THE REQUIREMENT FOR MYELOID CELL SUBSETS IN MEDIATING AN EFFECTIVE IMMUNE RESPONSE TO ECTROMELIA VIRUS INFECTION

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Microbiology and Immunology

by

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After a peripheral viral infection, a battle begins between the virus and the host’s immune system. The virus aims to replicate and spread despite the opposing immune response, which is deployed to contain the virus and mediate the host’s survival and recovery. Many viruses of importance to human health infect at a peripheral site and spread systemically. Early in the immune response to a peripheral viral infection, myeloid cells, including granulocytes, monocytes, monocyte-derived dendritic cells (mo-DC), macrophages, and dendritic cells (DC), respond to control the virus.

Ectromelia virus (ECTV) is an orthopoxvirus that naturally causes mousepox in the mouse. ECTV infects at a peripheral site in the footpad and spreads systemically, resulting in death of susceptible mice. Phagocytic cells are required early for survival of ECTV infection, but the identity of the requisite cell(s) is unclear. To isolate roles for myeloid cells in immunity to ECTV, we utilized multiple cell depletion systems.

DC were required for survival of ECTV infection, but granulocytes alone, monocytes and mo-DC, or macrophages alone were not required for survival of ECTV infection. Depletion of either plasmacytoid DC (pDC) or depletion of lymphoid-resident CD8α+ DC did not result in death following ECTV infection. However, mice depleted of both pDC and CD8α+ DC were susceptible to ECTV infection, which indicated that the presence of either pDC or CD8α+ DC was sufficient for survival of ECTV infection. When both pDC and CD8α+ DC were absent, mice died from an inability to control viral replication, which resulted in enhanced viral dissemination and viral load. This lack of viral control correlated with a deficiency in antiviral cytokine production because pDC and CD8α+ DC were producers of required antiviral cytokine in response to ECTV.
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Abbreviations

AIDS- Acquired Immunodeficiency Syndrome
ALT- Alanine Transaminase
APC- Antigen-Presenting Cell
AST- Aspartate Transaminase
Batf3- Basic Leucine Zipper Transcription Factor ATF-Like 3
BSA- Bovine Serum Albumin
(C)- Crude Virus Prep
CCR2- C-C Chemokine Receptor 2
cDC- Conventional Dendritic Cell(s)
CDC-Center for Disease Control and Prevention
cGAS- Cyclic GMP-AMP Synthase
CLL- Clodronate Liposomes
CMV- Cytomegalovirus
CpG- unmethylated 2'-deoxyribo(cytidine-phosphateguanosine)
CPXV- Cowpox Virus
cre- Cre Recombinase
CSF-1- Colony Stimulating Factor 1
DAI- DNA-Dependent Activator of IFN-Regulatory Factors
DC- Dendritic Cell
dDC- dermal Dendritic Cell
D-LN- Draining Lymph Node (Popliteal Lymph Node in Footpad Infection)
DMEM- Dulbecco’s Modified Eagle Medium
IFN- Interferon: includes IFNα (alpha), IFNβ (beta), and IFNγ (gamma)

IFNAR- Interferon α/β Receptor (Type-1-Interferon Receptor)

IFNγbp- Interferon Gamma Binding Protein

IL- Interleukin

IL-1R- Interleukin-1 Receptor

IMDM- Iscove's Modified Dulbecco's Medium

i.n.- intranasal(ly)

i.p.- intraperitoneal(ly)

IRF- Interferon Regulatory Factor

ISG- Interferon-Stimulated Gene(s)

ISRE- Interferon-Stimulated Response Element

i.v.- intravenous(ly)

JAK1- Janus Kinase 1

kg- Kilogram

KO- Knockout

LN- Lymph Node

LysM- Lysozyme M gene

MaFIA- Macrophage Fas-Induced Apoptosis

MAPK- Mitogen-Activated Protein Kinases

MAVS- Mitochondrial Antiviral Signaling Protein

M-CSFR- Macrophage Colony Stimulating Factor Receptor (also known as CD115)

MDA5- Melanoma-Differentiation-Associated Gene 5

mg- milligram
MHC- Major Histocompatibility Complex
min- Minute(s)
mo-DC- Monocyte-Derived Dendritic Cell
MOI- Multiplicity of Infection
MPXV- Monkeypox Virus
MyD88- Myeloid Differentiation Primary Response 88 gene/ signaling protein
NF-κB- Nuclear Factor-kappaB
ng- nanogram
NK- Natural Killer
NKG2D- Natural-Killer Group 2, Member D
OAS- 2'-5'-Oligoadenylate Synthetase
PAMPs- Pathogen-Associated Molecular Patterns
pAPC- Professional Antigen-Presenting Cell
PBS- Phosphate Buffered Saline
PCR- Polymerase Chain Reaction
pDC- plasmacytoid Dendritic Cell
PE- Phycoerythrin
PFU- Plaque Forming Units
PKR- IFN-Inducible Double-Stranded RNA-Dependent Protein Kinase
pLN- Popliteal Lymph Node
PRR- Pattern-Recognition Receptor
RIG-I- Retinoic Acid-Inducible Gene 1
RNA- Ribonucleic Acid
RT- Room Temperature
RT-qPCR- Real-Time Quantitative Reverse Transcription Polymerase Chain Reaction
SAMHD1- Sterile Alpha Motif and Histidine-Aspartic Domain-Containing Protein 1
s.c.- subcutaneous(ly)
SEM- Standard Error of the Mean
STAT- Signal Transducer and Activator of Transcription
Std. dev.- Standard Deviation
STING- Stimulator of Interferon Genes
T1-IFN- Type I Interferon
T1-IFNbp- Type I Interferon Binding Protein
T_{CD4^+}- CD4^+ T cells
T_{CD8^+}- CD8^+ T cells
TCR- T Cell Receptor
TIR- Toll/IL-1 Receptor Domain
TLR- Toll-like Receptor
TYK2- Tyrosine Kinase 2
UVC- Ultraviolet C (a Type of Ultraviolet Light)
VACV- Vaccinia Virus
VARV- Variola Virus
VIG- Vaccinia Immune Globulin
WHO- World Health Organization
WT- Wild-type
YFP- Yellow Fluorescent Protein
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Chapter I: Introduction

One-third of people infected with Variola virus (VARV), an orthopoxvirus and the causative agent of smallpox, succumbed to the disease before the eradication of smallpox by world health efforts. Following the eradication of smallpox, there has been a reduction in smallpox vaccination programs due to the morbidity and mortality caused by vaccination using live Vaccinia virus (VACV), another orthopoxvirus. Currently, there is an increasing incidence of zoonotic orthopoxvirus infections, including monkeypox and cowpox, for which effective therapeutics are not available. Besides the use of VACV as a vaccination for smallpox, orthopoxviruses are also being utilized as vectors for vaccines against other infectious diseases and as vectors in tumor immunity.

Orthopoxviruses, similar to many viruses of significance to human and animal health, including Enteroviruses (polio, coxsackie), Rubulavirus (mumps), Morbillivirus (measles), varicella zoster virus, and arboviruses, which include Flaviviruses (Dengue virus, West Nile Virus, Yellow fever virus), spread from a peripheral site of infection to become systemic. The studies presented here focus on the early effective immune response to ECTV, an orthopoxvirus and the causative agent of mousepox. ECTV spreads systemically following infection of the footpad of the mouse. Early in the immune response, there is a requisite deployment of phagocytic innate immune cells to control the virus and mediate survival of ECTV infection. The identification of these required cell subsets and the mechanism(s) deployed by these immune cell subsets to control the virus presents a potential early target for the development of therapeutics and rational vaccine design.
Objective #1: Determine the requirement for granulocytes, inflammatory monocytes, mo-DC, and macrophages in the immune response to ECTV infection.

Rationale:

Previous experiments by other groups have indicated a requisite role for phagocytic innate immune cells in the response to ECTV infection. The methods used, carrageenan treatment or clodronate liposome (CLL) injection, have been described as methods for depletion of macrophages with the potential to deplete other phagocytic immune cells. Using several cell depletion methods, we isolated the requirement for multiple phagocytic immune cell subsets: granulocytes, monocytes and mo-DC, and macrophages early during the immune response to ECTV infection to identify roles of these phagocytic myeloid cell subset(s).

Findings:

Through the use of multiple myeloid cell depletion methods, we found that although depleted by CLL, granulocytes, inflammatory monocytes and mo-DC, and macrophages were not required for survival of ECTV infection. Macrophages also produce only low levels of interferon-alpha (IFNα), a required antiviral cytokine, in response to ECTV. Some of the depletion methods using transgenic mice were also found to be less-specific for macrophage depletion than indicated by previous work in the literature.

Objective #2: Determine the requirement for and roles of DC subsets in effective immunity to ECTV infection.
Rationale:

As discussed above, phagocytic innate immune cell depletion by CLL caused resistant mice to become susceptible to ECTV infection. Although previous studies have focused on the depletion of macrophages by CLL, during the investigation of Objective #1 (see above), we also found depletion of multiple DC subsets by CLL injection. Although macrophages did not produce much IFNα, a required antiviral cytokine, in response to ECTV, DC were producers of IFNα during the response to ECTV. We then investigated the roles of DC subsets early during the immune response to ECTV infection.

Findings:

Bulk DC were required early in the immune response to ECTV to control the virus and mediate survival of infection. More specifically, there was a requirement for either pDC or a lymphoid-resident DC subset, CD8α+ DC, to mediate early control of ECTV during infection. The presence of at least one of the DC subsets, either pDC or CD8α+ DC, was sufficient to control the virus, potentially by the production of required antiviral cytokine.

To summarize, we have identified myeloid cell subsets required in vivo for the early immune response to a peripheral infection with ECTV. Granulocytes alone, inflammatory monocytes and mo-DC, and macrophages alone were not required for mediating survival of ECTV infection; however, DC were a requisite myeloid cell population in effective immunity to ECTV infection. We have found a redundant function of two DC subsets, pDC and CD8α+ DC, to ultimately control the virus and mediate
survival of ECTV infection. This requisite function of the DC subsets was redundant as the presence of either pDC alone or CD8α+ DC alone was sufficient for effective viral control and host survival of ECTV infection. As pDC and CD8α+ DC were the producers of a required antiviral cytokine in response to ECTV, viral control by these DC subsets was likely mediated by antiviral cytokine production. In the absence of both pDC and CD8α+ DC, mice succumbed to ECTV infection unless viral replication is controlled by antiviral drug treatment. To our knowledge, this is the first description of a redundant function between DC subsets, and the early requisite viral control mediated by these cells provides target mechanisms for therapeutics.
Chapter II: Literature Review

A. Orthopoxviruses

i. Orthopoxvirus overview

Orthopoxviruses are enveloped viruses that replicate in the cytoplasm of host cells and contain a linear, double-stranded DNA genome of 170-250 kilobases. Viruses classified within the Orthopoxvirus genus include VACV, Camelpox virus, Cowpox virus (CPXV), Monkeypox virus (MPXV), VARV, and ECTV. Infection or immunization with an orthopoxvirus produces a cross-protective immune response to other orthopoxviruses. The cross-protective immune response is exemplified by immunization with VACV to protect against VARV as discussed below.

ii. Human Diseases of the Orthopoxviruses

VARV, a human pathogen and the causative agent of smallpox, killed around one-third of infected people prior to global efforts to eradicate smallpox. The World Health Organization (WHO) officially declared smallpox eradicated in 1980, marking the first instance of an infectious disease being removed from the world by global health efforts (Strassburg 1982). The success of the smallpox eradication program was due, in part, to the immunity provided by the smallpox vaccine (the smallpox vaccine is reviewed in Chapter II-B), but also due to the lack of animal reservoirs of VARV and the identification of the distinct pox lesions on infected individuals (Fenner 1982). However, VARV remains a potential bioterrorism threat and is classified by the Centers for Disease Control and Prevention (CDC) as a Category A agent, one that transmits from person to person and results in high mortality (Riedel 2005) (Henderson, Inglesby et al.)
1999). The disease, smallpox, occurs following infection of the respiratory or oropharyngeal mucosa with VARV in aerosolized droplets (Riedel 2005). VARV travels using the lymphatic ducts to the draining lymph node (D-LN) where viral replication occurs (Riedel 2005). The ultimate outcome is either death or recovery, which is characterized by control and elimination of the virus and immunity to VARV re-infection (Riedel 2005). Following the eradication of smallpox, vaccination rates with the smallpox vaccine have dropped. In the United States prior to 1972, children received the smallpox vaccine at one year of age, and when smallpox was declared eradicated in 1980, the WHO recommended cessation of mass vaccination strategies worldwide (Henderson, Inglesby et al. 1999). The use of the smallpox vaccine has known risks of morbidity and mortality (adverse events from the smallpox vaccine are covered in Chapter II-B), and is no longer recommended for the general public after the eradication of smallpox.

There is now a growing concern over the increasing frequency of zoonotic poxvirus infections (Shchelkunov 2013), including infection with Camelpox virus, CPXV, and MPXV (Reynolds and Damon 2012) (Rimoin, Mulembakani et al. 2010). The incidence of monkeypox has increased approximately 20-fold from the 1980s to 2006-2007 in endemic areas of Africa (Rimoin, Mulembakani et al. 2010). This increasing incidence of poxvirus infections is correlated with the decrease in mass vaccination programs with the smallpox vaccine (Reynolds and Damon 2012) as infection or vaccination with one orthopoxvirus elicits an immune response that is cross-reactive and protective against other orthopoxviruses (Essbauer, Pfeffer et al. 2010). Previous vaccination with the smallpox vaccine is indicated to be 85% effective at preventing
monkeypox (Jezek, Marennikova et al. 1986). Treatment of MPXV infections in humans is currently limited, and MPXV infection has shown a case-fatality rate up to 10% in humans (Frey and Belshe 2004) (Essbauer, Pfeffer et al. 2010). Clinical illness includes headache, fever, sweats, chills, lymphadenopathy, and the development of the characteristic pustular rash (Reed, Melski et al. 2004) (Reynolds and Damon 2012). Monkeypox is endemic in the Democratic Republic of Congo (DRC) and has been found in neighboring countries in Africa (Reynolds and Damon 2012). Monkeypox has also spread outside of endemic areas. The first time monkeypox was diagnosed outside of the African continent was in 2003 when 71 people in the United States were reported to have monkeypox following exposure to infected prairie dogs, which had been exposed to infected African rodents (Reed, Melski et al. 2004) (Essbauer, Pfeffer et al. 2010). Of eleven initial cases recognized in the United States during the 2003 outbreak, four individuals were hospitalized (Reed, Melski et al. 2004).

B. Vaccines and anti-poxviral therapies

i. The history of vaccination

Early records indicate that inoculation against smallpox, also known as variolation, was done by injecting material from the lesions of smallpox-infected people into the skin of uninfected people (Riedel 2005). The use of this method developed after people noticed that once an individual survived smallpox, he or she did not suffer from the disease again. The case-fatality rate of smallpox in 18th Century England was 20% to 60%, but for infants, the case-fatality rate was even greater (Riedel 2005). Variolation also had a risk of death, but the case-fatality rate of variolation was around
2% to 3%, much lower than the risk of death from naturally contracting smallpox (Riedel 2005).

Edward Jenner, who was variolated as a child, took note that dairymaids who had suffered from cowpox were protected from smallpox (Riedel 2005). Jenner was not the only person to notice this protection by cowpox, but he began the investigation of vaccination through scientific methods and promoted the development of vaccination programs (Riedel 2005). In May of 1796, Jenner inoculated a young boy with material from a cowpox lesion of a dairymaid (Riedel 2005). In July of that year, Jenner then inoculated the boy with VARV and did not observe disease, declaring the boy protected (Riedel 2005). This inoculation with material from a cowpox lesion was termed vaccination, which eventually replaced variolation (Riedel 2005).

ii. The current smallpox vaccine

No longer part of a routine vaccination program or available to the general public, the smallpox vaccine is now approved by the Food and Drug Administration (FDA) for individuals who are at-risk, including people who are involved in research using orthopoxviruses, such as VACV. For vaccination, a bifurcated needle holding a droplet of live VACV is used to puncture the skin of the upper arm repeatedly (Henderson, Inglesby et al. 1999). The site is covered with a loose bandage to prevent transfer of the virus to other sites on the body or to unvaccinated people. The site of vaccination is often noted to be pruritic and becomes a papule then a pustule and eventually drains potentially infectious fluid. The site is no longer considered infectious once the site
dries up and the scab falls off, which takes an average of three weeks but could take longer (Henderson, Inglesby et al. 1999).

Complications caused by the smallpox vaccine have been part of the consideration in ending mass vaccination programs against orthopoxviruses. In general, potential complications of vaccination have included rashes and fever. In addition to the milder adverse events, there is also a risk of serious adverse events following vaccination with VACV. There is no treatment available for postvaccinial encephalitis, which occurs in 1/300,000 primary vaccinations and results in death in 25% of cases (Henderson, Inglesby et al. 1999). For individuals who survive, some people have permanent neurological changes (Henderson, Inglesby et al. 1999). A second potential life-threatening adverse event of vaccination in immunodeficient or immunocompromised individuals is progressive vaccinia, in which the virus spreads from the site of infection to neighboring skin and to internal organs (Henderson, Inglesby et al. 1999). Progressive vaccinia can also occur following the transfer of infectious material from a vaccination site to an immunocompromised person, which is termed inadvertent inoculation (Henderson, Inglesby et al. 1999). Inadvertant inoculation also includes the transfer of virus from the vaccination site to other areas of the body of the vaccinated individual. A treatment option for progressive vaccinia has been vaccinia immune globulin (VIG) (see Chapter II-B-iii below).

In people who have eczema, there is risk of eczema vaccinatum occurring due to the virus spreading from the vaccine site to cover the area of the skin affected by eczema (Henderson, Inglesby et al. 1999). VIG therapy is also a treatment option for eczema vaccinatum (Henderson, Inglesby et al. 1999). Another complication of the
smallpox vaccine is generalized vaccinia in which the virus spreads systemically from the site of infection and the characteristic pox lesions develop on other areas of the body (Henderson, Inglesby et al. 1999). People with generalized vaccinia frequently recover without medical intervention, but VIG can be used for treatment (Henderson, Inglesby et al. 1999). There is a consensus on the five groups of individuals who are at high-risk for complications from the smallpox vaccine: people with eczema or similar skin conditions, pregnant women, people with cancer undergoing chemotherapy or treatment with corticosteroids, people with genetic immunodeficiency conditions, and HIV+ individuals (Henderson, Inglesby et al. 1999).

iii. Anti-poxviral therapies

VIG is given if certain complications develop following receipt of the smallpox vaccine. As discussed previously, these complications include progressive vaccinia, eczema vaccinatum, generalized vaccinia, and inadvertent inoculation of the eye (Henderson, Inglesby et al. 1999). VIG has been made by pooling plasma from donors that received the smallpox vaccine, and availability is presently limited (Henderson, Inglesby et al. 1999). Currently, there are no available antiviral treatments for smallpox or monkeypox. In severe cases, treatment involves supportive therapy, including intravenous (i.v.) hydration. Although there are multiple anti-poxviral compounds that have been developed or are under development (De Clercq 2010, Prichard and Kern 2012, Smee 2013), only select current agents will be discussed below. Several anti-poxviral drugs under development could be considered for investigational treatment in the case of a severe orthopoxvirus infection, although these pharmaceuticals are not
FDA-approved for treatment of orthopoxvirus infections in humans. Three older anti-poxviral compounds include cidofovir, CMX001, and ST-246® (tecovirimat; SIGA Technologies, NY, USA) (Smee 2013). Cidofovir is a nucleotide analog that inhibits the viral DNA polymerase (Smee 2013). Currently, cidofovir is approved by the FDA for treatment of cytomegalovirus (CMV) retinitis in patients with Acquired Immunodeficiency Syndrome (AIDS). Even a single dose of cidofovir can rescue mice with a lethal ECTV infection (Israely, Paran et al. 2012). Cidofovir is given i.v. or topically, but has a significant side effect of nephrotoxicity and has limited oral bioavailability, so CMX001, which is converted to cidofovir in vivo, was developed to have improved oral bioavailability (Buller, Owens et al. 2004, Smee 2013).

Another antiviral with oral bioavailability and activity against multiple orthopoxviruses, including VACV, ECTV, VARV, CPXV, and MPXV, in vitro is ST-246 (Yang, Pevear et al. 2005). ST-246 inhibits the production of extracellular virus and can rescue mice from a lethal ECTV challenge (Yang, Pevear et al. 2005). In 2009, a recipient of the smallpox vaccine developed progressive vaccinia and was successfully treated with VIG along with both CMX001 and ST-246 (Lederman, Davidson et al. 2012). CMX001 and ST-246 were provided under emergency investigational new drug protocols marking the first case of CMX001 treatment of a human with an orthopoxvirus infection (Lederman, Davidson et al. 2012). Viral resistance to ST-246 was found late in the course of disease (Lederman, Davidson et al. 2012), indicating the importance of continued drug discovery and development for anti-poxviral pharmacologic agents.
iv. Poxviruses as vaccine vectors

As previously discussed, poxviruses have been used for many years as live vaccines to prevent smallpox. Poxviruses, however, are also being used in the development of vaccines against multiple viral, bacterial, and fungal pathogens (Sanchez-Sampedro, Perdiguero et al. 2015). Currently, vaccines using poxviruses as vectors are already in many preclinical and clinical trials (Sanchez-Sampedro, Perdiguero et al. 2015). The orthopoxviruses have large genomes that allow for insertion of foreign gene sequences, which can be utilized for the expression of foreign proteins for vaccination. Other advantages to the use of the poxvirus VACV as a viral vector include the ability to lyophilize the vector for storage and mass distribution (as has been done for the smallpox vaccine), low mutation rates of VACV, and the cytoplasmic location of the virus limiting potential effects on the host genome (Walsh and Dolin 2011). Additionally, VACV is being developed as a vector to deliver tumor-specific antigens for the induction of an immune response to tumor cells (Walsh and Dolin 2011) (Shen and Nemunaitis 2005). Replication-competent VACV vectors are also being designed for use as an oncolytic virus, designed to target, infect, and kill tumor cells (Shen and Nemunaitis 2005, Walsh and Dolin 2011). The research on VACV and the use of VACV as a vaccine against smallpox has provided an understanding of the safety profile and immune response as a basis for development of VACV vectors.
C. The orthopoxvirus ECTV

ECTV was first described in 1930 following observed disease in a laboratory animal facility (Marchal 1930). ECTV is the causative agent of mousepox in mice and does not infect humans. ECTV infection of the mouse is used to investigate the immune response to orthopoxvirus infection, the modification of the immune response by the virus, the efficacy of anti-poxviral therapeutics and treatments, and the immune response to a virus that spreads systemically from a peripheral site of infection. ECTV naturally infects the mouse via abrasions in the skin of the footpad and spreads systemically via the lymphohematogenous route (Fig. 2.1) (Fenner 1948) (Marchal 1930). The natural route of infection, through the footpad, is used to study the natural disease course and pathogenesis and the consequent natural immune response of the host and is the route of infection used in this dissertation. From the footpad, virus spreads initially to the draining lymph node (D-LN), which is the popliteal LN (pLN), where viral replication continues (Fig. 2.1). ECTV eventually spreads to the spleen and liver via the bloodstream (Fig. 2.1), and in resistant mice, viral titers peak at 5 days post-infection (d.p.i.) (Rubio, Xu et al. 2013). Following secondary viremia, the swelling of the site of infection (foot) becomes noticeable, and some mice will develop pox lesions on the skin (Fig. 2.1). By 30 d.p.i., virus can no longer be titered from the footpad, D-LN, spleen, or liver of resistant mice, and any pox lesions on the skin have scabbed and healed (Fenner 1948).

Multiple tissues and organs are affected during ECTV infection. In addition to the footpad, D-LN, spleen, and liver, infectious material is found in the lung tissue and pleural fluid (Marchal 1930). Tissues with histological changes include inclusion bodies
**Figure 2.1.** Pathogenesis of ECTV infection

Mice were inoculated with ECTV in the hind footpad and monitored for viral spread and morbidity.

Figure 2.1.

in the epithelium and connective tissues of the infected foot, the small intestine, congestion in the lung tissue, and necrotic areas within the liver and spleen (Marchal 1930). Following recovery from a primary infection with ECTV, mice are resistant to re-infection, which indicates the development of lasting immunity (Marchal 1930). Markers of disease progression and severity of disease during ECTV infection include elevated levels of alanine transaminase (ALT) and aspartate transaminase (AST) in the blood as indicators of liver damage, increased neutrophilia in the blood, and increased viral genome copies in the blood; however, weight loss is not predictive of ultimate susceptibility to ECTV infection (Parker, Schriewer et al. 2008).

Mouse strains differ in resistance to ECTV infection, and resistance is affected by the route of infection and viral strain. The ECTV strain initially described by Marchal in 1930 was the Hampstead strain of ECTV (Marchal 1930). Compared to the Hampstead strain, the Moscow strain is much more infectious and virulent (Andrewes and Elford 1947), and this virulent Moscow strain of ECTV is the virus used for the experiments in this dissertation. The C57BL/6 mouse strain is naturally resistant to footpad infection with ECTV. However, C57BL/6 mice are more susceptible to intranasal (i.n.) infection and die from an i.n. viral inoculum that is 10-fold lower than an inoculum that does not kill mice when injected into the footpad (Parker, Siddiqui et al. 2009). While C57BL/6 mice are naturally resistant to ECTV infection, DBA/2 mice and BALB/c mice are inbred mouse strains that are naturally susceptible to ECTV infection (Wallace, Buller et al. 1985, Brownstein, Bhatt et al. 1989). Crosses of resistant and susceptible strains have been used to analyze the genetics of ECTV resistance (Wallace, Buller et al. 1985, Brownstein, Bhatt et al. 1989). An example of a resistance gene of C57BL/6 mice is the
The Rmp-1 gene (resistance to mousepox), which is linked to the NK (natural killer cell) gene complex on chromosome 6 (Wallace, Buller et al. 1985, Delano and Brownstein 1995). NK cells are required for survival of ECTV infection (Parker, Parker et al. 2007, Fang, Lanier et al. 2008). By using the naturally resistant C57BL/6 mouse strain, components of the immune response can be analyzed for cells and functions of the immune response that are requisite during ECTV infection. Blocking or removal of a requisite immune response component in C57BL/6 mice results in susceptibility to ECTV infection.

D. An overview of antiviral immunity in the context of orthopoxvirus infection

The innate immune response acts quickly following viral infection to prevent viral replication and spread and to recruit and activate other effector immune cells, including the cells of the adaptive immune response, T cells and B cells. In the case of skin infection, such as with ECTV infection, intact skin acts as a barrier to infection, and infection occurs when this barrier is broken, such as by abrasions in the skin. The responding innate immune cells include myeloid cells, some of which are already present at the site of infection in the skin and other cells are rapidly recruited. The myeloid cells include: granulocytes (neutrophils, eosinophils, basophils), monocytes, mo-DC, macrophages, and DC subsets. An additional innate immune cell subset that responds to viral infection is the NK cell subset, which produces cytokines and kills virus-infected cells. The cells of the innate immune response recruit other immune cells, secrete cytokines, activate T cells by antigen presentation, and control virus early to allow time for development of the T cell and B cell responses.
i. Pathogen recognition

To identify pathogens, the innate immune system uses recognition mechanisms that rely on detecting pathogen-associated molecular patterns (PAMPs), which are conserved features of microorganisms. The recognition of the PAMPs gives the immune system information about the pathogen presence, location, virulence, and replicative ability, all of which are important for the development of the effector response. Depending on the pattern recognition receptors (PRRs) activated by the PAMPs, differing responses of the immune system result (Iwasaki and Medzhitov 2015). The effector response that develops is crucial as the immune system aims to control and eliminate the pathogen while minimizing the immunopathology.

Toll-like receptors (TLRs) are a group of PRRs that are type I transmembrane proteins. Recognition of the PAMPs by TLRs occurs at the ectodomain of the receptor. TLRs also contain a transmembrane region and a cytosolic Toll/IL-1 receptor (Reed, Melski et al) (Reed, Melski et al) domain. Once the TLR binds a PAMP, the TIR activates downstream signaling cascades. TLR activation results in the recruitment of adaptor molecules including MyD88 and/or TRIF to ultimately lead to responses to combat the pathogen including production of pro-inflammatory cytokines, production of chemokines, and activation of the T1-IFN response (Kawai and Akira 2011). TLRs detect different features of viruses, bacteria, fungi, and parasites as shown in Table 2.1, which provides examples of PAMPs recognized by TLRs, but is not an exhaustive list (Decker, Muller et al. 2005, Kawai and Akira 2011). TLRs recognize PAMPs at the cell surface and within intracellular vesicles of immune cells, including DC subsets and cells of the monocyte-macrophage lineage. TLR1, TLR2, TLR4, TLR5, and TLR6 are located
Table 2.1. Pathogen-Associated Molecular Patterns and TLR Recognition

<table>
<thead>
<tr>
<th>TLR</th>
<th>Pathogen</th>
<th>Pathogen-associated molecular pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR 2/1 or TLR 2/6</td>
<td>Bacteria</td>
<td>lipoproteins; lipoteichoic acid; peptidoglycans</td>
</tr>
<tr>
<td>TLR2</td>
<td>Viruses</td>
<td>structural proteins</td>
</tr>
<tr>
<td></td>
<td>Fungi</td>
<td>mannán; zymosan, β-glucan</td>
</tr>
<tr>
<td></td>
<td>Parasites</td>
<td>tGPI-mutin</td>
</tr>
<tr>
<td>TLR3</td>
<td>Viruses</td>
<td>dsRNA</td>
</tr>
<tr>
<td>TLR4</td>
<td>Viruses</td>
<td>structural proteins</td>
</tr>
<tr>
<td></td>
<td>Bacteria</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td></td>
<td>Fungi</td>
<td>mannán</td>
</tr>
<tr>
<td></td>
<td>Parasites</td>
<td>glycoinositolphospholipids</td>
</tr>
<tr>
<td>TLR5</td>
<td>Bacteria</td>
<td>flagellin</td>
</tr>
<tr>
<td>TLR6</td>
<td>Fungi</td>
<td>zymosan, β-glucan</td>
</tr>
<tr>
<td>TLR7</td>
<td>Bacteria</td>
<td>RNA</td>
</tr>
<tr>
<td></td>
<td>Viruses</td>
<td>ssRNA</td>
</tr>
<tr>
<td></td>
<td>Fungi</td>
<td>RNA</td>
</tr>
<tr>
<td>TLR8</td>
<td>Viruses</td>
<td>ssRNA</td>
</tr>
<tr>
<td>TLR9</td>
<td>Bacteria</td>
<td>DNA; unmethylated CpG</td>
</tr>
<tr>
<td></td>
<td>Viruses</td>
<td>DNA; unmethylated CpG</td>
</tr>
<tr>
<td></td>
<td>Fungi</td>
<td>DNA</td>
</tr>
<tr>
<td></td>
<td>Parasites</td>
<td>DNA; hemozoin</td>
</tr>
<tr>
<td>TLR11</td>
<td>Bacteria</td>
<td>profilin-like molecule</td>
</tr>
</tbody>
</table>
at the cell surface, TLR11 is found at the cell surface and within intracellular vesicles, and TLR3, TLR7, TLR8, and TLR9 are localized to intracellular vesicles (Kawai and Akira 2011). TLR13 is located intracellularly although the ligand for TLR13 remains unclear. There is redundancy in the recognition of PAMPs by TLRs and other groups of PRRs to activate responses specific to the PAMPs detected.

In the immune response to ECTV infection, TLR9 and the downstream signaling molecule myeloid differentiation primary response protein (MyD88) are essential for recognition of the virus to mediate survival of ECTV infection (Samuelsson, Hausmann et al. 2008, Sutherland, Ranasinghe et al. 2011, Rubio, Xu et al. 2013); whereas TLR2, TLR4, and TLR7 are not required for survival (Sutherland, Ranasinghe et al. 2011). Within an intracellular vesicle, TLR9 activation results in the recruitment of MyD88 for the production of pro-inflammatory cytokines via NF-κB and activation of the T1-IFN response via interferon regulatory factor 7 (IRF7) (Fig. 2.2) (Kawai and Akira 2011) (McNab, Mayer-Barber et al. 2015). NF-κB, IRF7, and a receptor for T1-IFN signaling (IFNAR1) are each required for survival of ECTV infection (Rubio, Xu et al. 2013), indicating the importance of these pathways in the immune response to ECTV.

ii. The T1-IFN and NFκB pathways

There are three IFN groups in the immune response. The T1-IFN family includes IFNα, of which there are 14 subtypes in mice, the single IFNβ, and several poorly defined single products including IFNε, IFNκ, and IFNδ (McNab, Mayer-Barber et al. 2015). The TII-IFN family consists of IFNγ, a cytokine primarily produced by T cells and NK cells. The TIII-IFN family includes IFNλ1, IFNλ2, IFNλ3, and IFNλ4. To inhibit the
Figure 2.2. Recognition of PAMPs via TLR9 and activation of T1-IFN signaling

Following recognition by PAMPs in the intracellular vesicle, such as an endosome, by TLR9, MyD88 is recruited and downstream signaling is activated to induce effects such as cytokine production via NF-κB and IRF7. The T1-IFN produced, IFNα and IFNβ, can signal by autocrine or paracrine signaling through the T1-IFN receptor (IFNAR) to activate interferon-stimulated response elements (ISRE) and induce transcription of interferon-stimulated genes (ISG).

Figure 2.2.

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McNab et. al. 15: 87-103, © 2015. (McNab, Mayer-Barber et al. 2015)
TII-IFN, IFNγ, ECTV encodes an IFNγ-binding protein (IFNγbp) that binds to IFNγ (Smith and Alcami 2002, Sakala, Chaudhri et al. 2007). Using an IFNγbp deletion mutant ECTV, the absence of the IFNγbp results in increased IFNγ and T cell responses in vivo (Sakala, Chaudhri et al. 2007). Additionally, mice normally susceptible to WT ECTV infection survive infection with the IFNγbp deletion mutant ECTV (Sakala, Chaudhri et al. 2007), indicating the importance of the IFNγbp in the pathogenesis of ECTV infection.

The remainder of this discussion will focus on the roles of IFNα/β that induce an antiviral state following detection of a viral infection. Following the production of T1-IFNs by the activation of PRR signaling pathways, the cytokines produced can interact with the T1-IFN receptor (IFNAR) on the surface of the cell releasing the cytokines or on neighboring cells (Fig. 2.2) (McNab, Mayer-Barber et al. 2015). The binding of IFNα/β to the T1-IFN receptor activates downstream signaling through the protein tyrosine kinases Janus kinase 1 (JAK1) and tyrosine kinase 2 (TYK2) leading to phosphorylation of the canonical signal transducer and activator of transcription 1 (STAT1) and STAT2. Phosphorylated STAT1 and STAT2 interact with IRF9 to form the ISG factor 3 complex that ultimately induces the activation of interferon-stimulated response elements (ISRE) and transcription of interferon-stimulated genes (ISG) (McNab, Mayer-Barber et al. 2015). The activation of hundreds of ISG results in an antiviral state of the cell (McNab, Mayer-Barber et al. 2015). Although the canonical pathway results in the expression of ISG, there are multiple pathways that mediate downstream signaling from the activated T1-IFN receptor (Fig. 2.2) (McNab, Mayer-Barber et al. 2015).
The ISG induced following recognition of a viral infection are dependent upon the virus detected. Some of the ISG induced as part of the antiviral response include IFN-inducible double-stranded RNA-dependent protein kinase (PKR) to inhibit translation, 2’-5’-oligoadenylate synthetase (OAS) that degrades viral RNA, and IFN-induced transmembrane proteins (IFITM) (McNab, Mayer-Barber et al. 2015). Additionally, the T1-IFN response affects myeloid cells, NK cells, T cells, and B cells by promoting migration and cross-presentation by DC and promoting activation, proliferation, and memory development (McNab, Mayer-Barber et al. 2015). The response is regulated to prevent damaging effects resulting in immunopathology.

Indicating the importance of the T1-IFN response following ECTV infection, ECTV encodes a T1-IFN binding protein (bp) that is encoded by EVM166 and specifically blocks the function of IFNα, not IFNβ (Xu, Rubio et al. 2012). The T1-IFNbp acts as a decoy to bind IFNα to prevent the binding of IFNα to the functional cellular receptor. Early after ECTV infection, treatment with antibody that blocks the function of the T1-IFNbp rescues ECTV-susceptible mice (Xu, Rubio et al. 2012), indicating the importance of the T1-IFNbp in the virulence of ECTV. An antibody response to the T1-IFNbp can be generated by immunizing with a recombinant T1-IFNbp, and this antibody response protects susceptible mice from lethal ECTV infection (Xu, Cohen et al. 2008). These findings support the potential for the targeting of virulence factors as part of vaccine design. This thesis discusses cytokine production, including IFNα, by subsets of the innate immune system.

Mice deficient in T1-IFN signaling or in NFκB signaling succumb to ECTV infection (Rubio, Xu et al. 2013). In addition to the T1-IFNbp, ECTV also encodes other
host-response modifiers, including p105bp encoded by EVM002 that inhibits NFκB and is important for ECTV virulence (Rubio, Xu et al. 2013). The p105bp inhibits the alteration of the p105 subunit of NFκB to the activated p50 subunit. The inhibition of NFκB mediated by the p105bp results in the reduction of proinflammatory mediators following ECTV infection (Rubio, Xu et al. 2013).

iii. Granulocytes

Granulocytes, which include neutrophils, basophils, and eosinophils, are recognized as CD11b+Ly6C+Ly6G+ cells. In response to an invading pathogen, granulocytes quickly respond to the site of infection to internalize cell debris and, in the case of bacterial infection, internalize the pathogen for intracellular killing; however, during viral infection, neutrophils have shown a role in antigen presentation (Hufford, Richardson et al. 2012). In response to influenza virus infection, neutrophils respond early by trafficking to the site of infection (Hufford, Richardson et al. 2012). In the infected respiratory tract, neutrophils express major histocompatibility class I (MHCI), but not MHCII, and play a role in the induction of the IFNγ response by T_{CD8}+ (Hufford, Richardson et al. 2012). Following VACV infection of the ear pinnae, responding Ly6C+Ly6G+ cells produce IFNα, IFNβ, and reactive oxygen species (Fischer, Davies et al. 2011). These Ly6C+Ly6G+ cells also are important for minimizing the size of the lesion and the amount of tissue damage during VACV infection (Fischer, Davies et al. 2011). In this dissertation, the involvement of granulocytes in mediating survival of ECTV infection will be discussed.
iv. Monocytes and mo-DC

There are two subsets of monocytes that circulate in the blood that are CD11b⁺. One subset of monocytes is identified as CX₃CR₁⁺CCR₂⁻ and is recruited into non-inflamed tissues in the steady-state (Geissmann, Jung et al. 2003). For extravasation into peripheral tissues from the blood in steady-state, this CX₃CR₁⁺CCR₂⁻ monocyte subset requires CX₃CR1, a receptor for the tethered transmembrane chemokine fractalkine (CX₃CL1) (Geissmann, Jung et al. 2003). The other subset of monocytes in the blood is CCR₂⁻CX₃CR₁⁻, and this monocyte subset is called inflammatory monocytes as they are recruited to sites of inflammation (Geissmann, Jung et al. 2003). The inflammatory monocytes also express Ly6C on the cell surface. Numbers of Ly6C⁺ monocytes in the blood are CCR2-dependent as CCR2 is required for the emigration of these inflammatory monocytes from the bone marrow into the blood in order to ultimately migrate to areas of inflammation (Boring, Gosling et al. 1997) (Dal-Secco, Wang et al. 2015) (Serbina and Pamer 2006) (Tsou, Peters et al. 2007, Tamoutounour, Guilliams et al. 2013) and for emigration from blood into inflamed/infected tissues (Iijima, Mattei et al. 2011).

In the steady-state, the Ly6C⁺ monocytes (inflammatory monocytes) extravasate into the peripheral tissues from the blood and develop into mo-DC (Geissmann, Jung et al. 2003), although these numbers of developing mo-DC are increased during inflammation/infection (Leon, Lopez-Bravo et al. 2007, Tamoutounour, Guilliams et al. 2013). As cells develop from monocytes into mo-DC, Ly6C expression decreases and CD11c expression increases (Tamoutounour, Guilliams et al. 2013). The mo-DC in the skin are phagocytic and able to migrate to the D-LN by a CCR7-dependent mechanism.
during skin inflammation (Randolph, Inaba et al. 1999, Tamoutounour, Guilliams et al. 2013). Following infection, mo-DC produce pro-inflammatory cytokines, including IL-12 (Leon, Lopez-Bravo et al. 2007). The mo-DC from the skin present antigen for proliferation and activation of effector functions of T_{CD8+} and T_{CD4+} (Tamoutounour, Guilliams et al. 2013), such as the production of cytokines, including IFNγ (Leon, Lopez-Bravo et al. 2007).

v. Tissue-resident macrophages

Macrophages internalize cell debris and pathogens, express CD11b, and are dependent on expression of the M-CSFR for development (Satpathy, Wu et al. 2012). The environment in which the cell resides plays an important role in controlling the macrophage phenotype (Dey, Allen et al. 2014, Davies and Taylor 2015). PRR activated by recognition of PAMPs results in the production of cytotoxic nitrogen and oxygen radicals and pro-inflammatory cytokines by macrophages for microbicidal activity. Macrophages resident in the skin have poor migratory ability in the steady-state and in inflammatory conditions (Tamoutounour, Guilliams et al. 2013). These cells have high phagocytic activity, but are poor at presenting exogenous antigen for T cell activation (Tamoutounour, Guilliams et al. 2013). In addition to the inflammatory response of macrophages, there is also a role for macrophages in the resolution of inflammation, including clearing debris and apoptotic cells, dampening the inflammatory response, and promoting healing of damaged tissue (Dey, Allen et al. 2014).
vi. DC and DC subsets

DC were first discovered as a stimulating cell in lymphoid tissues in the 1970s by Steinman et al. (Steinman and Witmer 1978). In addition to conventional DC (cDC) (including CD8α⁺ DC and CD11b⁺ DC), pDC are also dependent on FMS-related tyrosine kinase 3 ligand (Flt3L) for development (Satpathy, Wu et al. 2012). DC are now recognized as professional antigen-presenting cells (pAPC) that present antigen for activation of T cells of the adaptive immune response. In addition to the antigen-presenting function, DC also produce antiviral and proinflammatory cytokines in response to viral infection. The phenotypes and functions of the DC subsets CD11b⁺ DC, CD8α⁺ DC, and pDC are discussed in more detail below. CD11b⁺ DC, CD8α⁺ DC, and pDC are all infected in vivo by ECTV, and these cells are all efficient at directly presenting antigen for activation of T<sub>CD8⁺</sub> (Sei).

a. CD11b⁺ DC

As distinct from mo-DC, CD11b⁺ DC include migratory DC located within the skin and mucosal tissues. The CD11b⁺ DC are distinguished from CD64⁺ mo-DC as CD64⁻ (Langlet, Tamoutounour et al. 2012, Tamoutounour, Guilliams et al. 2013). CD64 is the IgG receptor FcγRI. CD11b⁺ DC internalize antigen in the skin and migrate into the D-LN using a CCR7-dependent mechanism (Nizza and Campbell 2014). CD11b⁺ DC in the skin include at least three subsets. There are two Langerin⁺ subsets: Langerhans cells and Langerin⁺ dermal DC (dDC) and a Langerin⁻ subset: Langerin⁻ dDC. Langerhans cells are present in the epidermis, and unlike Langerin⁺ dDC, Langerhans cells express the cell adhesion molecule EpCAM (Nagao, Ginthoux et al. 2009).
Langerin\(^+\) and Langerin\(^-\) DC in the skin are migratory and carry antigen from the skin to the D-LN to stimulate the T\(_{CD8^+}\) response (Nizza and Campbell 2014). During VACV infection, Langerin\(^+\) dDC are important for activation of the T\(_{CD8^+}\) response, including cell proliferation and the induction of effector functions, including IFN\(\gamma\) production (Seneschal, Jiang et al. 2014).

b. CD8\(\alpha^+\) DC

The development of the lymphoid-resident CD8\(\alpha^+\) DC is dependent on the transcription factor Batf3 (Hildner, Edelson et al. 2008). Batf3KO mice lack CD8\(\alpha^+\) DC, except during infection, CD8\(\alpha^+\) DC can develop by Batf3-independent mechanisms, but the development of CD8\(\alpha^+\) DC takes more than a week after infection (Tussiwand, Lee et al. 2012). For activation of the T cell response, CD8\(\alpha^+\) DC cross-present antigen to T\(_{CD8^+}\), which is important for the induction of the T\(_{CD8^+}\) response to West Nile Virus (Hildner, Edelson et al. 2008). During ECTV infection, CD8\(\alpha^+\) DC are infected by ECTV and, similar to CD11b\(^+\) DC and pDC, directly present antigen for activation of T\(_{CD8^+}\) (Sei). In contrast to CD11b\(^+\) DC and pDC, CD8\(\alpha^+\) DC internalize exogenous antigen and cross-present antigen for activation of T\(_{CD8^+}\) (Sei). Although the CD8\(\alpha^+\) DC are the cross-presenting DC during ECTV infection, the absence of the CD8\(\alpha^+\) DC does not alter antigen-specific T\(_{CD8^+}\) proliferation or T\(_{CD8^+}\) cytokine production to immunodominant peptide following ECTV infection (Sei).
c. pDC

PDC in the mouse were initially recognized as major T1-IFN producing cells with plasmacytoid morphology, a spherical shape with a smooth surface (Asselin-Paturel, Boonstra et al. 2001). TLR7 and TLR9 are preferentially expressed by pDC, and these PRR signal through MyD88 for production of IFNα/β via IRF7 activation (McNab, Mayer-Barber et al. 2015). PDC are recognized as CD11c<sup>lo</sup>CD11b<sup>+</sup>B220<sup>+</sup> and also express Ly6C (Asselin-Paturel, Boonstra et al. 2001). In response to influenza virus, these cells produce IFNα and the biologically active form of IL-12, IL-12p70, which is a pro-inflammatory cytokine that can induce IFNγ production, cell proliferation, and cytotoxic effector functions of NK and T cells (Asselin-Paturel, Boonstra et al. 2001). Antigen presentation by pDC results in T<sub>CD8+</sub> proliferation and activation to produce IFNγ (Asselin-Paturel, Boonstra et al. 2001, Takagi, Fukaya et al. 2011). During murine CMV infection, pDC are important for IFNα production and the virus-specific T<sub>CD8+</sub> response (Swiecki, Gilfillan et al. 2010).

vii. NK cells

NK cells are part of the innate immune response to viral infection and are identified as NK1.1<sup>+</sup>CD3<sup>e−</sup>. NK cell effector functions include IFNγ production and perforin-dependent cytolytic activity. In the case of viral infection, the cytolytic activity involves the killing of viral-infected cells to control the virus.

NK cells of the mouse have a 4-stage developmental process that is indicated by cell surface expression of CD11b and CD27: CD11b<sup>low</sup>CD27<sup>low</sup> → CD11b<sup>low</sup>CD27<sup>high</sup> → CD11b<sup>high</sup>CD27<sup>high</sup> → CD11b<sup>high</sup>CD27<sup>low</sup> (Chiossone, Chaix et al. 2009). In steady-state,
the CD11b^{low}CD27^{low} and CD11b^{low}CD27^{high} NK cell subsets have greater proliferative capacity than the CD11b^{high}CD27^{high} and CD11b^{high}CD27^{low} NK cell subsets (Chiossone, Chaix et al. 2009).

During ECTV infection, NK cells mediate viral control and are required early after infection for survival (Parker, Parker et al. 2007, Fang, Lanier et al. 2008). In response to ECTV infection, NK cells proliferate and numbers of NK cells increase in the D-LN, spleen, and liver (Parker, Parker et al. 2007). NK cells are recruited quickly, and cell numbers in the D-LN peak at 2 d.p.i. (Parker, Parker et al. 2007). Following ECTV infection, there is an increase in NK cells with a mature phenotype (CD11b^{high}CD27^{low}), and these mature phenotype NK cells have more IFNγ and granzyme B (GzmB) staining than NK cells with an intermediate (CD11b^{high}CD27^{high}) or immature (CD11b^{low}CD27^{high}) phenotype (Fang, Lanier et al. 2008). GzmB is secreted by cytotoxic NK cells to kill target cells by apoptosis. The state of NK cell activation is a balance between engagement of activating and inhibitory receptors. In response to ECTV, the activating receptor natural-killer group 2, member D (NKG2D), a type II transmembrane anchored C-type lectin-like receptor, is required for mediating the cytotoxic activity of NK cells to control the viral load (Fang, Lanier et al. 2008). The ligands for NKG2D include a family of ligands that are homologous to the MHCI proteins (Champsaur and Lanier 2010).

viii. T cells

T cells are identified as CD3e^{+} cells and, in this dissertation, are further identified as either CD8^{+} or CD4^{+}. CD3e is a subunit of the T cell receptor complex. T_{CD8^{+}} are
activated by antigen presentation on MHCI, and \( T_{CD4} \) are activated by antigen presentation on MHCII by APC. \( T_{CD8} \) are typically recognized as having cytolytic function while \( T_{CD4} \) are regulators of the immune response and helper cells for their effects on macrophages and the B cell response. Target cells are killed by \( T_{CD8} \) mainly by the release of perforin, which forms a pore in the membrane of the target cell, followed by release of GzB, which activates the apoptotic pathway.

During ECTV infection, the \( T_{CD8} \) response is required to control the viral load and mediate survival after peripheral infection with ECTV (Karupiah, Buller et al. 1996). Following ECTV infection, \( T_{CD4} \) respond to the D-LN, spleen, and liver, and many of these responding \( T_{CD4} \) express GzB (Fang, Siciliano et al. 2012). The \( T_{CD4} \) response controls the viral load and ultimately mediates survival of ECTV infection as mice deficient in the \( T_{CD4} \) response die at approximately 20 d.p.i. (Fang, Siciliano et al. 2012). In vivo, MHCII-restricted cell killing requires perforin (Fang, Siciliano et al. 2012), indicating an important role for the cytolytic effector function of \( T_{CD4} \) during ECTV infection.

Memory \( T_{CD8} \) generated by immunization with VACV rescue mice from ECTV infection when transferred into susceptible mice and result in reduced viral titers in the spleen and liver compared to susceptible mice receiving naïve \( T_{CD8} \) (Xu, Fang et al. 2007). To protect from lethal ECTV infection, the memory \( T_{CD8} \) require a functional perforin response (Remakus, Rubio et al. 2013). Immunization with VACV by skin scarification, i.p. injection, or subcutaneous injection each generates memory \( T_{CD8} \) (Hersperger, Siciliano et al. 2014). However, the memory \( T_{CD8} \) generated by skin scarification have better functionality in producing IFN\( \gamma \), controlling virus, and reducing
morbidity and mortality during infection with ECTV (Hersperger, Siciliano et al. 2014), indicating the importance of immunization via the natural route of infection for generation of T cell memory. While $T_{\text{CD8}^+}$ memory cells can rescue ECTV-susceptible mice following ECTV infection, susceptible mice require a functional B cell response for survival (Xu, Fang et al. 2007), indicating that memory $T_{\text{CD8}^+}$ can mediate survival long enough for other arms of the immune response to develop, including the requisite antibody response.

iv. B cells

B cells are part of the adaptive immune response to viral infection. Following recognition of antigen, B cells can develop into antibody-secreting plasma cells. The antibody produced may neutralize virus to protect cells from infection as part of viral control or antibody can be involved in activation of the complement cascade or antibody-dependent cellular cytotoxicity to lyse infected cells (Burton 2002). Mice deficient in the B cell response die from ECTV infection late after infection (more than 40 d.p.i.) (Fang and Sigal 2005, Fang, Siciliano et al. 2012), which indicates an inability to eliminate the virus in the absence of the antibody response.

E. Summary

In this dissertation, I will discuss the involvement of the myeloid cell subsets: granulocytes, monocytes and mo-DC, macrophages, and DC subsets in mediating survival of ECTV infection as requisite roles for these cell subsets during ECTV are unclear. The involvement of these cells in the response to a peripheral viral infection
with ECTV can provide knowledge for rational vaccine and antiviral therapy design. Important cells or their functions could be targeted by vaccines or mimicked by pharmacological agents to mediate survival and recovery of orthopoxvirus infections and potentially other peripheral viral infections. Currently, the smallpox vaccine has risks of morbidity and mortality, and therapeutics for treatment of vaccine adverse events and orthopoxvirus infections are only available under investigational drug protocols and their efficacy in humans is unclear.
Chapter III: Materials and Methods

Mice

C57BL/6 mice were purchased from Charles River Laboratories (cat. no. 027). CD11c::cre (cat. no. 008068) (Caton, Smith-Raska et al. 2007), LysM::cre (cat. no. 004781) (Clausen, Burkhardt et al. 1999), iDTR (cat. no. 007900) (Buch, Heppner et al. 2005), CCR2KO (cat. no. 004999) (Boring, Gosling et al. 1997), MyD88^fl/fl (cat. no. 008888) (Hou, Reizis et al. 2008), MaFIA (cat. no. 005070) (Burnett, Kershen et al. 2004), and Batf3KO (cat. no.013755) (Hildner, Edelson et al. 2008) were purchased from The Jackson Laboratory and bred at The Penn State College of Medicine. All mouse strains were on the C57BL/6 background. CD11c^wt/cre x iDTR^{+-} mice (CD11c::cre x iDTR) were generated by breeding CD11c^wt/cre mice with iDTR^{+-} mice, and genotype was verified by polymerase chain reaction (PCR) (Fig. 3.1). All PCR for genotype verification was performed on DNA obtained from tail snips and isolated using a DNeasy Blood & Tissue Kit (Qiagen). DNA was run on a 1.5% agarose gel and stained using ethidium bromide. LysM^{wt/cre} x iDTR^{+-} mice (LysM::cre x iDTR) were generated by breeding LysM^{cre/cre} mice with iDTR^{+-} mice, and genotype was verified by PCR (Fig. 3.2). CCR2KO mouse genotype was verified by PCR (Fig. 3.3). LysM^{wt/cre} x MyD88^{wt/fl} mice (LysM::cre x MyD88-floxed) were generated by breeding LysM^{cre/cre} mice with MyD88^{fl/fl} mice, and genotype was verified by PCR (Fig. 3.4). Mouse strain information is summarized in Table 3.1. Mice were maintained in a specific-pathogen free facility at The Penn State College of Medicine. All animal experiments were approved by the The Penn State College of Medicine IACUC.
**Table 3.1.** Mouse strains

<table>
<thead>
<tr>
<th>Mouse Strain</th>
<th>Information</th>
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| C57BL/6            | -Wild type  
|                    | -Background of all mouse strains used                                                   |
| CD11c:cre x iDTR   | -Express cre recombinase in CD11c+ cells  
|                    | -Cre recombinase removes floxed stop cassette resulting in expression of inducible diphtheria toxin receptor (iDTR)  
|                    | -Deplete CD11c+ cells by injection of diphtheria toxin (DT)                               |
| LysM:cre x iDTR    | -Express cre recombinase in LysM+ cells  
|                    | -Cre recombinase removes floxed stop cassette resulting in expression of inducible diphtheria toxin receptor (iDTR)  
|                    | -Deplete LysM+ cells by injection of diphtheria toxin (DT)                               |
| LysM:cre x MyD88-floxed | -Express cre recombinase in LysM+ cells  
|                    | -Cre recombinase removes floxed MyD88 gene in LysM+ cells                                |
| CCR2KO             | -Lack CCR2 which is required for trafficking of inflammatory monocytes from bone marrow into blood                                           |
| MaFIA              | -Express GFP and an inducible suicide gene in CD115+ cells  
|                    | -Injection of AP20187 results in apoptosis of CD115+ cells                               |
| Batf3KO            | -Lack transcription factor ATF3 necessary for development of CD8α+ DC                                                                     |
**Figure 3.1.** Genotyping CD11c:cre x iDTR mice.

(A) Primers used for amplification of CD11c:cre transgene from tail snip DNA. (B) Primers used for amplification of iDTR transgene or wild-type gene region from tail snip DNA. (C) No DNA amplification occurred for the no DNA control sample. The tail snip DNA sample from the C57BL/6 mouse had amplification of only the iDTR WT DNA, but the CD11c:cre x iDTR DNA sample had amplification of the iDTR WT, the iDTR mutant DNA (iDTR transgene), and the CD11c:cre mutant DNA (CD11c:cre transgene). Mice 1, 2, and 3 were CD11c:cre (-) and mice 4, 5, and 6 were CD11c:cre (+). Mice 1-6 were positive for the iDTR transgene.
Figure 3.1.

A  CD11c:cre transgene forward (oIMR7841): 5' ACT TGG CAG CTG TCT CCA AG 3'
     CD11c:cre transgene reverse (oIMR7842): 5' GCG AAC ATC TTC AGG TTC TG 3'

B  common primer (oIMR8545): 5' AAA GTC GCT CTG AGT TGT TAT 3'
     WT reverse primer (oIMR8546): 5' GGA GCG GGA GAA ATG GAT ATG 3'
     mutant primer (oIMR8128): 5' CAT CAA GGA AAC CCT GGA CTA CTG 3'

C  No DNA control
    C57BL/6 (negative control)
    CD11c:cre x iDTR (positive control)

1  2  3  4  5  6

603bp = iDTR WT band
313bp = CD11c:cre mutant band
242bp = iDTR mutant band
Figure 3.2. Genotyping LysM:cre x iDTR mice.

(A) Primers used for amplification of LysM:cre transgene or wild-type gene region from tail snip DNA. (B) Primers used for amplification of iDTR transgene or wild-type gene region from tail snip DNA. (C) No DNA amplification occurred for the no DNA control sample. The tail snip DNA from the C57BL/6 (WT) mouse had amplification of only the iDTR WT DNA and LysM:cre (-) wild-type DNA. The DNA from the LysM:cre x iDTR positive control mouse and the DNA from mouse samples 1, 2, and 3 had amplification of the iDTR WT and transgene DNA and the LysM:cre (-) (wild-type) and LysM:cre transgene DNA, indicating that mice 1, 2, and 3 were LysM:cre x iDTR.
Figure 3.2.

A LysM:cre mutant primer (oIMR3066): 5’ CCC AGA AAT GCC AGA TTA CG 3’
common primer (oIMR3067): 5’ CTT GGG CTG CCA GAA TTT CTC 3’
WT primer (oIMR3068): 5’ TTA CAG TCG GCC AGG CTG AC 3’

B common primer (oIMR8545): 5’ AAA GTC GCT CTG AGT TGT TAT 3’
WT reverse primer (oIMR8546): 5’ GGA GCG GGA GAA ATG GAT ATG 3’
mutant primer (oIMR8128): 5’ CAT CAA GGA AAC CCT GGA CTA CTG 3’

C

700bp= LysM:cre (+) mutant band
603bp= iDTR WT band
350bp= LysM:cre (-) band (wild-type)
242bp= iDTR mutant band
Figure 3.3. Genotyping CCR2KO mice.

(A) Primers used for amplification of mutant (knockout) gene region or WT gene region from tail snip DNA. (B) There was no amplification of DNA in the no DNA control sample. The tail snip DNA sample from the C57BL/6 (WT) mouse showed amplification of WT gene region. Tail snip DNA samples from mice 1-5 showed amplification of the mutant band only, indicating that mice 1-5 were CCR2KO.
**Figure 3.3.**

A  WT primer (olMR3375): 5’ CCA CAG AAT CAA AGG AAA TGG 3’
   WT primer (olMR3376): 5’ CCA ATG TGA TAG AGC CCT GTG 3’
   mutant forward primer (olMR6916): 5’ CTT GGG TGG AGA GGC TAT TC 3’
   mutant reverse primer (olMR6917): 5’ AGG TGA GAT GAC AGG AGA TC 3’

B  424bp = WT band
   280bp = mutant band
**Figure 3.4.** Genotyping LysM:cre x MyD88-floxed mice.

(A) Primers used for amplification of LysM:cre transgene or WT gene region from tail snip DNA. (B) Primers used for amplification of floxed or WT MyD88 gene. (C) There was no amplification of DNA in the no DNA control sample. Tail snip DNA from a C57BL/6 (WT) mouse had amplification of the MyD88 WT gene region and the LysM:cre (-) (WT) DNA. LysM:cre (-) (WT) DNA, LysM:cre (+) transgene DNA, and MyD88-floxed DNA region was amplified from the positive control sample (LysM:cre x MyD88-floxed) and from the tail snip DNA of mice 1-5 indicating that mice 1-5 are LysM:cre x MyD88-floxed mice.
Figure 3.4.

A LysM:cre mutant primer (oIMR3066): 5' CCC AGA AAT GCC AGA TTA CG 3'
common primer (oIMR3067): 5' CTT GGG CTG CCA GAA TTT CTC 3'
WT primer (oIMR3068): 5' TTA CAG TCG GCC AGG CTG AC 3'

B forward primer (oIMR9481): 5' GTT GTG TGT GTC CGA CCG T 3'
reverse primer (oIMR9482): 5' GTC AGA AAC AAC CAC CAC CAT GC 3'

C

<table>
<thead>
<tr>
<th>Lane</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>No DNA control</td>
</tr>
<tr>
<td>2</td>
<td>C57BL/6</td>
</tr>
<tr>
<td>3</td>
<td>LysM:cre x MyD88-floxed (positive control)</td>
</tr>
<tr>
<td>4</td>
<td>LysM:cre x MyD88-floxed (positive control)</td>
</tr>
<tr>
<td>5</td>
<td>LysM:cre x MyD88-floxed (positive control)</td>
</tr>
</tbody>
</table>

353bp = MyD88-floxed band
266bp = MyD88 WT band
700bp = LysM:cre (+) mutant band
350bp = LysM:cre (-) band (wild-type)
Viruses

For infections, 6- to 12-week-old mice were used. WT ECTV (Moscow) crude prep (C) stocks were produced in L929 cell monolayers. All experiments were done using WT ECTV (C). For intradermal (i.d.) hind footpad infection, mice were sedated using ketamine-xylazine and injected with 3,000 PFU of ECTV in a volume of 30µl HBSS/0.1% BSA into either the left or right hind footpad, as indicated. Mock-infected mice were injected with 30µl HBSS/0.1% BSA into one hind footpad. For survival experiments, mice were monitored daily for morbidity and mortality. Resistant mice were considered as having less than 25% mortality, intermediate mice as having between 25% and 75% mortality, and susceptible mice as having greater than 75% mortality. ECTV was inactivated by treatment with psoralen (conc. 10mg/ml) and treatment under a UVC lamp for 2 hours. This treatment renders the virus replication incompetent (Fischer, Tscharke et al. 2007). When indicated, mice were treated with 100mg/kg cidofovir (Mylan International) intraperitoneally (i.p.) in PBS at 3 and 6 d.p.i. Control mice received PBS i.p. at the same time points.

Plaque Assay for Ectromelia Virus Titers

Infected footpad, draining popliteal LN (D-LN), spleen, and liver were harvested at 5 d.p.i., weighed (except D-LN), and frozen in HBSS/0.1%BSA at -80°C. Titers were determined by plaque assay on VERO cells. Briefly, tissues were homogenized using a TissueLyser II (Qiagen). 10-fold serial dilutions in HBSS/0.1%BSA were used to infect confluent VERO cells in 24-well plates (3 wells/dilution). Cells were incubated at 37°C for 6 days. Media was then removed and cells were stained with 0.5% crystal violet.
The crystal violet was aspirated, the cells were air-dried, and the plaques were counted to calculate PFU per milliliter. For weighed tissues, PFU per gram tissue was calculated.

**Staining for Flow Cytometry of Cell Surface Markers**

D-LN or spleens were harvested at 2 or 3 d.p.i. as indicated and processed using 1mg/ml Collagenase D (Roche) digestion for 30 min at 37°C. Live cells were blocked and stained on ice in 2.4G2 supernatant/10% mouse serum (Sigma). Antibodies included biotin-conjugated CD19 (1D3), NK1.1 (PK136), CD90.2 (53-2.1) from eBioscience. Phycoerythrin (PE)-Cy7-streptavidin (eBioscience) was used to label biotin-conjugated antibodies. Other antibodies included CD45 (30-F11), CD11b (M1/70), B220 (RA3-6B2), CD8α (53-6.7), Ly6C (AL-21) from BD, CD11c (N418) and MHCII (I-A) from eBioscience, and CD64 (X54-5/7.1) and Ly6G (1A8) from Biolegend. Sample acquisition was performed with an LSRII flow cytometer (BD), and data were analyzed using FlowJo software (TreeStar). All myeloid cells were initially gated as live cells, singlets, CD45⁺, CD19⁻CD90.2⁻NK1.1⁻ (Fig. 3.5), and mo-DC, CD11b⁺ DC, pDC, and CD8α⁺ DC were identified as in Figure 3.5 unless otherwise indicated. Granulocytes were identified as: CD11c⁻CD11bʰ, and monocytes were identified as: CD11c⁻CD11bᵽ, unless otherwise indicated.

For NK cell and T cell analysis, cells were stained in 2.4G2 supernatant/mouse serum and 50µl/well BD Horizon Brilliant Stain Buffer (BD Bioscience). Unless otherwise noted, all antibodies were from BD Bioscience. Antibodies included CD45 (30-F11; Biolegend), CD8α (53-6.7; Biolegend), CD11b (M1/70; Biolegend), CD69
**Figure 3.5.** Flow cytometry gating strategy for identifying myeloid cell subsets.

Following infection in one hind footpad, spleens were harvested at 3 d.p.i. Splenocytes were stained for flow cytometry and gated as live cells, singlets, CD45⁺, then as NK1.1⁻ CD90.2⁻CD19⁻. Myeloid cell subsets were identified as follows: granulocytes (CD11c⁻CD11b⁺), monocytes (CD11c⁻CD11b⁻), mo-DC (CD11c⁺CD11b⁺CD64⁺), pDC (CD11c⁺CD11b⁻B220⁺), CD11b⁺ DC (CD11c⁺CD11b⁺CD64⁻CD8α⁻), and CD8α⁺ DC (CD11c⁺CD11b⁻B220⁻CD8α⁺). Numbers represent percentage of cells.
Figure 3.5.

Kaminsky et al, Figure S1.

FSC-A

SSC-A

74.3

FSC-W

95.9

SSC-A

CD45

93.7

NK1.1, CD90.2, CD19

SSC-A

8

CD11c

55.7 43.4

SSC-A

CD11b

14.9 7.5

granulocytes

monocytes

CD11b

SSC-A

27.3 70.3

CD64

78.8 16.3

CD11b+ DCs

mo-DCs

CD8a

SSC-A

61.4 35.9

CD8a

67

pDCs

CD8a+ DCs

B220
(H1.2F3; eBioscience), CD4 (RM4-5), CD3e (145-2C11), NK1.1 (PK136), CD27 (LG.3A10), CD44 (1M7), and CD62L (MEL-14). Cells were gated as live cells, singlets, and CD45+ populations. T cells were then gated as CD3e+NK1.1- with TCD8+ gated as CD8α+CD4+ (Total TCD8+) and TCD4+ gated as CD4+CD8α- (Total TCD4+). TCD8+ and TCD4+ were gated as naïve (CD44loCD62Lhi), effector (CD44hiCD62Llo), memory (CD44hiCD62Lhi) populations, and CD69+ populations. NK cells were initially gated as NK1.1+CD3e- (Total NK cells). NK cells were then gated as CD62L+, CD69+, immature (CD11b-CD27+), intermediate (CD11b+CD27+), and mature (CD11b+CD27-).

**Intracellular Cytokine Staining**

For *ex vivo* cell analyses, mice were infected with 3,000 PFU in the footpad, and the D-LN was harvested and stained for flow cytometry to initially exclude T cells, B cells, and NK cells. Cells were also stained with antibodies for CD11c and CD11b. For *in vitro* analyses, spleens were harvested from Flt3L-treated mice, digested using 1mg/ml Collagenase D (Roche), and DC were sorted on an autoMACS using panDC microbeads (MiltenyiBiotec). DC were then left untreated or were treated with WT ECTV (C) or UVC-inactivated WT ECTV (C) at an MOI=10 in DMEM/5% FBS for 6h. Cells were then incubated in 2.4G2 supernatant/10% mouse serum and stained using CD11c (N418), CD11b (M1/70), B220 (RA3-6B2), and CD8α (53-6.7) from BD Biosciences.

For both *ex vivo* and *in vitro* analyses, cells were incubated in 10µg/ml brefeldin A (Sigma) for 4h. Cell were stained for surface markers to identify myeloid cell subsets, fixed in 1% paraformaldehyde for 10min at RT and washed with PBS. Cells were
permeabilized with 0.5% saponin and stained with FITC-labeled anti-IFNα (Clone RMMA-1; R&D Systems). Samples were then washed and analyzed. Data acquisition was performed on the same day as tissue harvest as acquisition following overnight storage at 4°C has resulted in increased background and decreased IFNα signal.

**Cell Depletion**

**Clodronate liposome-based depletion.** Clodronate (dichloromethylene bisphosphonate) liposomes (CLL) from Boehringer Mannheim GmbH (Mannheim, Germany) were injected i.v. in a total volume of 200µl CLL solution at 1 d.p.i. for depletion. Control mice were injected with 200µl PBS i.v. at 1 d.p.i.

**DT-based depletion.** CD11c:cre x iDTR mice were injected with 20ng/g DT (Sigma) i.p. at 1 d.p.i. unless indicated otherwise. Control mice received PBS i.p. LysM:cre x iDTR mice were injected with 40ng/g DT (Sigma) i.p. at 1 d.p.i. unless indicated otherwise. Control mice received PBS i.p.

**AP20187 depletion in MaFIA mice.** To deplete CD115+ cells in MaFIA mice, mice were injected i.p. with 10mg/kg AP20187 (Clontech B/B homodimerizer S5030) on days -3, -2, -1, and 1 then injected with 1mg/kg i.p. every third day from day 1 to deplete CD115+ cells. Control mice were injected i.p. with vehicle (1.7% Tween20, 4% ethanol, 10% PEG-400 in sterile water) following the same schedule.

**Antibody-based depletion.** For depletion of Ly6G+ cells (granulocytes), C57BL/6 mice were injected i.v. on day -1 then every third day with 0.7mg anti-Ly6G antibody (1A8; BioXCell) in 500µl PBS. Control mice received 0.7mg Rat IgG2a isotype control
antibody (2A3; BioXCell) in 500µl PBS following the same schedule. Efficiency of depletion is shown in Figure 3.6A.

For depletion of pDC, mice were injected i.p. on days -2, -1, and then every third day from day -1 throughout the experiment with 500µg anti-pDC antibody (BX444; BioXCell) in 500µl PBS. Control mice were injected i.p. with 500µg Rat IgG1 anti-Horseradish Peroxidase antibody (HRPN; BioXCell) in 500µl PBS following the same schedule.

For a second method of depletion of CD115^+ cells, mice were injected i.p. every other day starting at day -5 with 3mg AFS98 antibody (anti-CD115 depleting antibody) in 200µl 0.1%BSA/HBSS. Control mice were injected with 200µl vehicle i.p. following the same schedule. Efficiency of depletion is shown in Figure 3.6B.

**Microscopy**

Mice were infected in the hind footpad with 1 x 10^6 PFU ECTV expressing NP-S-EGFP (Calvo, Yokoyama et al.). D-LN were harvested 12h post-infection, mounted in OCT, and frozen. 20µm sections were cut and incubated with F_{ab} donkey anti-mouse IgG (Jackson ImmunoResearch) then stained with directly-labeled Alexa647-conjugated anti-CD11c (N418) (eBioscience). Staining was visualized using an Olympus 1X81 DSU Spinning Disk microscope and Slidebook 5.0 software.
**Figure 3.6.** Depletion efficiency of granulocytes or macrophages by antibody-based methods.

(A) C57BL/6 mice were injected i.v. with 0.5ml of 1.4mg/ml anti-Ly6G depleting antibody (1A8) or with a control antibody (2A3) in PBS on day -1 then every third day and infected on day 0 with 3,000 PFU ECTV in the right hind footpad. Spleens were harvested at 21 d.p.i., processed, and cells were analyzed by flow cytometry. Cells were gated on live cells, singlets, NK1.1<sup>-</sup>CD90.2<sup>-</sup>CD19<sup>-</sup>, CD11b<sup>+</sup>, Ly6C<sup>+</sup>Ly6G<sup>+</sup>. The percentage of Ly6C<sup>+</sup>Ly6G<sup>+</sup> cells (of CD11b<sup>+</sup> cells) is shown. Data are representative of two independent experiments (mean +/- SEM). (B) C57BL/6 mice were injected with 3mg/ mouse anti-CD115 depleting antibody (AFS98) or vehicle control (PBS) i.p. every other day starting at day -5 and infected in the left hind footpad with 3,000 PFU ECTV. At 4 d.p.i., spleens were harvested and stained for flow cytometry. Macrophages were identified as: live cells, singlets, CD45<sup>+</sup>, B220<sup>-</sup>, Ly6C<sup>-</sup>, CD11b<sup>+</sup>F4/80<sup>+</sup>. Data are representative of two independent experiments (mean +/- SEM). ***p<0.001, Student’s unpaired t-test.
Figure 3.6.

A

% Ly6C+Ly6G+ cells

- 2A3 (IgG2a Control)
- 1A8 (anti-Ly6G)

B

Number of macrophages (x10^5)

- PBS
- AFS98

***
Data display and statistical analysis

Statistical analyses were performed using unpaired, two-tailed Student’s t-test as applicable. When applicable, data is displayed as mean +/- SEM. Log-rank (Mantel-Cox) test was used to determine significance of survival curves. Values of $p<0.05$ were considered significant. $p$ values are indicated as follows: *$p<0.05$, **$p<0.01$, ***$p<0.001$. 
Chapter IV: Granulocytes, inflammatory monocytes and mo-DC, and macrophages are not requisite in the immune response to ECTV infection

Introduction

Following a viral infection, cells of the innate immune response rapidly respond to prevent viral replication and spread. Some of these responding cells include cells of the myeloid subsets: granulocytes (neutrophils, eosinophils, basophils), monocytes, mo-DC, and macrophages. During infection and inflammation, these cell populations are altered from the steady-state in cell number and effector functions (Tamoutounour, Guilliams et al. 2013) (Leon, Lopez-Bravo et al. 2007). These innate immune cells not only activate and recruit cells of the adaptive immune response, these innate cell subsets also phagocytose pathogens and cell debris, produce pro-inflammatory cytokines, and produce antiviral cytokines to control the virus to allow time for the adaptive immune response to develop.

During footpad infection with ECTV, myeloid cells quickly respond to sites of viral infection. Granulocytes extravasate from the blood into the tissue to begin phagocytosing debris and containing pathogens. Inflammatory monocytes also enter the sites of infection/ inflammation from the circulation and can develop into mo-DC. Phagocytic macrophages and DC, however, are already present within the tissue. Monocytes, mo-DC, and macrophages are infected with virus after a peripheral infection with ECTV, and these cells directly present antigen for activation of $T_{CD8^+}$ (Sei). Neutrophils are also capable of presenting antigen, as during influenza virus infection,
neutrophils present antigen to activate the T_{CD8}+ response (Hufford, Richardson et al. 2012). The T_{CD8}+ response is required early after ECTV infection for viral control (Karupiah, Buller et al. 1996) (Fang and Sigal 2005). In addition, Ly6C^+Ly6G^+ cells (granulocytes) produce antiviral cytokines, such as IFNα and IFNβ, and are required for minimizing tissue damage during VACV infection (Fischer, Davies et al. 2011). One of the cytokines produced by these Ly6C^+Ly6G^+ cells in response to VACV (Fischer, Davies et al. 2011), IFNα, is an antiviral cytokine required for survival during ECTV infection (Xu, Rubio et al. 2012) (Rubio, Xu et al. 2013), indicating a potential important role for these cells.

The involvement of granulocytes, monocytes, mo-DC, and macrophages in producing cytokine and mediating survival following ECTV infection is currently not well-understood. Phagocytic cells of the immune response, which include monocytes, mo-DC, and macrophages as well as DC, are required early during infection for control of virus and, ultimately, for survival of ECTV infection. This involvement of phagocytic immune cells subsets in the response to ECTV has been investigated using carrageenan and CLL treatment to deplete phagocytic cells. Carrageenan consists of mucopolysaccharides from the cell walls of red algae. After carrageenan treatment to deplete phagocytes, including macrophages, mice were unable to control the virus and became susceptible to ECTV infection (Tsuru, Kitani et al. 1983). Carrageenan, however, has been shown to have other effects on the immune response, including activation of pathogen sensing pathways via TLR4 and aberrant activation of NK cells (Zhang, Tsai et al. 2010) (Abe, Kawamura et al. 2002). In addition to phagocyte depletion by carrageenan treatment, resistant mice become susceptible to ECTV
infection following treatment with CLL (Karupiah, Buller et al. 1996). After injection, CLL are internalized by phagocytic cells and are depleted by apoptotic mechanisms (Van Rooijen 1989). Although frequently described as depleting macrophages (Qian, Jutila et al. 1994) and monocytes (Sunderkotter, Nikolic et al. 2004), CLL have been shown to deplete other phagocytic cell populations, including DC (Leenen, Radosevic et al. 1998). During infection with another orthopoxvirus, VACV, CLL depletes monocytes and DC subsets in the spleen (Davies). Although indicated to deplete macrophages, carrageenan and CLL both have other effects on the immune response, including depletion of other phagocytic immune cells, which could be involved in mediating the susceptibility of mice to ECTV following treatment with these compounds.

Because carrageenan and CLL deplete phagocytic immune cells and not a specific myeloid cell subset, the roles of monocytes, mo-DC, and macrophages in ECTV infection have been unclear. Initially, we investigated the efficacy of CLL depletion on myeloid cell subsets during ECTV infection. To address specific roles of monocytes, mo-DC, and macrophages in effective immunity to ECTV, we utilized multiple depletion systems to isolate the requirement for these cell subsets in the immune response to ECTV infection. We then assessed the cytokine response of these myeloid cells to ECTV.

Results

*CLL deplete multiple myeloid cell subsets in vivo*

Phagocytes of the immune response are required for survival of ECTV infection as mice depleted of phagocytic cells by CLL injection die early from ECTV infection with
a mean time to death of 8.5 d.p.i. (Karupiah, Buller et al. 1996). Without these phagocytic cells, mice are unable to control ECTV as indicated by elevated viral titers in the spleen and liver compared to non-depleted mice (Karupiah, Buller et al. 1996). To investigate the phagocytic cells depleted by CLL during ECTV infection, mice were infected in the footpad with ECTV and injected i.v. at 1 d.p.i. with CLL for depletion. We assessed depletion during ECTV infection as viral infection alters the myeloid cell compartment from steady-state (Tamoutounour, Guilliams et al. 2013) (Leon, Lopez-Bravo et al. 2007), so cell depletion by CLL during infection has the potential to differ from cell depletion during homeostasis. Multiple myeloid cell subsets were depleted at 2 d.p.i. by CLL injection during ECTV infection including mo-DC, CD11b⁺ DC, pDC, CD8α⁺ DC, and monocytes (Fig. 4.1), which is similar to the depletion of monocytes and DC subsets in the spleen during VACV infection (Davies). (The depletion of DC by CLL is discussed in detail in Chapter V.) In contrast, although granulocytes are phagocytic, granulocytes were not depleted by CLL injection (Fig. 4.1).

*The non-requisite roles of granulocytes, inflammatory monocytes, mo-DC, and macrophages in the immune response to ECTV infection*

Although granulocytes were not depleted by CLL injection during ECTV infection (Fig. 4.1), granulocytes have been reported by another group to be required for effective immunity to ECTV (Tahiliani, Chaudhri et al. 2013). In addition, Ly6C⁺Ly6G⁺ cells are important for minimizing tissue damage during VACV infection (Fischer, Davies et al. 2011). Granulocytes express the cell surface marker Ly6G, so we targeted granulocytes for depletion using injection of anti-Ly6G antibody (1A8) or injection of an
Figure 4.1. Myeloid cell subset depletion by CLL injection.

C57BL/6 mice were infected in the left hind footpad with 3,000 PFU ECTV and injected with CLL or PBS i.v. at 1 d.p.i. Spleens were harvested at 2 d.p.i., processed, and cells were stained for flow cytometry. Cells were initially gated as live cells, singlets, CD45\(^+\), CD19\(^-\)CD90.2\(^-\)NK1.1\(^-\). Myeloid cell subsets were identified as in Figure 3.5. Data are pooled from two independent experiments (mean +/- SEM). ***p<0.001, Student’s unpaired t-test.
Figure 4.1.
isotype control antibody (2A3) (Fig. 3.6A). The 1A8 anti-Ly6G antibody is more specific for granulocyte depletion than the anti-granulocyte receptor-1 (anti-Gr-1) antibody, RB6-8C5, which targets cells expressing Ly6C and/or Ly6G, including granulocytes and monocytes (Daley, Thomay et al. 2008) and potentially other Ly6C⁺ cells, including pDC, mo-DC, and dermal macrophages (Asselin-Paturel, Boonstra et al. 2001, Tamoutounour, Guilliams et al. 2013). Mice depleted of granulocytes did not have greater than 25% mortality during ECTV infection and isotype control antibody-treated mice had 100% survival (Fig. 4.2), which indicated that granulocytes alone, including neutrophils, likely do not have a requisite role in the immune response to ECTV infection.

In contrast to granulocytes, monocytes and mo-DC were depleted by CLL injection during ECTV infection (Fig. 4.1), indicating that monocytes and mo-DC could have required roles in mediating the survival of ECTV infection. To investigate the involvement of inflammatory monocytes and mo-DC in the immune response to ECTV, we used CCR2KO mice, which lack CCR2. CCR2 is a chemokine receptor required for the recruitment of inflammatory monocytes from the bone marrow into circulation to ultimately traffic to sites of inflammation or infection (Boring, Gosling et al. 1997) (Dal-Secco, Wang et al. 2015) (Serbina and Pamer 2006) (Tsou, Peters et al. 2007). In addition to exit from the bone marrow, CCR2 is required for emigration of inflammatory monocytes from the blood to peripheral tissues (Iijima, Mattei et al. 2011). In CCR2KO mice, there is no defect in resident macrophages (Boring, Gosling et al. 1997). Because mo-DC develop at the site of inflammation or infection from circulating inflammatory monocytes (Tamoutounour, Guilliams et al. 2013), mo-DC are also unable
Figure 4.2. ECTV challenge of Ly6G^+ cell-depleted mice.

C57BL/6 mice received i.v. injections of 0.5ml of 1.4mg/ml anti-Ly6G depleting antibody 1A8 or with an IgG2a control antibody (2A3) in PBS on day -1 then every third day and were infected with 3,000 PFU ECTV in the right hind footpad on day 0. Survival was monitored to 21 d.p.i. Data are combined from two independent experiments. Comparison of the survival between the two groups is not significant, Log-rank test.
Figure 4.2.
to develop in CCR2KO mice (Lin, Suzuki et al. 2008). Following infection with ECTV, CCR2KO mice and control WT mice survived (Fig. 4.3), which indicated that neither inflammatory monocytes nor mo-DC play requisite roles in the early immune response to ECTV infection. We investigated the level of monocytes and mo-DC in the spleens of ECTV-infected CCR2KO mice at 8 d.p.i. and found a reduction in both monocytes and mo-DC compared to C57BL/6 mice (Fig. 4.4). Although granulocytes, inflammatory monocytes, and mo-DC were not required for survival of ECTV infection, macrophages are also depleted by CLL treatment (Qian, Jutila et al. 1994) (van Rooijen, Kors et al. 1989) and could be involved in the development of an effective immune response to ECTV.

To isolate the role of macrophages in the immune response to ECTV, we first utilized MaFIA mice. In MaFIA mice, the expression of GFP and a suicide gene are both driven by the c-fms (CD115/ M-CSFR) promoter (Burnett, Kershen et al. 2004). CD115+ cells are targeted for depletion by injection of AP20187, which dimerizes the suicide receptor leading to activation of the Fas pathway and apoptosis of the cells (Burnett, Kershen et al. 2004) (Clackson, Yang et al. 1998) (Amara, Clackson et al. 1997). MaFIA mice depleted of CD115+ cells succumbed to ECTV infection, while mock-infected and vehicle-treated MaFIA mice and control WT mice did not have greater than 20% mortality (Fig. 4.5A). AP20187-treated, uninfected controls were monitored for morbidity and mortality as AP20187 treatment i.p. has been shown to cause the development of peritoneal adhesions (Burnett, Beus et al. 2006). The death of CD115+ cell-depleted mice indicated that the cell populations depleted in MaFIA mice were required for survival of ECTV infection. Although the MaFIA depletion system has
**Figure 4.3.** ECTV challenge of CCR2KO mice.

C57BL/6 or CCR2KO mice were infected with 3,000 PFU ECTV in the right hind footpad and monitored for survival out to 21 d.p.i. Data are combined from two independent experiments.
Figure 4.3.
Figure 4.4. Monocyte and mo-DC numbers in CCR2KO mice.

C57BL/6 or CCR2KO mice were infected with 3,000 PFU ECTV in the right hind footpad. On day 8 p.i., spleens were harvested, processed, and cells were stained for flow cytometry. Cells were gated as: live cells, singlets, CD19^−NK1.1^−CD90.2^− then as monocytes: CD11c^−CD11b^+Ly6C^−Ly6G^− or mo-DC: CD11c^+CD11b^+CD64^+MHCII^+Ly6C^+.

Data are from one experiment with 2 mice/group and samples run as experimental duplicates (mean +/- SEM). **p<0.01, Student’s unpaired t-test.
Figure 4.4.
been used as a macrophage depletion method, some DC are also depleted by AP20187 injection of MaFIA mice (Burnett, Kershen et al. 2004), so we analyzed the depletion of myeloid cell subsets in depleted MaFIA mice. During ECTV infection, we did not find significant depletion of myeloid cell subsets in the D-LN (Fig. 4.5B). However, when we investigated the cell subsets depleted in the spleen by AP20187 compared to vehicle treatment of MaFIA mice during ECTV infection, we found depletion of mo-DC and DC at 5 d.p.i. (Fig. 4.5C). There was also an increase in the granulocytes and monocytes in the spleen at 5 d.p.i. (Fig. 4.5C). Because the MaFIA depletion system was not specific for depletion of only cells of the monocyte-macrophage lineage (Fig. 4.5C) and the cost of AP20187 became financially prohibitive, we stopped use of the MaFIA system. Instead, to isolate the involvement of cells of the monocyte-macrophage lineage in the innate immune response to ECTV, we next used the LysM:cre x iDTR mouse depletion system.

In LysM:cre x iDTR mice, Cre recombinase expression is driven by the endogenous promoter of M lysozyme (LysM) (Clausen, Burkhardt et al. 1999), which is active in granulocytes, monocytes, and macrophages (Faust, Varas et al. 2000) and some DC (Jakubzick, Bogunovic et al. 2008). Cre recombinase expression within the cell results in the removal of a loxP-flanked STOP cassette leading to expression of the simian iDTR (Buch, Heppner et al. 2005). Cells expressing the iDTR can then be targeted for depletion in vivo by injection of DT (Buch, Heppner et al. 2005). High doses and repeated dosing of DT can be toxic and ultimately lethal to mice (Goldwich, Steinkasserer et al. 2012), and DT can cause dysfunction of the podocytes of the kidney leading to proteinuria (Goldwich, Steinkasserer et al. 2012). We titrated the amount of
Figure 4.5. ECTV challenge of MaFIA mice.

(A) C57BL/6 or MaFIA mice were injected i.p. with vehicle or 10mg/kg AP20187 on days -3, -2, -1, and 1 and then injected with 1mg/kg AP20187 every third day from day 1. On day 0, mice were mock-infected or infected with 3,000 PFU ECTV in one hind footpad. Mice were monitored for survival out to 13 d.p.i. Data are combined from two independent experiments. ***p<0.001, Log-rank test (MaFIA:AP20187 + ECTV group compared to C57BL/6: AP20187 + mock group, C57BL/6: vehicle + ECTV group, or MaFIA: AP20187 + mock group). (B and C) MaFIA mice were injected with vehicle or AP20187 as in (A). On day 0, mice were infected with 3,000 PFU ECTV in the right hind footpad. At 5 d.p.i., D-LN (B) and spleens (C) were harvested, processed, and stained for flow cytometry. Cells were gated on live cells, singlets, and CD90.2^NK1.1^-CD19^- then for myeloid cell subsets: CD11b^{+}CD11c^{+}Ly6C^{+}Ly6G^{+} (granulocytes), CD11b^{+}CD11c^{+}Ly6C^{+}Ly6G^{-} (monocytes), CD11b^{+}CD11c^{+}MHCII^{+}CD64^{+} (mo-DC), and CD11b^{-}CD11c^{+} (cDC). Data are from one experiment with 2 or 3 mice per group (mean +/- SEM). *p<0.05, **p<0.01, ***p<0.001, Student’s unpaired t-test.
Figure 4.5.

A

- C57BL/6: AP20187 + mock-infected (6)
- C57BL/6: vehicle + ECTV (6)
- C57BL/6: AP20187 + ECTV (6)
- MaFIA: AP20187 + mock-infected (6)
- MaFIA: vehicle + ECTV (8)
- MaFIA: AP20187 + ECTV (8)

% Survival

Day post-infection

B

Vehicle + ECTV
AP20187 + ECTV

D-LN

Number of cells/D-LN (x 10^4)

C

Vehicle + ECTV
AP20187 + ECTV

Spleen

Number of cells/spleen (x 10^6)

granulocytes
monocytes
mo-DC
DC

granulocytes
monocytes
mo-DC
DC

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DT to minimize toxicity. To titrate the dose of DT that we could use to deplete myeloid cells while minimizing toxicity, we repeatedly treated WT and LysM:cre x iDTR mice with varying doses of DT. WT mice were resistant to DT treatment during ECTV infection and survived when given a dose of DT up to 40ng/g (Fig. 4.6A). LysM:cre x iDTR mice, however, were susceptible to repeated DT treatment of 50ng/g and died at 13-14 days after the first DT injection (Fig. 4.6B). LysM:cre x iDTR mice that received PBS or DT at 10ng/g or 20ng/g survived out to 20 days (Fig. 4.6B), indicating that we could use repeated DT treatment in this system for cell depletion during an ECTV challenge. We chose to use 40ng/g DT treatment for cell depletion during ECTV infection as this dose did not cause mortality in WT mice (Fig. 4.6A), and we expected LysM:cre x iDTR mice to survive DT treatment at this dose out to the planned 13 d.p.i. of an ECTV challenge. In addition to minimizing DT toxicity, we also chose 40ng/g DT to use the highest dose of DT to maximize cell depletion.

When LysM:cre x iDTR mice were injected repeatedly with 40ng/g DT to deplete cells of the monocyte-macrophage lineage, mice died at 7-9 d.p.i. after footpad infection with ECTV (Fig. 4.7). However, WT control mice or LysM:cre x iDTR mice receiving either DT or ECTV infection alone did not have greater than 40% mortality in multiple experiments (Fig. 4.7). These results indicated that 40ng/g DT was a nonlethal dose of DT, and that cells depleted in DT-injected LysM:cre x iDTR mice were required for mediating survival of ECTV infection. Because we used repeated injections of DT during ECTV infection, the period of time during which the depleted cells were required to mediate survival remained unclear. To isolate the time frame during which the cells were required, we injected LysM:cre x iDTR mice with DT on different days (day -1, 1, 3,
Figure 4.6. Titration of DT dose in C57BL/6 and LysM:cre x iDTR mice.

(A) C57BL/6 mice were injected i.p. with PBS or with 10ng/g DT, 20ng/g DT, 30ng/g DT, or 40ng/g DT on days -3, -2, -1 then every third day from day -1. On day 0, mice were infected with 3,000 PFU ECTV in the right hind footpad. Survival was monitored out to 21 d.p.i. Data are from one experiment. (B) LysM:cre x iDTR mice were injected i.p. with PBS or with 10ng/g DT, 20ng/g DT, or 50ng/g DT on days 1, 2, and 3 then every third day from day 3. Mice were monitored for survival out to 20 days. Data are from one experiment. *p<0.05, Log-rank test (50ng/g DT group compared to PBS group).
Figure 4.6.

A

B

% Survival

% Survival

Day post-infection

Day

0 3 6 9 12 15 18 21

0 4 8 12 16 20

PBS (4)

10ng/g DT (4)

20ng/g DT (4)

30ng/g DT (4)

40ng/g DT (4)

PBS (4)

10ng/g DT (5)

20ng/g DT (5)

30ng/g DT (4)

40ng/g DT (4)

*
Figure 4.7. ECTV challenge of LysM:cre x iDTR mice repeatedly treated with DT for depletion.

C57BL/6 or LysM:cre x iDTR mice were injected with PBS or 40ng/g DT i.p. on days -3, -2, -1, and then every third day from day -1 out to the end of the experiment. Mice were mock-infected or infected on day 0 with 3,000 PFU ECTV in the right hind footpad. Data are combined from four independent experiments. ***p<0.001, Log-rank test (LysM:cre x iDTR: DT + ECTV group compared to each control group).
Figure 4.7.

- C57BL/6: DT + mock-infected (12)
- C57BL/6: PBS + ECTV (12)
- C57BL/6: DT + ECTV (16)
- LysM:cre x iDTR: DT + mock-infected (11)
- LysM:cre x iDTR: PBS + ECTV (12)
- LysM:cre x iDTR: DT + ECTV (13)

% Survival vs Day post-infection
or 5 p.i.) during ECTV infection. Depletion of the requisite myeloid cells in LysM:cre x iDTR mice at 1 d.p.i. resulted in death of all ECTV-challenged mice; however, if cells were depleted at day -1 p.i. there was less than 60% mortality, at 3 d.p.i. there was less than 40% mortality, and at 5 d.p.i. all mice survived (Fig. 4.8). In addition, DT injection or ECTV infection alone were not lethal to the mice (Fig. 4.8). The mortality of the depleted and infected LysM:cre x iDTR mice indicated that the cells that are required for mediating survival of ECTV infection were required early during infection (around 1 d.p.i.).

We next investigated the myeloid cell subsets that are depleted by DT-injection of LysM:cre x iDTR mice during ECTV infection. For the analysis of myeloid cell depletion, we used DT injection only at 1 d.p.i., which had resulted in death of ECTV-infected LysM:cre x iDTR mice (Fig. 4.8). Following DT injection at 1 d.p.i., LysM:cre x iDTR mice had depletion of granulocytes, monocytes, CD11b+ DC, and CD8α+ DC in the D-LN at 2 d.p.i. compared to DT-treated WT mice, but this depletion was not significant in comparison to non-depleted LysM:cre x iDTR mice (Fig. 4.9A). However, in the spleen of depleted LysM:cre x iDTR mice at 2 d.p.i., there was depletion of mo-DC, CD11b+ DC, granulocytes, and monocytes compared to non-depleted LysM:cre x iDTR mice, and some depletion of CD8α+ DC compared to WT mice (Fig. 4.9B), indicating the nonspecific myeloid cell depletion in this system. Because multiple myeloid cell subsets were targeted in the depletion of LysM:cre x iDTR mice, we cannot make conclusions from these data about which cell subset(s) were requisite early in mediating the survival of mice infected with ECTV. However, we did investigate the mechanistic involvement of these requisite cells in mediating survival of ECTV infection.
**Figure 4.8.** ECTV challenge of LysM:cre x iDTR mice depleted once with DT.

LysM:cre x iDTR mice were injected i.p. with 40ng/g DT on days -1, 1, 3, or 5 and infected with 3,000 PFU ECTV on day 0 in the right hind footpad. Control mice were either (a) injected with DT on one day and mock-infected or (b) infected on day 0 with ECTV as above and injected with PBS on one day. Data are combined from two independent experiments. NS= not significant (Day 3 DT + ECTV group compared to DT only group or ECTV only group), *p<0.05 (Day -1 DT + ECTV group compared to DT only group or ECTV only group), ***p<0.001 (Day 1 DT + ECTV group compared to DT only group or ECTV only group and Day 1 DT + ECTV group compared to Day 3 DT + ECTV group), Log-rank test.
Figure 4.8.

- LysMcre x iDTR: DT (9)
- LysMcre x iDTR: ECTV (9)
- LysMcre x iDTR: Day -1DT + ECTV (10)
- LysMcre x iDTR: Day 1 DT + ECTV (10)
- LysMcre x iDTR: Day 3 DT + ECTV (10)
- LysMcre x iDTR: Day 5 DT + ECTV (10)

% Survival vs. Day post-infection

0 2 4 6 8 10 12 14 16

NS

***

*
Figure 4.9. Myeloid cell subsets depleted by DT injection of LysM:cre x iDTR mice.

C57BL/6 or LysM:cre x iDTR mice were infected in the right hind footpad on day 0 with 3,000 PFU ECTV and injected i.p. with 40ng/g DT or PBS at 1 d.p.i. D-LN and spleens were harvested at 2 d.p.i., processed, and stained for flow cytometry. Cells were gated on live cells, singlets, CD45+ cells, and CD19−CD90.2−NK1.1−. Myeloid cell subsets were gated as in Figure 3.5. Data are from one experiment.
Figure 4.9.

A

Draining pLN

B

Spleen

Number of cells/ draining pLN (x 10^4)

Number of cells/ spleen (x 10^6)

- mo-DC
- CD11b+ DC
- pDC
- CD8a+ DC
- granulocyte
- monocyte

Legend:
- C57BL/6: PBS + ECTV (3)
- C57BL/6: DT + ECTV (3)
- LysM:cre x iDTR: PBS + ECTV (3)
- LysM:cre x iDTR: DT + ECTV (3)
Death in the ECTV-infected LysM:cre x iDTR mice depleted of requisite immune cells could have been due to an aberrant, damaging immune response or a lack of viral control in the absence of the requisite immune cells. To investigate the level of viral control in the cell-depleted LysM:cre x iDTR mice, we analyzed viral titers at 5 d.p.i. Viral titers were analyzed at 5 d.p.i. because this is the time-point of peak viral titers in resistant WT mice (Rubio, Xu et al. 2013). There was no difference in the ECTV titers in the infected footpads of depleted LysM:cre x iDTR mice compared to control PBS-treated mice or WT mice (Fig. 4.10A); however, there was a significantly greater titer in the D-LN of depleted LysM:Cre x iDTR mice compared to each of the three control groups (PBS-treated WT mice, DT-treated WT mice, PBS-treated LysM:cre x iDTR mice) (Fig. 4.10B). There were also greater viral titers in the spleens (Fig. 4.10C) and livers (Fig. 4.10D) of cell-depleted mice compared to PBS-treated and WT control mice at 5 d.p.i., although these differences were not significant. Overall, the elevated viral titers in the tissues of cell-depleted LysM:cre x iDTR mice as compared to non-depleted mice indicated that in the absence of requisite immune cells, mice were unable to control ECTV during infection.

Following ECTV infection, virus is recognized by the TLR9/ MyD88 pathway as both TLR9KO mice and MyD88KO mice are susceptible to ECTV infection (Samuelsson, Hausmann et al. 2008) (Sutherland, Ranasinghe et al. 2011) (Rubio, Xu et al. 2013). This viral recognition through TLR9/ MyD88 results in the activation of an effective immune response to ECTV infection, so we investigated the requirement for MyD88 signaling in the requisite cells that are depleted in DT-injected LysM:cre x iDTR
Figure 4.10. Viral titers following ECTV infection of DT-depleted LysM:cre x iDTR mice.

(A, B, C, and D) C57BL/6 and LysM:cre x iDTR mice were injected i.p. with 40ng/g DT or PBS at 1 d.p.i. All mice were infected with 3,000 PFU ECTV in the right hind footpad on day 0. Infected footpads (A), D-LN (B), spleens (C), and livers (D) were harvested at 5 d.p.i., processed, and plated in serial dilutions on cell monolayers for viral titers. Data are combined from two individual experiments.
Figure 4.10.

(A) Infected footpad

(B) Draining pLN

(C) Spleen

(D) Liver

Titer/g infected footpad

Titer/g draining pLN

Titer/g spleen

Titer/g liver

C57BL/6 + PBS
C57BL/6 + DT
LysM:cre x iDTR + PBS
LysM:cre x iDTR + DT
mice during ECTV infection. To knockout MyD88 signaling specifically in these myeloid cells, we used LysM:cre x MyD88-floxed mice. The LysM:cre x MyD88-floxed mice express Cre recombinase under control of the LysM promoter, which results in the removal of the loxP-flanked MyD88 gene only in LysM-expressing cells. LysM:cre x MyD88-floxed mice challenged with ECTV survived infection as did the WT control mice (Fig. 4.11), indicating that although the LysM-expressing cells were required to mediate survival of ECTV infection, their requisite function is not dependent on functional MyD88 signaling within these cells.

Both MaFIA and LysM:cre x iDTR systems were not specific for investigating only the involvement of the monocyte-macrophage lineage in the requisite immune response to ECTV as other myeloid cell subsets were also depleted (Figs. 4.5B, 4.5C, 4.9A, 4.9B). We then utilized anti-CD115 antibody (AFS98) treatment of WT mice to investigate the involvement of tissue-resident macrophages in the immune response to ECTV (Hashimoto, Chow et al. 2013) (Hume and MacDonald 2012) (Hashimoto, Chow et al. 2011). AFS98 has been shown to deplete macrophages in the spleen and LN, deplete circulating monocytes, but not affect the numbers of neutrophils or DC (Hashimoto, Chow et al. 2011). Following injection of the anti-CD115 antibody, there was depletion of macrophages in the spleen, although this depletion was not complete (Fig. 3.6B). We next used the anti-CD115 antibody to deplete the tissue-resident macrophages during ECTV challenge. Both anti-CD115 antibody-treated mice and vehicle-treated mice survived the challenge with ECTV (Fig. 4.12), indicating that tissue-resident macrophages were not required for mediating survival of ECTV infection.
Figure 4.11. ECTV challenge of LysM:cre x MyD88-floxed mice.

C57BL/6 or LysM:cre x MyD88-floxed mice were infected with 3,000 PFU ECTV in the right hind footpad on day 0 and monitored for survival out to 21 d.p.i. Data are combined from two independent experiments.
Figure 4.11.

![Graph showing % survival vs. day post-infection for C57BL/6 (6) and LysM:cre x MyD88-floxed (9) groups.](image-url)
Figure 4.12. ECTV challenge of CD115⁺ cell-depleted mice.

C57BL/6 mice were injected i.p. with 3mg/mouse anti-CD115 depleting antibody (AFS98) or vehicle control every other day starting at day -5 and infected with 3,000 PFU ECTV in the left hind footpad on day 0. Survival was monitored out to 22 d.p.i. Data are combined from two independent experiments.
Figure 4.12.

Experiment by Nikhil J. Parekh, 2015.
**Myeloid cell cytokine production in response to ECTV**

In the effective immune response to ECTV, T1-IFN, specifically IFNα, is produced and required for viral control and mediating survival of ECTV infection (Xu, Rubio et al. 2012) (Rubio, Xu et al. 2013). However, the identities of the cell populations producing IFNα during ECTV infection are not clear. We analyzed the level of IFNα production by myeloid cell subsets, including CD11b+CD11c− cells (likely includes macrophages and granulocytes), CD11b+CD11c+ cells (likely includes CD11b+ DC and mo-DC), and CD11b−CD11c+ cells (likely includes pDC and CD8α+ DC). The CD11b+CD11c− cells, which include macrophages and granulocytes, did not produce much IFNα in response to ECTV (Fig. 4.13). However, the CD11b−CD11c+ cells were the main producers of IFNα at 3 d.p.i. in the D-LN (Fig. 4.13), indicating a potential requisite role for DC subsets as cytokine producers in the immune response to ECTV infection. (The involvement of DC in the immune response to ECTV will be discussed in Chapter V.)
Figure 4.13. IFNα production myeloid cell subsets following ECTV infection.

(A) Mice were infected with 3,000 PFU ECTV in the footpad. At 2 d.p.i., D-LN were harvested, and cells were stained for flow cytometry. Cells were gated on live cells, singlets, NK cell and T cell negative, then as CD11b⁺CD11c⁻ (macrophages and granulocytes), CD11b⁺CD11c⁺ (conventional DC), or CD11b⁻CD11c⁺ (pDC and CD8α⁺ DC). Representative histograms of IFNα levels are shown. The percentage of IFNα⁺ cells was quantified and shown in (B). Data are representative of two independent experiments. Experiment by Dr. Janet J. Sei. Data analyzed by Dr. Chris C. Norbury.
Figure 4.13.

Experiment by Dr. Janet J. Sei. Data analyzed by Dr. Chris C. Norbury.
Discussion

ECTV infection is characterized by systemic spread of virus from a peripheral site of infection in the footpad and results in death of susceptible mice. Phagocytic cells are required for survival of ECTV infection based on depletion by carrageenan or CLL treatment (Tsuru, Kitani et al. 1983) (Karupiah, Buller et al. 1996), but these depletion systems affect multiple subsets of immune cells. During ECTV infection, we found depletion of monocytes and multiple DC subsets by CLL injection, supporting the non-specific depletion of phagocytic myeloid cell subsets by this method, which is similar to the depletion of multiple myeloid cell populations by CLL injection during VACV infection (Davies). Although phagocytes are required for survival of ECTV infection, we did not find requisite roles for granulocytes alone, inflammatory monocytes and mo-DC, or macrophages alone in effective immunity to ECTV infection. However, we did not investigate the requirement for multiple subsets, such as both granulocytes and macrophages, in the immune response to ECTV. The function of one subset could compensate for an absence of another subset, and depletion of multiple cell subsets could be utilized to investigate potential compensation.

Even though neutrophils were previously reported to be necessary for mediating survival of ECTV infection (Tahiliani, Chaudhri et al. 2013), we found that mice depleted of granulocytes (including neutrophils) survived ECTV infection. Neutrophils act as APCs for activation of the T_{CD8+} response to influenza virus infection (Hufford, Richardson et al. 2012), and during VACV infection granulocytes minimize tissue damage and produce T1-IFNs (Fischer, Davies et al. 2011), indicating the potential for an important role for granulocytes during ECTV infection. In our work, we utilized anti-
Ly6G antibody (1A8) for granulocyte depletion, but the previous work by another group used the anti-Gr-1 antibody, RB6-8C5 (Tahiliani, Chaudhri et al. 2013). Anti-Ly6G antibody (1A8) is much more specific for granulocyte depletion than the anti-Gr-1 antibody, RB6-8C5 (Daley, Thomay et al. 2008). In contrast to 1A8, RB6-8C5 not only depletes granulocytes, which express Ly6G, but also depletes subsets of Ly6C⁺Ly6G⁻ cells, including monocytes and circulating lymphocytes (Daley, Thomay et al. 2008). In addition, Ly6C is also expressed on TCD8⁺ (Jaakkola, Merinen et al. 2003), which are required for viral control and survival early after ECTV infection (Karupiah, Buller et al. 1996) (Fang and Sigal 2005). Thus, the depletion of cells expressing Ly6C and/or Ly6G by use of RB6-8C5 with the goal of targeting only granulocytes (Ly6G⁺ cells) confounds the interpretation of data.

To investigate the involvement of macrophages in the protective immune response to ECTV, we used the MaFIA, LysM:cre x iDTR, and anti-CD115 antibody (AFS98) depletion systems. The depletion of CD115⁺ cells in MaFIA mice resulted in susceptibility to ECTV infection, which indicated that these cells are requisite in the immune response to ECTV. However, cells depleted in the MaFIA system have been shown to include not only cells of the monocyte-macrophage lineage, but also DC (Burnett, Kershen et al. 2004). We also found DC depletion in the MaFIA system and depletion of mo-DC, but we saw an increase in granulocytes and monocytes in the spleen 24 h after an injection of depleting drug (AP20187). This increase in granulocytes and monocytes was potentially due to increased emigration of these cells from the bone marrow; however, because of the depletion of multiple subsets of myeloid
cells and the rising cost of AP20187, we did not investigate the depletion, increase in granulocytes, or monocytosis any further.

We then used the LysM:cre x iDTR transgenic mouse system to investigate the involvement of the monocyte-macrophage lineage in the immune response to ECTV. Cells depleted in LysM:cre x iDTR mice were required early during infection to control virus and mediate survival. Similar to carrageenan treatment, CLL injection, and the MaFIA system, the LysM:cre x iDTR system also was not specific to the monocyte-macrophage lineage as we also found depletion of DC subsets during ECTV infection. As previously reported, when GFP is expressed under the LysM promoter, thus marking cells currently expressing LysM as GFP+, then GFP is expressed in granulocytes, monocytic cells, and macrophages as expected (Faust, Varas et al. 2000) (Ye, Iwasaki et al. 2003).

However, in the LysM:cre x iDTR system that we used, whenever Cre recombinase is expressed under the LysM promoter, the loxP-flanked target gene (STOP cassette) is deleted permanently, whether or not LysM continues to be expressed in the cells. Therefore, if LysM is ever expressed in the cell, the loxP-flanked gene is removed, and all progeny cells will lack that gene. When LysM:cre mice are crossed with mice with loxP-flanked target genes, the removal of the target gene occurs in macrophages and granulocytes as well as in some splenic DC (Clausen, Burkhardt et al. 1999). These results support our finding of depletion of multiple cell subsets, including some DC, in LysM:cre x iDTR mice. In addition, the crossing of LysM:cre mice with yellow fluorescent protein (EYFP) reporter mice (LysM:cre x ROSA26 EYFP mice) results in loxP-flanked STOP cassette removal resulting in expression of YFP.
(Ye, Iwasaki et al. 2003). Cells expressing YFP are then currently expressing LysM or have expressed the gene previously to allow for YFP expression (Ye, Iwasaki et al. 2003). As expected in this system, granulocytes, macrophages, and monocytes express YFP; however, some DC, B cells, and T cells also express YFP (Abram, Roberge et al. 2014) (Ye, Iwasaki et al. 2003). Unexpectedly, some hematopoietic stem cells (HSC) also express YFP in this system, and all progeny cells derived from these HSC will also be labeled (Ye, Iwasaki et al. 2003), which could include the DC, B cells, and T cells described above. Currently, the reason for expression of LysM in HSC is unknown. The finding of YFP expression in HSC indicates a potential reason for the expression of YFP in multiple immune cell subsets in the LysM:cre x ROSA26 EYFP mice. The expression of LysM in HSC would also support the results that multiple cell subsets are depleted in LysM:cre x iDTR mice; however, we did not investigate the removal of the loxP-flanked STOP cassette in HSC of LysM:cre x iDTR mice or the expression of iDTR on HSC. Overall, the depletion systems have effects on multiple cell types that need to be considered when utilizing these systems for experiments. Although these effects can limit interpretation of data, the use of a depletion time course, multiple depletion systems, and a broad analysis of cell subsets affected can allow interpretations to be made and direct future experiments.

The depletion of DC subsets that we observed, and the potential for targeting other immune cells in the LysM:cre x iDTR system limit our conclusions on cells of the monocyte-macrophage lineage in this system. However, cells that were depleted in this system are required for survival of ECTV infection. This mechanism of target cell deletion would also affect the LysM:cre x MyD88-floxed mice. MyD88 mediates
signaling through TLRs (except TLR3) and the IL-1R (Janssens and Beyaert 2002, Kawai and Akira 2011). As an adapter molecule, MyD88-mediated signaling includes NF-κB activation for inflammatory responses and signaling via the mitogen-activated protein kinase (MAPK) pathway for activation of the transcription factor AP-1 that regulates cell proliferation and death (Janssens and Beyaert 2002). LysM:cre x MyD88-floxed mice, however, survived ECTV infection, indicating that although MyD88 signaling is required for mediating survival of ECTV infection (Sutherland, Ranasinghe et al. 2011) (Rubio, Xu et al. 2013) and LysM-expressing cells are required, MyD88 signaling is not required in cells that have expressed or are currently expressing LysM. The requisite MyD88 signaling as part of viral recognition and activation of the antiviral response is likely then mediated by cells not targeted in this system, which could include remaining DC. The LysM-expressing cells, however, could still be activated by pathogen and damage sensing pathways distinct from MyD88 signaling. Alternatively, the detection of virus by LysM$^+$ cells could result in cytokine secretion that activates/induces an antiviral state in the LysM-expressing cells that lack MyD88.

Following treatment of mice with anti-CD115 antibody (AFS98), mice survived ECTV infection. We found that injection of AFS98 depleted macrophages in the spleen; however, we did not look at depletion of macrophages in LN or other peripheral tissues, including the skin. This system has been indicated to be more specific for affecting cells of the monocyte-macrophage lineage. Macrophages in the spleen and LN are depleted along with circulating monocytes, but there is no effect on neutrophils or DC (Hashimoto, Chow et al. 2011), unlike in the MaFIA or LysM:cre x iDTR depletion systems. The blockade of CD115 potentially leads to increased circulating CSF-1, and
when AFS98 levels drop, the enhanced levels of CSF-1 result in greater signaling and rebound monocytopoiesis (Hume and MacDonald 2012); however, we did not investigate potential monocytopoiesis in this system. From the results of the AFS98 system, we concluded that tissue-resident macrophages were not required for mediating survival of ECTV infection.

T1-IFN, specifically IFNα, has previously been shown to be an antiviral cytokine required for survival of ECTV infection (Xu, Rubio et al. 2012) (Rubio, Xu et al. 2013), but the cell subsets responsible for production of IFNα in response to ECTV were unclear. We investigated the potential for granulocytes and cells of the monocyte-macrophage lineage to produce IFNα. However, we found that IFNα produced in response to ECTV was produced by some DC subsets (CD11c+CD11b− cells) not by granulocytes and macrophages (CD11c−CD11b+ cells) or by mo-DC and CD11b+ DC (CD11c+CD11b+ cells). The production of the required cytokine IFNα by DC combined with the depletion of DC by CLL injection in ECTV-infected mice, which results in susceptibility to infection (Karupiah, Buller et al. 1996), led us to investigate the involvement of DC in the immune response to ECTV infection (Chapter V). I will speculate on the requisite functions of the phagocytic cells necessary for mediating survival of ECTV infection in the Chapter VI: Overall discussion and future directions.
Chapter V: DC subsets are required for mediating protective immunity to ECTV infection

Introduction

Following skin infection of the footpad of the mouse with ECTV, the natural route of infection, the virus initially spreads to the D-LN. ECTV then spreads systemically via the lympho-hematogenous route and replicates in the spleen and liver (Fenner 1948). Cells of the immune response act quickly to combat and contain the viral infection to mediate survival of resistant mice. Control of the virus and survival of ECTV infection require cytolytic effector functions of the innate immune response by NK cells (Parker, Parker et al. 2007, Fang, Lanier et al. 2008) and the adaptive immune response by T_{CD8+} (Karupiah, Buller et al. 1996) (Fang and Sigal 2005) and T_{CD4+} (Karupiah, Buller et al. 1996, Fang, Siciliano et al. 2012). Phagocytic cells of the innate immune response are also required for controlling the virus and mediating survival of ECTV infection (Tsuru, Kitani et al. 1983) (Karupiah, Buller et al. 1996). Multiple cell populations of the responding innate immune system are phagocytic, including: granulocytes, monocytes, mo-DC, macrophages, and DC subsets. Other than phagocytosis, functions of these cells include antigen presentation for activation of T cell responses and cytokine and chemokine production for antiviral and pro-inflammatory responses and immune cell recruitment. Because several cell subsets are targeted by methods to deplete phagocytes, the identities of phagocytic cells that are required for survival of ECTV infection and the functions of these cells have remained unclear.

Following phagocytic cell depletion by CLL injection during ECTV infection, we found depletion of monocytes, mo-DC, and DC subsets (see Chapter IV; Fig. 4.1), and
others have described macrophage depletion by CLL (Qian, Jutila et al. 1994). Although phagocyte depletion by CLL renders mice susceptible to ECTV infection (Karupiah, Buller et al. 1996), we did not find requisite roles for granulocytes, monocytes, mo-DC, or macrophages in survival of ECTV infection (see Chapter IV). Although depleted by CLL injection, the involvement of DC in mediating survival of ECTV infection had not yet been investigated.

Requisite effector functions utilized by the phagocytic cells during ECTV infection also remained unclear. We assessed antiviral cytokine production, and we found that DC, not monocytes, macrophages, or granulocytes produced IFNα in response to ECTV (see Chapter IV; Fig. 4.12). IFNα is a cytokine required for viral control and effective immunity to ECTV (Xu, Rubio et al. 2012) (Rubio, Xu et al. 2013). In addition to cytokine production, innate immune cells also recruit and activate cells of the adaptive immune response following infection. During ECTV infection, DC, macrophages, and B cells are infected by virus and directly present antigen for activation of the T<sub>CD8+</sub> response (Sei), which is required early after ECTV infection (Karupiah, Buller et al. 1996) (Fang and Sigal 2005). In addition to direct presentation for T<sub>CD8+</sub> activation, a subset of DC, the lymphoid-resident CD8α<sup>+</sup> DC, also internalize exogenous antigen and cross-present this antigen to activate T<sub>CD8+</sub> during ECTV infection (Sei). This ability of DC to activate T<sub>CD8+</sub> during ECTV infection along with the depletion of DC found in ECTV-susceptible CLL-injected mice, and the production of IFNα by DC suggest that DC could have an important role in the immune response to ECTV infection.

To isolate the roles of DC during ECTV infection, we used multiple methods of DC depletion. We found that DC were required early for viral control to mediate survival
of ECTV infection. More specifically, either pDC or CD8α⁺ DC were required to control ECTV for survival of infection. The presence of at least one of the two subsets was necessary for survival of infection. The production of antiviral cytokine by these DC subsets, pDC and CD8α⁺ DC, correlated with survival of ECTV infection. The effector function utilized by pDC and CD8α⁺ DC for viral control was sufficient if either DC subset, pDC or CD8α⁺ DC, was present during ECTV infection.

Results
The involvement of bulk DC in mediating viral control and survival of ECTV infection

To investigate the role of DC in the immune response to ECTV infection, we utilized CD11c:cre x iDTR mice to deplete bulk DC. In CD11c:cre x iDTR mice, Cre recombinase expression is driven by the CD11c promoter (Caton, Smith-Raska et al. 2007). CD11c is an integrin expressed on the cell surface of DC and is used as a marker to identify DC. Following Cre recombinase expression in CD11c⁺ cells, a loxP-flanked STOP cassette is removed allowing expression of the iDTR (Buch, Heppner et al. 2005). CD11c⁺ cells are then targeted for depletion by injection of DT, which binds to the DTR and results in apoptosis of the cell (Buch, Heppner et al. 2005). The dose of DT used was initially titrated for use in the CD11c:cre x iDTR system. The amount of DT was optimized to deplete CD11c⁺ cells in the LN, spleen, and skin with minimal toxicity. CD11c:cre x iDTR mice were injected with DT on three consecutive days then every other day. Mice treated with 50ng/g DT died at day 6, mice treated with 20ng/g DT died at day 15, and mice treated with 10ng/g DT survived to the end of the experiment at 4 weeks (Fig. 5.1A). Using the dose of 20ng/g DT, mice were depleted
of CD11c+ cells in the skin, spleen, and multiple LN (Fig. 5.1B), indicating that we could use 20ng/g DT to deplete CD11c+ cells while avoiding mortality from DT before 10-11 d.p.i. When CD11c:cre x iDTR mice received injections of DT on day -3, -2, -1 then every other day from day -1 to deplete DC during ECTV infection, mice succumbed to infection (Fig. 5.2). Control WT mice and CD11c:cre x iDTR mice treated with DT alone or only infected survived (Fig. 5.2). The death of DC-depleted mice during ECTV infection indicated a requirement for DC in the immune response to ECTV. Because we used injection of DT throughout the challenge, the timeframe for the requirement of DC in response to ECTV infection remained unclear. To investigate the timing of the requirement for DC, we injected DT for DC depletion at day -1, 1, 3, or 5 post-ECTV infection. CD11c:cre x iDTR mice receiving either DT or ECTV infection survived, and mice depleted of DC at day -1, 3, or 5 p.i. had 45%, 50%, and 50% survival of ECTV infection, respectively (Fig. 5.3). Mice depleted of DC at 1 d.p.i. succumbed to ECTV infection (Fig. 5.3), indicating a requirement for DC very early in the immune response to ECTV infection.

Although we found this early requirement for DC in the immune response to ECTV infection, the reason for death in the absence of DC remained unclear. Without the requisite DC, mice could have been dying from an out-of-control, damaging immune response as the DC could have been involved in induction of an effective, not excessive, immune response to ECTV. As another possibility, the requisite DC were potentially involved in control of the virus prior to the development of the adaptive
Figure 5.1. Titration of DT dose in CD11c:cre x iDTR mice

(A) CD11c:cre x iDTR mice were injected i.p. with 10ng/g, 20ng/g, or 50ng/g DT on days 1, 2, 3 and then every other day and monitored for survival. Data are from one experiment with 2 mice/group. (B) CD11c:cre x iDTR mice were injected i.p. with PBS (black bars) or with 20ng/g DT (gray bars) as in (A) and tissues: cervical LN (cLN), popliteal LN (pLN), inguinal LN (iLN), skin, and spleen were harvested, processed, and stained for flow cytometry. Cells were gated on live cells, singlets, and as CD11c\(^+\). The percentage of CD11c\(^+\) cells is shown. Samples for LN and skin were pooled from 3 mice and run as experimental duplicates. Spleen samples were from 3 mice and each run as experimental duplicates. Data are from one experiment (mean +/- Std. dev.). p-values: \(*p<0.05, **p<0.01, ***p<0.001,\) Student’s unpaired t-test. Experiments by Dr. Janet J. Sei.
Figure 5.1.

Experiments by Dr. Janet J. Sei.
Figure 5.2. ECTV challenge of CD11c:cre x iDTR mice depleted by repeated DT injections.

C57BL/6 mice and CD11c:cre x iDTR mice were injected i.p. with 20ng/g DT or PBS on days -3, -2, -1, and every other day from day -1. On day 0, mice were infected with 3,000 PFU ECTV in the left hind footpad or mock-infected. Survival was monitored out to 11 d.p.i. Data are combined from 4 independent experiments. NS= not significant (CD11c:cre x iDTR: DT group compared to C57BL/6:DT group or C57BL/6:ECTV group), ***p<0.001 (CD11c:cre x iDTR:DT + ECTV group compared to CD11c:cre x iDTR:DT group, C57BL/6:DT group, C57BL/6:ECTV group, or CD11c:cre x iDTR:ECTV group), Log-rank test. One experiment was done by Dr. Janet J. Sei.
Figure 5.2.

- C57BL/6: DT (9)
- C57BL/6: ECTV (9)
- C57BL/6: DT + ECTV (12)
- CD11c:cre x iDTR: DT (8)
- CD11c:cre x iDTR: ECTV (9)
- CD11c:cre x iDTR: DT + ECTV (12)

% Survival vs. Day post-infection

*** NS
Figure 5.3. ECTV challenge of CD11c:cre x iDTR mice depleted at single timepoints.

CD11c:cre x iDTR mice were either (a) injected with DT i.p. on day 1 and mock-infected on day 0, (b) injected with PBS i.p. at 1 d.p.i. and infected with 3,000 PFU ECTV in the left hind footpad on day 0, or (c) infected with 3,000 PFU ECTV in the left hind footpad on day 0 and injected with DT i.p. at -1, 1, 3, or 5 d.p.i. Survival was monitored out to 15 d.p.i. Data are combined from four independent experiments, except data for DT injections at 3 or 5 d.p.i. are combined from two independent experiments. *p<0.05 (DT group compared to -1 DT + ECTV group, 3 DT + ECTV group, or 5 DT + ECTV group), ***p<0.001 (DT group compared to 1 DT + ECTV group). Not shown: p<0.05 (1 DT + ECTV group compared to -1 DT + ECTV group or 5 DT + ECTV group), not significant (1 DT + ECTV group compared to 3 DT + ECTV group), Log-rank test.
Figure 5.3.
immune response, and mice could have been dying from a lack of viral control. To investigate viral control in the absence of requisite DC, mice were infected with ECTV on day 0 and depleted of DC at 1 d.p.i., the timepoint that resulted in death (Fig. 5.3), and virus was titered from tissues at 5 d.p.i. As the virus spreads from the site of infection (footpad) to the D-LN and then systemically to the spleen and liver, we harvested these tissues for titering. There was no difference in the viral titers in the infected footpads and D-LN of DC-depleted mice compared to non-depleted mice (Fig. 5.4A, B). Systemically, there was an increase in viral titers in the livers of DC-depleted mice compared to non-depleted mice (Fig. 5.4C), although this difference was not significant; however, there was a significantly greater (~ 2 log) viral titer in the spleens of DC-depleted mice compared to non-depleted mice (Fig. 5.4D).

The greater viral titers found in the livers and spleens in the absence of requisite DC suggested that these DC were involved in mediating control of ECTV. If DC-depleted mice were dying from uncontrolled viral replication and spread, control of the virus should rescue these mice following ECTV infection. To pharmacologically control ECTV, we used cidofovir, a nucleotide analog that also inhibits the viral DNA polymerase (Smee 2013) and has antiviral activity against ECTV (Fang, Siciliano et al. 2012, Israely, Paran et al. 2012). As we previously found, DC-depleted mice died from ECTV infection, but when treated with cidofovir at 3 and 6 d.p.i. to control the virus, DC-depleted mice survived ECTV infection (Fig. 5.5). The ability of cidofovir to rescue DC-depleted mice indicated that the DC-depleted mice were dying from ECTV infection due to an inability to control the virus. However, whether the enhanced replication of virus

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**Figure 5.4.** Viral titers following ECTV infection of DT-depleted CD11c:cre x iDTR mice.

(A, B, C, and D) C57BL/6 or CD11c:cre x iDTR mice were injected i.p. with 20ng/g DT or PBS at 1 d.p.i. All mice were infected with 3,000 PFU ECTV in the left hind footpad on day 0. Infected footpads (A), D-LN (B), livers (C), and spleens (D) were harvested at 5 d.p.i., processed, and plated in serial dilutions on cell monolayers for viral titers. Data are combined from two individual experiments. Data points represent individual mice. *p<0.05, Student’s unpaired t-test.*
Figure 5.4.
Figure 5.5. Cidofovir treatment of DT-depleted CD11c:cre x iDTR mice following ECTV infection.

CD11c:cre x iDTR mice were infected with 3,000 PFU ECTV in the left hind footpad and injected i.p. with DT at 1 d.p.i. Mice were injected with either 100mg/kg cidofovir or PBS i.p. on both 3 and 6 d.p.i. Survival was monitored to 21 d.p.i. Data are combined from two independent experiments. ***p<0.001, Log-rank test.
Figure 5.5.

- DT + ECTV + PBS (N=8)
- DT + ECTV + cidofovir (N=8)

% Survival vs. Day post-infection

***
or a stronger immune response to virus due to enhanced replication ultimately mediated susceptibility of DC-depleted mice remains unclear.

The death of bulk CD11c⁺-depleted mice from ECTV infection implicated a general requirement for DC in the response to ECTV, but roles for specific DC subsets were unclear. We next investigated which specific subsets of DC were depleted in the CD11c:cre x iDTR system as effects observed within DT-mediated CD11c⁺ cell depletion systems can differ based on the specific transgenic mouse strains used (Caton, Smith-Raska et al. 2007, Tittel, Heuser et al. 2012, Abram, Roberge et al. 2014). Additionally, DT-injection during ECTV infection has the potential to have a cell depletion profile that differs from depletion in steady-state due to changes in cell numbers and trafficking during infection (Tamoutounour, Guilliams et al. 2013) (Leon, Lopez-Bravo et al. 2007). Mice were injected with DT for DC depletion at 1 d.p.i., and at 2 d.p.i., we found depletion of pDC and CD8α⁺ DC, but not CD11b⁺ DC in both the D-LN and spleens in comparison to non-depleted mice (Fig. 5.6A, B). These results indicated that during ECTV infection, DT injection in these CD11c:cre x iDTR mice targeted both pDC and a lymphoid-resident DC subset, CD8α⁺ DC, but CD11b⁺ DC numbers were not affected.

Cytokine production by DC subsets in response to ECTV

Although we found that bulk CD11c⁺ cells were required for controlling the virus to mediate survival of ECTV infection, the effector functions utilized by these DC to control the virus remained unclear. We found that DC (CD11c⁺CD11b⁻ cells), not granulocytes, monocytes, or macrophages (CD11c⁻CD11b⁺) or mo-DC or CD11b⁺ DC
Figure 5.6. DC subsets depleted by DT injection of CD11c:cre x iDTR mice.

(A and B) C57BL/6 or CD11c:cre x iDTR mice were infected with 3,000 PFU ECTV in the left hind footpad on day 0 and injected with 20ng/g DT or PBS i.p. at 1 d.p.i. D-LN (A) or spleens (B) were harvested at 2 d.p.i., processed, and cells were stained for flow cytometry. Cells were gated as live cells, singlets, CD45⁺, CD19⁻CD90.2⁻NK1.1⁻. DC subsets were identified as follows: CD11b⁺ DC (CD11c⁺CD11b⁺CD8α⁻CD64⁻), pDC (CD11c⁺CD11b⁻B220⁺), and CD8α⁺ DC (CD11c⁺CD11b⁻B220⁻CD8α⁺). Data are representative of two independent experiments (mean +/- SEM). *p<0.05, **p<0.01, ***p<0.001, Student's unpaired t-test.
Figure 5.6.
(CD11c⁺CD11b⁺) produced IFNα in response to ECTV (Fig. 4.12). The T1-IFN response is required for survival of ECTV infection as mice lacking T1-IFN signaling die from ECTV infection (Rubio, Xu et al. 2013). ECTV encodes a T1-IFNbp that inhibits the T1-IFN response by binding IFNα, not IFNβ (Xu, Rubio et al. 2012). Blocking the function of the T1-IFNbp rescues susceptible mice following ECTV infection (Xu, Rubio et al. 2012), indicating the importance of the IFNα response.

To investigate the CD11c⁺ subsets producing IFNα, WT mice were treated with Flt3L to enhance development of DC, and splenocytes from these mice were then harvested and sorted for DC. These DC were left untreated (naïve), treated with live WT ECTV (WT ECTV), or treated with replication-incompetent UVC-inactivated WT ECTV (UVC ECTV) (Fischer, Tscharke et al. 2007). UVC/psoralen treatment induces DNA damage in the virus that inhibits the expression of viral genes (Fischer, Tscharke et al. 2007). The inhibition of viral gene expression, including the expression of host-response modifier proteins that are produced to evade the host’s immune response, could result in altered recognition of and response to viral infection. In response to both WT ECTV and UVC ECTV, pDC and CD8α⁺ DC produced IFNα, although production by CD8α⁺ DC was not as great in response to UVC ECTV compared to live WT ECTV (Fig. 5.7). In contrast, CD11b⁺ DC only produced IFNα above background in response to UVC ECTV, not live WT ECTV (Fig. 5.7). The pDC and CD8α⁺ DC, not CD11b⁺ DC, were producers of IFNα, a required cytokine (Xu, Rubio et al. 2012) (Rubio, Xu et al. 2013), in response to live WT ECTV, and these two DC subsets were depleted in ECTV-susceptible DT-injected CD11c:cre x iDTR mice, indicating a potential requisite role for one or both of these DC subsets in mediating survival of ECTV infection. The
**Figure 5.7.** IFNα production by DC subsets following treatment with ECTV.

Spleens were harvested from C57BL/6 mice at 10-14 days after s.c. injection of B16-Fit3L tumor cells. Splenocytes were processed, and DC were sorted on an AutoMACS using panDC microbeads. DC remained untreated (naïve) or were treated with live WT ECTV (WT ECTV) or UVC-inactivated WT ECTV (UVC ECTV) at an MOI=10 for 6h. Cells were stained for flow cytometry and gated for pDC (B220⁺) then CD8α⁺ DC (CD8α⁺CD11b⁻) and CD11b⁺ DC (CD11b⁺CD8α⁻) that were positive for IFNα production. Data are representative of four independent experiments with experimental duplicates (mean +/- SEM). **p<0.01, ***p<0.001, Student’s unpaired t-test. Experiments by Dr. Janet J. Sei. Data analyzed by Dr. Chris C. Norbury.
Experiments by Dr. Janet J. Sei. Data analyzed by Dr. Chris C. Norbury.
CD8α⁺ DC is a lymphoid-resident DC subset that presents antigen for activation of the T<sub>CD8⁺</sub> response. In addition to direct antigen presentation, the CD8α⁺ DC internalize exogenous antigen for cross-presentation to activate T<sub>CD8⁺</sub> (Sei, Hildner, Edelson et al. 2008). Although also able to present antigen, pDC are major cytokine producing cells, especially T1-IFN, and pDC also produce pro-inflammatory cytokines (Asselin-Paturel, Boonstra et al. 2001, Takagi, Fukaya et al. 2011). CD11b⁺ DC carry antigen from the site of infection to present to T cells in the D-LN for induction of T cell responses (Nizza and Campbell 2014, Seneschal, Jiang et al. 2014).

**The involvement of DC subsets in viral control and survival of ECTV infection**

Initially, we found a requirement for bulk DC in the response to ECTV infection using the CD11c:cre x iDTR depletion system. Although this system targets DC, other cells are also depleted. Caton et. al. and Abram et. al. crossed CD11c:cre mice with EYFP reporter mice, in which a loxP-flanked STOP cassette prevents expression of YFP until removed by Cre recombinase (Caton, Smith-Raska et al. 2007, Abram, Roberge et al. 2014). In this system, YFP is expressed in DC in the spleen, but some monocyte/macrophage populations and a small percentage of NK cells, T cells, and B cells express YFP (Caton, Smith-Raska et al. 2007, Abram, Roberge et al. 2014). Additionally, depletion of CD11c-expressing T<sub>CD8⁺</sub>, which are activated T<sub>CD8⁺</sub>, occurs in a CD11c-DTR transgenic mouse depletion system (Jung, Unutmaz et al. 2002).

NK cells, T cells, and B cells are all required for survival of ECTV infection although T<sub>CD4⁺</sub> and B cells are required later after infection than NK cells and T<sub>CD8⁺</sub> (Karupiah, Buller et al. 1996, Parker, Parker et al. 2007, Fang, Lanier et al. 2008) (Fang
and Sigal 2005) (Karupiah, Buller et al. 1996, Fang, Siciliano et al. 2012). NK cells are required prior to day 6 p.i., and mice lacking the $T_{CD8^+}$ response die from ECTV infection at approximately 8-10 d.p.i.; whereas, mice lacking the $T_{CD4^+}$ response die from ECTV infection at approximately 20 d.p.i., and without the B cell response, mice die after 40 d.p.i. (Karupiah, Buller et al. 1996, Parker, Parker et al. 2007, Fang, Lanier et al. 2008) (Fang and Sigal 2005) (Karupiah, Buller et al. 1996, Fang, Siciliano et al. 2012).

Depletion of some of these requisite immune cells, specifically the cells required early, NK cells or $T_{CD8^+}$, could have been contributing to death from ECTV in the CD11c:cre x iDTR depletion system. In some systems of DT depletion of CD11c$^+$ cells, neutrophilia and monocytosis also occur (Tittel, Heuser et al. 2012) further confounding interpretation of bulk CD11c$^+$ depletion data, which led us to investigate the role of DC using more specific DC subset depletion systems, Batf3KO mice and antibody-based pDC depletion.

To isolate the roles of CD8$\alpha^+$ DC and pDC in the immune response to ECTV, we utilized Batf3KO mice and depletion of pDC by antibody-based methods. Batf3KO mice lack a transcription factor necessary for the development of a lymphoid-resident DC subset, CD8$\alpha^+$ DC (Hildner, Edelson et al. 2008). Batf3KO mice challenged with ECTV had greater than 80% survival in multiple experiments, and WT mice had 100% survival of ECTV infection (Fig. 5.8A), indicating that CD8$\alpha^+$ DC were not required for survival of ECTV infection. CD8$\alpha^+$ DC were not necessary for survival of ECTV infection, but pDC were also depleted in ECTV-susceptible DT-injected CD11c:cre x iDTR mice and produced IFN$\alpha$ in response to ECTV and thus could have a requisite role in response to ECTV. We investigated the involvement of pDC in the response to ECTV infection by
**Figure 5.8.** ECTV challenge of Batf3KO mice, pDC-depleted mice, and pDC-depleted Batf3KO mice.

(A) C57BL/6 or Batf3KO mice were either mock-infected or infected with 3,000 PFU ECTV in the right hind footpad on day 0. Survival was monitored out to 21 d.p.i. Data are pooled from three independent experiments. NS=not significant (Batf3KO:ECTV group compared to each control group), Log-rank test.  

(B) C57BL/6 mice were injected with pDC-depleting antibody (BX444) or isotype control antibody (HRPN) i.p. on days -2 and -1 then every third day from day -1 and either mock-infected or infected with 3,000 PFU ECTV in the left hind footpad on day 0. Data are pooled from three independent experiments with one of these experiments done by Dr. Janet J. Sei. NS= not significant (anti-pDC Ab + ECTV group compared to each control group), Log-rank test.  

(C) Batf3KO mice were injected with pDC-depleting antibody or control antibody as in (B) and either mock-infected or infected with ECTV as in (B). Survival was monitored out to 14 d.p.i. Data are pooled from three independent experiments. ***p<0.001 (anti-pDC Ab + ECTV group compared to each control group), Log-rank test.
Figure 5.8.

A

- C57BL/6: mock (N=15)
- C57BL/6: ECTV (N=15)
- Batf3KO: mock (N=14)
- Batf3KO: ECTV (N=15)

% Survival vs. Day post-infection

B

- C57BL/6: control Ab + ECTV (N=11)
- C57BL/6: anti-pDC Ab + mock (N=10)
- C57BL/6: anti-pDC Ab + ECTV (N=11)

C

- Batf3KO: anti-pDC Ab + mock (N=9)
- Batf3KO: control Ab + ECTV (N=12)
- Batf3KO: anti-pDC Ab + ECTV (N=12)

% Survival vs. Day post-infection

NS

***
depleting pDC in WT mice using injection of anti-PDCA-1 antibody (BX444). Following depletion of pDC, mice had greater than 80% survival of ECTV infection (Fig. 5.8B). In addition, mice receiving control antibody and mock-infected mice also survived out to the end of the experiment (Fig. 5.8B). The survival of pDC-depleted mice following ECTV infection indicated that, along with CD8α+ DC alone, pDC alone were also not required for survival of ECTV infection. We isolated the involvement of pDC alone and CD8α+ DC alone and found that neither subset is required for survival of ECTV infection; however, potentially the presence of at least one of the subsets, pDC or CD8α+ DC, is sufficient for mediating survival of ECTV infection.

To investigate whether the presence of at least one DC subset, pDC or CD8α+ DC, is sufficient for mediating survival of ECTV infection, we depleted pDC in Batf3KO mice for an absence/depletion of both pDC and CD8α+ DC. Batf3KO mice receiving pDC-depleting antibody only or ECTV infection only survived; however, 100% of the pDC-depleted Batf3KO mice died early following ECTV infection (Fig. 5.8C). These results indicated that the presence of at least one DC population, either pDC or CD8α+ DC, is required for survival of ECTV infection as without both pDC and CD8α+ DC, mice die from ECTV infection.

Because CD8α+ DC have been shown to develop during parasite infection in Batf3KO mice by a Batf3-independent pathway (Tussiwand, Lee et al. 2012), we investigated the level of CD8α+ DC in Batf3KO mice and the efficacy of pDC depletion during ECTV infection in these mice. In the spleen at 3 d.p.i., CD8α+ DC were reduced in the Batf3KO mice, whether injected with anti-PDCA1 or control antibody, compared to WT mice (Fig. 5.9). Injection of anti-PDCA1 antibody resulted in depletion of pDC in
Figure 5.9. Depletion of pDC and CD8α⁺ DC in pDC-depleted Batf3KO mice.

C57BL/6 or Batf3KO mice were injected with pDC-depleting antibody (BX444) or isotype control antibody (HRPN) i.p. on days -2 and -1 then every third day from day -1 and either mock-infected or infected with 3,000 PFU ECTV in the left hind footpad on day 0. Spleens were harvested at 3 d.p.i., and cells were stained for flow cytometry. Cells were gated as live cells, singlets, CD45⁺, CD19⁻CD90.2⁻NK1.1⁻. DC subsets were identified as follows: pDC (CD11c⁺CD11b⁻B220⁺) and CD8α⁺ DC (CD11c⁺CD11b⁻B220⁻ CD8α⁺). Data are representative of two independent experiments (mean +/- SEM).
*p<0.05, **p<0.01, ***p<0.001, Student’s unpaired t-test.
Figure 5.9.
WT and Batf3KO mice compared to isotype antibody-injected control mice (Fig. 5.9). These DC subset numbers confirmed the reduction of pDC or CD8α⁺ DC in these depletion systems.

Mice with ablation of both pDC and CD8α⁺ DC, the pDC-depleted Batf3KO mice, died from ECTV infection; however, the reason for the susceptibility of these mice was unclear. With the depletion/absence of both pDC and CD8α⁺ DC, mice could have been dying from ECTV infection due to a harmful, aberrant immune response if pDC and CD8α⁺ DC were necessary for controlling the immune response. Death mediated by excessive immunopathology would be indicated by a lack of an increase in ECTV titers of susceptible pDC-depleted Batf3KO mice compared to resistant mice. Other than control of the immune response, pDC and CD8α⁺ DC could have been controlling ECTV to prevent death of mice due to an overwhelming viral load. We investigated the ability of susceptible pDC-depleted Batf3KO mice to control ECTV by titering virus from multiple tissues at 5 d.p.i. There was no difference in viral titer in the infected footpad of pDC-depleted Batf3KO mice compared to mice lacking CD8α⁺ DC alone, mice lacking pDC alone, or WT mice (Fig. 5.10A). In D-LN, there was a greater viral titer in Batf3KO mice, whether injected with anti-PDCA1 or control antibody, compared to WT mice (Fig. 5.10B). However, there was no difference in the viral titer in D-LN of susceptible pDC-depleted Batf3KO mice compared to resistant Batf3KO mice (Fig. 5.10B), indicating that the increased viral titer in the D-LN of pDC-depleted Batf3KO mice does not represent an inability to control ECTV. The viral titers in the spleen of pDC-depleted Batf3KO mice were elevated compared to the other three groups of resistant mice: WT, pDC depletion alone, Batf3KO mice (Fig. 5.10C), which indicated an inability to control
Figure 5.10. Viral titers and cidofovir treatment in pDC-depleted Batf3KO mice.

(A, B, and C) C57BL/6 or Batf3KO mice were injected with anti-PDCA1 antibody (BX444) or control antibody (HRPN) i.p. on days -2 and -1 then every third day from day -1 and infected on day 0 with 3,000 PFU ECTV in the left hind footpad. Infected footpads (A), D-LN (B), and spleens (C) were harvested at 5 d.p.i. Tissues were then processed and plated in serial dilution on cell monolayers for viral titers. Data are combined form two independent experiments. *p<0.05, **p<0.01, ***p<0.001, Student’s unpaired t-test. (D) Batf3KO mice were injected with anti-PDCA1 antibody and infected with 3,000 PFU ECTV in the left hind footpad. Mice received 100mg/kg cidofovir or PBS i.p. at 3 and 6 d.p.i. Survival was monitored to 17 d.p.i. Data are combined from two independent experiments. ***p<0.001, Log-rank test.
Figure 5.10.

A

C57BL/6: Control Ab
C57BL/6: anti-pDC Ab
Batf3KO: Control Ab
Batf3KO: anti-pDC Ab

Titer/g

Infected Footpad

B

C57BL/6: Control Ab
C57BL/6: anti-pDC Ab
Batf3KO: Control Ab
Batf3KO: anti-pDC Ab

Titer/draining pLN

C

C57BL/6: Control Ab
C57BL/6: anti-pDC Ab
Batf3KO: Control Ab
Batf3KO: anti-pDC Ab

Titer/g

Spleen

D

Batf3KO: anti-pDC Ab + ECTV + PBS (N=6)
Batf3KO: anti-pDC Ab + ECTV + cidofovir (N=6)

% Survival

Day post-infection
the virus in mice lacking both pDC and CD8α+ DC. To investigate whether this increased viral load in the pDC-depleted Batf3KO mice was mediating death of the mice, we again used cidofovir to pharmacologically control ECTV. Similar to our previous results, pDC-depleted Batf3KO mice died from ECTV infection, but treatment with cidofovir at 3 and 6 d.p.i. rescued ECTV-infected pDC-depleted Batf3KO mice (Fig. 5.10D), supporting that mice lacking both pDC and CD8α+ DC were dying due to uncontrolled viral replication. However, whether the death from the inability to control the virus is mediated by damage from enhanced virus replication or from a stronger immune response to the greater viral load is unclear.

Mice with depleted/absent pDC and CD8α+ DC died from an inability to control ECTV, but the mechanism(s) utilized by the pDC and CD8α+ DC to control the virus remained unclear. Previously, we found that pDC and CD8α+ DC were the DC subsets producing IFNα, a required antiviral cytokine (Xu, Rubio et al. 2012) (Rubio, Xu et al. 2013), in response to ECTV (Fig. 5.7), indicating a potential method to control virus. However, during viral infection, DC are also involved in recruitment and activation of lymphocytes. Following infection with a virus, pDC are important for NK cell and T_{CD8+} recruitment (Swiecki, Gilfillan et al. 2010), and both NK cells and T_{CD8+} are required for viral control and survival of ECTV infection (Karupiah, Buller et al. 1996, Parker, Parker et al. 2007, Fang, Lanier et al. 2008) (Fang and Sigal 2005). Additionally, DC are infected and present antigen for activation of T_{CD8+} during ECTV infection (Sei). PDC can also present antigen to T_{CD4+} (Sapoznikov, Fischer et al. 2007), which is another cell of the adaptive immune response required for survival of ECTV infection.
When we investigated numbers of total NK cells and of NK cell subsets: CD62L⁺, CD69⁺ (recently activated), immature, intermediate, and mature in the spleen at 4 d.p.i., we found that pDC depletion, whether in WT or Batf3KO mice slightly reduced numbers of total NK cells and NK subsets (Fig. 5.11A). However, there was no significant difference between NK cell numbers in pDC-depleted Batf3KO mice compared to NK cell numbers in each of the control groups of resistant mice: WT, pDC-depleted, Batf3KO (Fig. 5.11A), indicating that a deficiency of NK cells was likely not playing a role in the susceptibility of pDC-depleted Batf3KO mice to ECTV infection. Next, we investigated the T\textsubscript{CD4⁺} response to ECTV infection in pDC-depleted Batf3KO mice. There were no differences in the numbers of total, naïve, effector, memory, or CD69⁺ T\textsubscript{CD4⁺} in pDC-depleted Batf3KO mice compared to numbers of T\textsubscript{CD4⁺} in all groups of resistant control mice (Fig. 5.11B). Additionally, although there was a slight reduction in total T\textsubscript{CD8⁺} numbers in pDC-depleted Batf3KO, this difference was not significant compared to the total T\textsubscript{CD8⁺} numbers in all of the groups of resistant control mice (Fig. 5.11C). There were no differences in numbers of T\textsubscript{CD8⁺} subsets: naïve, effector, memory, CD69⁺ in pDC-depleted Batf3KO compared to all of the resistant control mice (Fig. 5.11C), indicating that a T\textsubscript{CD8⁺} deficiency was not likely involved in the susceptibility of pDC-depleted Batf3KO mice to ECTV infection. Overall, a deficiency in the requisite lymphocyte response to ECTV infection is likely not mediating the lack of viral control and, ultimately, the death of mice lacking both pDC and CD8α⁺ DC.
Figure 5.11. NK cells, T_{CD4+}, and T_{CD8+} in pDC-depleted Batf3KO mice following ECTV challenge.

(A, B, and C) C57BL/6 or Batf3KO mice were injected with pDC-depleting antibody (BX444) or control antibody (HRPN) on days -2 and -1 then every third day from day -1 and infected with 3,000 PFU ECTV in the left hind footpad on day 0. At 4 d.p.i., spleens were harvested, processed, and stained for flow cytometry. Cells were gated on live cells, singlets, and CD45^+. For NK cell subsets (A), cells were gated as CD3e^-NK1.1^+ (Total) then CD62L^+, CD69^+, CD11b^-CD27^+ (immature), CD11b^+CD27^+ (intermediate), or CD11b^+CD27^- (mature). For T_{CD4+} subsets (B), cells were gated on CD3e^-NK1.1^-CD4^+CD8^α^- (Total) then on CD44^{lo}CD62L^{hi} (Naïve), CD44^{hi}CD62L^{lo} (Effector), CD44^{hi}CD62L^{hi} (Memory), or CD69^+. For T_{CD8+} subsets (C), cells were gated on CD3e^-NK1.1^-CD4^-CD8^α^+ (Total) then on CD44^{lo}CD62L^{hi} (Naïve), CD44^{hi}CD62L^{lo} (Effector), CD44^{hi}CD62L^{hi} (Memory), or CD69^+. Data are representative of two independent experiments (N= 2 or 3) with experimental duplicates. Student's unpaired t-test was used to compare cells of the pDC-depleted Batf3KO group to either cells of the pDC-depleted C57BL/6 group or cells of the control antibody Batf3KO group. Statistics would have been shown only if both of these comparisons had a significant p value (p<0.05).
Figure 5.11.

A

B

C

NK cell subsets

CD4+ T cell subsets

CD8+ T cell subsets

Number of NK cells/ spleen (x10^6)

Number of CD4+ T cells/ spleen (x10^7)

Number of CD8+ T cells/ spleen (x10^7)
Discussion

Following footpad infection with ECTV, the virus rapidly begins replicating and spreading. Although deployed in response to ECTV, granulocytes, monocytes, mo-DC, and macrophages are each not required for mediating survival of ECTV infection. Instead, we found an early requisite role for DC in controlling the virus to mediate survival of ECTV infection. Specifically, the presence of at least one of two DC subsets, either pDC or CD8α⁺ DC, was required for survival of infection. Mice with an absence/depletion of either pDC or CD8α⁺ DC survived ECTV infection, but when both pDC and CD8α⁺ DC were lacking, mice succumbed to ECTV infection. These DC subsets, pDC and CD8α⁺ DC, produced IFNα, a required cytokine in response to ECTV infection (Xu, Rubio et al. 2012) (Rubio, Xu et al. 2013). Requisite cytokine production to control ECTV was likely mediating survival of infection.

Previously, another group found an early requisite role for only pDC in mediating survival of ECTV infection (Tahiliani, Chaudhri et al. 2013), which is not consistent with our results of pDC-depleted mice surviving ECTV infection in multiple experiments. In our experiments, we titrated the dose of anti-PDCA1 antibody effective for depletion as 0.5mg/mouse. At doses greater than 0.5mg/mouse, we did not see improvement in the level of depletion (Norbury lab, data not shown). Our dosing schedule was also optimized to give antibody injections every third day throughout the experiment. Tahiliani et. al. instead used injections of a larger dose more frequently, 1mg/mouse every other day (Tahiliani, Chaudhri et al. 2013), which could have had more detrimental effects from increased handling and stressing of the mice than our dosing regimen. Additionally, a cutoff of 25% weight loss during ECTV challenge was used by
Tahiliani et. al. as the cutoff for euthanizing the mice and marking them as dead in the experiment (Tahiliani, Chaudhri et al. 2013); however, weight loss is a poor marker of disease severity during ECTV infection and is not predictive of susceptibility to ECTV infection (Parker, Schriewer et al. 2008). Mice infected with ECTV, whether ultimately resistant or susceptible, lose weight early after infection (Parker, Schriewer et al. 2008). While certain susceptible mice die at 8-10 d.p.i., resistant mice begin regaining weight at this timepoint following the initial weight loss after ECTV infection (Parker, Schriewer et al. 2008). Weight loss is a common symptom of systemic infection and could be caused by loss of appetite due to the immune response including pro-inflammatory cytokines of the immune response (Kamperschroer and Quinn 2002), although mechanisms underlying weight loss are unclear.

For functions of the DC subsets, we found that pDC and CD8α⁺ DC produced IFNα in response to live WT ECTV and UVC ECTV. In contrast, CD11b⁺ DC produced IFNα in response to UVC ECTV, indicating the ability of CD11b⁺ DC to produce IFNα, but did not produce IFNα above background in response to live WT ECTV. UVC/psoralen treatment of virus results in DNA damage that inhibits the expression of viral genes, such as the host-response modifiers (HRM) (Fischer, Tscharke et al. 2007). Potentially, the production of cytokine by CD11b⁺ DC is inhibited by live WT ECTV due to a HRM produced by the virus, and this mechanism of inhibition is inactivated by UVC/psoralen treatment of virus. Orthopoxviruses encode many host-response modifiers, including proteins that bind cytokines and chemokines, including the T1-IFNbp, and signaling inhibitors, such as inhibitors of the TIR signaling domain which is present in TLRs, IL-1R, and MyD88 (Bowie, Kiss-Toth et al. 2000, Chen, Buller et al.
TLR9/MyD88 signaling is required in the recognition of ECTV infection as both TLR9 and MyD88 are required for survival of ECTV infection (Samuelsson, Hausmann et al. 2008) (Sutherland, Ranasinghe et al. 2011) (Rubio, Xu et al. 2013). Signaling through MyD88 ultimately results in an antiviral response, including the production T1-IFNs and pro-inflammatory cytokines. Multiple HRM produced by ECTV, including those that block signaling and others that bind cytokines, could be blocking T1-IFN production by CD11b+ DC. The CD11b+ DC could potentially sense viral infection early after infection prior to the production of the HRM.

To investigate the response of CD11b+ DC, we could treat isolated DC with live WT ECTV or UVC ECTV and measure IFNα by flow cytometry over a time course to determine the timeframe of IFNα production. Additionally, we have multiple knockout viruses that lack genes encoding specific HRM. We can treat DC subsets with these knockout viruses and compare the cytokine production to the cytokine levels following treatment with live WT ECTV or UVC ECTV. Production by CD11b+ DC in response to ECTV lacking a specific host-response modifier would indicate the involvement of that HRM in inhibiting the production of IFNα.

There was no deficiency in the cell numbers of NK cells, TCD4+, or TCD8+ in the spleens of mice lacking both pDC and CD8α+ DC compared to resistant control mice, which indicated that the lack of these two DC subsets did not affect these requisite NK and T cell responses (Karupiah, Buller et al. 1996) (Fang and Sigal 2005) (Karupiah, Buller et al. 1996, Parker, Parker et al. 2007, Fang, Lanier et al. 2008, Fang, Siciliano et al. 2012). A defect in the TCD4+ response is also not likely involved in mediating the death of mice lacking pDC and CD8α+ DC as pDC-depleted Batf3KO mice die from
ECTV infection prior to 10 d.p.i., but mice lacking an effective $T_{CD4^+}$ response die later after infection (~20 d.p.i.) (Karupiah, Buller et al. 1996, Fang, Siciliano et al. 2012). A deficient $T_{CD8^+}$ response is also not likely involved in the susceptibility of ECTV-infected pDC-depleted Batf3KO mice as during ECTV infection, multiple immune cells, including DC, macrophages, and B cells, are infected by ECTV and present antigen for activation of $T_{CD8^+}$ (Sei), which supports a redundancy in direct antigen presentation for effective $T_{CD8^+}$ activation. Additionally, in the absence of CD8α+ DC, a DC specialized in cross-presentation of antigen, there is no defect in $T_{CD8^+}$ proliferation or the $T_{CD8^+}$ response to immunodominant peptide, supporting that antigen presentation by cell subsets other than CD8α+ DC is effective for induction of the $T_{CD8^+}$ response.

To our knowledge, these are the first findings of redundancy in the functions of two DC subsets, pDC and CD8α+ DC, that is required for survival of a peripheral viral infection. The requisite function of these DC subsets is to ultimately control ECTV to survive infection. In the absence of one subset or the other, either pDC or CD8α+ DC, the remaining DC subset is able to sufficiently control the virus for survival of ECTV infection. This is an example of a built-in backup for the immune system. If some of these DC, pDC and CD8α+ DC, are dysfunctional or infected and killed, either by the virus or by the cytolytic immune response, an effective level of function still remains to control the virus. These results also highlight the importance of early viral control for mediating survival of viral infection prior to the development of an effective adaptive immune response, which has implications for the development of therapeutics.

These DC can potentially be targeted for activation of their antiviral functions during viral infection, or pharmacological agents can be developed to mimic the virus-
controlling functions of these cells. As we found with the use of cidofovir treatment of pDC-depleted Batf3KO mice, early control of virus resulted in survival of ECTV-susceptible mice. This viral control likely held back the virus long enough for the development of a protective adaptive immune response. However, the development of the adaptive immune response in these cidofovir-treated mice would need to be analyzed to confirm that pharmacological control of the virus allowed time for the adaptive immune cells to respond.

Although our data analysis of cytokine production and the depletion of both pDC and CD8α+ DC in ECTV-susceptible CD11c−-depleted mice led us to investigate the roles of both pDC and CD8α+ DC during ECTV infection, we did not investigate double or triple depletions of other myeloid cell subsets.
Chapter VI: Overall discussion and future directions

Smallpox was previously a major world health concern due to high rates of morbidity and mortality prior to the eradication of smallpox by global health efforts. A contributing factor to smallpox eradication was the use of live VACV as a vaccine against smallpox. The health risks from VACV vaccination combined with the reduced benefit of the vaccine following the eradication of smallpox led to the end of many smallpox vaccination programs. Currently, there is an increasing incidence of morbidity and mortality resulting from zoonotic orthopoxvirus infection, for which safe and effective therapeutics are not available (2008). This rise in the incidence of zoonotic orthopoxvirus infections and the continued concern of the use of VARV, the causative agent of smallpox, as a bioterrorism agent support the development of safer vaccines and safe and effective pharmacological agents. However, in order to rationally develop these therapeutics, the basic understanding of the development and functions of a protective immune response to orthopoxvirus infection is important. The knowledge of the effective immune response can indicate potential cell targets, methods to limit viral replication and spread, and ultimately requisite factors for elimination of virus and host recovery.

To study the immune response to orthopoxvirus infection, we have used infection of the mouse with its natural pathogen, ECTV. ECTV causes mousepox following infection through abrasions of the skin in the footpad. Intradermal (i.d.) footpad infection is the natural route of infection of ECTV (Fenner 1948). ECTV replicates at the site of infection, spreads to the D-LN, and then becomes systemic and can be titered from the spleen and liver (Fenner 1948). Other routes of infection with ECTV, such as i.n. and
i.v., are lethal in mice resistant to footpad infection (Parker, Siddiqui et al. 2009) (Parker, Siddiqui et al. 2010), indicating the importance of using the natural route of infection when studying the immune response. Another orthopoxvirus, VACV, is also used to study the immune response to an i.d. infection in mice as immunization with VACV (smallpox vaccine) is given i.d. However, VACV is not a natural pathogen of the mouse and does not lead to systemic infection following i.d. infection of the ear pinnae (Tscharke and Smith 1999). In addition to using ECTV to investigate the mechanisms utilized by the immune response to control and eliminate an orthopoxvirus, ECTV is also an example of a peripheral viral infection that spreads systemically as part of the natural host-pathogen interaction. In addition to orthopoxviruses, many viruses of concern to human health spread systemically from a peripheral site of infection including: measles virus, arboviruses (WNV, dengue virus), varicella zoster virus (chickenpox).

The involvement of phagocytic cells of the innate immune response in the development of effective immunity to ECTV

Following viral infection, responding innate immune cells can produce cytokines and activate an antiviral state to contain the virus and limit viral replication. Responding myeloid cells also recruit and activate other cells of the immune response, including NK cells and T cells. Following infection with ECTV, the phagocytes of the innate immune response have an early requisite role in controlling the virus and mediating survival of infection (Karupiah, Buller et al. 1996). The role of phagocytes in the immune response to ECTV infection was isolated using CLL depletion methods (Karupiah, Buller et al. 1996). However, the identities of phagocytic cells depleted by CLL during ECTV
infection were unclear. In addition to macrophage depletion by CLL (Qian, Jutila et al. 1994), we found depletion of monocytes, mo-DC, and DC subsets: CD11b+ DC, pDC, and CD8α+ DC following CLL injection during ECTV infection. Our lab also found similar depletion of monocytes and DC subsets by CLL treatment during skin infection with another orthopoxvirus, VACV (Davies).

Although phagocytes depleted by CLL injection are required for survival of ECTV infection (Karupiah, Buller et al. 1996), when we isolated the involvement of monocytes and mo-DC, granulocytes alone, and macrophages alone using multiple depletion methods, we found no requirement for these cells in the immune response to ECTV. Instead, we found an early requirement for CD11c+ cells to control the virus and mediate survival of ECTV infection. Upon exposure to ECTV, the DC, not the macrophages, monocytes, mo-DC, or granulocytes were the cells producing T1-IFN, a required antiviral cytokine (Xu, Rubio et al. 2012) (Rubio, Xu et al. 2013).

The requirement for bulk CD11c+ cells in the effective immune response to ECTV was found using the CD11c:cre x iDTR transgenic mouse system. DT injection in the CD11c:cre x iDTR depletion system also depletes subsets of monocytes/macrophages, NK cells, T cells, and B cells in addition to DC (Caton, Smith-Raska et al. 2007, Abram, Roberge et al. 2014). After finding the requirement for bulk CD11c+ cells, we initially tried to investigate the involvement of DC subsets while also ruling out death of CD11c+-depleted mice due to depletion of immune cells other than DC. We tried to do this by adoptively transferring DC from WT mice into DC-depleted mice to rescue these susceptible mice during ECTV infection. The transfer of more than 10^7 bulk DC was unable to rescue the DC-depleted mice following ECTV infection (Norbury lab, data not
shown), so we were unable to use this method to investigate specific DC subsets. Potentially, the adoptively transferred WT DC did not survive once injected into the mouse, did not traffic to appropriate sites, or, if DC localized to required sites, they did not function normally. Because the successful adoptive transfer of WT DC was not our focus, we did not further explore the inability of WT DC to rescue DC-depleted mice during ECTV infection. We instead used pDC-depleting antibody to specifically target pDC and Batf3KO mice to isolate the role for CD8α⁺ DC. We chose to target both pDC and CD8α⁺ DC because both of these DC subsets produced a requisite cytokine, IFNα (Xu, Rubio et al. 2012) (Rubio, Xu et al. 2013), in response to live WT ECTV, and both pDC and CD8α⁺ DC, but not CD11b⁺ DC, were depleted in ECTV-susceptible DT-injected CD11c:cre x iDTR mice.

Mice lacking either pDC or CD8α⁺ DC, survived ECTV infection; however, mice lacking both pDC and CD8α⁺ DC succumbed to ECTV infection. In the absence of both pDC and CD8α⁺ DC, the immune response was no longer able to control the virus, and mice died as a consequence of an overwhelming viral load. The two DC subsets, pDC and CD8α⁺ DC, but not CD11b⁺ DC, produced IFNα. Additionally, in preliminary experiments, pDC and CD8α⁺ DC produced pro-inflammatory cytokines IL-6 and IL-12 in response to live WT ECTV (Norbury lab, data not shown). In contrast, CD11b⁺ DC produced IL-12 in response to UVC ECTV, but not in response to live WT ECTV (Norbury lab, data not shown). To our knowledge, this is the first description of this functional redundancy between pDC and CD8α⁺ DC to control virus in a peripheral viral infection. With at least one of these subsets present, either pDC or CD8α⁺ DC, the level of the ultimate function is sufficient to control ECTV.
Similar to ECTV, many viruses infect in the periphery but establish a systemic infection, including other poxviruses, such as VARV and MPXV. These results support the importance of early viral control in a peripheral viral infection. When we treated mice lacking pDC and CD8α⁺ DC with cidofovir, an anti-poxviral drug, to control viral replication, mice survived ECTV infection. This requirement for immediate antiviral activity provides a rationale for the development of antiviral therapeutics for use early after viral infection.

The control of the virus, whether by the function of pDC and CD8α⁺ DC or by cidofovir treatment, likely mediates survival of the mouse long enough to allow for the development of the adaptive immune response. The adaptive immune response would then take over to effectively control and eliminate the virus for survival of infection. We did not verify, however, that cidofovir treatment of mice lacking pDC and CD8α⁺ DC allowed for development of the adaptive immune response. At 6 or 7 d.p.i., we could harvest spleens and LN from pDC-depleted Batf3KO mice treated with either PBS or cidofovir to investigate the development of the adaptive immune response. As mice lacking pDC and CD8α⁺ DC died from ECTV infection at 8-9 d.p.i., tissues should be harvested prior to this expected time of death of the susceptible mice. Because mice lacking T_{CD8⁺} die early after ECTV infection (~10 d.p.i.) (Karupiah, Buller et al. 1996, Fang, Siciliano et al. 2012), but mice lacking the T_{CD4⁺} response die later (~20 d.p.i.) and (Karupiah, Buller et al. 1996, Fang, Siciliano et al. 2012) mice lacking the B cell response die much later (~60 d.p.i.) after ECTV infection (Karupiah, Buller et al. 1996, Fang, Siciliano et al. 2012), the T_{CD8⁺} response and possibly the T_{CD4⁺} response could be investigated early at 6-7 d.p.i. Flow cytometry could be utilized to analyze T cell
numbers, subset numbers, and activation markers. Additionally, we can measure proliferation of OT-I T cells, $T_{CD8^+}$ with a TCR specific for the peptide SIINFEKL, following infection with SIINFEKL-expressing ECTV. Proliferation of OT-I T cells would be indicative of antigen-specific activation of $T_{CD8^+}$ via antigen presentation. Intracellular cytokine staining could be used to assess the effector functions of the T cells in response to peptide stimulation. As the cytotoxic function of T cells is important in ECTV infection, cells can be stained for GzmB or assessed for level of cytolytic specific killing.

Additionally, to confirm the development of an effective immune response following control of virus, mice lacking pDC and CD8α+ DC and treated with cidofovir can be monitored for survival for a longer period of time. These data would indicate the potential for therapeutics that control a virus to be sufficient during viral disease to mediate recovery by allowing time for the adaptive immune response to develop. As the B cell response is the aspect of adaptive immunity required latest (~60 d.p.i.) (Karupiah, Buller et al. 1996, Fang, Siciliano et al. 2012), mice can be monitored for survival beyond 60 d.p.i. We also did not confirm the level of viral control in cidofovir-treated mice lacking pDC and CD8α+ DC, but this could be done by titering virus from multiple tissues at 6 or 7 d.p.i. from pDC-depleted Batf3KO mice treated with PBS compared to mice treated with cidofovir.

An effector function utilized by pDC and CD8α+ DC was cytokine production. These two DC subsets produced IFNα, a requisite antiviral cytokine (Xu, Rubio et al. 2012) (Rubio, Xu et al. 2013), in response to live and UVC/psoralen-inactivated ECTV. Although we investigated the impact of the absence of pDC and CD8α+ DC on cell types
that could be recruited and activated, we did not find differences in NK cell, T\textsubscript{CD8+}, and T\textsubscript{CD4+} numbers, maturation, and activation in mice lacking both pDC and CD8\textalpha+ DC compared to resistant mice (WT, pDC-depleted, and Batf3KO). These results did not support a role for pDC and CD8\textalpha+ DC in directly affecting NK and T cell numbers and recruitment.

To further analyze cytokine production in mice lacking both pDC and CD8\textalpha+ DC compared to resistant mice, we utilized RT-qPCR for RNA expression of a panel of cytokines and chemokines in the D-LN at 2 d.p.i. For this analysis, we utilized a Mouse Cytokines & Chemokines RT\textsuperscript{2} Profiler PCR Array (Qiagen) that profiles the expression of 84 chemokines and cytokines. From this analysis, we did not find any significant differences in the levels of RNA of these 84 chemokines and cytokines, including IFN\textalpha2, in ECTV-infected pDC-depleted Batf3KO mice compared to infected resistant mice (Norbury lab, data not shown). We did not investigate the level of cytokine and chemokine expression in uninfected mice. Although we measured RNA levels for IFN\textalpha2 in the array, there are fourteen types of IFN\textalpha in the mouse, so we cannot rule out that one or several of the other types of IFN\textalpha could be involved in the response to ECTV. We could analyze multiple IFN\textalpha subtypes by RT-qPCR or instead utilize ELISA to measure serum IFN\textalpha as the antibody in ELISA binds to the IFN\textalpha protein. We would expect these assays to show a reduction in IFN\textalpha in the absence of both pDC and CD8\textalpha+ DC compared to WT mice during ECTV infection as the pDC and CD8\textalpha+ DC produce IFN\textalpha in response to ECTV. Potentially, we would find an intermediate level of IFN\textalpha in pDC-depleted mice and Batf3KO mice compared to WT mice and pDC-depleted Batf3KO mice. Additionally, recombinant IFN\textalpha could be used to treat mice lacking both
pDC and CD8α+ DC to determine if IFNα can rescue these mice. Rescue of susceptible mice by IFNα treatment would indicate that a deficiency in IFNα plays a role in the death of these ECTV-infected mice. To our knowledge, there is no method currently available to specifically knockout IFNα production in both pDC and CD8α+ DC because a promoter specific to both pDC and CD8α+ DC that can be used to regulate cre expression does not exist to my knowledge. Additionally, IFNAR-floxed mice exist, but this would knockout T1-IFN signaling, not T1-IFN production, and as far as I know, mice with loxP-flanked genes of the IFNα subtypes do not exist.

After ECTV infection i.d. in the footpad, virus replicates and initially spreads to the D-LN then disseminates systemically as indicated by replication of virus in spleen and liver (Fenner 1948). In titering ECTV from the tissues of susceptible bulk CD11c+ cell-depleted mice, we did not find differences in viral titer in the footpad or D-LN, but we did find elevated viral titers systemically, in the spleen and liver, in comparison to resistant mice. Additionally, viral titers in mice lacking both pDC and CD8α+ DC were not elevated in the footpad or D-LN, but were elevated in the spleen as compared to all other groups of resistant control mice. As similar levels of viral replication occur at the site of infection in resistant and susceptible mice, the level of viral control does not seem to be at the footpad. Viral titers are also not different in the D-LN of susceptible mice compared to resistant mice; however, systemic viral titers are elevated in susceptible mice compared to resistant mice. The elevated systemic viral titers could be due to a lack of viral control in the D-LN of susceptible mice. Although viral titers were not elevated in the D-LN, a lack of viral control in the D-LN could result in earlier systemic dissemination of virus or enhanced viral dissemination from the D-LN. Earlier
viral dissemination from the D-LN could be investigated by titering virus from systemic tissues, liver and spleen, over a time course; however, we titered virus only after virus had spread systemically at 5 d.p.i. In contrast, the level of viral control could be systemic as differences in viral titers were found in the spleen and liver, but not the D-LN, of susceptible mice compared to resistant mice. The level of viral control is difficult to investigate as experiments utilizing removal of the D-LN would completely alter normal viral dissemination and methods to specifically measure virus disseminating from the D-LN are not available.

**Methods of innate immune cell depletion and potential off-target effects**

In order to isolate the roles and functions of multiple myeloid cell subsets in the immune response to ECTV infection, we utilized multiple myeloid cell depletion methods. The mouse systems of depletion included MaFIA mice, LysM:cre x iDTR mice, CD11c:cre x iDTR mice, and Batf3KO mice. In the MaFIA mice and LysM:cre x iDTR mice, we expected depletion of cells of the monocyte-macrophage lineage, but we also found depletion of some DC subsets. In the CD11c:cre x iDTR mice, we found the opposite effect in which CD11b⁺ DC were not depleted although cells were CD11c⁺. As an example of other unexpected effects in these systems that could affect the outcome of experiments, Batf3KO mice, which lack CD8α⁺ DC, develop CD8α⁺ DC through a Batf3-independent pathway in a model of parasite infection, although the development takes longer than a week after infection (Tussiwand, Lee et al. 2012). As another unexpected effect, the injection of DT in CD11c:cre x iDTR mice depletes bulk DC but also results in a neutrophilia that improves clearance of bacteria from the kidney in a
mouse model of pyelonephritis (Tittel, Heuser et al. 2012). There are also descriptions in the literature of other immune cells unexpectedly depleted or increased in these systems. These off-target changes display the importance of assessing for potential unexpected effects of these methods and the efficacy of depletion within each experimental system being used, whether viral infection, steady-state, or autoimmunity (Hume 2011, van Blijswijk, Schraml et al. 2013), so that they can be considered during interpretation of the data. Off-target effects present potential problems with use of the depletion systems and should be kept in mind when designing experiments as well as when interpreting data.

For the LysM:cre x iDTR depletion system, work from other investigators supports the expression of LysM and thus Cre recombinase in HSC (Ye, Iwasaki et al. 2003). The removal of the LoxP-flanked STOP cassette in these cells is then permanent, resulting in the expression of iDTR in these HSC as well as their progeny (immune cells: myeloid cells, T cells, NK cells). This expression of LysM in HSC provides a potential mechanism for our findings of depletion of multiple myeloid cell subsets, including granulocytes, monocytes, and DC subsets in the LysM:cre x iDTR system during ECTV infection. We did not investigate the effects of DT injection in the LysM:cre x iDTR system on HSC or adaptive immune cells, but we could assess the depletion of HSC, NK cells, and T cells following DT injection of ECTV-infected LysM:cre x iDTR mice. To our knowledge, there is no antibody available to measure the expression of the iDTR on cells using flow cytometry. Measurement of removal of the STOP cassette would rely on sorting of cell subsets followed by PCR to detect STOP cassette removal in a small percentage of cells.
Immune response to peripheral poxvirus infection: ECTV versus VACV

Following peripheral infection with ECTV or VACV, myeloid cells migrate to the site of infection. Early after infection, the number of myeloid cells at the site of infection is greater after VACV infection than after ECTV infection (Norbury lab, data not shown). During VACV infection, Ly6C+Ly6G+ cells respond to the site of infection, produce T1-IFN and reactive oxygen species, and are required to minimize tissue damage at the site of infection (Fischer, Davies et al. 2011). However, we did not find a requisite role for Ly6G+ cells alone in the response to ECTV infection, although this does not rule out a role for Ly6G+ cells or redundancy in function with another cell subset. While ECTV spreads to the D-LN then becomes systemic and can be titered from the spleen and liver in resistant and susceptible mice, VACV spreads to the D-LN and some of the initial inoculum of virus briefly becomes systemic. VACV, however, cannot be titered from the ovaries unless systemic macrophages are depleted, such as by CLL injection (Davies). During VACV infection, the internalization of virus by splenic macrophages does not seem to result in productive infection; instead, virus is filtered from the blood of the host. The lack of productive infection of VACV in macrophages could be the result of activity of sterile alpha motif and histidine-aspartic domain-containing protein 1 (SAMHD1). The replication of VACV is inhibited in macrophages that express SAMHD1 (Hollenbaugh, Gee et al. 2013). SAMHD1 is a deoxynucleotide triphosphate (dNTP) triphosphohydrolase that depletes cellular dNTPs during VACV infection (Hollenbaugh, Gee et al. 2013). In contrast, during ECTV infection, virus infects multiple cell types of the skin and immune cells, including macrophages, DC, and B cells (Sei), but viral replication is productive and virus spreads systemically.
For recognition of virus for induction of an effective immune response, MyD88 is important for recognition of both VACV (Davies, Sei et al. 2014) and ECTV, and TLR9 is also required for survival of ECTV infection (Samuelsson, Hausmann et al. 2008) (Sutherland, Ranasinghe et al. 2011) (Rubio, Xu et al. 2013). The cell subsets required to have functional MyD88 signaling in orthopoxvirus infection remains unclear. We investigated the requirement for MyD88 signaling in cells that expressed LysM, but we found that although LysM-expressing cells are required for survival of ECTV infection, these cells do not need to have MyD88 expression. This indicates that other immune cells are able to recognize ECTV via the MyD88 pathway in the absence of MyD88 signaling in LysM-expressing cells. However, the identities of cells required to have functional MyD88 signaling remain unclear. To further investigate identities of cells required to have functional MyD88 pathway signaling, we could cross the MyD88-floxed mice with other cre recombinase expressing mouse strains starting with CD11c:cre to ablate MyD88 signaling in bulk DC. Additionally, there are Langerin:cre mice (targets Langerhans cells and Langerin+ dDC), Clec9a:cre mice (targets conventional DC including CD8α+ DC), and CD11b:cre mice (targets granulocytes, monocytes, macrophages, mo-DC, CD11b+ DC). Similar to the other loxP/cre systems that we utilized, such as the LysM:cre x iDTR mice, we would need to investigate the cells with cre-mediated recombination in these systems during ECTV infection. This could be done by crossing the cre-expressing mice with reporter mice, such as the ROSA26 EYFP mice.
The involvement of CD11b⁺ DC in the immune response to ECTV infection

We investigated the involvement of bulk DC in the immune response to ECTV infection using DT injection of CD11c:cre x iDTR mice, and we found a requirement for DC. CD11b⁺ DC, however, were not depleted in this system for reasons that remain unclear. Although we found a required role for other DC subsets, that does not rule out an important role for CD11b⁺ DC in the immune response to ECTV infection. Results from the use of CD11b-DTR/CD11b:cre x iDTR mice to investigate the role of CD11b⁺ DC would be difficult to interpret as CD11b⁺ DC would not be the only cells targeted. Other cells expressing CD11b include granulocytes, monocytes, macrophages, mo-DC, and subsets of NK cells.

CD11b⁺ DC in the skin are migratory and consist of Langerin⁺ and Langerin⁻ subsets. These migratory DC internalize antigen in the skin and traffic to the D-LN to present antigen and activate the T_{CD8⁺} response. Migratory DC are required to traffic into the D-LN with antigen from topical skin application and during viral infection of the skin for T_{CD8⁺} proliferation (Schell, Granger et al. 2010, Nizza and Campbell 2014). Migration of these cells into the D-LN requires CC-chemokine receptor 7 (CCR7) (Forster, Schubel et al. 1999). In the absence of CCR7, DC in the D-LN (lacking migratory DC) are not able to activate T_{CD8⁺} proliferation (Nizza and Campbell 2014). In preliminary experiments, we found that CCR7KO mice die from ECTV infection (Norbury lab, data not shown), which suggests that the migratory DC from the skin are required for survival of ECTV infection. CCR7, however, is also involved in other aspects of the immune response, including T cell development and homing (Forster, Schubel et al. 1999, Forster, Davalos-Misslitz et al. 2008). There is impaired
lymphocyte migration in CCR7KO mice, altered organization of LN, and reduced naïve T cells in the LN with a failure to mount a primary T cell response (Forster, Schubel et al. 1999), which confounds the interpretation of data using CCR7KO mice.

Alternatively, Lang-DTR mice have been developed and characterized in which the DTR is inserted into the Langerin locus (Kissenpfennig, Henri et al. 2005). The Langerin+ DC can then be targeted for depletion by DT injection. This system targets Langerhans cells, Langerin+ dermal DC (dDC), but not Langerin− dDC. In response to skin scarification with VACV, Langerin+ dDC are important for the activation and proliferation of TCD8+ and for TCD8+ effector function (Seneschal, Jiang et al. 2014). In addition to these Langerin+ cells, these CD11b+ Langerin− dDC have also been shown to stimulate TCD8+ proliferation in a model of topical immunization (Nizza and Campbell 2014), but we would not be able to isolate the role of Langerin− dDC in the Lang-DTR system. The Lang-DTR mice would allow us to distinguish roles for Langerhans cells and Langerin+ dDC based on the timing of the injection (Nagao, Ginhoux et al. 2009, Seneschal, Jiang et al. 2014). Following depletion with DT, the Langerin+ dDC (~13 d) repopulate much more rapidly than the Langerhans cells (~6 weeks) (Nagao, Ginhoux et al. 2009, Seneschal, Jiang et al. 2014).

In contrast to the pDC and CD8α+ DC, CD11b+ DC produced IFNα in response to UVC/psoralen-inactivated ECTV, but not in response to live WT ECTV. The response of CD11b+ DC to live WT ECTV could be inhibited by the production of HRM by the virus. UVC/psoralen inactivation of ECTV prevents the expression of viral HRMs. As the viral proteins, including HRMs, are produced within the cytosol of the infected host
cell, HRMs could be inhibiting cytosolic recognition of the virus, which would prevent cytokine production.

Overall, there was not an increase in viral titer in the infected footpad or in the D-LN of pDC-depleted Batf3KO mice compared to resistant control mice; however, there was an increased viral titer in the spleen in the absence of pDC and CD8α⁺ DC. The increased viral titers in the spleen indicated a lack of systemic control of the virus, although we cannot rule out earlier spread of virus from and/or spread of greater viral load from the D-LN. We also found no difference in the viral titers in the livers of pDC-depleted Batf3KO mice compared to controls. CD8α⁺ DC are a lymphoid-resident DC subset and potentially more important for viral control in lymphoid tissues compared to other tissues, such as the liver.

For recognition of ECTV, TLR9 and its adapter protein, MyD88 are both required to ultimately mediate survival (Samuelsson, Hausmann et al. 2008, Sutherland, Ranasinghe et al. 2011, Rubio, Xu et al. 2013). TLR9 recognizes CpG DNA within endosomes, and within the cytosol, recognition of ECTV could result from multiple PRRs. The RIG-I-Like receptors, retinoic acid-inducible gene-1 (RIG-I) and melanoma differentiation-associated protein 5 (MDA5), detect cytosolic viral RNA and signal through the adaptor protein mitochondrial antiviral-signaling protein (MAVS); however, MAVS is not required for survival of ECTV infection (Luis Sigal lab, unpublished data). In addition to cytosolic RNA sensors, there are cytosolic PRRs that detect DNA. Stimulator of interferon genes (STING) is an adapter for DNA-sensing PRRs and is required for survival of ECTV infection (Luis Sigal lab, unpublished data). However, cyclic GMP-AMP synthase (cGAS), which is involved upstream of STING signaling, is
not required for survival of ECTV infection (Norbury lab, unpublished data). DAI, a cytosolic DNA sensor, is also not required for survival of ECTV infection and is therefore not required to signal through STING for survival of ECTV infection (Luis Sigal lab, unpublished data). The required signaling through STING during ECTV infection is potentially both cGAS-independent and DAI-independent and could be mediated by other PRR pathways or recognition and signaling via STING is mediated by multiple, redundant pathways. Following viral recognition, the activation of IRF7 and the production of IFNα are ultimately required for survival of ECTV infection (Rubio, Xu et al. 2013) (Xu, Rubio et al. 2012). As for which cell subsets require the requisite signaling mechanisms for recognition of ECTV, our lab found that MyD88 signaling was not required in LysM-expressing cells. However, TLR9 and MyD88 signaling are required in HSC and, more specifically, in CD11c+ cells (Luis Sigal, unpublished data).

Conclusions

In response to peripheral infection with ECTV, a natural pathogen of the mouse, we did not find requisite roles for granulocytes alone, monocytes and mo-DC, or macrophages alone; however, this does not rule out important roles for these myeloid cell subsets during ECTV infection. We did not investigate depletions of multiple subsets, such as double or triple depletions, which could indicate a requirement for redundant functions of these cells. We did, however, find a requirement for bulk CD11c+ cells during ECTV infection for mediating viral control and survival. By utilizing multiple cell depletion methods, we found a requirement for both pDC and CD8α+ DC for survival of ECTV infected mice.
To our knowledge, this is the first example of redundancy between two myeloid cell subsets in mediating survival of viral infection. Both pDC and CD8α⁺ DC are important for controlling the virus, and these DC subsets produce antiviral and proinflammatory cytokines in response to ECTV. When treated with a pharmacological agent, cidofovir, to control the virus in the absence of pDC and CD8α⁺ DC, mice lacking pDC and CD8α⁺ DC survived ECTV infection. The rescue of mice lacking pDC and CD8α⁺ DC by cidofovir indicates the importance of viral control in survival and suggests that the adaptive immune response developed in mice lacking pDC and CD8α⁺ DC when virus was controlled by cidofovir. We would need to further investigate the development of the adaptive immune response in cidofovir-treated pDC-depleted Batf3KO mice. Additionally, we did not find differences in NK cell, TCD8⁺, or TCD4⁺ subsets in pDC-depleted Batf3KO mice, which argue against a requisite role for the pDC and CD8α⁺ DC in recruiting and activating NK cells, TCD8⁺, or TCD4⁺. The pDC and CD8α⁺ DC could mediate control of ECTV, such as by proinflammatory and antiviral cytokine production, long enough for the response of the adaptive immune system to develop to control the virus and mediate survival. This would indicate the importance of early viral control following peripheral viral infection and support the development of antiviral therapeutics for early viral control. Initial control of virus may be enough to allow for an effective immune response to develop in the context of a functional adaptive immune response.
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