative pathway convertase, C3bBb. The alternative pathway amplifies C3b deposition initiated by any pathway. C3 activation leads to important antiviral effector

functions. Release of the anaphylatoxins C3a and C5a recruits inflammatory cells. C3b and C4b opsonize viral particles or infected cells, leading to neutralization or destruction by phagocytosis. C3b also leads to activation of C5 and formation of the MAC, which disrupts virions or infected cells. Complement activation is nature's adjuvant to trigger the adaptive immune response.

A. DAF: Decay Accelerating Activity



B. MCP: Cofactor Activity

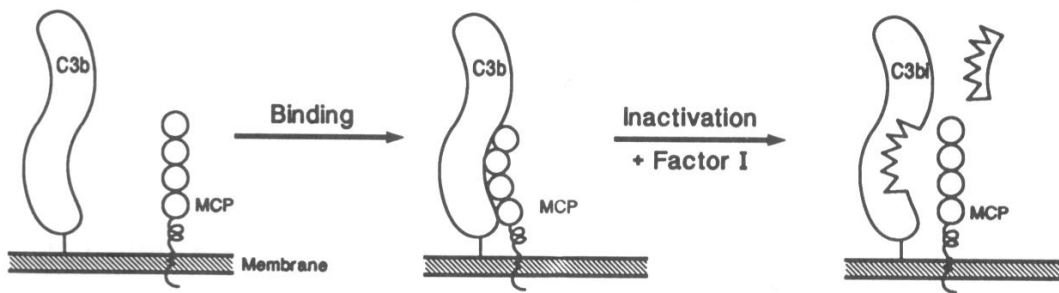


Figure 1-4: Complement regulatory activity

(A) Decay Accelerating Activity: the regulator binds to the C3 and C5 convertases and displaces the catalytic subunits.

(B) Cofactor Activity: the cofactor protein binds to C3b or C4b and recruits factor I to cleave the protein and irreversibly inactivate the convertases.

```

CCP1:SPICE CCTIPSRPINM*KFKNSVETDANANYNIGDTIEYLCLPGYR*KOKMGPYAKCTGTGWTLFNQCI*KRR
VCP
MOPICE Y          --
IMP          G
EMICE          N G DT-- KV          V          S V N          K

CCP2:SPICE C P S P R D I D N G H L D I G G V D F G S S I T Y S C N S G Y Y L I G E Y K S Y C K L G S T G S M V W N P K A P I C E S V K
VCP          Q          H S E E
MOPICE       Q          H S E E
IMP          Q I E Q S E Y E
EMICE       Q T Q S E E

CCP3:SPICE C Q L P P S I S N G H N G Y N D F Y T D G S V V T Y S C N S G Y S L I G N S G V L C S G G E W S N P P T C Q I V K
VCP S E D
MOPICE S E I I M
IMP P S V T E E IV D
EMICE S E E V D

CCP4:SPICE C P H P T I L N G Y L S S G F K R S Y S Y N D N V D F T C K Y G Y K L S G S S S T C S P G N T W Q P E L P K C V R -
VCP S K K
MOPICE I S N G K L L A A -----
IMP S T H R H
EMICE S K E K E

```

Figure 1-5: Homology of the Poxviral Inhibitors of Complement Enzymes (PICEs)

Color code: blue, differing residues; red, conserved cysteines; green star, putative heparin binding site (K/R-X-K/R). A frame-shift mutation in CCP-4 of MOPICE produces a truncated protein.

Table 1-1: Effect of genetic or induced deficiency on ECTV infection in resistant mouse strains

| Deficiency | Mortality | Route | Day of Death | Reference |
|-------------------------------------|------------------|--------------|---------------------|------------------|
| CD8 | 100% | fp | 8-10 | [23,24] |
| β2m (Lack MHC I) | 100% | fp | 14-18 | [19,24] |
| NK Cells | 80-100% | fp | 7-10 | [14,26] |
| Perforin | 100% | fp | 4-10 | [19-22] |
| Granzyme A & B | 100% | fp | 8-11 | [19,27] |
| IFN-γ | 100% | fp | 3-8 | [17-20] |
| | | | | |
| Nitric oxide synthase 2 | 100% | iv | 6-8 | [29] |
| Macrophages | 100% | fp | 8.5± 0.5 | [24] |
| TLR9 | 100% | in | 7-9 | [25] |
| TNFR p55 & p75 | 60% | fp | 10-18 | [28] |
| | | | | |
| MHC-II | 100% | fp | 12-35 | [19,20] |
| μMT | 100% | fp | 14-30 or ~60 | [19,20,23] |
| CD40 | 100% | fp | 40±16 | [23] |
| CD154 | 100% | fp | ~40 | [23] |
| Depletion of CD4⁺ | 0% at D21 | fp | | [24] |

fp, footpad; iv, intravenous; in, intranasal; D, day post-infection.

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Chapter 2

Multiple complement pathways required to survive ectromelia virus infection

Portions of the chapter are excerpted from:

Moulton E.A., Atkinson J.P., and Buller R.M. (2008) **Surviving mousepox infection requires the complement system.** *PLoS Pathogens*. In Press.

Introduction

To better understand the virulence of smallpox, investigators have turned to related poxviruses like ectromelia virus (ECTV), the causative agent of mousepox. Variola virus and ECTV have a narrow host-range and cause significant morbidity and mortality [3,4]. The numerous available mousepox-susceptible and -resistant mouse strains allow the components of the protective immune response to poxviruses to be dissected in the natural host.

Disease severity varies among inbred mouse strains, and comparisons of these strains have elucidated factors essential for survival. Mice naturally acquire ECTV via cutaneous abrasions, which is most commonly mimicked experimentally with footpad inoculation [4]. Through this route, ECTV infection is 100% lethal in susceptible strains (BALB/c, DBA/2, and A/J) but asymptomatic in the resistant C57BL/6 strain. The C57BL/6 strain has a stronger T_H1 type cytokine response and a more robust cytotoxic lymphocyte response than susceptible strains [5]. Lethal infection occurs in C57BL/6 mice that lack $CD8^+$ T cells [6,7], B cells [7,8], macrophages [6], natural killer (NK) cells [9,10], interferon (IFN)- γ [11-13], IFN α/β receptor [13], perforin [14,15], and granzyme A or B [16]. Survival, therefore, requires both the adaptive and innate immune response.

The innate immune system defends the host during the early phase of an infection and shapes the adaptive response [17-19]. The complement system is an essential component of the innate immune system, and evidence from human disease and animal models implicates complement as a critical part of host defense against several virus families [20-24].

Complement influences poxviral infections, but an essential role for survival has not been demonstrated. One study described increased inflammation at the inoculation site of cowpox virus in $C5^{-/-}$ mice; however, no mortality occurred in these mice [48]. Additionally, an allele for genetic resistance to ECTV mapped to the chromosomal region containing C5 [49].

Using complement-deficient mice, the mousepox model offers an opportunity to characterize the role of this system during infection in the natural host. Use of a model where the host and pathogen have co-evolved is particularly important given the species specificity of many poxviruses and of complement proteins, regulators, and receptors [3,50]. In this study, we focused on the role of C3, the complement cascade's central component. Resistant C57BL/6 mice that genetically lack C3 inadequately control ECTV infection and have increased morbidity, viral burdens, and mortality. Similar increases in mortality were observed in FB- and C4-deficient mice, indicating a role for multiple complement activation pathways in protection.

Methods

Virus production

Plaque-purified Moscow strain ECTV was propagated in murine L929 cells. Intracellular mature viral stocks were purified through a sucrose cushion as described [51] and titrated on BS-C-1 cells, an African green monkey kidney cell line [52]. Both cell lines were cultured in Dulbecco's modified Eagle's media (DMEM, BioWhittaker) supplemented with 10% heat-inactivated fetal calf serum (FCS, HyClone), 2 mM L-glutamine, and antibiotics.

Mice

The following strains on a C57BL/6 background were acquired: C3^{-/-} [53,54] and FB^{-/-} [55,56] from H. Molina, Washington University Medical School; C4^{-/-} [57] from M. Carroll, Harvard Medical School; and wild-type from Jackson Laboratories. Age-matched mice of both sexes were used in the footpad and ear pinna studies (6–11 weeks-old). Male mice were used in the intranasal (8–12 weeks) studies. Some wild-type mice used in the footpad studies were purchased from Jackson Laboratories. The rest of the mice were bred at Washington University in a specific pathogen-free facility. The animals were transported to the biohazard suite at Saint Louis University at least a week prior to infection. All experiments were performed following the animal care guidelines of the two institutions.

***In vivo* studies**

Mice were inoculated with 10 µl ECTV diluted in PBS to the indicated dose using a 29 gauge insulin syringe into the ear pinna and hind footpad or a 20 µl pipettor for the intranasal route.

Mice were anesthetized for inoculation using CO₂/O₂ for the footpad route and ketamine/xylazine for the ear pinna and intranasal routes. Individual mice were marked by ear punching or shaving. After infection and before sacrifice in the mortality studies, mice were manipulated only to obtain weights. Serum was collected from surviving animals at the end of the experiment. The survival curves include only animals that generated an antiviral antibody response, which was detected by ELISA in >95% of the mice [58].

Tissue titer

Blood was collected via cardiac puncture. Spleen and liver tissues were harvested aseptically, frozen immediately on dry ice, and stored at -70°C. Tissues were homogenized in PBS-1% FCS to ~10% (weight/vol) using 1 ml glass homogenizers. They were frozen and thawed three times, sonicated, and titrated on BS-C-1 monolayers [52].

Viremia analysis

DNA was isolated from whole blood collected in EDTA microtainer tubes (BD) using the High Pure PCR Template Preparation Kit (Roche). The kit's whole blood protocol was used with the following modifications. The 40 µl Proteinase K, 200 µl Binding Buffer, 150 µl PBS, and 50 µl of whole blood in EDTA were added sequentially and then vortexed. The incubation at 70°C was extended to 12 min. The sample was applied to the column by centrifugation at 8,000 g for 2 min and eluted in 50 µl.

Quantitative PCR was performed on viral DNA using Power SYBR Green PCR Master Mix on a 7500 Real Time PCR System (Applied Biosystems, Foster City, CA) [59]. The primers (10 pmol) SP028 (GTAGAACGACGCCAGAAT AAGAATA, 5' at 120627

bp) and SP029 (AGAAGATATCAGACGATCCACAATC, 5' at 120462 bp) were used to amplify 165 bp of gene EV107. The amplification product cloned into a plasmid vector (pGEM-T, Promega) was used as a standard to estimate copies of DNA/ μ l in blood. Three to four wells were used for each sample.

Histology

Tissue samples were fixed in 10% buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin by the Digestive Diseases Research Core Center, Washington University. The number of inflammatory foci and the magnitude of tissue necrosis were evaluated in blinded samples. Inflammatory foci in a 10 \times visual field were counted for \sim 7 fields/mouse liver.

Liver enzymes

AST and ALT levels were measured in samples of frozen sera by the Department of Comparative Medicine at Saint Louis University using a standard clinical analyzer.

Statistical analysis

All statistical analysis was performed using GraphPad Prism software version 5.01 (GraphPad Software, San Diego, CA). The survival curves were analyzed by the log-rank test. The Mann-Whitney test was used to determine the statistical significance of the viral titers, viremia, liver histology, and liver enzymes.

Results

C3 deficiency increased morbidity and mortality of ECTV inoculated by multiple routes

The route of infection influences the interaction between poxviruses and the host [60]. Half of the 16 mutant vaccinia viruses assessed using two routes of inoculation, ear pinna and intranasal, had a detectible phenotype by only one route. ECTV infections of C57Bl mice by the intranasal, intraperitoneal, or intravenous routes result in severe disease and mortality, while the footpad and intradermal routes cause minimal disease [61]. To examine the role of complement *in vivo*, wild-type and C3^{-/-} mice were infected by three routes: footpad, ear pinna, and intranasal.

Approximately 95% (54 of 57) of the wild-type mice survived when inoculated with 40,000 pfu of ECTV, the highest dose employed in the footpad infections (Figure 2-1A). In contrast, C3^{-/-} mice had about 90% mortality at that dose. They also had significantly increased mortality ($P < 0.0001$) at lower doses, even if inoculated with only 4 pfu. The median time to death increased as the dose decreased from 7 days at 40,000 pfu to 9, 10, or 13 days at the lower doses of 4,000, 400, or 4 pfu, respectively.

The C3^{-/-} mice also showed increased morbidity over the course of the infection. Unlike the wild-type mice on day 7 post-infection with 40,000 pfu, the C3^{-/-} mice displayed clinical signs of infection, including fur ruffling and hunchbacked posture. Consistent with these observations, C3^{-/-} mice lost more weight at 400 and 40,000 pfu than wild-type (Figure 2-1B). The few surviving C3^{-/-} mice at the 400 pfu dose required ~3 additional weeks,

compared to the wild-type mice, to return to their initial weight. All surviving mice in Figure 2-1A were held for at least 40 days to monitor recovery, and a subset of C3^{-/-} mice (*n*=4 at 400 pfu) were held to day 119 post-infection. The mice that survived the acute illness steadily recovered weight and showed no signs of relapse.

The ear pinna studies used a dose of 700 pfu to mimic the low inoculum thought to transmit the natural poxvirus infection [2]. The infection caused 72% mortality in the C3^{-/-} mice (28 of 39) (Figure 2-1C), compared to 25% in the wild-type mice (6 of 24, *P*=0.0002). The surviving C3^{-/-} mice lost less weight and recovered to the initial weight earlier with inoculation by the ear pinna compared to the footpad route (Figure 2-1B & D). In contrast to the increased morbidity and mortality observed, C3 deficiency caused no gross differences in the primary lesion; C3^{-/-} and wild-type mice had similar levels of footpad swelling or necrosis at the ear pinna inoculation site (data not shown).

To examine the role of C3 in intranasal infection, the dose was lowered to 100 pfu due to the increased susceptibility of the wild-type mice with this route. C3 deficiency increased the mortality rate from 40% to 80% (*P*<0.0001, Figure 2-1E). Similar to the other routes, the surviving C3^{-/-} mice had more severe disease than wild-type, as they had greater weight loss and took longer to recover (Figure 2-1F).

C3 deficiency resulted in earlier dissemination of ECTV to the target organs, higher peak viral titers, and delayed viral clearance

ECTV replicates at the inoculation site and in the draining lymph node to generate the primary viremia that infects the spleen and liver [4]. Virus released from these target organs

causes a secondary viremia, which seeds distal sites like the skin to generate the characteristic pox lesions.

To begin to dissect how C3 contributed to protection against ECTV, we examined viral burden in two key tissues, the spleen and liver. Wild-type and C3^{-/-} mice were inoculated in the footpad with either 400 or 40,000 pfu, and then spleen and liver tissue were collected on day 7 post-infection. All animals had detectible virus in either the spleen or liver. At the two doses, the C3^{-/-} mice had a 1-2 log higher mean titer than wild-type mice in both tissues (Figure 2-2A & B).

In wild-type mice, both doses produced similar maximal tissue titer; however, the higher dose increased the uniformity of the group and, thereby, increased the mean titer. At the 40,000 pfu dose, the splenic viral burden in the C3^{-/-} mice was ~150-fold higher ($P=0.0002$, Figure 2-2A). Reducing the dose to 400 pfu resulted in ~25-fold lower viral titer in the C3^{-/-} mice, yet it was still ~25-fold higher than the wild-type controls ($P=0.03$). In contrast, both doses produced similar liver titers in the C3^{-/-} mice. The lower dose revealed an 80-fold increase in the liver titer of the C3^{-/-} mice compared to the wild-type mice ($P=0.01$, Figure 2-2B), while the higher dose showed less of a difference between the strains (15-fold) due to the increased titer in the wild-type mice. Illustrative of the impact of C3, the C3^{-/-} mice at 400 pfu had higher titers than the wild-type mice given 40,000 pfu, a 100-fold more virus.

These increases in viral titer prompted further exploration of how C3 deficiency impacts viral spread. C3 could control viral replication early at the inoculation site by directly inactivating free virus or by recruiting inflammatory cells through release of anaphylatoxins.

The lack of C3 in the blood to neutralize or opsonize the virus could also result in greater viremia, thereby producing the higher titers observed in the target organs on day 7. Alternatively, C3's well-established ability to facilitate induction of antibody and T cell responses could explain the observed difference [21,22,54,62-65]. To elucidate when the infections in the C3^{-/-} and wild-type mice diverge, we inoculated via the ear pinna route and examined the viral burden in the blood, spleen, and liver on days 2, 4, 7, and 10 post-infection. The ear pinna route was selected for further analysis because it is a cutaneous route of inoculation that mimics a natural infection of the epithelium, where complement may promote containment.

Using whole blood enables an unbiased detection of all virus, whether free in the plasma or in infected cells. Quantitative PCR was employed to detect viral DNA in blood on days 2, 4, and 7 (Figure 2-2E). A few day 2 samples contained viral DNA, but most were below the detection limit. The C3^{-/-} mice had 2.0- and 2.5-fold higher levels of viral DNA than wild-type mice had on days 4 and 7 ($P=0.004$ and 0.03 , respectively).

Despite the low levels of viremia on day 2, infectious virus was present in the spleen of over 70% of the C3^{-/-} mice (13 of 18) compared to 28% of the wild-type mice (5 of 18, $P=0.006$, Figure 2-2C). By day 7 post-infection, the C3^{-/-} mice had 45-fold higher viral titers in the liver ($P=0.01$, Figure 2-2D), and there was also a similar trend in the spleen (6-fold, $P=0.09$). The wild-type mice regained weight starting on day 10 (Figure 2-2-1D), and by then over 80% had cleared the virus from the spleen or liver (9 of 11, Figure 2-2C & D). In contrast, less than half of the C3^{-/-} mice survived to day 10 (Figure 2-1C), and of these, over 75% had ongoing infection of the spleen and liver (7 of 9, $P=0.008$ & 0.01 , respectively).

In summary, C3 deficiency resulted in earlier dissemination to spleen and in higher peak titers in the liver. The viral infection also continued to day 10 in the C3^{-/-} when it had been cleared by most wild-type mice.

C3 deficiency increased hepatic inflammation and necrosis

In susceptible mouse strains, ECTV causes extensive hepatic and splenic necrosis [66,67]. We compared C3^{-/-} and wild-type mice for histopathological changes in the liver on days 4, 7, and 10 post-infection.

On day 4, the liver histopathology appeared normal in 4 of 5 wild-type and 3 of 4 C3^{-/-} mice (data not shown). By day 7, all animals had a diffuse lymphocytic infiltrate in addition to discrete inflammatory foci (Figure 2-3). These lesions varied in size and were smaller and less frequent in the wild-type (Figure 2-3A & B) compared to the C3^{-/-} mice (Figure 2-3C & D). They often occurred near the portal triad, and some contained areas of coagulative necrosis. An inflammatory infiltrate encircled the discrete necrotic foci (Figure 2-3B & C) and bordered the areas of bridging necrosis (Figure 2-3D). In contrast to the liver, no major differences were observed in the spleen at this time (data not shown). Using blinded samples, we counted the necrotic and non-necrotic foci and evaluated the location and severity of the necrosis in the liver (Figure 2-4).

There were prominent differences between the C3^{-/-} and wild-type mice relative to the number inflammatory foci and in the degree of necrosis. The C3^{-/-} mice had twice as many total foci (8 vs. 18 per field, $P=0.02$, Figure 2-4A) and 5-fold more foci containing regions of necrosis (3 vs. 15 per field, $P=0.03$, Figure 2-4B). The majority of inflammatory foci contained necrotic areas in two-thirds of the C3^{-/-} mice compared to only one-fifth of

the wild-type mice (Figure 2-4C). The C3^{-/-} mice had larger foci with more extensive necrosis ($P=0.02$, Figure 2-4D). Most wild-type mice had small foci with either no necrosis or only piecemeal necrosis (0 & 1 on necrosis severity scale, Figure 2-3A & B, respectively). In contrast, the C3^{-/-} mice had confluent areas of necrosis that coalesced into bands of bridging necrosis (2 & 4 on the necrosis severity scale, Figure 2-3C & D, respectively). Given that necrosis most frequently occurred in zone 1 of the liver, it likely originated there and then extended into zones 2 and 3 (Figure 2-4E).

The increased hepatic necrosis in the C3^{-/-} mice resulted in higher levels of liver enzymes, aspartate aminotransferase (AST) and alanine aminotransferase (ALT), in the serum on day 7 ($P=0.008$, 0.0503 , respectively, Figure 2-4F). The AST and ALT levels positively correlated with the viral burden (Figure 2-4G).

Most C3^{-/-} mice died between day 7 and 10 (Figure 2-1C). Two C3^{-/-} mice that were sacrificed on day 10 had ~5-7 inflammatory foci per field, while the 5 wild-type mice had only occasional foci (data not shown). At this time point, infectious ECTV persisted in the C3^{-/-} mice; whereas, wild-type mice had cleared the infection (Figure 2-2D).

Surviving ECTV infection required multiple complement pathways

To examine the importance of the classical and lectin vs. alternative pathways *in vivo*, we compared C4^{-/-} and FB^{-/-} mice to C3^{-/-} and wild-type mice. We challenged C4^{-/-} mice via the ear pinna route and monitored survival and weight loss. Over 90% of the C4^{-/-} mice succumbed to the infection ($P<0.0001$, Figure 2-5A). The C4^{-/-} and C3^{-/-} mice had comparable mortality and weight loss (Figure 2-5C & 2-1D).

Intranasal ECTV infection also produced similar results in the C3^{-/-}, C4^{-/-}, or FB^{-/-} mice. Each complement-deficient strain had a higher mortality rate compared the wild-type mice ($P<0.0001$), and there were no significant differences among the three strains (Figure 2-5B). The complement-deficient strains also lost weight at a similar rate (Figure 2-5D & 2-1F). Thus, control of ECTV *in vivo* required both the alternative and classical pathways.

Discussion

We investigated the impact of complement deficiency using the ECTV mouse model. Specifically, C3 deficiency permitted ECTV to disseminate earlier, reach a higher viral titer in the target organs, and induce greater liver damage. Deficiency of C3, C4, or FB resulted in acute lethal infection, establishing a requirement for multiple complement pathways in host defense against this pathogen.

The ECTV model system provides several advantages for analyzing the role of complement in poxviral pathogenesis. First, the mouse-specific pathogen ECTV has coevolved with and causes severe disease in the natural host, analogous to variola virus in humans. Second, the role of complement and the pathways involved can now be more rigorously dissected *in vivo* and *in vitro* with the availability of complement-deficient mice. Third, viral pathogenesis, morbidity, and mortality can be assessed by multiple routes of infection and across a range of viral inoculum to demonstrate a broad requirement for complement.

Complement-deficient mice succumbed to acute ECTV infection with the majority of deaths occurring between days 6-10. Based on time to death following footpad inoculation (Table 1-1), C3 deficiency resembled immunodeficiencies of other important components of the antiviral response, specifically CD8⁺ T cells [6,7], NK cells [9,10], and IFN- γ [12]. In contrast, mice deficient in CD4⁺ T cells [6], CD40, or CD40 ligand (CD154) [7] survive the acute phase but do not clear the virus. The CD40^{-/-} and CD154^{-/-} mice ultimately die ~4 to 8 weeks post-infection. This differs from surviving C3^{-/-} mice, which

recovered and did not show signs of ongoing illness for up to 4 months of observation. The early death of the complement-deficient mice highlights the complement system's essential contribution to survival during the first few days of infection.

To characterize how complement protects the host from lethal infection, we analyzed the impact of C3 deficiency on the kinetics of viral spread. ECTV replication at the inoculation site and in the draining lymph node produces a viremia that seeds the primary target organs, the liver and spleen [4]. Several observations from this study increase our understanding of complement's role in controlling poxviral infection.

First, as early as day 2, C3 deficiency allowed for greater spread of ECTV from the inoculation site to the spleen. This indicates that complement is a key player in the initial hours of infection, likely to control ECTV at the inoculation site. Second, we detected higher levels of viral DNA in the blood on days 4 and 7, which establishes that C3^{-/-} mice poorly control viral dissemination through the bloodstream. This higher viremia could result from increased replication in tissues and/or decreased clearance of virus from the bloodstream. Third, the liver viral titers on day 7 were ~50-fold higher in the C3^{-/-} mice. The greater viremia likely produced more extensive infection, but a delayed adaptive immune response may also have contributed to this observation. The viral titer correlated with serum levels of ALT, which suggests that ECTV caused hepatic necrosis either directly through lytic infection or indirectly through the antiviral immune response. An inflammatory infiltrate surrounded the necrosis in the C3^{-/-} mice, which contrasts with susceptible Balb/c mice where necrosis occurs in the absence of a lymphocytic infiltration [68]. In summary, we propose that mice lacking C3 have reduced ability to control ECTV locally and in the

bloodstream, leading to higher levels of infection greater tissue damage in the liver, and mortality.

Figures

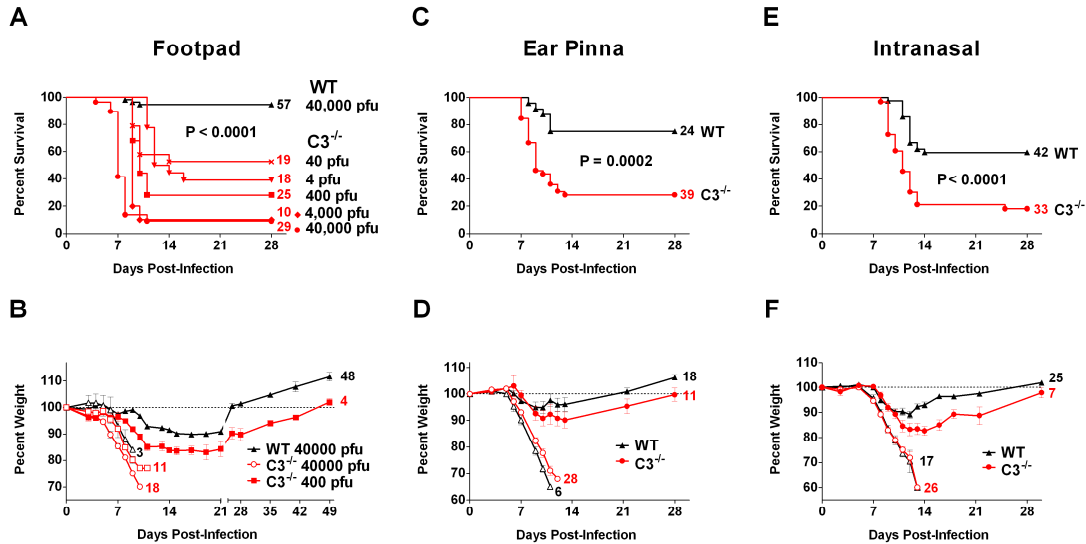


Figure 2-1: C3 deficiency increased mortality and morbidity in C57BL/6 mice

(A, C, & E) Greater mortality was observed in C3^{-/-} compared to wild-type (WT) C57BL/6 mice by multiple routes of inoculation. The number of animals in each group is to the right of the line, and the statistics compare the survival of C3^{-/-} and wild-type mice.

(B, D, & F) C3^{-/-} mice have increased morbidity following infection by the indicated route as monitored by weight loss. Weight loss for individual animals was normalized to their starting weight, and mean±SEM is shown. The graph segregates the mice that survived the infection (filled symbols) from those that succumbed (open symbols). The number of animals in each group is presented at the end of the line.

(A & B) Mice were inoculated in the footpad with the dose listed to the right of the survival curve or in the legend. The survival curves were generated from 2 or 3 separate experiments

(except for a single experiment at 4,000 pfu). The survival of the $C3^{-/-}$ mice significantly differed from the wild-type mice at all doses ($P < 0.0001$). (B) The one $C3^{-/-}$ mouse that survived at 40,000 pfu is not shown for clarity. $C3^{-/-}$ mice alive at day 49 continued to gain weight until day 119 post-infection.

(C & D) Mice were inoculated with 700 pfu ECTV via the ear pinna. The data are combined from four separate experiments. ($P = 0.0002$).

(E & F) Mice received 100 pfu of ECTV intranasally. The curves are generated from at least seven separate experiments ($P < 0.0001$).

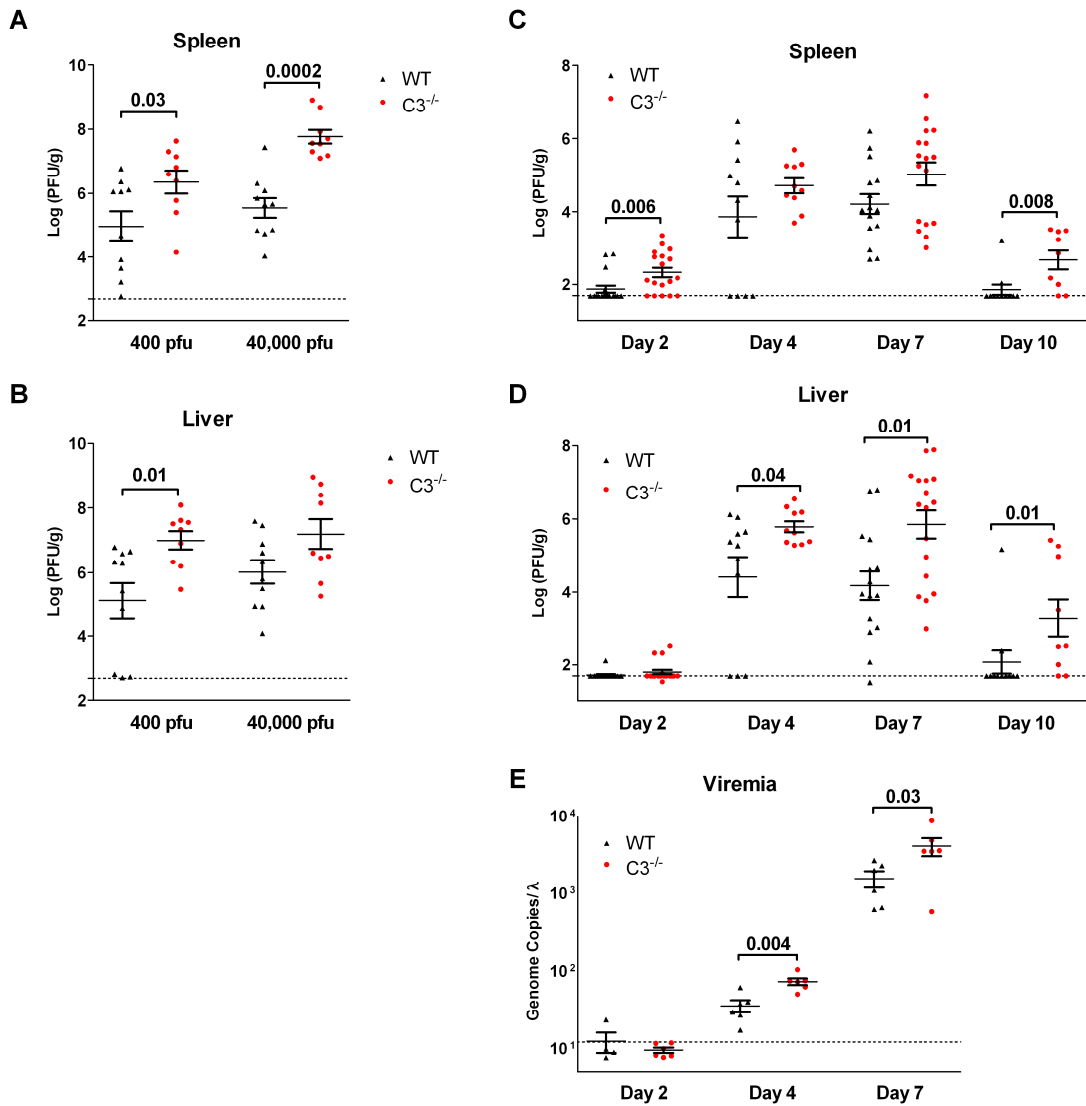


Figure 2-2: C3 deficiency promoted earlier dissemination to and increased viral titers in the target organs

C3^{-/-} and wild-type mice were infected by the footpad or ear pinna route and sacrificed on day 2, 4, 7 or 10 post-infection. The viral titer of the spleen (A, C) or liver (B, D) was determined by direct plaque assay and is expressed as the log₁₀ plaque forming units (PFU)/g tissue. The level of viral DNA in whole blood was measured using quantitative PCR (E).

The scatter plots show the viral burden of each wild-type (WT, black, ▲) and C3^{-/-} (red, ●) animal. The error bars indicate SEM. The wild-type and C3^{-/-} mice were compared, and *P* values <0.05 are reported above the bracket. The dotted line represents the limit of detection for the assay.

(A, B) Mice were infected with 400 or 40,000 pfu of ECTV in the footpad and sacrificed on day 7 post-infection. At 400 pfu, all animals had virus detected in either the spleen or liver.

(C-E) Animals were infected with 700 pfu of ECTV via the ear pinna.

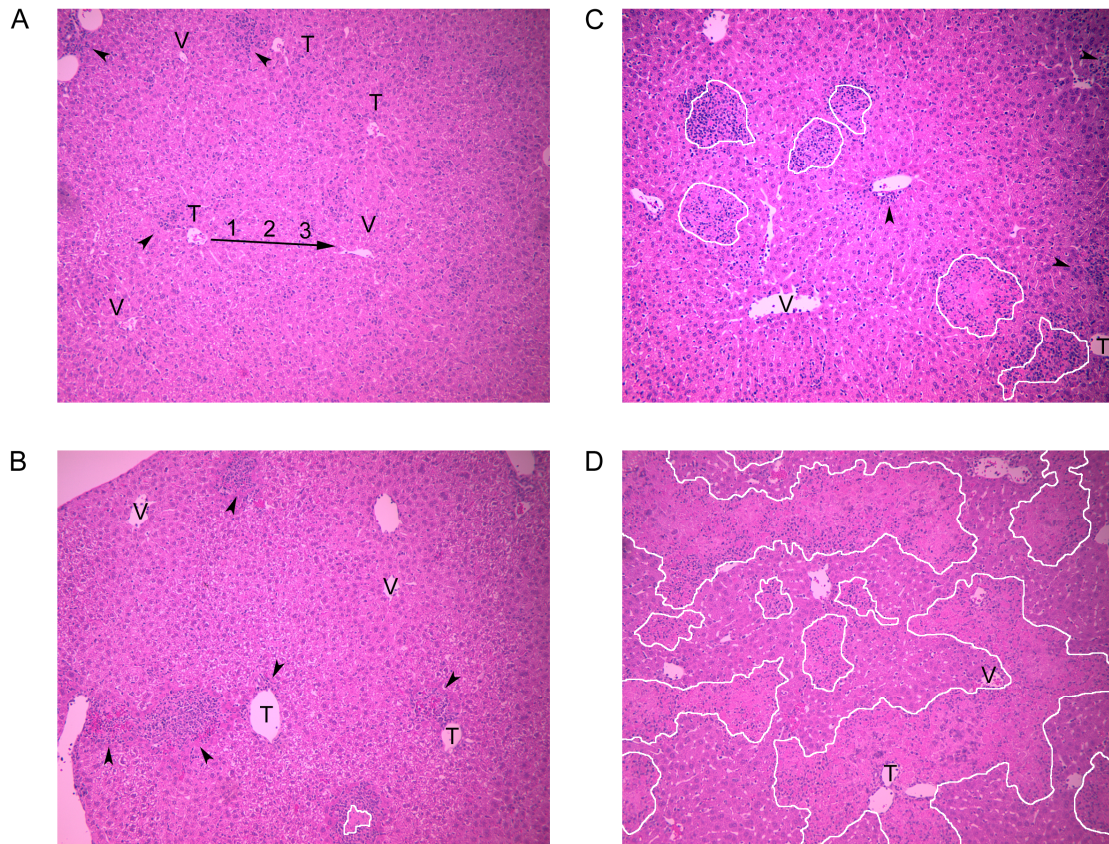


Figure 2-3: Extensive liver necrosis occurred in C3-deficient mice

Liver samples were taken from mice 7 days after infection with 700 pfu via ear pinna, fixed, sectioned, and stained with hematoxylin and eosin. Representative images show the range of differences between the strains. White lines border coalesced areas of necrosis. Arrowheads point to non-necrotic inflammatory foci. T, portal triad; V, central vein.

(A) Wild-type—Blood flows through the liver from zone 1 to 3, as indicated by the arrow. Zone 1 encircles the portal triad; zone 3 encircles the central vein, and zone 2 occurs between zones 1 and 3. There are small inflammatory foci adjacent to two portal triads.

- (B) Wild-type—There are inflammatory foci adjacent to portal triads, and one focus has a small area of confluent necrosis.
- (C) $C3^{-/-}$ —Larger inflammatory foci with areas of confluent necrosis.
- (D) $C3^{-/-}$ —An inflammatory infiltrate borders the extensive necrosis that bridges across all three zones ($T \rightarrow V$).

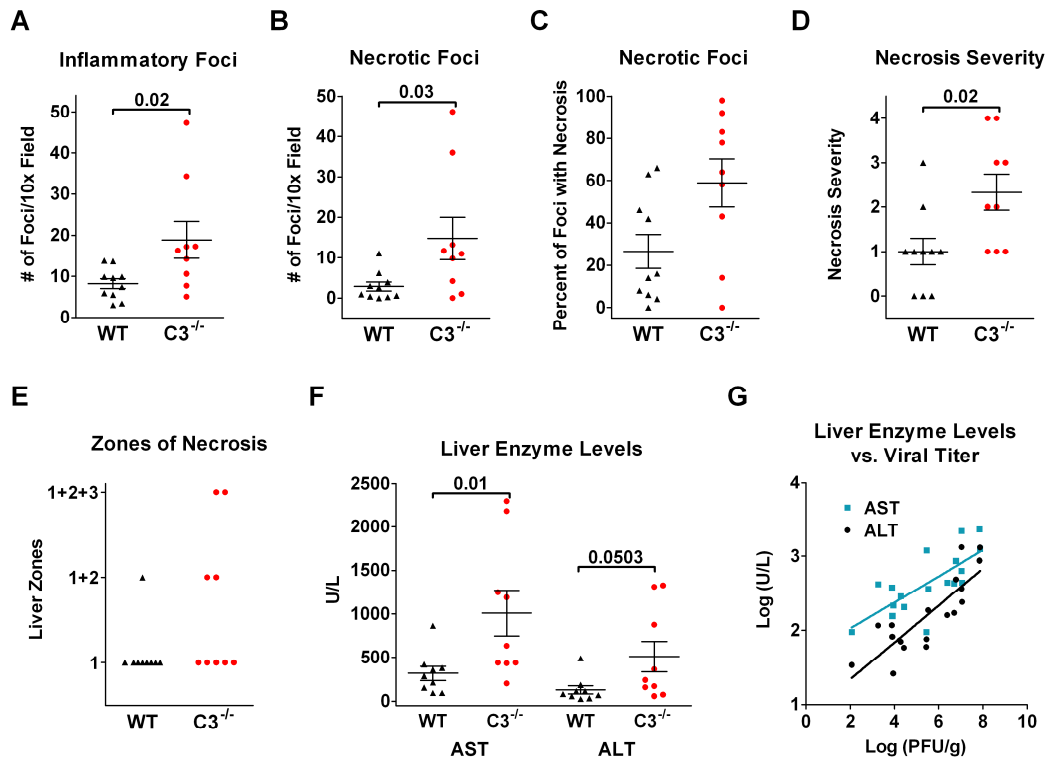


Figure 2-4: C3-deficient mice had a greater number of inflammatory foci with more extensive necrosis

(A-F) Wild-type (WT, black, ▲) and C3^{-/-} (red, ●) mice were compared, and *P* values are above the bracket. Error bars are SEM.

(A) The number of inflammatory foci/visual field was counted. Each point represents the mean from ~7 fields for an individual mouse. The graph plots the mean number of foci for each animal.

(B) The number of inflammatory foci containing necrosis was counted and displayed as described in (A).

(C) The percentage of foci that contained areas of necrosis.

- (D) The severity of the necrosis was quantitated using a 0-4 scale: 0, none; 1, piecemeal necrosis; 2, confluent areas of necrosis; 3, confluent areas of necrosis that extend beyond a single zone; and 4, bridging necrosis.
- (E) The zones of the liver (1, 2, & 3) where necrosis occurred. Zones are described in (Figure 2-3A).
- (F) The serum levels of the liver enzymes in uninfected mice were <100 for AST and <50 for ALT.
- (G) AST (blue) and ALT (black) levels positively correlate with liver viral titer ($r^2=0.54$ and 0.69 , respectively). The log of these parameters for both $C3^{-/-}$ and wild-type mice was used for linear regression.

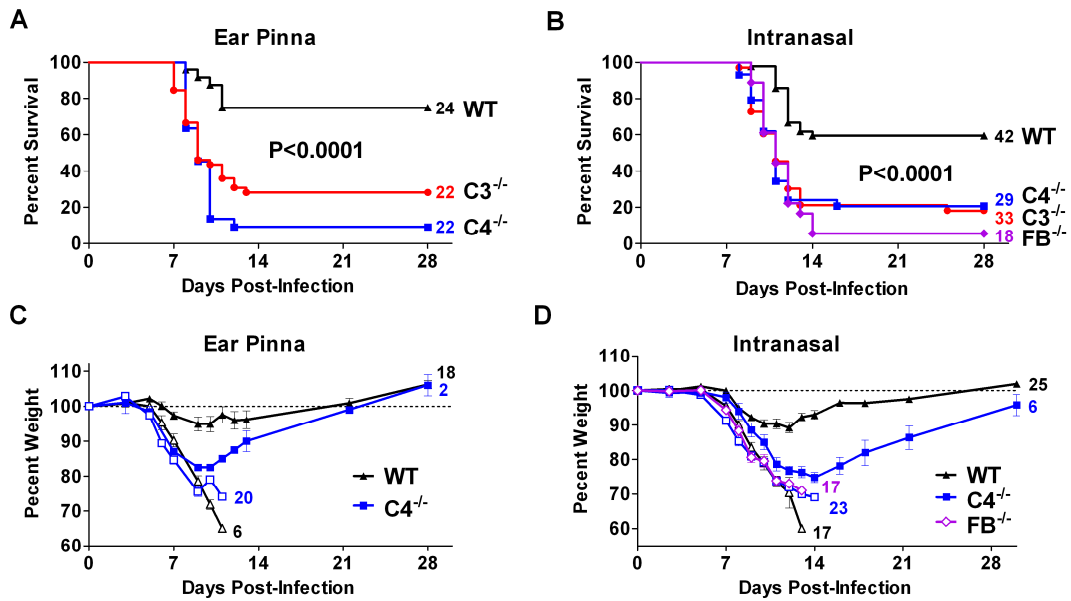


Figure 2-5: Deficiency of classical or alternative pathway components reduces survival

(A & C) $C4^{-/-}$ mice have greater mortality than wild-type (WT, $P<0.0001$). $C4^{-/-}$ mice were compared to $C3^{-/-}$ and wild-type mice following inoculation with ~ 700 pfu ECTV via the ear pinna. Weight loss for individual animals was normalized to their starting weight, and mean \pm SEM is shown. The weights of the surviving animals (filled symbols) are separated from those that succumbed (open symbols). The number of animals in each group is at the right of the line. The data combine four separate experiments.

(B & D) Increased mortality in $C4^{-/-}$, $FB^{-/-}$, and $C3^{-/-}$ mice compared to wild-type ($P<0.0001$). Mice received 100 pfu intranasally. Data are displayed as described above. Curves are generated from multiple experiments (n for $C4=6$, $FB=4$, $C3=7$, $WT=8$). The surviving $FB^{-/-}$ mouse was omitted from (D) for clarity.

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Chapter 3

Mouse complement neutralizes ectromelia intracellular mature virus

Portions of the chapter are excerpted from:

Moulton E.A., Atkinson J.P., and Buller R.M. (2008) **Surviving mousepox infection requires the complement system.** *PLoS Pathogens*. In Press.

Introduction

The complement system consists of cell-surface and serum proteins that interact to destroy invading microorganisms and infected host cells [1-4]. Three distinct pathways activate this cascade: classical, lectin, and alternative. Antibody binding to antigen triggers the classical pathway. Mannan-binding lectin (MBL) and related proteins recognize repetitive carbohydrate motifs on pathogens and infected cells to initiate the lectin pathway [5]. Spontaneously activated C3 initiates the alternative pathway, especially if deposited on surfaces deficient in regulatory proteins [6]. The alternative pathway also serves as a positive feedback loop by forming additional C3 convertases from the C3b produced by any pathway. In the absence of essential activating components, the slower and less efficient bypass pathways can activate the classical and lectin pathways at high serum concentrations [7]. All three pathways converge at the step of C3 cleavage to C3a and C3b, and they share a common terminal pathway that generates the C5a anaphylatoxin and the membrane attack complex (MAC).

Complement activation could delay viral dissemination by opsonizing or lysing, and thereby neutralizing, virions at the inoculation site or in the circulation and by promoting the inflammatory response, including the recruitment of phagocytic cells. To assess if complement could directly neutralize ectromelia virus (ECTV), we examined the interaction between purified ECTV and mouse complement *in vitro*. Our *in vitro* and *in vivo* evidence suggests that the complement system, in the presence of natural antibody, neutralizes ECTV early in infection and contributes to survival.

Methods

Virus production

Plaque-purified Moscow strain ECTV was propagated in murine L929 cells. Intracellular mature viral stocks were purified through a sucrose cushion as described [8] and titrated on BS-C-1 cells, an African green monkey kidney cell line [9]. Both cell lines were cultured in Dulbecco's modified Eagle's media (DMEM, BioWhittaker) supplemented with 10% heat-inactivated fetal calf serum (FCS, HyClone), 2 mM L-glutamine, and antibiotics.

Mice

The following strains on a C57BL/6 background were acquired: $C3^{-/-}$ [10,11] and $FB^{-/-}$ [12,13] from H. Molina, Washington University Medical School; $C4^{-/-}$ [14] from M. Carroll, Harvard Medical School; B cell-deficient μ MT [15] from H. W. Virgin, Washington University Medical School; $C1q^{-/-}$ [16] from M. Botto, Imperial College School of Medicine; $FD^{-/-}$ [17] from Y. Xu, University of Alabama, Birmingham; and $MBL A^{-/-} \times MBL C^{-/-}$ (B6.129S4-*Mbl1tm1Kata Mbl2tm1Kata/J*) and wild-type from Jackson Laboratories. The $C5^{+/+}$ and $C5^{-/-}$ C57BL/10 mice (B10.D2-*Hc1 H2d H2-T18c/nSn*], B10.D2-*Hc0 H2d H2-T18c/oSn*) were also obtained from Jackson Laboratories. Age-matched mice of both sexes were used in the μ MT survival experiments (10-11 weeks-old). Male μ MT mice were used in the sera transfer (10-12 weeks) studies. Some wild-type and μ MT mice were purchased from Jackson Laboratories. The other mice were bred at Washington University in a specific pathogen-free facility. The animals were transported to the biohazard suite at Saint Louis

University at least a week prior to infection. All experiments were performed following the animal care guidelines of the two institutions.

***In vivo* studies**

Mice were inoculated with 10 μ l ECTV diluted in PBS to the indicated dose using a 29 gauge insulin syringe into the hind footpad. Mice were anesthetized for inoculation using CO₂/O₂. Individual mice were marked by ear punching or shaving. After infection and before sacrifice in the mortality studies, mice were manipulated only to obtain weights. In the passive transfer experiment, mice received intraperitoneally 1 ml of wild-type or μ MT C57BL/6 sera on day -1 and 0.5 ml every two days starting on day 0.

Complement neutralization assay

Mouse EDTA plasma and sera were collected on ice from male C57BL/6 mice in microtainer tubes (BD), separated by centrifugation, and then pooled, aliquoted, and frozen at -70°C. Plasma and sera were diluted on ice into GVB \pm Ca⁺⁺/Mg⁺⁺ (B102, B103, Complement Technology) or GVB without Ca⁺⁺/Mg⁺⁺ (GVB^o), respectively, to 2 \times the desired final concentration (vol/vol). Purified ECTV was sonicated and diluted in PBS (without Ca⁺⁺/Mg⁺⁺) to $\sim 5 \times 10^4$ pfu/ml. A 1:10 dilution in the buffer used to dilute the complement source, GVB \pm Ca⁺⁺/Mg⁺⁺, produced a final concentration of 5 pfu/ μ l. An equal volume of virus (30 μ l \approx 150 pfu) was added rapidly to the diluted complement at RT. Samples were vortexed, centrifuged for 5 sec, and incubated at 37°C for 60-90 min. Samples were diluted by addition of 700 μ l of DMEM-2% FCS, vortexed, and applied to BS-C-1 monolayers in 6-well plates. After 1 h, 3 ml/well of 37°C overlay media

(1% carboxymethylcellulose in culture media) was added. After 3-5 days, the cells were fixed with 1 ml/well of an 11% formaldehyde / 0.13% crystal violet / 5% ethanol solution for over 1 h, rinsed, and dried. The number of plaques was scored visually using a light box. The EDTA plasma or sera data were normalized to the buffer only control or heat-inactivated sera, respectively.

Statistical analysis

All statistical analysis was performed using GraphPad Prism software version 5.01 (GraphPad Software). Either 1-way ANOVA followed by Tukey multiple comparisons test or 2-way ANOVA was used for the analysis of the complement neutralization assays. The survival curves were analyzed by the log-rank test.

Results

Mouse complement neutralized ECTV intracellular mature virions

To explore the interaction between C3 and ECTV *in vivo*, we examined how mouse complement affects ECTV virions *in vitro*. Purified intracellular mature virus (IMV) was incubated with either EDTA-treated plasma or sera from naïve C57BL/6 mice. Infectious virus was detected as plaques on a BS-C-1 monolayer. EDTA-treated plasma was reconstituted with a buffer containing calcium and magnesium to allow for complement activation.

Reconstituted wild-type plasma neutralized approximately 90% of the virus (Figure 3-1A, $P < 0.001$). Heat inactivation or buffer lacking calcium and magnesium abolished neutralization. Wild-type sera concentrations of 10, 25, or 50% neutralized 70-80% of the ECTV (Figure 3-1B, $P < 0.0001$). These observations implicate the complement system in neutralizing ECTV.

Classical pathway initiated neutralization

To further define how complement neutralized ECTV, sera from mice genetically deficient in complement components or antibody were used (Figures 3-2F). The neutralizing activity was reduced by ~50% with deficiency of either C3 or C4 (Figure 3-2A). Mixing C3^{-/-} and C4^{-/-} sera produced results equivalent to wild-type sera.

This requirement for C4 for full ECTV neutralization was further dissected. The C1q subunit of C1 interacts with antibody to trigger the classical pathway. MBL, a C1q analog, initiates the lectin pathway. MBL A^{-/-} × MBL C^{-/-}, C1q^{-/-}, and antibody-deficient

(μ MT) sera were compared (Figure 3-2B). μ MT or C1q^{-/-} sera only partially neutralized ECTV, comparable to C4^{-/-} sera. Conversely, wild-type levels of neutralization occurred independent of MBL A and C. These data establish that natural antibody activated the classical complement pathway to neutralize ECTV.

μ MT sera neutralized ECTV when combined with natural antibody

Further analysis revealed three key points relative to natural antibody. First, heat-inactivated wild-type sera behaves like buffer alone, which indicates that natural antibody alone lacks neutralizing activity; instead, complement activity was required to neutralize ECTV (Figure 3-1A & 3-2E). Second, heat-inactivated wild-type sera, as a source of natural antibody, restored the neutralizing activity of μ MT sera (Figure 3-2C). Consistent with this finding, μ MT or heat-inactivated wild-type serum did not effectively neutralize ECTV independently, but they did so in combination. Third, the modest but significantly greater neutralization in the normal compared to heat-inactivated μ MT sera suggests that antibody-independent (alternate pathway) complement activation also occurred.

Alternative pathway contributed to neutralization

C3b deposited by any pathway can then interact with factor B (FB) and factor D (FD) to generate the alternative pathway C3 convertase, which amplifies C3b production (positive feedback loop). Alternative pathway activation itself likely explains the neutralization observed in the μ MT, C1q^{-/-}, or C4^{-/-} sera. FB^{-/-} or FD^{-/-} sera neutralized less ECTV than wild-type sera (Figure 3-2D), which indicates that the alternative pathway enhanced complement-mediated neutralization initiated by the classical pathway.

Opsonization neutralized most ECTV, but lytic activity also had a role

C3b could neutralize ECTV by directly preventing attachment to or entry into the cell or by disrupting the virion's membrane through formation of the C5 convertase and the MAC. C5b initiates the terminal pathway that forms the MAC, and no lytic activity occurs in the absence of C5. C5^{-/-} sera from C57BL/10 mice were used to define the contribution of the MAC to neutralization (Figure 3-2E). C5^{-/-} sera neutralized a significant portion of virus ($P < 0.001$), however, less than C5^{+/+} sera ($P < 0.05$). These findings suggest that opsonization by C4b and C3b mediated most of the neutralization; although, the MAC also contributed.

To conclude, these findings demonstrate that naïve wild-type mouse sera neutralized ECTV. We propose that natural antibodies bound to ECTV and triggered the classical pathway. This led to C4b deposition, formation of the C3 convertase, and C3b deposition on the virus. The alternative pathway amplified the C3b placed on the virion by the classical pathway. Most ECTV neutralization occurred through opsonization by C4b and C3b, with a minor contribution from the MAC.

Natural antibody delayed mortality in μ MT mice

Complement poses a barrier to the systemic spread of pathogens, particularly through the bloodstream [18]. The major role of complement could be to neutralize ECTV upon recognition by natural antibody. Our prior experiments established that B cell-deficient μ MT mice challenged with a high dose of ECTV by the footpad route all died early in infection (94% by day 8) (Figure 3-3A). Their early death suggests that B cells contribute to survival prior to the rise of specific antibody on day 7 [19].

Based on our *in vitro* data and the data of others [20,21], we hypothesized that natural antibody is primarily responsible for this early protection. Consequently, providing μ MT mice with natural antibody should prolong their survival. Based on the work of Ochsenbein *et al.* [21], μ MT mice infected with a high dose of ECTV were treated with naïve sera from either μ MT or wild-type mice (Figure 3-3B). Treatment with wild-type sera increased the median day of death from 7 to 9; however, sera lacking natural antibodies (μ MT) had no effect. On day 8 post-infection, over half of the mice receiving wild-type sera outlived both other groups and 16 of 17 mice from the prior experiments (Figure 3-3C). Thus, natural antibody delayed, but did not prevent, lethal ECTV infection in μ MT mice.

Discussion

Naïve plasma or sera neutralized ECTV in a complement-dependent manner, even at a concentration as low as 10%. Sera from mice deficient in specific complement components demonstrated that maximal neutralization required both the classical and alternative pathways. μ MT sera, lacking antibody, resembled the sera deficient in the classical pathway components, C1q or C4, and addition of natural antibody restored neutralization activity. Opsonization led to neutralization of the majority of viral particles; however, the modest but significant difference between the $C5^{-/-}$ and $C5^{+/+}$ sera indicated that the MAC also contributed to viral damage. Interestingly, no complement component deficiency tested fully abolished neutralization. The residual activity suggests that the classical and alternative pathways functioned independently, likely because both C4b and C3b opsonized and, consequently, neutralized ECTV. However, the system was most effective when the two pathways and the MAC worked cooperatively.

The reconstitution of the neutralization activity in the μ MT sera with heat-inactivated wild-type sera suggests that natural antibody is important in the neutralization process. Consistent with this observation and prior studies with other viruses [20,21], natural antibody passively transferred into μ MT mice lengthened survival during the acute infection. In our experiments, most μ MT mice died early, with 100% mortality by day 9 at the highest inoculum. The mice that survived the acute infection eventually died at about 2 months post-infection. Our findings differ from prior studies, which described mortality at either 2-4 weeks [22] or 2 months [23] post-infection. More of our mice survived the acute infection at

the lower doses. This suggests that the observed discrepancy could be secondary to differences in the viral stock or dose, as both differed among the three groups. The death of the μ MT mice, despite natural antibody treatment, indicates that B cells help control the infection by additional mechanisms.

Our *in vitro* experiments provide a model for understanding the fate of the viral inoculum in our *in vivo* experiments, since they both used the same stock of purified IMV. To understand the spread of infection, a second infectious form must be considered. During viral replication in the host cell, extracellular enveloped virus (EEV) is produced by enveloping the IMV with an additional unique membrane derived from the Golgi complex and late endosomal compartment (Figure 1-1)[24]. In studies of vaccinia virus, the host's complement regulators, present in the outermost membrane, protect the EEV from human and rabbit complement; in contrast, the IMV is sensitive to complement [25]. Our study builds on this observation by determining the contribution of each complement activation pathway to the neutralization of IMV infectivity, and it implicates natural antibody as the primary initiating factor [21]. We also show that natural antibody by itself is ineffective and requires augmentation by the complement system to neutralize ECTV. Additionally, the neutralization observed with vaccinia virus and ECTV points to the IMV form being inherently susceptible to complement-mediated neutralization.

The relative importance of IMV vs. EEV during infection *in vivo* is not well established. However, the IMV's sensitivity to complement neutralization suggests that ECTV likely travels through areas featuring efficient complement activation, such as the blood stream, in the EEV form or within infected cells. At extravascular sites, where

complement levels are lower than in circulation, infected cells may produce sufficient soluble poxviral complement regulatory protein to protect the IMV.

Most poxvirus disease models initiate infection with the complement-sensitive IMV. If complement activity in the mouse behaves as it does *in vitro*, then inoculated ectromelia IMV should be recognized by natural antibody and coated with C4b and C3b, resulting in neutralization of viral infectivity at the site of injection and inhibition of spread. This line of reasoning could explain why the mortality increases in the wild-type mice as the invasiveness of the route decreases [26]. Percutaneous inoculation would likely result in neutralization, while application to the mucosal membranes might enable ECTV to enter host cells before being neutralized by complement. Once internalized, ECTV produces its regulatory protein and EEV to evade complement and propagate the infection. Additionally, based on the *in vitro* data, complement deficiency would greatly limit this initial neutralization, which likely contributes to the early spread and greater mortality observed in the complement-deficient mice.

A sub-neutralizing concentration of complement opsonins could target the virion for immune adherence and phagocytosis *in vivo*, particularly in blood with its high complement levels. Furthermore, the liver sinusoids are lined with Kupffer cells bearing CR1g (complement receptor of the Ig superfamily), which mediates phagocytosis of C3-opsonized pathogens [27]. Indeed, the liver clears over 95% of intravenously administered ECTV from the circulation within 5 min of injection [28]. In the following hour, most of the viral antigen in the liver becomes undetectable by immunofluorescence, and viral infectivity decreases by over 90%. This rapid removal suggests that the virus has been recognized as foreign and

tagged for immune adherence and phagocytosis. Opsonization by complement followed by uptake via the recently described CRIg provides a mechanistic explanation for these important observations made nearly five decades ago [28].

These observations influence the interpretation of poxviral infections initiated with an IMV-rich inoculum by the intravenous route. The liver's Kupffer cells may sequester most of the inoculated virus within minutes and destroy much of it within an hour, thereby inhibiting systemic dissemination. Not only is the dose effectively reduced by ~10-fold, but the neutralized IMV also provides the immune system with an immediate source of antigen. These issues have particular relevance for the monkeypox and variola virus non-human primate models that commonly use the intravenous route to test vaccines and antivirals for human use [29-34].

Figures

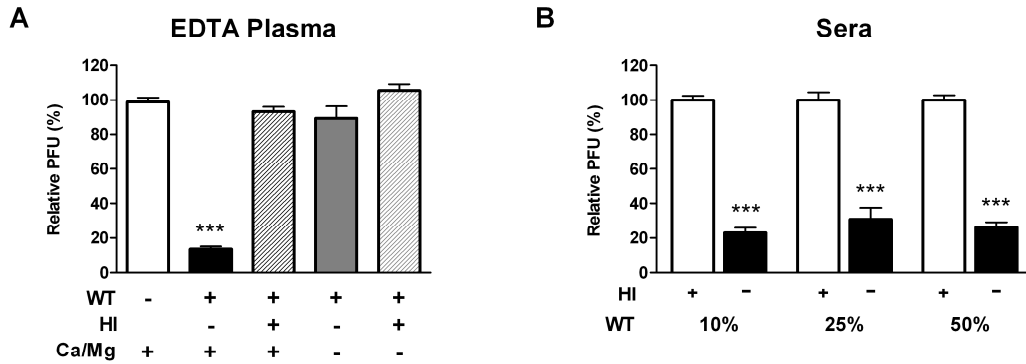


Figure 3-1: The murine complement system neutralizes ECTV virions

(A-B) ECTV was incubated with a mouse complement source at 37°C for 1 hour and then added to BS-C-1 monolayers. The absolute number of plaques was normalized to the appropriate controls (white bars) to give the relative number of plaques. Graphs display mean±SEM from multiple experiments.

(A) Mouse plasma neutralizes ECTV. ECTV was combined with 10% wild-type (WT) mouse EDTA-treated plasma or heat inactivated (HI) plasma in gelatin veronal buffer in the presence or absence of calcium and magnesium (Ca/Mg). The active plasma (in the presence of Ca/Mg) differed from all other groups (***, $P < 0.001$). These data were combined from 9 independent experiments using EDTA plasma collected on 3 separate days. The total number of replicates for each column from left to right follows: $n=25, 29, 17, 7, 3$. Data were analyzed using 1-way ANOVA followed by the Tukey multiple comparison test.

(B) Mouse serum neutralizes ECTV (***, $P < 0.0001$) at multiple concentrations. ECTV was incubated with increasing concentrations of HI (white) or active (black) WT mouse sera.

Data represent 3-4 independent experiments performed in duplicate with 4 different collections of sera. A 2-way ANOVA was used to analyze concentration and activity.

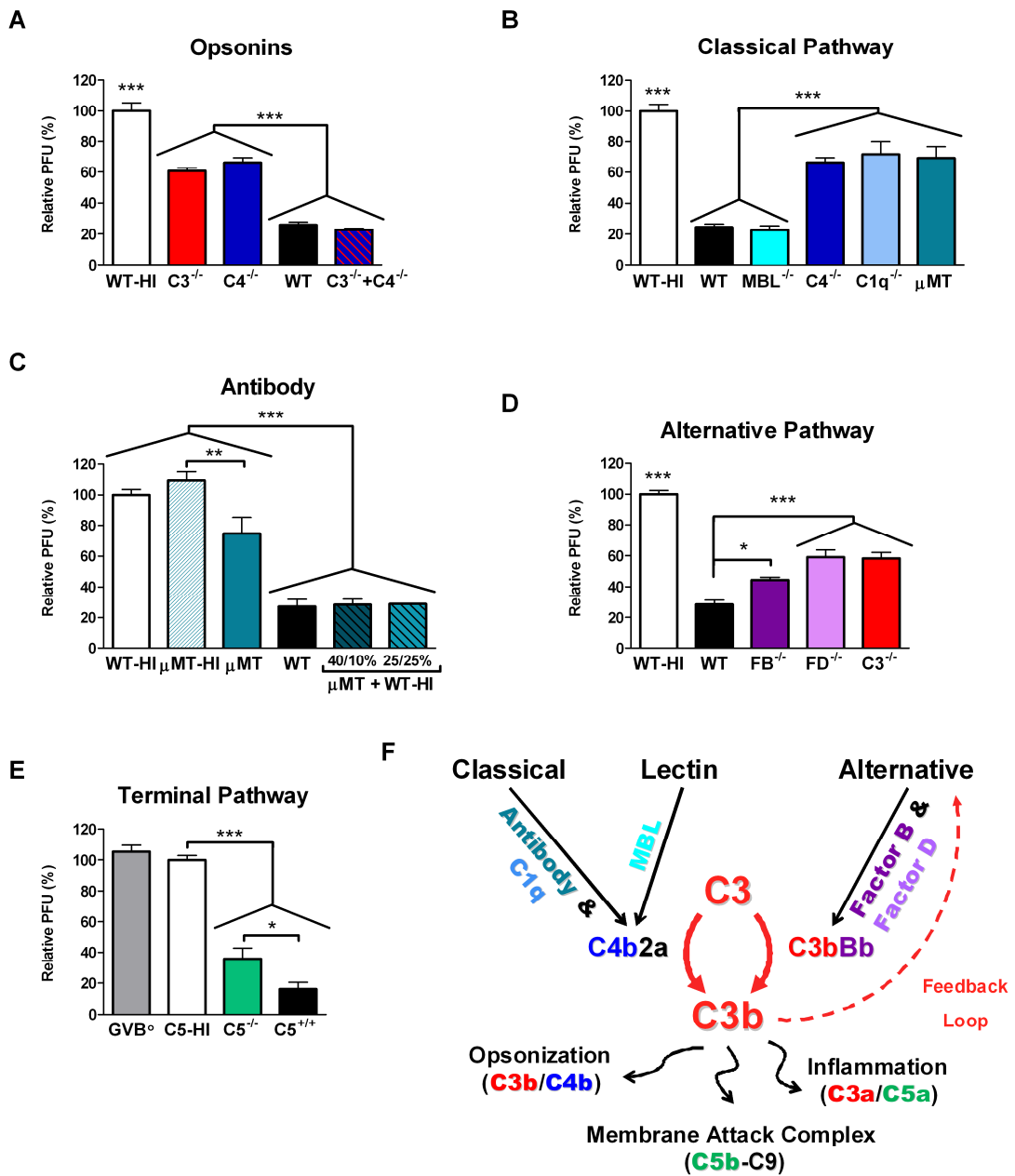


Figure 3-2: Maximal neutralization requires multiple complement pathways

The relative roles of the complement pathways were analyzed using sera from mice genetically deficient in complement components (C3, C4, C1q, MBL A × MBL C, FB, FD,

or C5) or antibody (μ MT). ECTV was incubated at a final concentration of 50% sera at 37°C for 1 hour and then added to BS-C-1 monolayers. The absolute number of plaques was normalized to heat-inactivated sera (HI, white bars) to give the relative number of plaques. Graphs display mean \pm SEM from multiple experiments. Data were combined from at least 2 experiments performed in duplicate with independent collections of sera. All data were analyzed using 1-way ANOVA followed by the Tukey multiple comparison test, and any significant differences among grouped bars are noted on the graph. Unless noted specifically below, the significant differences are displayed on the graph as follows: *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

(A) C3^{-/-} or C4^{-/-} sera have reduced neutralizing capacity. Combining C3^{-/-} and C4^{-/-} sera restored neutralizing activity to WT levels. WT-HI differed from the other conditions (***). Data were from at least 3 experiments performed in duplicate.

(B) Deficiencies in the classical pathway (C4, C1q) and antibody (μ MT), but not the lectin pathway (MBL), reduce neutralization. WT-HI differed from C1q^{-/-} ($P<0.01$) and differed from the rest of the conditions ($P<0.001$). Data were from at least 2 experiments performed in duplicate.

(C) Neutralizing activity requires the alternative pathway components: factor B (FB), factor D (FD), and C3. WT-HI differed from the other conditions (***). Data were from at least two experiments performed in duplicate.

(D) Addition of either 10% or 25% WT-HI sera restores the neutralizing activity of antibody-deficient μ MT sera to WT capacity. Heat inactivation of μ MT sera reduces neutralization. Data were from 2 experiments done in duplicate.

(E) MAC (C5b-9) formation enhances neutralization but is not required. Both the C5^{+/+} and C5^{-/-} sera had greater neutralization than HI-C5^{+/+}. The buffer only control (GVB^o) is equivalent to WT-HI. Data combined from 3 experiments performed in triplicate.

(F) The classical, lectin, and alternative pathways each initiate the complement cascade by forming a C3 convertase that cleaves C3 to C3a and C3b. Antibody triggers the classical pathway through C1q. The carbohydrate motifs on pathogens activate the lectin pathway through MBL. The classical and lectin pathways form the C4 containing convertase, C4b2a. C3 deposited from these two pathways or from spontaneous activation of C3 initiates the alternative pathway. Upon binding the activated C3 molecule, FB is cleaved to Bb by FD, which forms the alternative pathway convertase, C3bBb. The alternative pathway amplifies the C3b deposited by any pathway. C3 activation leads to important antiviral effector functions. Release of the anaphylatoxins C3a and C5a recruits inflammatory cells. C3b and C4b opsonize viral particles or infected cells, leading to neutralization or destruction by phagocytosis. C3b also leads to activation of C5 and formation of the MAC which disrupts virions or infected cells.

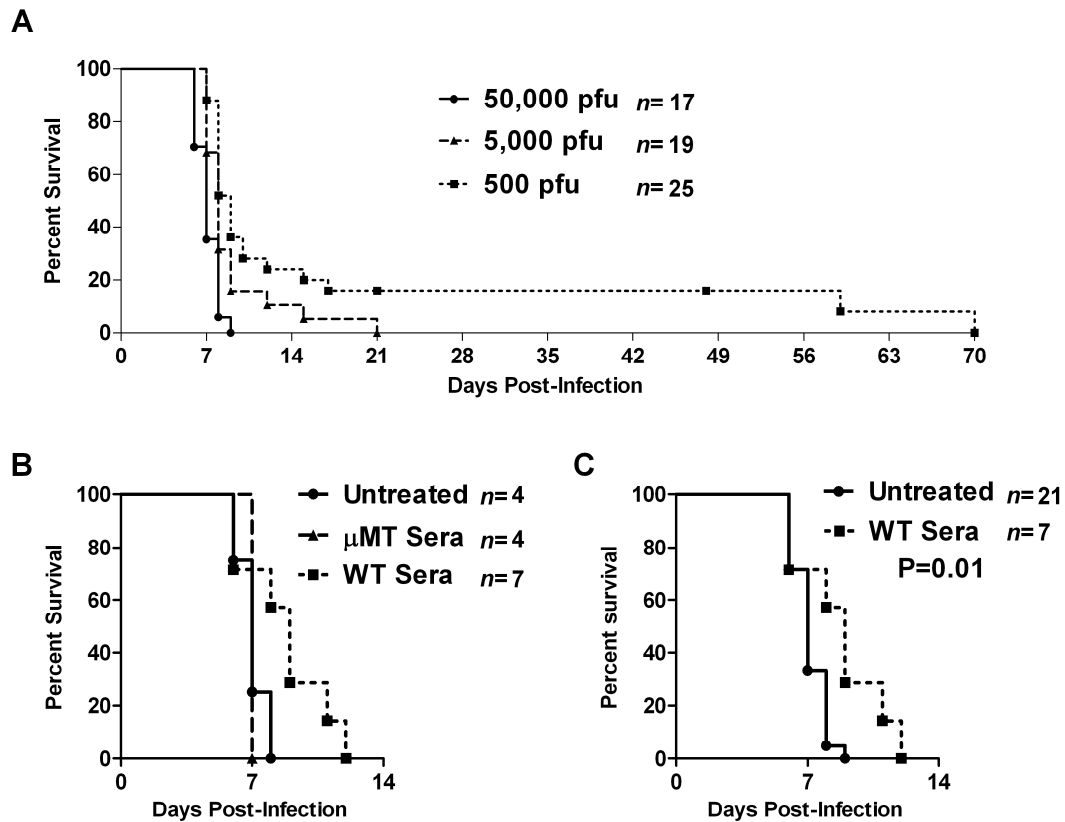


Figure 3-3: Natural antibody delays mortality in μ MT mice

(A) μ MT mice are susceptible to ECTV via the footpad route. μ MT mice were challenged with ECTV, and a dose dependent increase in mortality was observed. The 50,000 pfu dose differs from 5,000 pfu ($P=0.006$) and 500 pfu ($P<0.0001$), and the lower doses differ from each other ($P=0.045$). Survival curves were constructed from 4 separate experiments, and the number of animals is to right of the legend.

(B & C) Treating μ MT mice with natural antibody delays mortality. μ MT mice were challenged with high doses of ECTV (100,000 pfu) via the footpad route in two separate experiments. Some mice received wild-type sera as a source of natural antibody, while others

received μ MT sera (1 ml on day -1 followed by 0.5 ml on days 0, 2, 4, 6, 8, 10). The untreated curve in (G) includes the historical control data from the untreated mice at 50,000 pfu in (A). The number of animals in each group is next the legend.

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Chapter 4

Ectromelia Inhibitor of Complement Enzymes

(EMICE) protects intracellular mature virus

from neutralization by mouse complement

Introduction

In their large double-stranded DNA genomes, poxviruses encode multiple factors that modify the immune system [1,2]. Study of these molecules has provided insights into the balance between viral pathogenesis and the host's immune response [3-6]. Viruses have evolved multiple strategies to evade the complement system [7]. They may hijack the host's complement regulatory proteins by incorporating them in their envelopes [8-11], binding them in the serum [12,13], or upregulating them on the infected cell's surface [14]. In other cases, viruses produce their own complement regulatory proteins to protect themselves [15-18].

Variola virus, vaccinia virus, monkeypox virus, and ectromelia virus (ECTV) each produce an orthologous complement regulatory protein (poxviral inhibitors of complement enzymes, PICE) that has structural and functional homology to host proteins [19-24]. Like the host regulatory proteins membrane cofactor protein (MCP, CD46) and decay accelerating factor (DAF, CD55), the PICEs are composed of four complement control protein (CCP) domains except for MOPICE (monkeypox inhibitor of complement enzymes), which has a frame-shift mutation in CCP-4 that results in premature truncation. The complete loss of MOPICE may account for the reduced virulence observed in the West African compared to Congo basin strains of monkeypox virus [24,25]. The limits of the monkeypox animal models, however, have made this a difficult hypothesis to test. While PICE-deficient mutants have been generated in vaccinia and cowpox viruses, neither of the parent viruses causes a lethal infection in the tested models [20,26]. The loss of the regulatory protein resulted in smaller local lesions with vaccinia [20] and in greater inflammation in the case of

cowpox [26]. An incomplete understanding of the role of the complement system during poxviral infections complicates interpretation of these results.

In vitro, the PICEs regulate complement activation through a combination of decay accelerating activity and cofactor activity. The co-evolution of variola virus with its only natural host, humans, may explain the enhanced activity against human complement observed with SPICE (smallpox inhibitor of complement enzymes) compared to the other PICEs [19,27]. The most well characterized PICE is VCP (vaccinia virus complement control protein) [20,21,28-38]. Originally described as a secreted complement inhibitor [21], VCP also attaches to the surface of infected cells (*in vivo* and *in vitro*) through a covalent interaction with the viral membrane protein A56 [33]. The ability of VCP and SPICE to interact with cells through their heparin-binding sites is also well documented *in vitro* [23,30,31,35].

In this study of the PICE produced by ECTV, the ability of EMICE (EctroMelia Inhibitor of Complement Enzymes) to regulate complement activation was assessed. Recombinant EMICE (rEMICE) reduced activation of both human and mouse complement. Murine cells produced measurable EMICE 4–6 h post-infection. The level of EMICE in the supernatant reached 1 µg/ml by 16 h, which is prior to the release of the majority of the complement-sensitive intracellular mature virus (IMV) from infected cells. At a concentration of 1 µg/ml, rEMICE protected ECTV IMV from complement-mediated neutralization.

Methods

rEMICE production

Using EMICE cDNA (in pSG5) as a template, the coding sequence of EMICE was generated by PCR using the following primers: 5'- CCGGAATTTCGGAATGTGCTGTACTATTCCGTCACG-3' and 5'- ATAAGAATGCGGCCGCTTATTCGCGTACACATTTTGG-3'. The resulting PCR fragment was ligated into the EcoR1 and NotI sites of pET28a-2 [23], a derivative of pET28a (EMD/Novagen) generated in our laboratory. For recombinant protein production, the following method was developed [23]. First, 25 ml of an overnight culture of *E. coli* containing the construct was inoculated into 500 ml of LB containing kanamycin (30 mg/ml) and chloramphenicol (34 mg/ml) and grown to an OD₆₀₀ of 0.6–0.8 followed by induction with 1 mM isopropyl-β-D-thiogalactopyranoside at 37°C for an additional 3–5 h. Cells were harvested and pellets were frozen at -80°C until needed. For inclusion body protein purification, pellets were thawed and resuspended in 50 ml of solution buffer (50 mM Tris (pH 8.0), 25% sucrose, 1 mM EDTA, 0.01% NaN₃, 10 mM DTT) to which 0.8 ml of freshly prepared 50 mg/ml lysozyme (Sigma-Aldrich), 1250 U of benzonase nuclease (Novagen), and 1 ml of 1 M MgCl₂ were added. An equal volume of lysis buffer (50 mM Tris (pH 8.0), 1% Triton X-100, 0.1 M NaCl, 0.01% NaN₃, 10 mM DTT) was added, and the solution was stirred gently at room temperature for 1 h. After cooling, the suspension was sonicated with three 15 s bursts (Fisher Scientific model 500 Sonic Dismembrator) at 50% amplitude followed by the addition of 5 ml of 0.5 M EDTA. The lysate was then centrifuged at 6000 × g for 30 min at 4°C. The resulting

inclusion body pellet was washed (50 mM Tris (pH 8.0), 0.5% Triton X-100, 0.1 M NaCl, 1 mM EDTA, 0.01% NaN₃, 1 mM DTT) followed by a second wash with the same buffer but without Triton X-100. For solubilization of the inclusion bodies, the pellet was resuspended in 6 M guanidine HCl, 10 mM Tris (pH 8.0), and 20 mM 2-ME and centrifuged at 14,000 × g for 10 min. A second high-speed centrifugation at 100,000 × g for 30 min at 4°C was performed to remove any insoluble material. For protein refolding, solubilized inclusion body protein was added dropwise in three injections over 36 h at a final concentration of 10–100 µg/ml in refolding buffer (100 mM Trizma base, 400 mM L-arginine-HCl (Sigma-Aldrich), 2 mM EDTA, 0.02 M ethanolamine, 0.5 mM oxidized glutathione (Sigma-Aldrich), and 5 mM reduced glutathione (Sigma-Aldrich)). The refolding solution was concentrated in a Millipore stirred filtration cell followed by buffer exchange with 20 mM Tris (pH 8.0).

EMICE quantification and detection

rEMICE was quantified using carbonic anhydrase (C5024, Sigma) as a standard on a Coomassie-stained SDS polyacrylamide gel. EMICE in tissue culture samples was compared to rEMICE via Western blotting [24]. Non-reduced and reduced samples were electrophoresed in 12% SDS-polyacrylamide gels (Invitrogen), transferred to nitrocellulose membranes, and probed with 1:5000 dilution of a previously described rabbit anti-VCP antibody [24] followed by HRP goat anti-rabbit IgG (GE Healthcare). rEMICE was also detected via flow cytometry [23]. Cells were incubated with a 1:5000 dilution of rabbit anti-VCP for 30 min at 4°C followed by FITC-donkey anti-rabbit IgG secondary antibody diluted to 1:100 for 30 min at 4°C (Sigma). After washing, the cells were resuspended in 0.5% paraformaldehyde and analyzed with a BD Biosciences FACSCalibur system.

C3b and C4b binding assay

Binding to C3b and C4b was detected using a previously described ELISA format [39]. Briefly, wells were coated overnight at 4°C with human C3b or C4b (Complement Technologies, CompTech) diluted in PBS to 5 µg/ml followed by blocking for 1 h at 37°C in 1% BSA, 0.1% Tween 20 in PBS. EMICE was diluted in low salt ELISA buffer (10 mM Tris (pH 7.2), 25 mM NaCl, 0.05% Tween 20, 4% BSA, 0.25% Nonidet P-40) and incubated for 1.5 h at 37°C. Wells were washed with low salt ELISA buffer between each step. Incubation with rabbit anti-VCP antibody diluted 1:5000 in low salt ELISA buffer for 1 h at 37°C was followed by peroxidase-coupled donkey anti-rabbit IgG (Jackson ImmunoResearch) at 1:10,000 for 1 h at 37°C. The 3,3',5,5'-tetramethylbenzidine substrate was added, and the OD was determined at 630 nm. Binding assays employed serially diluted samples in at least three separate experiments.

C3b and C4b cofactor assay

The cofactor assays were previously described [40]. In brief, biotinylated ligands were combined with human factor I (100 ng) and varying concentrations of the cofactor proteins in low salt cofactor buffer (10 mM Tris (pH 7.2), 25 mM NaCl, 1% Nonidet-P40). Cleavage products were evaluated using 10% reducing gels (Invitrogen) followed by transfer and Western blotting with ExtrAvidin-HRP (E2886, Sigma). Detection was with SuperSignal Substrate (34380, Pierce). Assays were performed in duplicate at least three times.

Decay-accelerating assay

Decay-accelerating assays for the classical pathway C3 convertase were performed as previously described [24] with modifications for reading absorbance via microtiter dishes. Briefly, Ab-coated sheep erythrocytes (EA, CompTech) were diluted in dextrose gelatin veronal buffer (DGVB⁺⁺) to 1.7×10^8 cell/ml. The cells were washed by centrifugation for 5 min at $2000 \times g$ and resuspended in DGVB⁺⁺ between sequential incubations with the following human proteins (CompTech): C1 at 1 $\mu\text{g}/\text{ml}$ for 15 min at 30°C, C4 at 2.2 $\mu\text{g}/\text{ml}$ for 15 min at 30°C, and C2 at 0.25 $\mu\text{g}/\text{ml}$ for 4 min at RT. After a brief chilling on ice, the cells were washed and resuspended at 1.7×10^8 cell/ml in DGVB⁺⁺. To assess decay accelerating activity, 50 μl of inhibitor (diluted in DGVB⁺⁺) was combined with an equal volume of prepared cells for 10 min at 30°C. Subsequently, 250 μl of guinea pig serum (CS1662, Colorado Serum Company) diluted 1/20 in 40 mM EDTA-GVB (CompTech) was added and incubated for 30 min at 37°C. After centrifugation for 3 min at $2000 \times g$, the supernatant was removed and the absorbance at 405 nm was determined. Cells were incubated in distilled water to produce the 100% lysis control, while cells incubated only in buffer were the background control. Assays were performed at least four times with each condition in duplicate.

Complement challenge assay

Chinese hamster ovary cells (CHO), the American Type Culture Collection CHO-K1 line, were obtained from the Washington University Tissue Culture Support Center. They were cultured in Ham's F12 medium supplemented with 10% heat-inactivated (HI) fetal calf

serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin G, and 100 µg/ml streptomycin sulfate.

The standard procedure for initiating the complement pathways [41] was modified to use mouse sera as the source of complement. CHO cells (about 70% confluence) were freed using the cell detachment buffer (4mM EDTA (Sigma) and 10% HI-FCS in PBS) and collected and washed in PBS-1% FCS. To allow rEMICE to bind the CHO cells, 1×10^6 cells were mixed with 20 µg of rEMICE in a total volume of 100 µl in 96 well V bottom plate (1×10^6 /well) and incubated at 30°C in an Eppendorf Thermomixer for 30 min (300 rpm). Following incubation, cells were placed on ice and washed with PBS-1% FCS. The sensitizing antibody, IgG from rabbits injected with whole CHO cells (Harlan Bioproducts for Science), was added to the cells, and the mixture was incubated for 20 min at 4°C on a shaker (600 rpm). The cells were centrifuged at $466 \times g$ for 5 min; the supernatant was discarded.

For complement activation, the cells were washed in 100 µl gelatin veronal buffer with Ca^{++}/Mg^{++} (GVB⁺⁺, G-6514, Sigma) and then resuspended in 50 µl GVB⁺⁺. An equal volume of freshly isolated mouse sera diluted into the same buffer to a 20% concentration was added and mixed thoroughly. Mouse sera were collected in eppendorf tubes, clotted on ice for 30 min, and the supernatant was stored at 4°C until use. After 45 min incubation at 37°C (300 rpm) in the Thermomixer, the samples were washed with 100 µl of PBS-1% FCS. Cells were stained with a 1:100 dilution of FITC conjugated goat F(Ab')₂ fragment to mouse C3 (Cappel 55510) for 30 min at 4°C with shaking (600 rpm), washed in PBS-1% FCS, and

resuspended in 0.5% paraformaldehyde in PBS. C3 deposition was detected by flow cytometry.

Virus production and culture

Plaque-purified Moscow strain ECTV was used to generate the EMICE-deficient virus (Δ EMICE). The left and right flanking segments of the EMICE gene (017) were selected to produce a central 600 bp deletion. They were amplified by PCR using EVM 017 LF-5' (GCGGGCGCCGTGGAGTTTATACCACGTATGAG) with EVM017 LF-3' (GCGACG CATTGCGTCGACGCTAGCGGACGTGACGGAATAGTACAG) and EVM017 RF-5' (GCGACCGTACTCGAGGGCGGCCGCAAGCTTGATCATACTCATAACAAGCACAAT G) with EVM 017 RF-3' (GCGGAATTCCGTATCTCCGACA AGCACGTAG) and then ligated into pNCEV017. This plasmid was recombined into ECTV as previously described [42]. Briefly, six-well plates of CV-1 cells were infected with ECTV (2×10^7 pfu/well) and then transfected with 2 ng of pNCEV017 using Lipofectamine 2000 (Invitrogen). After 48 h, cell lysates were collected and isolates were subject to two rounds of plaque purification in the presence of mycophenolic acid followed by three rounds without mycophenolic acid. The EMICE gene was reintroduced to Δ EMICE using a similar protocol and a 10 kb PCR product from genomic DNA (23179–33190 bp). Crude Δ EMICE stock (4×10^6) was combined with 2 μ g psoralen, 120 μ g bovine serum albumin, and DMEM to a total volume of 1 ml. After 10 min incubation, the mixture was exposed to a UV lamp in a 6-well tissue culture plate and applied to a BS-C-1 monolayer. The 10 kb PCR product and viral DNA from Δ EMICE were transfected with Lipofectamine 2000 at a 1:40 molar ratio. The resulting virus was collected and subject to four rounds of plaque purification on BS-C-1

cells. The deletion and restoration of EMICE was detected by PCR using the primers EVM 017 LF-5' and EVM 017 RF-3' and confirmed by Western blotting. Viral DNA was isolated from infected BS-C-1 cells, an African green monkey kidney cell line, using the DNeasy Blood and Tissue Kit (cultured cell protocol, Qiagen).

Plaque-purified ECTV strains were propagated in murine L929 cells. Intracellular mature viral stocks were purified through a sucrose cushion as described [43] and titrated on BS-C-1 cells [44]. A single stock of each virus was aliquoted, titrated, and used for all experiments. Both cell lines were cultured in Dulbecco's modified Eagle's media (DMEM, BioWhittaker) supplemented with 10% HI-FCS (HyClone), 2 mM L-glutamine, and antibiotics.

In the EMICE production studies, 24-well plates of L929 with 10^6 cells/well were infected at an MOI of 1 with Δ EMICE or +EMICE ECTV for 1 hr. Each well was washed with 1 ml of 37°C DMEM two times. Finally, 1000 μ l of DMEM was added to each well, and this marked the 0 h time point. At each time point, 800 μ l of supernatant was collected for analysis, and the remaining supernatant was discarded. The cell layer was washed two times with 1 ml DMEM; 100 μ l of DMEM was added; the cells were scraped off, resuspended, and collected. 200 μ l of supernatant was used to determine the viral titer. At early times, 500 μ l of supernatant was concentrated 10-fold in a Millipore concentrator unit (UFV5BCC25). All samples were stored at -70°C. Frozen samples were thawed, mixed 1:1 with Laemmli Sample Buffer (Bio-Rad), boiled for 10 min, and transferred on dry ice to Washington University where they were analyzed by Western blotting.

Mice

The following strains on a C57BL/6 background were acquired: C3^{-/-} [45,46] and FB^{-/-} [47,48] from H. Molina, Washington University Medical School; C4^{-/-} [49] from M. Carroll, Harvard Medical School; B cell-deficient μ MT [50] from H. W. Virgin, Washington University Medical School; C1q^{-/-} [51] from M. Botto, Imperial College School of Medicine; FD^{-/-} [52] from Y. Xu, University of Alabama, Birmingham; and MBL A^{-/-} \times MBL C^{-/-} (B6.129S4-*Mbl1tm1Kata Mbl2tm1Kata*/J) and wild-type from Jackson Laboratories. The C5^{+/+} and C5^{-/-} C57BL/10 mice (B10.D2-*Hc1 H2d H2-T18c*/nSnJ, B10.D2-*Hc0 H2d H2-T18c*/oSnJ) were also obtained from Jackson Laboratories.

Complement neutralization assay

Mouse sera were collected on ice from male C57BL/6 mice in microtainer tubes (BD) and then pooled, aliquoted, and frozen at -70°C. EMICE or a control protein was serially diluted 1:10 in GVB without Ca⁺⁺/Mg⁺⁺ (GVB^o, B103, CompTech) producing a final volume of 20 μ l. Purified ECTV was sonicated and diluted in PBS (without Ca⁺⁺/Mg⁺⁺) to 5×10^4 pfu/ml. A 1:10 dilution in GVB^o produced a final concentration of 5 pfu/ μ l. Wild-type mouse sera were diluted to 60% in GVB^o, and 10 μ l of this was added to the protein containing tubes. An equal volume of virus (30 μ l \approx 150 pfu) was added rapidly to the diluted complement/protein at RT. Samples were vortexed, centrifuged for 5 sec, and incubated at 37°C for 2 min in water bath and for 60–90 min in an incubator. Samples were diluted by the addition of 700 μ l of DMEM-2% FCS, vortexed, and applied to BS-C-1 monolayers in 6-well plates. After 1 hr, 3 ml/well of 37°C overlay media (1% carboxymethylcellulose in culture media) was added. After 3–5 days, the cells were fixed with 1 ml/well of

11% formaldehyde / 0.13% crystal violet / 5% ethanol solution for over 1 hr, rinsed, and dried. The number of plaques was scored visually using a light box. The data were normalized to the HI-sera control.

***In vivo* studies**

Mice were anesthetized with ketamine/xylazine and inoculated intranasally with 10 μ l ECTV diluted in PBS to the indicated dose using a 20 μ l pipettor. The 100 pfu experiments used male mice (8–12 weeks). Wild-type male and female mice (10–12 weeks-old) received 500 pfu or 250 pfu, respectively. Individual mice were marked by ear punching or shaving. After infection and before sacrifice, mice were manipulated only to obtain weights.

Statistical analysis

All statistical analysis was performed using GraphPad Prism software version 5.01 (GraphPad Software, San Diego, CA). The CHO complement deposition used an unpaired t test (two-tailed). The EC₅₀ was determined using non-linear regression (log(agonist) vs. normalized response—variable slope). The survival curves were analyzed by the log-rank test.

Results

Characterization of rEMICE's complement regulatory activity

Similar to our previous work with recombinant PICEs (rVCP, rMOPICE, and rSPICE) [23,24], we produced rEMICE in *E. coli* and analyzed its regulatory activity. Throughout these experiments, rEMICE was compared to rSPICE, a potent regulator of the human complement system that served as a positive control. The regulation of the complement system by PICEs requires an ability to bind the key components of the C3 convertases, C3b and C4b. rEMICE's interaction with these two proteins was assessed using an established ELISA protocol [39].

In the case of human C3b, rEMICE bound approximately 10-fold less than rSPICE (Figure 4-1A). rSPICE has the highest affinity for C3b of the PICEs tested [24]. Upon comparison to published data, rEMICE's binding ability most closely resembled that of rMOPICE and was greater than rVCP, which is about 100-fold less than rSPICE. In contrast, rEMICE and rSPICE bound C4b similarly (Figure 4-1B). The higher affinity for human C4b compared to human C3b was also observed for rMOPICE and rVCP [24].

Given the efficient binding to C4b and previous reports of decay accelerating activity being mediated by the PICEs [24,27,34], we explored the ability of rEMICE to dissociate (decay) the human classical pathway C3 convertase (Figure 4-1C). In this experimental method, the convertase (C4bC2a) was assembled on the surface of antibody-coated erythrocytes using purified human complement components. The cellular intermediate was then incubated with the regulatory protein followed by C3 and the late components of the

complement system. If the regulatory protein efficiently dissociates the convertases, then there is less hemolysis of the cells. rEMICE decayed the human classical pathway C3 convertase, being about 50% as active in this regard as rSPICE.

In contrast to decay accelerating activity, cofactor activity permanently deactivates the convertases. Factor I, in the presence of a cofactor like the PICEs, cleaves the α chain of C3b or C4b, and the resulting cleavage fragments can no longer form a convertase. rEMICE's ability to serve as a cofactor protein was assessed by combining it with the protease factor I and biotinylated C3b or C4b. Similar to rSPICE, rEMICE serves as a cofactor protein for the cleavage of C3b and C4b at concentrations as low as 3.3 ng/ml (Figure 4-1D), and no cleavage occurred in the absence of factor I.

rEMICE bound CHO cells and protected against mouse complement

The three heparin binding sites that mediate SPICE's interaction with the surface of cells are present in EMICE [23,24]. Secreted EMICE could limit complement deposition on infected cells by binding to their surface. Similar to rSPICE, rEMICE bound to CHO cells (Figure 4-2A). This enabled us to next assess how well rEMICE regulated mouse complement on a cell surface.

CHO cells, with or without prior exposure to rEMICE, were mixed with an anti-CHO antibody, and then mouse sera was added. Flow cytometry was employed to measure the quantity of C3b deposited (Figure 4-2B). Since the classical pathway was activated (wild-type or FB^{-/-} sera) by the antibody in this assay, it most closely mimics an infection process upon the production of specific antiviral IgG antibody. In this setting, rEMICE did not impact C3 deposition. However, if only the alternative pathway was activated (C4^{-/-}sera),

rEMICE significantly reduced C3 deposition by about 90% ($P=0.002$, Figure 4-2C). EMICE's ability to regulate the alternative pathway may help ECTV evade the innate and early adaptive immune response; however, after the induction of a strong humoral response, classical pathway activation may overwhelm EMICE's regulatory capacity.

Generation of EMICE-deficient virus

Loss of the complement regulatory protein affects local lesion size of cowpox and vaccinia virus [20,26]; however, these non-lethal infection models cannot address the hypothesis that loss of MOPICE produces the reduced mortality observed in the West African strains of monkeypox [25]. To attempt to address this, an EMICE-deficient ECTV (Δ EMICE) was produced by deleting 600 bp from the middle of the EMICE gene in the wild-type Moscow strain of ECTV (WT). The functional EMICE gene was restored in the Δ EMICE strain to produce the rescue strain (+EMICE). EMICE-containing clones were selected by PCR, and EMICE production was confirmed by Western blotting (Figure 4-3).

EMICE secreted early during infection, prior to the release of virus

To understand when and where EMICE might influence the infection, murine L929 cells were infected at an MOI of 1, washed, and then cultured in 1000 μ l of media. The EMICE produced was assessed in the supernatants and cells via Western blotting. Given the low levels of EMICE observed at the earliest time points in prior experiments, the supernatants at initial time points were concentrated 10-fold (Figure 4-4A). The blots of concentrated and neat supernatant show the EMICE contained in 10% or 1% of the total supernatant, respectively (Figure 4-4A & B). To enable comparison, 10% of the total cells were also

analyzed (Figure 4-4C). The quantity of EMICE produced was approximated using rEMICE as a standard (Figure 4-4D).

EMICE was undetectable in the supernatant (Figure 4-4A) and cells (data not shown) at 0 h and in the samples from the Δ EMICE virus, which served as a control for potential cross-reaction of the antibody with other viral proteins. In the concentrated supernatants at 4 h, greater than 1 ng of EMICE was detected, indicating that the concentration was at least 10 ng/ml. In the next 2 h, the concentration of EMICE increased 10-fold to 100 ng/ml, as indicated by the 10 ng in the concentrated sample (Figure 4-4A) and about 1 ng in the unconcentrated supernatant (Figure 4-4B). An additional 10-fold increase in EMICE concentration to 1000 ng/ml (1 μ g/ml) occurred between 12–16 h since there was greater than 10 ng of EMICE in the unconcentrated supernatant at 16 h. In contrast, minimal quantities of EMICE were detected in the cells at all time points (Figure 4-4C), which indicates that EMICE is predominately a secreted protein.

The largest increase in infectious virus in the supernatant occurred between 16–18 h post-infection (Figure 4-4E). The release of IMV from infected cells likely accounts for much of this increase, since the majority of the virus detected at 18 and 24 h post-infection was IMV (data not shown). At 16 h, the EMICE level was 1000 ng/ml (Figure 4-4D), and most of rEMICE's complement regulatory activity occurred *in vitro* at lower concentrations (Figure 4-1). The large quantity of EMICE secreted from infected cells prior to the release of IMV could provide protection from complement-mediated destruction.

Soluble rEMICE protected ECTV IMV

As described in chapter 3, mouse complement neutralizes the IMV form of ECTV effectively *in vitro*, even at serum concentrations as low as 10%. Sera from mice genetically deficient in antibody or either classical or alternative pathway components had decreased activity. Our prior data indicate that natural antibody initiates the classical complement cascade, and the subsequent engagement of the alternative pathway amplifies opsonization, the predominant process of neutralization in wild-type mice.

To test EMICE's ability to protect IMV, rEMICE or a control protein was mixed with wild-type mouse sera just prior to the addition of purified IMV. The IL-18 binding protein of ECTV was used as a control because it was also produced in *E. coli* and is similar in size. Sera (10%) neutralized 87% of the virus in the absence of recombinant protein. The IL-18 binding protein did not affect neutralization, except slightly at the highest condition when the IL-18 binding protein and its buffer were 40% of the reaction volume ($\leq 4\%$ in all other conditions). In contrast, rEMICE provides greater protection than the control with 1000-fold less protein. At the highest concentration, rEMICE reduced neutralization by 85%. However, protection of 50% of the virus occurred at a lower concentration, EC_{50} of 280 ng/ml, as determined by non-linear regression. This level is about 4-fold lower than the 1 $\mu\text{g/ml}$ concentration of EMICE observed *in vitro* (Figure 4-4D).

Δ EMICE and +EMICE ECTV were attenuated in wild-type mice and shared a similar change in plaque morphology

The intranasal route of inoculation can produce a lethal infection in the normally resistant C57BL/6 mice. This route was used to determine if a loss of EMICE expression would

reduce mortality *in vivo*. The 100 pfu dose selected in the first set of experiments was intended to produce 50% mortality in the wild-type mice. Ideally, this dose would be sensitive to changes in virulence and would enable detection of increased susceptibility in the complement-deficient mice.

This dose did produce significantly greater mortality in all three complement-deficient strains compared to the wild-type mice ($P < 0.0001$, Figure 2-5B). However, a mortality difference between WT and Δ EMICE ECTV was not observed in wild-type mice at 100 pfu (Figure 4-6A). Despite the similar mortality, the mice that received Δ EMICE ECTV appeared healthier 9 days post-infection, and had less weight loss compared to the mice that received the WT ECTV (data not shown). Reproducible attenuation was demonstrated in wild-type mice at higher infectious doses (Figure 4-7). At 100 pfu, Δ EMICE ECTV was attenuated in the $C3^{-/-}$ ($P = 0.02$, Figure 4-6B) and $FB^{-/-}$ ($P = 0.002$, Figure 4-6C) mice. In contrast, the two viruses produced equivalent mortality in the $C4^{-/-}$ mice (Figure 4-6D). These data suggest that regulation of C4 may be EMICE's primary role *in vivo*. This ability to control classical pathway activation may be particularly advantageous as the virus confronts the host's antiviral antibodies, either natural antibody in the first days of the infection or the initial adaptive response.

At higher doses of WT ECTV that cause a uniformly lethal infection, significant attenuation was observed with the Δ EMICE ECTV (Figure 4-7). Unfortunately, virulence was not consistently restored by replacing the EMICE gene (+EMICE ECTV). During the course of these experiments, an increase in plaque size in the Δ EMICE and +EMICE ECTV compared to the WT ECTV was noticed when the cultures were accidentally allowed to

overgrow for several days. It therefore seems likely that the mutation responsible for the change in plaque size influenced the *in vivo* results and produced the attenuation observed in the Δ EMICE and +EMICE ECTV. Alternatively, the attenuation observed in +EMICE ECTV could result from a second mutation acquired during the plaque purification process when generating the rescue strain.

Discussion

At the amino acid (aa) sequence level, EMICE most closely resembles VCP (18 aa differ & 2 aa deletion), then MOPICE (18 aa differ prior to the frame shift mutation), and finally SPICE (26 aa differ & 2 aa deletion) (Figure 1-5). Over half (10 of 18) of the residues that are unique to EMICE occur in CCP-1, where the other PICEs are essentially identical. This concentration of differences likely results from the co-evolution of ECTV with its host. The changes presumably improve viral fitness by increasing EMICE's ability to regulate mouse complement, similar to SPICE's enhanced activity (vs. VCP) for human complement [19]. Alternatively, natural ECTV infection is not subject to the selective pressures that have preserved CCP-1 in SPICE, VCP, and MOPICE.

While several monoclonal antibodies to CCP-1 of VCP do not block functional activity [37], this domain is required for binding to C3b and C4b [28,34]. EMICE's ability to bind C3b mirrored what was previously observed with MOPICE [24]. This similarity is particularly interesting given the structural variation between the two proteins: the 18 aa differences in addition to the truncation of CCP-4 in MOPICE. Based on this line of reasoning, CCP-2,3 are the most important for ligand binding, which is consistent with electrostatic modeling [53] as well as a report that function blocking antibodies recognize either CCP-2 or CCP-3,4 [37]. Moreover, the differences in CCP-2 mediate SPICE's enhanced activity compared to VCP [27,53].

While PICEs bind both C3b and C4b, they bind C4b with higher affinity [24,34,54]. Additionally, they have decay accelerating activity for the C4b containing convertases

generated by the classical and lectin pathways. The pressures that have selected for this enhanced activity against C4 suggest that C4 has an important role in the immune response to a poxviral infection. Since complement activation has antiviral effects that enhance host survival (Figures 2-1 & 2-5), the ability to inactivate C4b would be advantageous if the classical or lectin pathway initiates complement activation. As indicated in chapter 3, natural antibody initiates the complement-mediated neutralization of IMV.

Interestingly, EMICE protected CHO cells against complement activation by the alternative pathway using antibody as the activator. Cofactor activity mediates this protection, since the other PICEs have limited or absent decay accelerating activity for the alternative pathway convertases [24,34,54]. EMICE's regulation of the alternative pathway (and potentially the classical pathway when activated at a lower level by natural antibody) may help ECTV evade the innate and early adaptive immune response.

A high-affinity rabbit IgG directed to CHO cells efficiently engages the classical pathway and overwhelmed the regulatory capacity of EMICE. Prior reports using similar methods showed that SPICE decreased C3b deposition from the classical pathway by about 50% [23]; however, this decrease would likely be insufficient to protect the cells from the detrimental results of complement activation (mimics the setting of abundant high-affinity antibody in an adaptive immune response). The impact of the PICEs during infection likely occurs prior to the generation of a strong humoral response.

EMICE's ability to protect IMV from the complement-mediated neutralization initiated by natural antibody demonstrates its potential to regulate mouse complement *in vivo* and influence events early during infection. Protection occurred at concentrations of

EMICE that were produced *in vitro* before most of the IMV was released. The concentrations of EMICE reached *in vivo* may be lower, but EMICE's ability to dimerize and bind to cell surfaces may enhance its regulatory activity in the microenvironment of the infected cell. While these experiments can only model what occurs *in vivo*, they demonstrate that infected cells produce large quantities of EMICE, and EMICE impedes neutralization of ECTV.

ECTV infection produced multiple forms of EMICE. The unpaired N-terminal cysteine of VCP economically mediates both dimer formation [24] and cell surface expression via its interaction with A56 [33]. Not only is this single residue responsible for tethering the complement inhibitor to the surface of infected cells, and possibly as well to the extracellular enveloped virus (EEV) form, but it also produces dimers with enhanced regulatory activity [24]. The multiple forms of the PICEs appear advantageous to the virus, as the only PICE that lacks a free N-terminal cysteine, MOPICE, has a free cysteine in the truncated CCP-4 that mediates dimerization [24]. The generation of ECTV mutants carrying EMICE lacking the N-terminal cysteine could address this question *in vivo*; however, differentiating between the effects of dimerization vs. A56 conjugation could be difficult. This potential interaction between EMICE and A56 and its contribution to the complement resistance of EEVs as well as virulence *in vivo* require future investigation.

Generation of anti-PICE antibodies may help to clear the infection, and the passive administration of function-blocking antibodies could reduce virulence and aid recovery. These antibodies could enhance complement activity by disabling the inhibitor, while

simultaneously tagging infected cells that express PICEs on their surface for destruction by complement or other immune-mediated mechanisms.

The increased mortality in the complement-deficient mice indicates how important this system is to antiviral defense, and it also underscores how delicate the balance between survival and mortality is in poxviral infections. A lethal infection is not necessarily advantageous to the virus. With a virus that primarily causes acute infections, evolution may favor viruses that replicate at high levels (to promote dissemination) without killing the host before transmission. Since ECTV has coevolved with its murine host, the interactions between the two are likely more nuanced compared to other experimental models of poxviral infection.

The difference between Δ EMICE and WT ECTV observed in the FB^{-/-} mice supports an intriguing hypothesis about the delicate balance between the host's defenses and a viral virulence factor. While FB deficiency increased mortality from WT ECTV, natural antibody may still trigger the intact classical pathway in these mice and produce C4b and C3b deposition, albeit less efficiently than in a wild-type mouse. Given the lack of the alternative pathway feedback loop in FB^{-/-} mice, EMICE could better control complement activation in this setting, resulting in increased virulence. In the absence of EMICE, unchecked complement activation by natural antibody could exert its antiviral activities, particularly the neutralization by the C4b and C3b opsonins, and thereby reduce the virulence of the infection. The FB^{-/-} mice infected with Δ EMICE ECTV have a mortality rate similar to wild-type mice infected with WT ECTV. This similarity suggests that the combination of

these two deficiencies (FB^{-/-} and ΔEMICE) maintains the equilibrium present in the wild-type infection.

While these data showing attenuation of ΔEMICE ECTV in FB^{-/-} and C3^{-/-} but not C4^{-/-} mice are suggestive, a new ΔEMICE and rescue ECTV must be generated for a definitive analysis. The similar mortality observed with the ΔEMICE and +EMICE viruses in the wild-type mice complicates interpretation of the *in vivo* data. These results, combined with the change observed in plaque morphology of these two viruses, indicate that a second mutation was inadvertently selected during the generation of the ΔEMICE virus. We cannot distinguish between this mutation being solely responsible for the attenuation observed or just masking the true effect of EMICE.

Figures

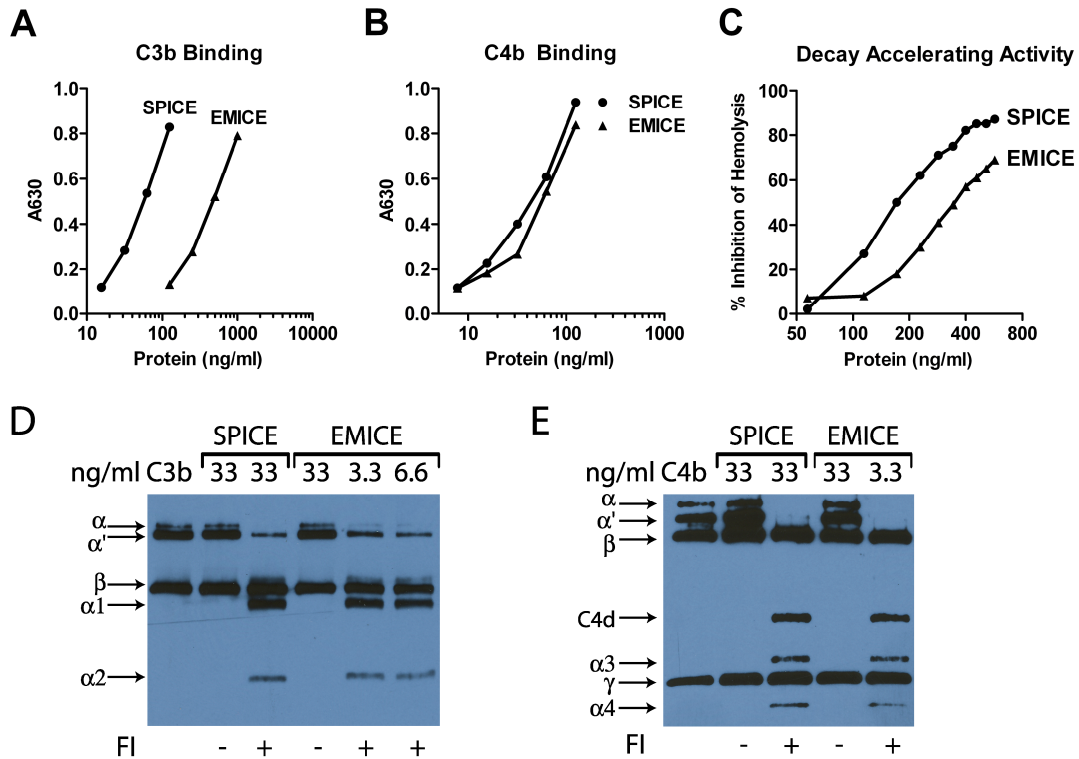


Figure 4-1: rEMICE bound and regulated human complement activation fragments, C3b and C4b

EMICE's ability to interact with human C3b (A) or C4b (B) was measured using an ELISA format.

(C) The classical pathway C3 convertase was assembled on the surface of antibody-coated erythrocytes using purified human complement components. The ability to dissociate the convertase was detected as decreased hemolysis when the cells were exposed the terminal complement components.

(D) During C3b cofactor activity, Factor I (FI) inactivates C3b by cleaving the α or α' chain into two fragments, α_1 and α_2 .

(E) With C4b cofactor activity, factor I cleaves the α or α' chain twice to release C4d from C4c. C4c comprises the α chain fragments, α_3 and α_4 , and the β and γ chains.

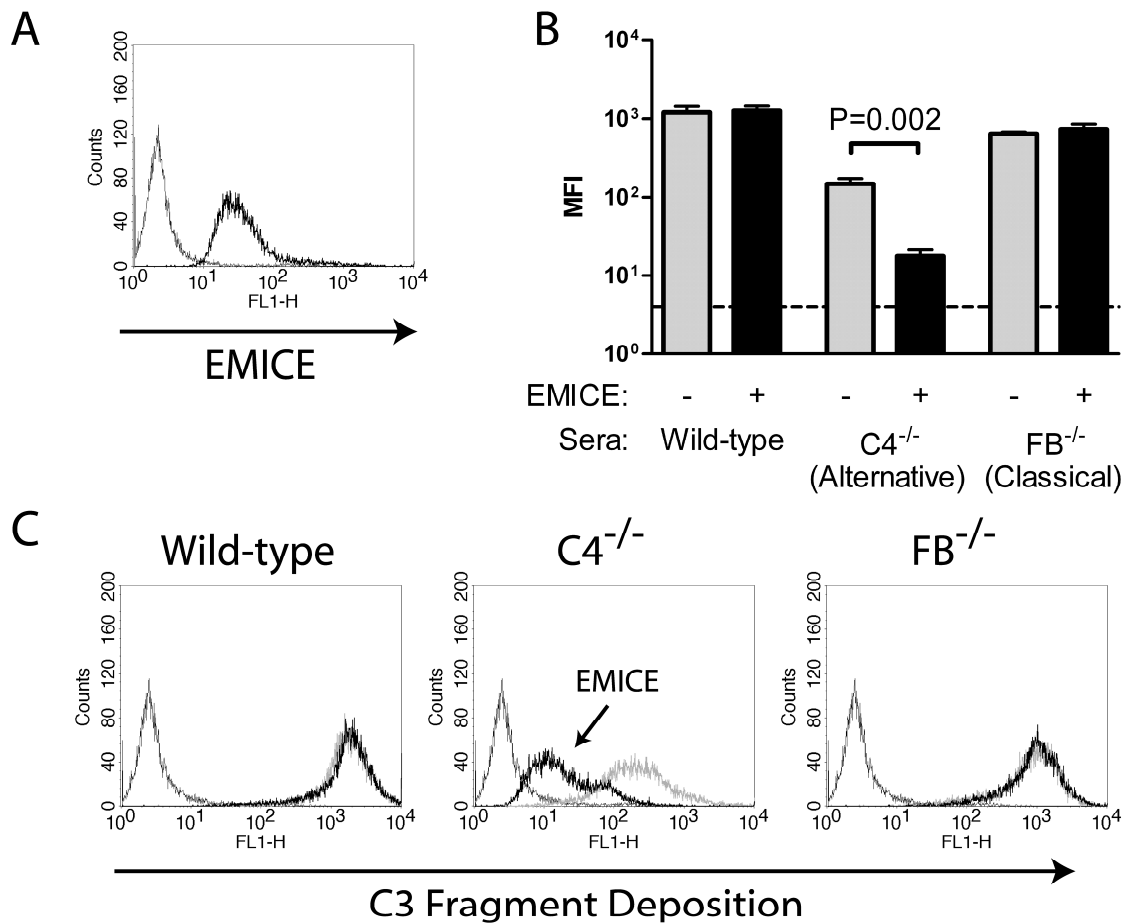


Figure 4-2: rEMICE bound cells and regulated the alternative pathway in mouse sera

(A) rEMICE bound CHO cells. 10^6 CHO cells were mixed with 20 μ g of EMICE (heavy line), and rEMICE was detected with a polyclonal rabbit anti-VCP antibody. Thin line represents cells not exposed to rEMICE.

(B) rEMICE significantly reduced C3 deposition by the alternative pathway. CHO cells (gray) or CHO cells with rEMICE bound (black) were coated with anti-CHO antibody and exposed to sera from the indicated strains of mice. C3 deposition was measured as mean fluorescence intensity (MFI) from two experiments performed in duplicate, and images from

one are shown (C). In $C4^{-/-}$ sera, only the alternative pathway functions, and the decreased C3 deposition is indicated by the arrow. Thin line represents cells not exposed to sera.

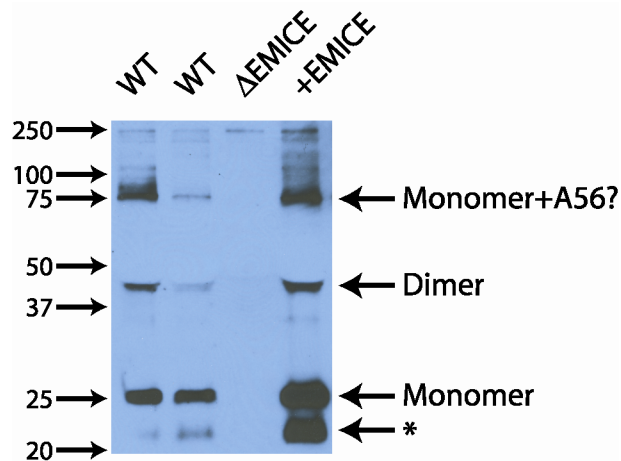


Figure 4-3: EMICE-deficient (Δ EMICE) ECTV produced and then EMICE was restored (+EMICE)

Two different crude lysates from the wild-type (WT) ECTV are shown. Δ EMICE ECTV was generated using a construct with a 600 bp deletion in the middle of the EMICE gene. The wild-type genomic sequence was added back to Δ EMICE, and EMICE producing clones were selected by PCR and confirmed by Western blotting a crude stock of infected cells collected in supernatant. The multiple forms of EMICE are labeled based on observations of VCP [33]. Some VCP is covalently bound to the viral membrane protein A56, and a similar interaction of EMICE with A56 may explain the largest band observed. A smaller antigenically related protein of unknown significance is indicated by *.

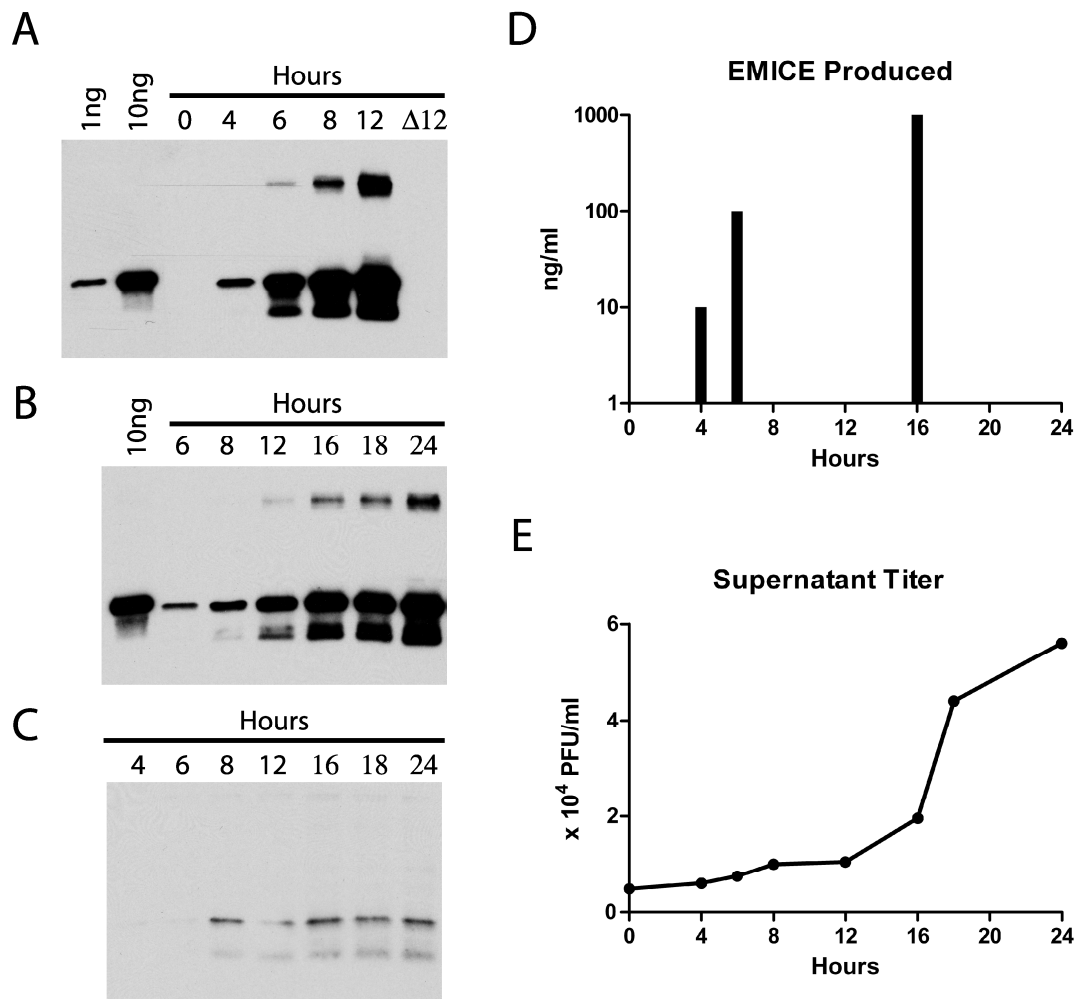


Figure 4-4: Secretion of EMICE occurred early during infection

(A-C) The production of EMICE was followed over time in murine L929 cells infected at an MOI of 1. After infection for 1 hour, the cells were rinsed to remove free virus and then cultured in 1000 μ l of media. The EMICE produced was assessed via Western blotting in (A) 10-fold concentrated supernatants (10% of total), (B) unconcentrated supernatants (1% of total), or (C) washed cells (10% of total). The recombinant EMICE is labeled with the

amount of loaded (1 or 10 ng). The numbers (0–24) indicate the time (h) when the samples were obtained. Δ indicates the Δ EMICE ECTV sample.

(D) The concentration of EMICE in the supernatant based on comparison to the recombinant EMICE.

(E) The supernatant was titrated on BS-C-1 cells at the time of collection.

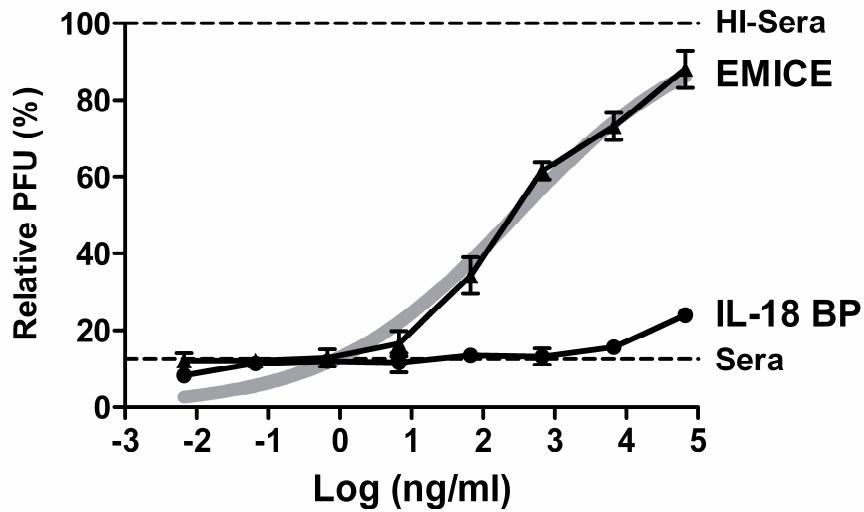


Figure 4-5: Soluble EMICE protected IMV from complement-mediated neutralization

Prior to the addition of purified IMV, mouse sera from naïve wild-type C57BL/6 mice were mixed with recombinant protein, EMICE or the ECTV IL-18 binding protein (IL-18 BP) as a control. The lower dashed line shows the level of neutralization (87%) by the sera at a final concentration of 10%. The data are normalized to heat-inactivated (HI) sera, and the EMICE line displays mean \pm SEM from two experiments performed in duplicate. The gray curve was produced using non-linear regression.

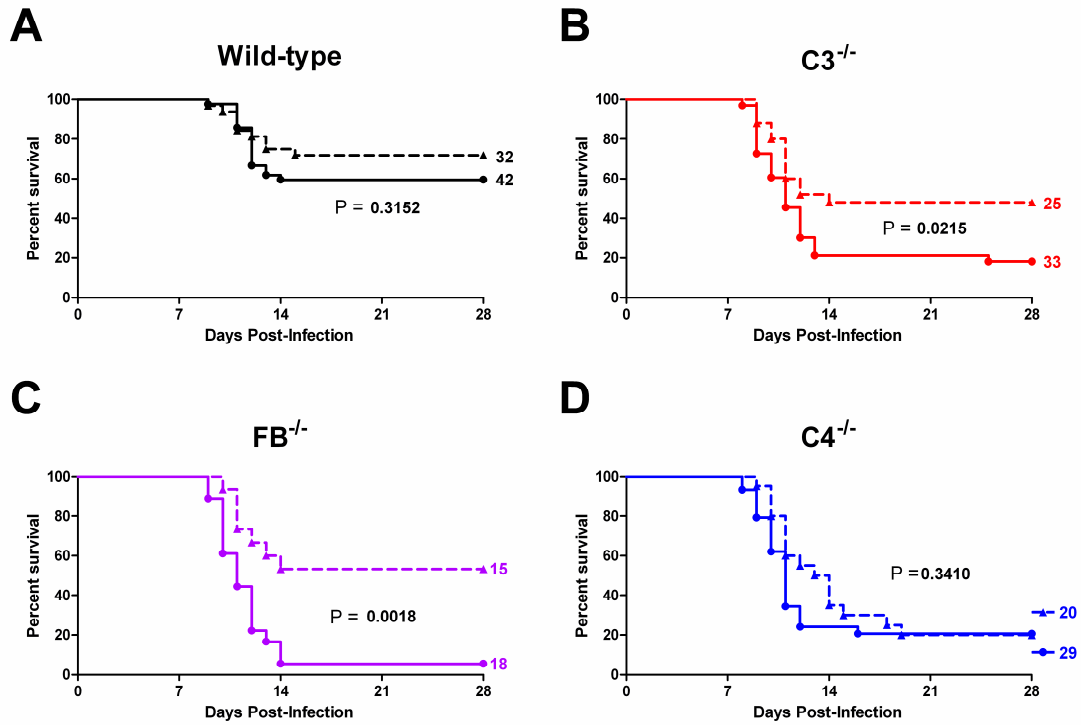


Figure 4-6: The Δ EMICE mutation attenuated ECTV in C3^{-/-} and FB^{-/-} mice but not in C4^{-/-} and wild-type mice

The indicated strains of wild-type or complement-deficient male mice were inoculated intranasally with 100 pfu of wild-type (●, solid) or Δ EMICE (▲, dashed) ECTV. The numbers to the right of the curves indicate the number of animals in each group. Survival curves were compiled from 3–7 separate experiments.

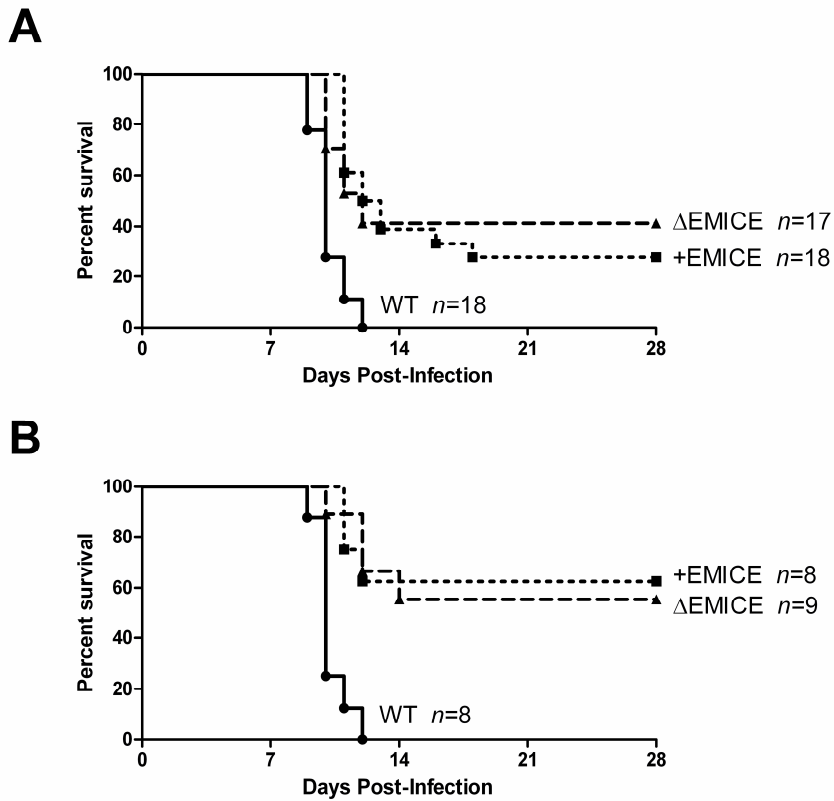


Figure 4-7: Attenuation observed in Δ EMICE remains in +EMICE ECTV

Mice were inoculated intranasally with wild-type (●), Δ EMICE (▲), or +EMICE (■) ECTV.

(A) Male mice received 500 pfu.

(B) Female mice received 250 pfu.

The survival curves were constructed from four (A) or two (B) separate experiments.

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Chapter 5

Conclusions and Future Directions

Conclusions

The mousepox model offered an uncommon opportunity to characterize the role of the complement system in the host-poxvirus relationship using a pathogen and a host that have co-evolved. The major goal of this thesis was to test the hypothesis that the complement system plays a role in defense against ectromelia virus (ECTV) infection and that ECTV counters the effects of complement by producing EMICE (Ectromelia Inhibitor of Complement Enzymes). Exploring aspects of both the host and the virus provided a better understanding of the delicate balance that occurs in this relationship.

The *in vivo* studies employing C3^{-/-}, C4^{-/-}, and FB^{-/-} mice establish that multiple complement activation pathways are required to survive ECTV infection initiated by three routes of inoculation. In the C3-deficient mice, ECTV disseminated from the inoculation site earlier and produced higher viral titers in the target organs. Additionally, the minority of C3^{-/-} mice that survived to day 10 post-infection had an active infection in these organs, while most wild-type mice had cleared the infection. The early dissemination observed in the C3^{-/-} mice prompted an investigation into the ability of the murine complement system to directly neutralize ECTV.

The *in vitro* experiments employed sera from the same strains used to characterize the effect of complement deficiency *in vivo*, and the neutralizing capacity *in vitro* paralleled the *in vivo* mortality observed. Naïve mouse sera neutralized the intracellular mature virus (IMV) of ECTV *in vitro*, and sera lacking either classical or alternative pathway components had decreased activity. Several lines of evidence indicate that natural antibody initiated the classical complement cascade in the wild-type mouse. Substantial neutralization occurred in

sera without lytic activity, which points to opsonization as the predominant mechanism of neutralization. Based on these results, we propose that natural antibody binds viral antigen to activate the classical pathway, followed by engagement of the alternative pathway's feedback loop to further opsonize the virus. The delayed mortality observed in the μ MT mice treated with naïve sera underscores the importance of natural antibodies to protection. The ability of natural antibody to neutralize ECTV in turn depends upon its ability to activate the complement system. This likely accounts for why the virus encodes a complement regulatory protein.

EMICE was obtained by using recombinant protein technology as well as through infection of a murine cell line. Recombinant, and presumably murine, EMICE had both cofactor and decay accelerating activity for human complement, and it protected cells from the activation of mouse complement via the alternative pathway. It also prevented complement-mediated neutralization of the IMV at concentrations produced by the infected cell *in vitro*.

Given that poxviruses must face both natural antibody and the complement system to establish an infection, a virus lacking its regulator would be at a greater risk of being neutralized by complement activity. Our results support the supposition that the loss of this regulator contributes to the reduced virulence of some strains of monkeypox virus [1]. From the host's perspective, the complement system is critical to the innate immune response to poxviruses. It reduces viral spread and decreases tissue titers and, therefore, cellular damage in target organs.

Future Directions

This characterization of the role of the complement system during ECTV infection has raised multiple questions that are now under investigation. The availability of genetically modified mouse strains makes the ectromelia model a powerful one for addressing these issues.

Complement effector mechanisms *in vivo*

The early mortality of the C3^{-/-}, C4^{-/-}, and FB^{-/-} mice demonstrates an essential role for the classical and alternative pathways in the initial stages of a poxvirus infection. Despite equivalent mortality levels, further analysis will define distinct functions for each complement pathway *in vivo*, as such differences exist in the immune response to other viruses [2]. Undoubtedly, complement deposition triggers other effector functions, such as recruiting inflammatory cells, promoting phagocytosis, and priming the adaptive immune response. The precise contribution of each of these to protection *in vivo* remains unexplored.

The similarity between the *in vivo* mortality and *in vitro* serum neutralization experiments suggests that complement neutralizes ECTV and, thereby, limits its spread. Determining if, when, where, and how this neutralization occurs would provide insight into the roles for each of the pathways. The ability of C3 to reduce or delay the spread of ECTV from the inoculation site to the spleen on day two post-infection suggests three sites where this neutralization likely occurs: the inoculation site/primary lesion, the draining lymph node (memory CD8⁺ T cells control infection here [3]), and the bloodstream.

An initial investigation would examine the amount of infectious virus at the inoculation site and in the draining lymph node immediately, a few hours, and a day after infection of wild-type and complement-deficient mice. Should the viral titer at the inoculation site be significantly reduced in the wild-type mice, as we predict, it would be interesting to assess if inclusion of recombinant EMICE would protect the inoculated IMV from neutralization. Additionally, if complement-mediated neutralization at the inoculation site is an important mechanism of C57BL/6 resistance, then reducing complement activation through inclusion of EMICE may enhance the virulence of the infection. Similar increases in virulence could result from inoculation with extracellular enveloped virus (EEV), as this form resists complement activation through incorporation of complement regulators in its outermost viral membrane. Thus, these approaches could provide a method for initiating virulent infection in other poxvirus models that typically use IMV, such as the non-human primate modes for monkeypox or variola virus.

Our *in vitro* data demonstrate the susceptibility of the IMV form to complement-mediated neutralization, and this strongly suggests that ECTV disseminates through the bloodstream either inside infected cells or in the EEV form. Determining which of these is the case should provide insight into viral pathogenesis. If EEVs are primarily responsible for the viremia, this would explain the ability of anti-EEV antibodies, either passively transferred [4] or generated to recombinant protein [5-7], to protect against a lethal infection.

Alternatively, if infected cells mediate systemic dissemination, then this raises the question of what types of cells are responsible, when and where they acquired the infection, and what is their final destination. A logical extension of our work would be to examine the

participation of complement receptors in this process. A consideration is that a limited amount of complement deposition could aid viral entry into cells bearing complement receptors, like B cells, dendritic cells, and macrophages [8]. The fate of IMV bearing sub-neutralizing levels of complement could be followed when combined with phagocytic cells that possess or lack complement receptors. We hypothesize that phagocytosis would result in destruction and decreased viral titers; however, its potential to enhance infection under certain conditions cannot be ruled out. Infection studies in mice deficient in complement receptors could address the importance of these proteins *in vivo*. Of particular interest are CR1/2, due to its expression on cell types that are infected *in vivo* [9], and CR1g, for its potential to mediate the hepatic clearance of ECTV introduced intravenously [10].

Role of antibody during ECTV infection

The ability of heat-inactivated wild-type sera to restore the neutralizing activity of μ MT sera *in vitro* indicates that natural antibody is important in the neutralization process. The acute mortality observed in the μ MT mice combined with the ability of the passive transfer of normal sera to lengthen survival suggests that natural antibody helps control the acute infection *in vivo*. However, the eventual death of these animals demonstrates that the other activities of B cells are also required for survival of ECTV infection. Mice deficient in secreted IgM, yet retaining B cells and some ability to generate an antiviral humoral response, would help distinguish between the role of natural antibody vs. these other activities [11,12].

Since human sera also neutralize IMV, characterization of the natural antibody population present in C57BL/6 mice may provide insight into human poxviral infection. An initial step would be to determine the isotype of the natural antibody population that

recognizes poxviruses. Natural antibody presumably reacts with an epitope found on the IMV particle based on the neutralization observed. Natural antibody may also bind EEV antigens given that a polyreactive repertoire mediates the antibacterial activity of natural antibody when combined with complement [13]. The regulatory proteins present in the membrane of the EEV contribute to its resistance to complement [14]; however, lower levels of natural antibody to the EEV compared to the IMV may also reduce complement activation. Determining the viral antigens recognized would contribute to our understanding of the role of the EEV form *in vivo*.

A similar analysis of the earliest humoral response would provide additional perspective on this topic. Examining when neutralizing antibodies to IMV and EEV develop would provide perspective on what antigens have stimulated the immune response. The addition of complement to the EEV neutralization assay may enhance detection. Additionally, the ability of complement to hasten the development of the EEV neutralizing response should also be assessed. In preliminary experiments, $C3^{-/-}$ mice have lower levels of anti-ECTV IgG on day 10 with a significant difference on day 16.

Characterization of EMICE *in vivo*

The large quantities of EMICE produced by infected cells along with its ability to protect ECTV IMV in the fluid phase *in vitro* prompt, as a logical extension of our work, further investigation into EMICE's ability to bind virus particles, protect infected cells, and modulate the local environment. Of specific interest are the EMICE levels systemically (in the bloodstream) and at the primary lesion, as well as EMICE's effect on complement activation at the inoculation site. Complement activation and release of anaphylatoxins is important for

recruiting inflammatory cells to the site of infection [15]. If EMICE reduced complement activation at the inoculation site, then detectable changes may occur in the inflammatory response, either in quantity or in character.

EMICE may also protect infected cells, which could be assessed using infected mouse cells and a protocol similar to the CHO cell assay. Hypothetically, host cells may decrease production of their regulators during infection to increase their susceptibility to complement, and EMICE may be expressed on the cells' surfaces to compensate. Flow cytometry could assess changes in the levels of the host regulators and EMICE after infection. If the regulator levels decrease, then the ability of recombinant protein to bind to or protect cells infected with Δ EMICE ECTV would mimic the ability of secreted EMICE to protect surrounding infected cells during this period. EMICE has conserved the heparin binding sites in CCP-1, despite the numerous other changes in this domain, which may enhance its protective abilities.

Similar to VCP [16], cell surface expression of EMICE may result from an interaction with the viral protein A56, which is suggested by the high molecular weight band present on Western blots of infected cell cultures. Such an interaction may also target EMICE to the EEV. Proteomic analysis of IMV and EEV from vaccinia and monkeypox viruses produced in HeLa cells suggests that these viruses use different mechanisms to protect EEV from complement [17]. The IMV forms of both viruses had neither the viral nor the host regulators. Vaccinia EEV contained VCP and high levels of A56, while monkeypox EEV lacked MOPICE and had only minimal A56. Instead, monkeypox EEV had high levels of the host regulators (DAF and CD59), which were absent in the vaccinia

EEV. Given that VCP forms a covalent linkage to A56 on the surface of infected cells, it seems likely that this mechanism explains VCP's presence in the EEV [16]. EMICE's influence on the complement resistance of the EEV remains to be determined.

Along the same lines, the influence of the dimerized form of EMICE or the other PICEs *in vivo* is unknown. Unfortunately, a single cysteine mediates dimer formation and A56 conjugation, which complicates assessment of the significance of the various forms *in vivo*. While our *in vivo* data are suggestive, to definitely examine the role of EMICE *in vivo*, a new Δ EMICE ECTV mutant must be produced. Versions of EMICE lacking the N-terminal cysteine or the heparin binding sites, in addition to the wild-type protein, could be reintroduced to the Δ EMICE mutant. The collection of resulting viruses could begin to address the role of the A56 conjugation/dimer formation and heparin binding *in vivo*. If Δ EMICE ECTV is attenuated, then genetic complementation should occur in a $C3^{-/-} \times C4^{-/-}$ mouse, since these are the two complement proteins that EMICE targets. Further analysis of this attenuation could reveal when Δ EMICE ECTV deviates from a normal infection. How EMICE deficiency changes the pathogenesis and virulence of ECTV has implications for targeting the PICEs to therapeutically modulate the virulence of human poxviral infections.

References

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