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ADVANCEMENTS IN MAMMALIAN COMPARATIVE IMMUNOLOGY-
DEVELOPING NEW TOOLS AND INVESTIGATING THE INNATE IMMUNE
RESPONSE TO CEDAR VIRUS.

by

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DEDICATION

Mum and Dad,

If there was a PhD in parenthood, you'd have earned it.

This is for you.

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May 15th, 2020

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ABSTRACT

Advancements in mammalian comparative immunology-developing new tools and investigating the innate immune response to Cedar virus

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Emerging viruses pose a serious threat to public health. Virtually all of the recent emerging virus outbreaks have come from human-wildlife interactions. These wildlife animals that harbor a virus are referred to as reservoirs. When the same virus then enters a human host however, overwhelming pathogenesis is often observed. Bats are the reservoirs for many of these zoonotic viruses. For example, *Pteropus sp* bats are the source of Hendra (HeV) and Nipah virus (NiV). Both of these viruses cause severe disease with high mortality rates that are often a result of immunopathogenesis. Cedar virus (CedV) belongs to the same genus as HeV and NiV but is non-pathogenic. Here, CedV is used as a model for HeV and NiV to study human-bat comparative immunology.

Because bats are the natural reservoirs of so many viruses, it is important to investigate how they are able to tolerate virus infections. A major goal of the present research is to develop a new technology that could allow a comparison of the human immune response to that of the viral reservoir. We use lentiviral transduction of two viral oncogenes, v-Raf and v-Myc, to immortalize macrophages isolated from murine blood. The resulting cell lines exhibit surface markers consistent with mature murine macrophages, most notably CD11b and F4/80. These cells are IFN γ responsive and capable of phagocytosis. Further, the cell lines are cytokine independent, which is important when working with species for which commercially available cytokines do not exist.

We also investigate the NF- κ B response to CedV. This pathway is activated during many types of infections and is responsible for the subsequent activation of many pro-inflammatory genes. To compare the NF- κ B response in *P. alecto* (reservoir) to that of humans (host) we use kidney fibroblast cell lines from *P. alecto* (PaKi and PaKiT) and the human cell lines HEK 293T and MRC-5. We find that the activation of NF- κ B in PaKi cells is overall less robust than in the human cell lines. Additionally, we find activation of NF- κ B occurs sooner after CedV infection in PaKiT cells than in HEK 293T cells. Moreover, we detect more viral protein in PaKi cells than MRC-5 cells. This is consistent with our data that show blocking the NF- κ B pathway yields a higher viral titer and more detectable virus within the cells.

A20 expression is activated by the NF- κ B pathway. Upon expression, A20 serves as a negative regulator of the pathway. We demonstrate that CedV induces robust A20 expression, and upon further analysis, that the CedV phosphoprotein (P), is sufficient for this activation. Remarkably, this CedV induced expression is independent of the

activation of NF- κ B. Alternative splicing is a process by which mRNA is edited to include or exclude different introns and exons of the RNA transcript to create a unique protein isotype. We determine that this CedV mediated A20 expression occurs even in biallelic A20 exon 3 knockout cells. This suggests the A20 we are detecting during infection is a unique isoform of A20 which does not contain exon 3. The abundance of different protein isotypes from a given gene can be altered during infections. However, virus mediated splicing that results in a unique host protein is novel. Thus, through this dissertation work, I have developed a new tool with comparative immunology potential, contributed to henipavirus comparative immunology with existing tools, and described a novel way viruses may alter a host's cellular biology.

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CHAPTER 1: INTRODUCTION

ZOONOTIC DISEASE

The incidence of emerging infectious diseases is increasing. While there are numerous causes for the rise in outbreaks, most notable is humans' continued encroachment on land that was previously inhabited only by wildlife (222). These events lead to microbes passing from animals to humans and causing disease. Infections caused by this type of transmission are known as zoonoses or zoonotic diseases. Many zoonotic microbes have evolved with the wildlife and formed commensal relationships, while in humans they are novel and often pathogenic (51). In fact, nearly three quarters of emerging infectious diseases are zoonotic and the majority of these are caused by viruses (21). Among these zoonotic, emerging viruses are some well-known examples including human immunodeficiency virus (HIV), Nipah virus (NiV), Hendra virus (HeV), Hantaviruses, Marburg Virus, SARS-Coronavirus (SARS-CoV), and Avian influenza (21; 73; 79; 92; 141). The mortality rates in these emerging infectious viral diseases are usually high and can reach up to 100% (10; 53). Even now, we are in the midst of an unprecedented outbreak (COVID-19) and the data suggests this disease is almost certainly a zoonosis (105). Studying these pathogens and looking for patterns and commonalities must be a top priority if we are to be equipped for treating these emerging diseases.

Viral reservoirs

As with most zoonotic diseases, the wildlife animal involved harbors a pathogen but often does not develop disease. These animals are known as reservoirs. Relatively

little is known about most reservoirs of emerging viruses as they are typically not standard laboratory animals, and are thus not easily studied. Among these reservoirs are various water birds (influenza virus), voles and deer mice (hantaviruses), multimammate mice (Lassa virus), and fruit bats (henipaviruses) (16; 21). Although rodent species carry a lot of the zoonotic viral burden, upon a comprehensive analysis, it was found that within the mammalian order bats are the reservoir to the highest proportion of zoonotic viruses (167). One way we can begin to understand these emerging, zoonotic viruses better is to examine what is occurring in bats on an immunological level while they are harboring an emerging virus. By determining ways in which the immunological response to a given virus in humans differs from the bat reservoir, one can better understand what needs to be targeted in order to have a “reservoir-like” infection. In other words, the characteristics of viral reservoirs can inform ways in which pathology can be decreased or avoided in the host. When a new outbreak then occurs from bats, we have a better understanding of what we aiming to target when considering therapeutics.

RNA viruses found in bats

Ebolaviruses

The six species of *Ebolavirus* genus belong to the *Filoviridae* family. These viruses are enveloped, negative, single-stranded RNA viruses. Pathogenic *Ebolaviruses* cause Ebola virus Disease (EVD) which typically begins with flu like symptoms and vomiting (208). Coagulopathy is common but in more recent outbreaks severe hemorrhaging has been rare (204). Instead, uveitis, hypovolemia, and rash are more often observed. Death is usually the result of multi-organ system failure and typically

occurs around days 7-16 (208). After the 2014 outbreak, rheumatic and ocular sequelae were observed frequently(214).

The first recorded outbreak occurred in 1976 in Sudan and was followed several months later by an outbreak in the Democratic Republic of Congo. These two outbreaks were caused by *Ebolavirus Sudan* and *Zaire*, respectively (176). Most recently, the *Ebolavirus* outbreaks have occurred in several countries in West Africa and affected individuals in the United States and Europe as well. The largest outbreak occurred from 2013-2016 and primarily affected New Guinea, Liberia, and Sierra Leone. The suspected case number totaled over 28,000 with over 11,000 confirmed deaths (120).

Bats can be linked to most of the *Ebolavirus* outbreaks. In the initial outbreak in 1976, workers who became sick noted bats were often present in the factory where they were employed (176). More recently, in the West African outbreak, the index case was a young boy who spent time playing near a tree that housed a colony of bats (120). Several years later, this species of bat was found to harbor the newest species of *Ebolavirus*, *Bombali ebolavirus* (108). Investigators have also shown through biosurveillance, that some bat species are seropositive for *Ebolavirus Zaire* and *Sudan* in Africa (55). Another group showed that insectivorous and frugivorous bats can be experimentally infected with Ebola virus and circulate the virus without necessarily developing illness (201). The experimental evidence combined with the anecdotal evidence during outbreaks strongly suggests that bats are the reservoir for at least some Ebolaviruses and that their biological response to these infections is important to consider.

Coronaviruses

Coronaviruses are large, enveloped, positive sense, single-stranded RNA viruses. Of the six coronaviruses that cause human disease (HCoVs), the most notable are Severe Acute Respiratory Syndrome-associated coronavirus (SARS-CoV), Middle East Respiratory Syndrome Coronavirus (MERS-CoV), and Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2). SARS-CoV was identified in 2002 in South China. The virus infected over 8000 people in nearly 30 different countries. SARS-CoV was followed by MERS-CoV in 2012 in the Middle East with most cases in Saudi Arabia. This outbreak lasted nearly five years and resulted in over 2000 lab-confirmed cases. Most recently, SARS-CoV-2 emerged in Wuhan, China. The number of cases far surpassed that of SARS and as of this writing total at more than 820,000 confirmed cases and over 40,000 deaths. The United States and Italy have the most confirmed cases with approximately 175,000 and 105,000, respectively (1). While case number and total number of deaths caused by the SARS-CoV-2 are highest, SARS and MERS both have higher case fatality rates at 10% and 40%, respectively (41). All three of these coronaviruses viruses cause a wide range of disease severity influenced by age and co-morbidities. The most common symptoms are fever, fatigue, and respiratory distress. Disease for all three viruses can manifest as Acute Respiratory Distress Syndrome (ARDS) which can require mechanical ventilation and ultimately lead to death (94; 97; 228).

The outbreaks of SARS-CoV and MERS-CoV were associated with human-animal interactions; civets at an open market and dromedary camels kept for husbandry and competition, respectively (13; 63; 71; 228). Studies eventually showed that both of these animals were intermediary hosts to humans. In other words, they served as the

animal in between the viral reservoir and the terminal human host. Through experimental infections and deep sequencing investigators concluded that bats are the reservoirs for both of these pathogenic coronaviruses as well as closely related coronaviruses (16; 77; 101; 128; 158). The above facts, combined with the high rates of mutations found in RNA viruses means the possibility of spillover of additional CoVs from bats that can infect and cause disease in humans almost certainly exists (96; 228). In China alone, at least 17 species of coronaviruses have been found circulating in bats (70). Although it has not yet been confirmed, the SARS-CoV-2 likely originated in bats. In approximately 1/3 of the original 41 2019-nCoV cases, the patient reported having visited a seafood market where bats and other wildlife were sold (104; 239).

Molecular evidence also supports bats as the reservoir for SARS-CoV-2. Both SARS-CoV-2 and SARS-CoV use the ACE2 receptor for entry into human cells (239). These two viruses also have 82% sequence similarity. An 89% sequence identity between SARS-CoV-2 and a SARS-related CoV (CoVZXC21) isolated from bats, also supports this hypothesis (39). Considering the severity and pandemic potential all three of these coronaviruses, studying the bat reservoirs in the context of coronavirus infections is an important focus.

Henipaviruses

Henipaviruses are enveloped, single-stranded RNA viruses belonging to the *Paramyxoviridae* family. The number of henipavirus species continues to grow, but to the date of this writing there are a recorded 5 species. Only two of these species, HeV and NiV virus, are known to be pathogenic in humans (53; 81).

HeV was first identified in 1994 in Hendra, Australia. The first cases involved horses which were later determined to be the intermediary hosts to the first human case. HeV has resulted in the death of over 100 horses and the disease it causes is severe in both horses and humans (159). The virus causes neurological and respiratory disease in both humans and horses and in fatal cases disease progresses rapidly. A vaccine that protects horses from HeV, Equivac HeV, was approved for use in 2012 (154).

Four years after the first HeV outbreak, the first recorded NiV outbreak occurred in Kampung Sungai Nipah, Malaysia. This outbreak also included humans and an intermediary host, which in this case was swine. This initial outbreak not only resulted in close to 300 human fatalities, but also required upwards of 1,000,000 pigs to be culled (43; 156). Several outbreaks have occurred since 1998 (9; 38; 42; 93). Most recently, in 2018, 17 people died during an outbreak in Kerala, India (194). NiV, like HeV, causes respiratory and neurologic disease; though it tends to manifest more often as neurological disease. Severe encephalitis is common and can reoccur even years after the initial onset of disease. There is no approved vaccine for NiV (8).

For both pathogenic henipaviruses, investigators found *Pteropid* sp. bats were the reservoir and that the intermediary hosts had confirmed or probable access to these species of bats (82; 219; 229). A third species of henipavirus, Cedar virus (CedV), has not been shown to cause disease in humans nor in henipavirus disease model animals, which include Syrian hamsters, ferrets, and guinea pigs (148; 189). CedV was, like HeV and NiV however, isolated from *Pteropid* bats (148). No known henipaviruses cause pathogenesis in bats (147).

COMPARATIVE IMMUNOLOGY

Comparative immunology generally requires utilizing a species that is not a standard or typical laboratory animal. It is often used as a way to relate an animal that is relatively easy to work with in the lab, such as an invertebrate, to a more complex animal, like humans (59). Immunology arose as a field of study in the late 1800s when Élie Metchnikoff described phagocytes in sea urchins (78). In this way, Metchnikoff used a non-standard laboratory animal (sea urchins) by relying on their representativeness or likeness to humans and other animals (they all contain phagocytic cells). Comparative immunology however, can extend beyond the mere substitution of a species for ease of study. This subset immunology also encompasses studies that use non-laboratory animals to highlight a difference from humans or another species of interest (59). For this reason, comparative immunology is an especially relevant field when studying zoonotic disease. Researchers can compare the immune response from a reservoir species (typically an animal found only in wildlife and is often a bat) to that of a human or agricultural animal. In other words, they compare the immune response of a reservoir which remains healthy, to that of a host where the same pathogen causes disease. Looking for the difference between the immune response in these two species can inform what needs to be changed or targeted in order to avoid pathogenesis in the host.

INNATE IMMUNE RESPONSE

The innate immune response is the branch of the immune system that mounts the initial reaction to a pathogen. This is contrast to the adaptive immune response, which although more pathogen specific, requires a longer period of time before becoming effective. The innate immune response encompasses two branches: humoral and cellular. The humoral immune response consists of complement activation, naturally occurring

antibodies, coagulopathy, and acute phase proteins (180). The first of these, the complement pathway, can be activated in response not just to pathogens, but also to damaged cells. There are several different initiation pathways that lead to complement activation but all of these pathways converge at assembly of C5 convertase and ultimately, result in the activation of the membrane attack complex (MAC) (197). Upon MAC activation, cytolysis of damaged cells and some bacteria can occur (152).

Additionally, one of the proteins involved in the complement cascade, C3b, can coat the surface of a pathogen or damaged cell. Receptors found on phagocytes are able to bind to these proteins and this leads to an enhanced likelihood of phagocytosis (68).

Coagulopathy cascades, both intrinsic and extrinsic, are a series of proteolytic reactions which converge at cleavage of prothrombin (II) and ultimately result in formation of a thrombin clot. These clots can protect vasculature from invading pathogens (197).

Another important facet of the humoral innate immune response is production of natural antibodies. These antibodies, unlike antibodies produced during the adaptive immune response, do not react to a single unique epitope (206). Instead, naturally occurring antibodies recognize a broad range of antigens and thus maintain the role of the innate immune in generating an immediate reaction to a foreign body (20). In certain cases, these antibodies must already be present when the pathogen enters the host. For this reason, natural antibodies are often involved in clearing and preventing chronic infections (178).

The last major subset within humoral innate immunity is production of pentraxins or acute phase proteins such as C-reactive protein (174). Much like the above described natural antibodies, these recognize conserved patterns of both pathogens and damaged

cells (197). Similar to complement C3b-coated pathogens, cells or pathogens coated with certain acute phase proteins facilitate their phagocytosis by macrophages (157).

The cellular innate immune response is equally as important as the humoral innate response for mounting an initial reaction to a pathogen or damage. Macrophages, granulocytes (eosinophils, neutrophils, and basophils), mast cells, and dendritic cells are the major cellular components of the innate immune response. Some of the humoral responses described above work together with the processes these cell subsets perform early after pathogen entry or tissue damage. One important example was discussed above, whereby complement activation and acute phase proteins cooperatively promote phagocytosis of pathogens and damaged cells. Phagocytosis occurs canonically in macrophages and is an innate cellular response by which these pathogens and damaged cells are internalized in phagosomes. The phagosomes formed after initiation of phagocytosis merge with lysosomes and the pathogen or apoptotic cell is degraded by presence of a low pH, hydrolysis, and free radical oxygen species in the resulting phagolysosome. This is one process by which presentation of a foreign antigen can begin and is a meeting point between the adaptive and innate immune responses (88).

Neutrophils are the most prevalent of granulocytes and have an important role in cellular innate immunity. Neutrophils are able to release neutrophil extracellular traps (NETs) which are composed of cellular chromatin and proteins. The cellular chromatin essentially entangles pathogens while the proteins have bactericidal properties (107). Granulocytes more generally however, share the ability to degranulate. This involves the release of a myriad of bactericidal proteins and enzymes stored within secretory vesicles

in the cell (37). As such, these cell subtypes have non-pathogen specific properties that help eliminate or alleviate an infection early on.

Last of the major innate cell subgroups are dendritic cells. These are perhaps the most important cells in bridging the innate and adaptive immune response. Dendritic cells play an important role in constantly sampling antigens present in their tissue-resident environments and presenting antigen to cells of the adaptive immune response so that they may mount an antigen-specific response to a particular pathogen (102).

Molecular Mediators of the Innate Immune Response

Although the various innate cell types have their own specific roles, they also share some functions. Notably, all these cells produce cytokines as an early response to infection. There are many types of cytokines and they play various roles during infections and allergic responses. Their expression, along with many other anti-pathogen pathways and genes, first require innate immune cell recognition of a pathogen by a pattern recognition receptor (PRR) (151). PRRs can be membrane bound or cytosolic and belong to one of the following four main groups: toll-like receptors (TLRs), C-type receptors (CLRs), nucleotide-binding domain leucine-rich repeat-containing receptors (NLRs), and RIG I like receptors (RLRs). These pattern recognition receptors recognize properties that are unique to pathogens and are known as pattern associated molecular patterns (PAMPs) (7). PAMPs are found on all types of pathogens and include lipopolysaccharide (LPS) in bacteria, double stranded RNA (dsRNA) in viruses, GPI anchor in parasites, and zymosan in fungi (233). As the focus of this work is viruses, the PRRs and PAMPs associated with this type of pathogen will be highlighted.

There are over ten TLRs found in mammals. Among them the endosomal TLRs 3, 7, and 9 recognize viral nucleic acids. TLR9 recognizes DNA while TLR3 and 7 recognize viral RNA. The membrane bound TLR2 and 4 are also involved in PAMP recognition during viral infections (7). For example, TLR4 responds to a membrane protein of Respiratory Syncytial virus (RSV) (115). The MAP kinase pathway, NF- κ B pathway, and the IRF pathway are the 3 main pathways activated in response to TLR activation. The activation of these pathways post TLR stimulation requires the signaling adaptor molecule Myeloid differentiation primary response 88 (MYD88), with the exception of TLR3 and 4 which use the adaptor molecule TIR-domain-containing adapter-inducing interferon- β (TRIF). In some circumstances, additional adaptor molecules like TIRAP or TRAM are recruited. All signaling adaptors are recruited to the cytoplasmic TIR tail domains of each TLR (40). The MAP kinase and NF- κ B pathways lead to activation of many pro-inflammatory cytokines such as TNF α and IL-6. On the other hand, activation of IRF3 and IRF7 lead to production of IFN β (interferon β) and IFN α , respectively (Figure 1). These two types of interferons belong to the Type I Interferon family and can be produced in most cell types. These interferons activate interferon stimulatory genes (ISGs) which are identified by the interferon-stimulated response elements (ISRE) in their promoters. These ISGs encode for the production of hundreds of proteins that aide in the clearance of viruses by a myriad of mechanisms (188).

Retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated protein 5 (MDA-5) belong to the RLR family of PRRs and can also induce interferon production. These cytoplasmic receptors recognize single- and double-stranded RNA,

respectively (203). Most RLRs contain a caspase activation and recruitment domain (CARD) as does the protein mitochondrial activator of virus signaling (MAVS). Upon RNA-mediated activation of RIG-I or MDA-5, CARD-CARD interactions occur between MAVS and the RLR. These interactions lead to a signaling cascade that results in activation of the NF- κ B and IRF pathways. (90; 111) (Figure 2).

PRRs in the NLR family are particularly important during virus infections in the context of inflammasome activation. Most data produced to date focus on the NLRP3 inflammasome, though others exist. These protein complexes contain a caspase and the adaptor protein, ASC (adaptor apoptosis-associated speck-like protein containing a CARD), as well as other proteins that vary with the specific inflammasome type. Upon recognition of a PAMP and a second signal (often ROS or ion fluxes), the inflammasome is assembled and is able to cleave the inactive pro-IL-1 β to the active pro-inflammatory cytokine IL-1 β (139).

The PRRs and the signaling pathways they activate are imperative to the host's primary reaction to a virus. Although the antiviral immune mechanisms are vast, viruses have evolved measures that permit them to evade virtually all of them. In other cases, viruses are able to manipulate the host's innate antiviral pathways in order to increase their own fitness. Some of these strategies will be discussed later.

NF- κ B

There are several methods by which NF- κ B can be activated, some of which were just described. Before activation, these NF- κ B transcription factors are located in the cell cytoplasm in complex with the negative regulator, I κ B (inhibitor of κ B). Depending on the initiation factor and subsequent signaling cascade, the NF- κ B pathway is classified as

canonical or non-canonical. Three of the NF- κ B transcription factors are associated with the canonical NF- κ B pathway: p65/RelA, c-rel, and p105/p50 (NF- κ B₁). These transcription factors form heterodimers and homodimers. As was discussed previously, this pathway can be activated by TLR3 or RIG-I stimulation. Additionally, during the innate immune response, inflammatory cytokines like Tumor necrosis factor alpha (TNF- α) and Interleukin-1 (IL-1) can activate the canonical NF- κ B pathway, while T-cell receptor (TCR) and B-cell receptor (BCR) stimulation activates the canonical NF- κ B pathway in the adaptive immune response (164).

After the canonical pathway is stimulated, the IKK (I κ B kinase) complex must be activated. This complex is made up of 3 subunits: IKK α , IKK β , and NEMO (NF- κ B essential modifier). A wide variety of signaling adaptors can activate the IKK complex in the canonical pathway (200). During TNF- α induced NF- κ B signaling, TGF β -activated kinase 1 (TAK1) is often responsible for activating the IKK complex (164). Regardless of the specific players involved, the IKK complex must be phosphorylated at two distinct serines for downstream activation to occur. Depending on the original stimulus, the catalytic subunits of the IKK complex (IKK α and IKK β) then phosphorylate I κ B or p105. This targets I κ B or p105 for degradation by the proteasome. Once degraded, the NF- κ B transcription factor dimers are no longer sequestered in the cytoplasm and thus move to the nucleus of the cell (Figure 3A). Once there, the dimer is able to activate its target genes which contain a conserved recognition site, known as a κ B site, in their promoter (91). This canonical pathway is involved in regulation of a many genes involved in the immune response. Pro-inflammatory cytokines, chemokines, inflammatory enzymes,

adhesion molecules, receptors, and apoptosis are some of the broader categories of genes controlled by this branch of the NF- κ B pathway (137; 210)

The remaining two members of the NF- κ B transcription factor family are associated with the non-canonical NF- κ B pathway. These two factors, RelB and p100/p52 (NF- κ B2), form a dimer much like the subunits in the canonical pathway. The alternative pathway is restricted to activation via certain TNF superfamily members, including, CD40 ligand and Receptor activator of nuclear factor kappa-B ligand (RANKL). Unlike the canonical pathway, this pathway requires NF- κ B-inducing kinase (NIK) to activate the IKK complex (200). IKK α is the IKK complex subunit responsible for continuation of the non-canonical pathway post-NIK activation. This is achieved by IKK α direct phosphorylation of p100 (193). In the non-canonical NF- κ B pathway p100 serves a role similar to that of I κ B in the canonical pathway. When IKK α phosphorylates p100, p100 undergoes selective degradation. This results in production of the p52 subunit which in complex with RelB, then translocates to the nucleus where this heterodimer acts as a transcription factor (193) (Figure 3B). The alternative NF- κ B pathway plays important roles in generation and maintenance of T-cells, maturation and survival of B-cells, and mediating secondary lymphoid organ development (153; 200; 221).

NF- κ B activation during virus infection

Activation of the canonical and non-canonical NF- κ B pathways occurs during virtually all types of viral infections. Some investigators have found that activation can help the host clear a viral infection while other investigators have shown that the

pathway's activation promotes viral fitness. Despite this variability, it is apparent that, as Hippocrates is noted for saying, "everything in excess is opposed by nature (172)."

Because NF- κ B activation promotes the transcription of genes that encode for many antiviral proteins, studies exist that exemplify the importance of the pathway in viral clearance. For the *Flavivirus*, Dengue virus (DENV), one group demonstrated that infection of *Drosophila* brains induced the NF- κ B response and that this induction aided in control of the infection (136). In other cases, NF- κ B can be important to developing the ideal antibody response. This is the case for Influenza A virus (IAV) whereby mice lacking RelB could clear infection but developed a poor humoral response (117).

Immunopathogenesis is a hallmark of many infections. Sometimes the NF- κ B response appears to serve a detrimental role. Rhinoviruses are the causative agents of the common cold. Mice deficient in p65 for example, have significantly decreased pro-inflammatory cytokines but have an interferon response that is sufficient for the host to control infection. This is a case which indicates blocking NF- κ B may be beneficial for disease progression in the host (18). Similar results were found in swine testicular and epithelial cell lines infected with Transmissible gastroenteritis virus (TGEV), a virus that causes disease in many sucking pigs (217). More examples, however, exist in which NF- κ B may mediate the immunopathogenesis but it has not been determined if blocking this pathway would result in an uncontrollable infection.

Experiments on EBOV have demonstrated that virus like particles (VLPs) expressing glycoproteins (GPs) lead to a TLR4-mediated NF- κ B response in human monocytic cells. Investigators determined that this induction is a major mediator of some of the pro-inflammatory cytokines produced and particularly of IL-6 (2). Later, more

evidence for a role in immunopathogenesis due to NF- κ B activation was produced. Reston virus is a non-pathogenic member of the *Ebolavirus* genus. Live Reston virus (RESTV) as well as VLPs containing the RESTV GPs, had a nearly inexistent TLR4 mediated NF- κ B activation in primary human macrophages. On the other hand, these experiments recapitulated the TLR4 mediated NF- κ B activation associated with the GPs from EBOV (166). Looking at human liver biopsies also allowed Hepatitis B virus (HBV) disease to be attributed, in part, to the NF- κ B response (231).

The role of the NF- κ B response can also change during infection. Human Papilloma virus (HPV) is a DNA virus which can cause cervical cancer. Early after infection, NF- κ B is important for HPV persistence. Later in infection however, it appears to be more involved in tumor progression. In both cases the NF- κ B pathway drives virus productivity but the manifestation is different (47).

Virus induced manipulation of the NF- κ B pathway

Viruses have evolved mechanisms to manipulate nearly every step of the NF- κ B pathway [Reviewed in (57)]. Regulation can be manipulated such that an increase in the activation of the pathway occurs. In other cases blocking or decreasing activation of the pathway is beneficial to the virus. These modifications can also affect apoptosis and cell cycle regulation. In all cases, the virus-mediated NF- κ B modifications are attempts to increase the fitness of the virus (90).

Use of κ B transcription factors is one mechanism by which viruses take advantage of the NF- κ B pathway. HIV-1 for example, contains two κ B sites close to the region where its genomic transcription begins. In this way the virus is able to drive transcription by taking advantage of p50-p50 or p50-p65 κ B dimers present in T-cells (90; 116).

In other cases, viruses encode for proteins that can directly interact with members of the NF- κ B signaling pathway. Human T-cell Leukemia/Lymphotropic Virus Type-1 (HTLV-1) encodes for an oncogenic protein, Tax, that is required for the virus to replicate and is also required for transformation of T-cells (5). Tax interacts with many members of the NF- κ B pathway. In the cytoplasm, the constitutive NF- κ B activation found in HTLV-1 infected cells is the result of constitutive phosphorylation of the IKK signaling complex. This occurs when Tax associates with NEMO, leading to degradation of I κ B α and I κ B β (84). The result is constitutive NF- κ B activation which inhibits cell cycle checkpoint regulation, DNA repair, and ultimately leads to transformation of infected T-cells (146).

Poliovirus also encodes for a protein that can directly act on proteins of the NF- κ B pathway. This protein, a protease referred to as 3C, is capable of modifying the NF- κ B response. Early in infection, NF- κ B signaling occurs through the canonical pathway. Later in infection however, 3C protease can cleave the p65-RelA dimer resulting in a truncated protein. In contrast to the Tax protein of HTLV-1, this protein results in inactivation of the NF- κ B pathway and acts as a way for the virus to evade one component of the host innate immune response (161).

In other circumstances, viruses will induce upregulation of the NF- κ B pathway as a way to aide in its replication (90). IAV protein M2 induces downregulation of G protein pathway suppressor 1 (GPS1) expression. This protein is a member of the COP9 signalosome which is involved in decreasing NF- κ B expression. This decrease in a negative regulator of NF- κ B means that the net NF- κ B activation is increased in the cell. This combined with the observation that cells with a downregulation of GPS1 have virus

with less active polymerases, suggests that the M2 mediated increase in NF- κ B activity is involved in promoting virus replication (202).

Diversity in Paramyxovirus induced NF- κ B responses

The viruses belonging to the paramyxovirus family have varying effects on the NF- κ B pathway as well as different mechanisms to manipulate it. Among this diverse family is: mumps virus (MuV), Sendai virus (SeV), measles virus (MeV), respiratory Syncytial virus (RSV), and the aforementioned Hendra virus (HeV) (169).

RNA encoding for the small hydrophobic protein (SH) is not found in all viruses in the family Paramyxovirus. SH is found in the Rubulavirus, Pnuemovirus, and Metapneumovirus genera. MuV, a member of the *Rubulavirus* genus, can inhibit NF- κ B activation through the action of its SH protein. After constructing a mutant MuV that lacks the SH protein, Franz et al. demonstrated that the SH protein decreases phosphorylation of IKK β and its downstream effectors. This ultimately leads to decreased translocation of p65 to the nucleus. They found that SH co-immunoprecipitates with the TNF- α receptor and suggested that this may be the mechanism by which the protein is able to decrease and thus evade the NF- κ B response (185).

SeV, a member of the genus *Respirovirus*, naturally infects mice and is often used as a representative paramyxovirus in laboratory studies as it cannot cause human disease. Investigators showed that accessory protein C of SeV is involved in Nitric oxide (NO) regulation in mouse macrophages. NO is produced by macrophages during SeV infection by the enzyme inducible Nitric oxide Synthase (iNOS). This production of NO helps the cell clear SeV. NF- κ B is involved in activating transcription of iNOS but cells infected

with viruses lacking the C protein of SeV had significantly higher NF- κ B production than those infected with WT SeV. This suggests that the C protein of SeV is preventing NF- κ B activation as a way to avoid production of the antiviral NO (66). NiV C protein is likely also involved in ameliorating the NF- κ B response as mutant viruses lacking the C protein sequence in their genome produced significantly less of several cytokines activated by the NF- κ B pathway (149).

Data on the morbillivirus, MeV, provide examples of both pro and antiviral effects of NF- κ B pathway activation. Co-immunoprecipitation experiments showed the c-terminus of the MeV V protein binds to p65. This binding prevents NF- κ B dimers containing p65 from entering the nucleus and activating κ B-containing promoters (190). Still, MeV replication is enhanced by activation of NF- κ B. The increased activation is dependent on a MeV-mediated increase in sphingosine kinase 1 (SK1) (215). There is likely a balancing act whereby too much NF- κ B is harmful to the virus, so it has evolved a mechanism to slow it down. At the same time, the virus evolved such that its replication is improved by the host's natural NF- κ B response.

It has been shown that HeV replication also benefits from NF- κ B activation. miRNA-146a is elevated in the blood of ferrets and horses that have been infected with HeV. This miRNA, *in silico*, could target the mRNA for RNF11, a negative regulator of the NF- κ B response. siRNA was used to knockdown RNF11 which in turn, led to an increase in activation of NF- κ B. This increase resulted in higher HeV titers (198). The henipaviruses HeV and NiV cause severe pathogenesis in humans, and so it is not surprising they would have evolved mechanisms to thrive under pro-inflammatory conditions (173).

It has been well noted that patients with RSV, a member of the genus *Pneumovirus*, exhibit pathogenesis mediated by the immune response. As such, NF- κ B activation is especially robust in the airway, where most of the immunopathogenesis is found. Recently, it was shown that mice lacking RelA in non-ciliated epithelial cells have decreased disease severity scores as well as decreased cytokine production and neutrophil inflammation. Though disease is ameliorated when NF- κ B is decreased, viral replication is increased creating a difficult situation to address, immunopharmacologically (14).

The pro-inflammatory cytokine IL-1 β is also responsible for the airway immunopathology attributed to RSV. This cytokine is processed by the NLRP3 inflammasome and requires a two-signal activation (187). It was shown that activation of the NF- κ B response was required for the two-signal inflammasome activation to occur during RSV infection of macrophages (192).

A20

A20, also known as tumor necrosis factor alpha inducible protein 3 (TNFAIP3), is a 90 kDa protein with two domains. The N terminus contains an ovarian tumor (OTU) domain with deubiquitinating properties, while the C-terminus contains 7 zinc fingers (ZnF). One of these zinc fingers, ZnF4, contains a ubiquitin ligase domain (Figure 4) (69; 224). Generally speaking, A20 plays a role in preventing over-activation of the immune response. Mice without A20 die young due to inflammation-mediated organ failure (123). In humans, mutations rendering A20 non-functional is reported alongside allergy, tumor formation, and autoimmune disorders (4; 138; 140; 177).

Ubiquitin, a 76 amino acid peptide, is often used in immune signaling to control signaling pathways via post-translational modification. The addition or removal of ubiquitin signals the cell to perform a particular action. Ubiquitination occurs at lysine residues of the target protein. Ubiquitin is often added in the form of a polymeric chain which can serve varying functions, based the configuration in which it is added. New ubiquitin peptides can be added to lysines of the previously added ubiquitin at position 48 (K48) or position 63 (K63) (as well as at other lysines, which will not be discussed here) (171). K48 linkages target the protein to undergo proteasome-mediated degradation (211). Proteins with K63 linked ubiquitin chains are not usually proteasome directed. These proteins typically act as scaffolds for other proteins with ubiquitin binding domains (UBD) and as such drive the formation of multi-protein complexes (209).

As mentioned, The N-terminus of A20 contains an OTU domain which has deubiquitinating activity and as such, can remove K63 linked ubiquitin as a mechanism for disrupting a signaling pathway (69). A20 ZnF4, mentioned above, is able to add K48 linked ubiquitin chains as a mechanism for degrading specific target proteins (224).

In NF- κ B signaling

The transcription of A20 is activated by NF- κ B. In turn, this protein then acts as a negative regulator of NF- κ B and serves to dampen the pro-inflammatory response (224). Thus, these two proteins act in a negative feedback loop.

A20 associates with the IKK complex where it is phosphorylated by IKK β (98). A20 then disrupts signaling proteins upstream of the IKK complex to down-regulate the NF- κ B response (28; 224). One of these signaling proteins, Receptor Interacting Protein 1 (RIP1) is recruited after TNF α receptor 1 (TNFR1) is stimulated by its ligand. RIP1

requires K63 ubiquitination in order to transmit the signal downstream to the IKK complex (67). A20, utilizing its OTU domain, removes these K63 linked ubiquitin from RIP1 and then adds K48 linked ubiquitin to it. In the first step the signal transduction is terminated and in the second step, the signal transducing protein is targeted for degradation. This enzyme's dual activity therefore, terminates NF- κ B activation (224).

In interferon signaling

A20 is also involved in interferon (IFN) signaling. Interferons, alongside NF- κ B, promote an antiviral state in the cell and are regulated by interferon regulatory factors (IRFs) (89). The mechanisms behind A20's regulation of interferons is much less understood. One group demonstrated that a lack of A20 expression led to a decrease in virus mediated interferon activation and its overexpression led to an inhibition in activation of the IRF3 through RIG-I. It was the ZnF7 domain of A20, however, that mediated these responses. This suggests that targeting RIG-I for degradation is not the mechanism by which interferon activity is decreased. Once activated by double stranded RNA, RIG-I signals through to the mitochondrial antiviral signaling protein (MAVS). The same group also demonstrated that A20 inhibited MAVS signaling but not through a degradation-mediated mechanism (132). More work remains to determine the exact mechanism by which A20 mediates a decrease in the antiviral immune response.

During virus infections

Viruses evolve mechanisms to both manipulate the NF- κ B response or to use its activation to their advantage, as was described previously. A20 is a regulator of NF- κ B and examples exist where viral proteins have evolved to modify A20 expression. The role of A20 in virus infections is still not well understood and conflicting information exists

about its roles. In a human lung cell line, for example, the non-structural protein NS1 of Influenza A virus (IAV) induced a robust A20 response. In fact, when A20 was overexpressed in these cells, the replication of the virus increased and the IRF3 pathway and ISGs were decreased. This suggests A20 may be involved in the negative regulation of the antiviral response

Another group, however, demonstrated that mice lacking cell type specific A20 had less severe disease and better outcomes (142; 143). From studies where the A20 deletion was specific to myeloid cells, it was clear that A20 mediated the inflammatory response during IAV infection. When there was increased inflammation due to the loss of the negative regulator, the pro-inflammatory response was more robust. This helped the mice survive a lethal infection (143). In another paper however, this same group found something different. First, despite what was seen in the human lung cell line mentioned previously, virus replication was not affected by A20 deletion specific to airway epithelial cells. The absence of a negative regulator of the pro-inflammatory response would suggest an overall more robust immune response. In fact, this is what the group found in myeloid cells. These experiments in mice with airway epithelial cell specific A20 deletion, however, showed the opposite. The adaptive immune response and monocyte recruitment to the lungs was dampened in mice with the A20 deletion. This resulted in less lung damage and better infection outcome (142).

Investigations into the role of A20 during Hepatitis C virus (HCV) have also been conducted. In this case, one group found that A20 induction during HCV infection was likely mediated by the proteasomal degradation of upstream inhibitory factor 1 (USF-1). USF-1 is responsible for inhibition the A20 promoter. When A20 was overexpressed in a

hepatocarcinoma cell line it enhanced the production of HCV viral proteins via internal ribosome entry site (IRES) stimulation. This suggests A20 may play a role in increasing initiation of virus translation (125).

In paramyxoviruses, investigators showed that SeV induces a robust response at the protein level in monocytes but not in HEK cells (218). This was recapitulated with MeV. In fact, MeV phosphoprotein alone, was reported to be sufficient to induce A20 (230). In vivo, A20 was one of 13 significantly upregulated genes in peripheral mononuclear cells of MeV infected children (240). In all, a role for A20 exists during virus infection but the exact role is unclear and may be both virus and cell type specific.

IMMUNOPATHOLOGY IN ZOONOTIC DISEASE

Many highly pathogenic and ultimately lethal virus infections cause strong immune responses sometimes referred to as a “cytokine storm.” In these cases the host’s attempt to clear the pathogen ultimately leads to the pathogenesis. This immune system-mediated disease is termed immunopathogenesis and is found during EBOV, HCoV, Hantavirus, HeV, and IAV, to name a few (19; 58; 95; 133; 236). The classic example is IAV.

During IAV infection, epithelial cells and alveolar macrophages mount the initial reaction to virus infection (133; 205). Many cytokines are then released; most prominent are interferons. Inflammatory cell infiltration and apoptosis then begin to contribute to tissue damage (133). As more inflammatory cells continue to be recruited in an attempt to clear the infection, the cytokines they produce can begin to enter the blood stream and lead to multi-organ inflammation (207). The most severe cytokines storms from IAV are

from highly pathogenic strains such as H5N1 and are rarely seen in seasonal influenza infections (22; 99).

Using bats in comparative immunology

By studying bats, reservoirs of many viruses, we can begin to elucidate differences in their immune responses that may contribute to their lack of pathogenesis during infection. Immunomodulatory therapy is already used during IAV infections in humans (133). If we can better understand what the best target for immunomodulation is, we could perhaps apply this to many of the recently emerged viruses. Using bats as a comparison then, provides a unique way to determine which immune pathways should be targeted during emerging virus infections (133). Investigators have already shown, for example, *Pteropus alecto* cell lines infected with Australian Bat Lyssavirus (ABLV) are more resistant to cell death (119). A comparative study of 2 bat genomes with 8 mammalian species showed certain receptors associated with natural killer cells were completely absent in the bat genomes. This study also identified several genes associated with the DNA damage control and the innate immune responses that were under positive selection (234). Still, not many direct human to bat comparative studies that involve pathogenic viruses exist.

What makes bats unique?

Bats have unique characteristics that could tie into the control of their immune system. First, bats are the only mammals capable of sustained flight, which use this as the primary means of movement. Flight in bats requires a 3-5 fold increase in metabolism compared to any other exercise among animals approximately the same size (144). This means for a large portion of their lives, bats are in a high metabolic state.

Most of the adenosine triphosphate (ATP) used to power flight comes from oxidative phosphorylation (OXPHOS) so it is unsurprising that evolution within OXPHOS genes was what allowed the evolution of flight to occur (50). This evolution occurred at the same time as important immune system adaptations (234). This is probably because metabolic stress leads to a pro-inflammatory state (34). For example, exercise induced OXPHOS and flight can lead to the production of reactive oxygen species (ROS) (31; 52; 74). ROS are able to activate the inflammasome and lead to the production of the pro-inflammatory cytokine IL-1 β (192).

Another characteristic of bats that may have affected the evolution of their immune system is their roosting habits. Bats live in very close proximity to one another throughout their life. Some *Pteropus* roosts reach 20,000 individuals (80). These dense living conditions combined with the bat's capacity for flight and long distance travel means they are continuously in contact with many different antigens. Mounting an immune response is energy expensive and as such bats would need to have evolved mechanisms to avoid a hyper-inflammatory state caused by the above factors (56).

Available tools

There is good evidence that unique immunological characteristics derived during the evolution of flight contributed to bats' ability to avoid pathogenesis during many viral infections (147). Still, comparative immunology in bats remains relatively unstudied. This is largely due to the inherent complications involved when studying a non-traditional laboratory animal. Very few locations exist where *in vivo* infection experiments in bats can be conducted. This means almost all initial work must be done *in vitro* with cell lines derived from bat tissue. Although fibroblast cell lines from several

species do exist, most of them are immortalized using hTert or SV40 antigen oncogenic transformation (17; 46; 86; 121). *Pteropus alecto* fibroblasts that were immortalized by serial passaging also exist and these provide a non-transformed alternative (unpublished data). Even so, it would be beneficial to have immune cells to work with.

Primary immune cell work from bats exists but is limited. Obtaining primary immune samples from bats it requires either terminal procedures or provides very limited sample. When primary bone marrow from *P. alecto* was obtained, one group was able to derive dendritic cells (DC) and macrophages. Though this resulted in cells with monocytic morphology and function, it also required significant production of recombinant *P. alecto* growth factors and cytokines (238). A bat-mouse model was currently developed in which NOD-scid IL-2R^{-/-} (NSG) mice were transplanted with bone marrow from *Eonycteris spelaea*. Immune cells expanded post-transplantation and no clinical abnormalities were seen in the mice (232). This has the potential to serve as a possible *in vivo* model of bat immunology.

MACROPHAGES

Multipotent progenitor cells reside in the bone marrow. Specific transcription and growth factors allow these multipotent hematopoietic cells to differentiate to a common lymphoid or common myeloid progenitor. The common lymphoid progenitor will give rise to specialized lymphocytes like T and B-cells. Monocytes, as well as other innate immune cells like neutrophils, however, are derived from the common myeloid progenitor. Monocytes that have taken on a functional and definitive role are typically known as macrophages (223) (Figure 5). Several classification systems for defining different types of monocytes and macrophages exist. These classifications can be based

on the expression of certain surface receptors and/or based on the function the macrophage will perform (109).

One classification system separates macrophages into subsets known as M1 (classically activated) and M2 (alternatively activated) (Figure 6). Macrophages that are responding to pathogens are typically polarized to be M1 macrophages, while those that are involved in tissue repair and homeostatic clearance of apoptotic cells are polarized to be M2 macrophages (162; 165). M1 macrophages are vital for large scale production of pro-inflammatory cytokines and ROS which help them to recruit more pro-inflammatory cells and to activate genes which will aide in clearance of the pathogen. This is in contrast to M2 macrophages, which are anti-inflammatory in nature (135). M1 macrophages can also function as antigen presenting cells. When activated by IFN γ , for example, macrophages can upregulate expression of major histocompatibility complex II (MHC II) and are therefore able to present antigens to T-cells (183). Thus, they have a role in bridging the innate immune response with the adaptive immune response. The importance of macrophages during virus infections, specifically in the context of NF- κ B activation, was exemplified in many specific examples discussed earlier.

Oncogenes raf and myc

A proto-oncogene is a gene that has the potential to cause cancer. These genes are typically involved in cell division or growth. When these genes are mutated or over expressed they can lead to uncontrolled proliferation and malignancy (44). When attempting to create an immortalized cell line from bats, or in any species, the goal is infinite cycles of cell proliferation. For this reason, oncogenes are purposefully manipulated to induce uncontrolled cell proliferation during cell line production. Some

viruses have natural oncogenic potential. The most direct examples of this are viruses that contain their own oncogenic proteins. One example of this is HPV's E6/E7 proteins. They are able to disrupt several important tumor suppressor genes and can therefore lead to malignancy. Many retroviruses also have oncogenic properties (75).

The proto-oncogene Raf exists in the host as a family of kinases that initiate the ERK pathway. This pathway ultimately leads to growth, progression of the cell cycle, and proliferation (64). Overexpression of Raf is associated with several human cancers and is widely studied. A murine sarcoma virus and an avian carcinoma virus, both retroviruses, are where raf was originally described (225). The viral homolog of Raf is referred to as v-Raf.

Myc is another proto-oncogene found in many animals, with a viral homolog called v-Myc. In its proto-oncogenic state, myc is activated by many signal transduction pathways including Wnt and ERK. Upon activation it acts as a transcription factor for genes involved in cell proliferation and growth (48). This viral oncogene can transform some cell types on its own but can also work in tandem with other oncogenes (122).

v-Raf and v-Myc have been used together in mice in vivo in early studies of oncogenic transformation. Investigators constructed a virus (J2) using pieces of three other myc, and mil (avian raf homolog) containing viruses. These experiments showed that the virus expressing both oncogenes together caused neoplasia in newborn mice (179). Later, they used this virus to infect bone marrow and they found that this resulted in selective transformation of macrophages (25; 26). Similarly, they were able to transform microglia in primary culture (24). It appears as though together, these

oncogenes immortalize myeloid cells (25). This may be because the two oncogenes affect different components related to growth regulation (45).

PURPOSE OF RESEARCH AND PROPOSED SPECIFIC AIMS

Bats are key reservoirs for many emerging RNA viruses (167). Cedar virus (CedV) is a non-pathogenic henipavirus and is used as a model for the deadly Hendra (HeV) and Nipah (NiV) viruses (118). In *Pteropus sp.* bats, these viruses cause no disease. In humans and economically relevant livestock however, they manifest as severe disease and are burdensome biothreats (81). Since the initial outbreaks of HeV and NiV, new viruses continue to emerge from bats. In each instance, no clear pathogenesis is seen in the bat species that acts as the viral reservoir. In humans, however, disease is often severe (16). As more of these outbreaks occur a clear pattern is emerging. The severe disease in humans caused by zoonotic viruses is associated with robust inflammatory responses mounted in an attempt to clear the infection (236). An attractive hypothesis is that bats may be asymptomatic because of a unique ability to control this immunopathogenesis.

One of the biggest obstacles that exists in the field of bat immunology and comparative immunology in general, is the near absence of immune cell lines for *in vitro* work. Macrophages are important for the secretion of many pro-inflammatory cytokines, clearance of the pathogen, and recruitment of other classes of inflammatory cells (207). The ability to study this immune cell in almost all zoonotic reservoirs as well as the downstream spillover hosts is key. Yet no system exists that allows for broad-species immortalization of macrophages. Without this, comparative macrophage studies can only be done *in vivo* or in primary cells. Uncovering the role macrophages play in

immunopathogenesis during zoonotic viral infections in humans and other important host animals will be easier to elucidate if construction of comparative reservoir cell lines is feasible. Investigations in these cell lines could then lead to the understanding of immunopathogenesis patterns seen in many zoonotic viral infections and lead to immunotherapeutics that are applicable to multiple diseases.

Many have proposed that bats may be unique in their ability to respond to pro-inflammatory signals in part, because of responses that they may have evolved in order to manage the metabolic stress of flight (163). The byproducts of this physiological process are pro-inflammatory in nature and thus tightly controlled inflammatory regulation must exist. The transcription factor NF- κ B promotes expression of many genes involved in inflammation and cell survival. With NF- κ B having such global effects, there is good reason to study the signaling pathway that leads to its activation. What is more, this pathway mediates inflammatory responses found in macrophages but also in non-immune cells.

A20 is an important negative regulator of this pathway and existing research shows it serves to down-regulate the pro-inflammatory response (224). Robust activation of A20 in *Pteropus alecto* kidney cells infected with Hendra virus was observed previously. In this transcriptomic to proteomic study, KEGG (Kyoto encyclopedia of genes and genomes) pathway analysis demonstrated that genes associated with the NF- κ B pathway were significantly upregulated during infection (226). This is more evidence that indicates a potentially important role for NF- κ B and A20 in regulation of the pro-inflammatory response during zoonotic virus and, specifically, HeV infection. Because Hendra virus is a BSL-4 (Biosafety level 4) pathogen we propose to examine the role of

NF- κ B and A20 during henipavirus infection by using the non-pathogenic CedV as a model. To address these deficiencies and observations we proposed the following specific aims:

Specific aim 1: Create a method for macrophage immortalization with comparative immunology potential.

Subaim 1.1: Construct a safe and less complicated J2 based lentivirus

Subaim 1.2: Develop a protocol to immortalize mature macrophages using the improved virus

Subaim 1.3: Characterize the functionality and cell type of the resulting cell line made with the J2 based virus

Specific aim 2: Examine the roles of the NF- κ B signaling pathway and its negative regulator A20 during CedV infection in humans and *P. alecto*.

Subaim 2.1: Determine if CedV induces the NF- κ B response in humans and *P. alecto* cell lines

Subaim 2.2: Determine how A20 expression is induced during CedV infection

Subaim 2.3: Characterize the phenotype of CedV induced A20.

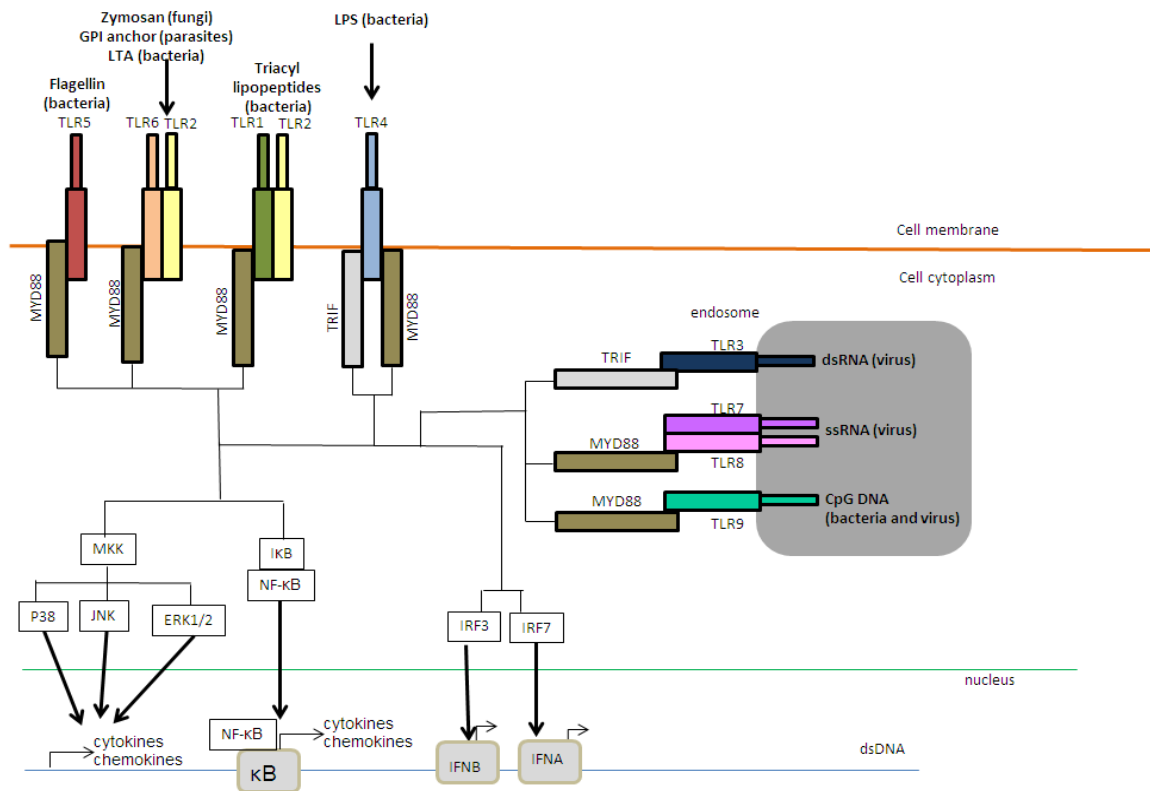


Figure 1: Toll like receptors and their signaling pathways

TLRs recognize different PAMPs. TLR 2 can interact with both TLR 1 or TLR6. When TLR2 interacts with TLR6 they recognize bacterial lipoteichoic acid (LTA), the GPI anchor in some parasites, and zymosan found in yeast, while when TLR2 interacts with TLR1 they recognize triacyl lipopeptides found on bacteria. TLR5 recognizes flagellin and TLR4 recognizes lipopolysaccharide (LPS), both found on bacteria. TLR3, 7/8 and TLR9 are found within cytoplasmic endosomes and recognize double stranded RNA (dsRNA), single stranded RNA (ssRNA) and CpG DNA, respectively. When TLRs located in the cell membrane signal through MYD88, induction of the NF- κ B and MKK pathways can occur leading to the induction of cytokines and chemokines. While they can still signal to NF- κ B under certain scenarios, TLRs 3 and 4 when signaling through TRIF, activate the interferon regulatory factor 3 (IRF3) pathway. This activation results in the production of IFN β . Generally, TLR7/8 and TLR9 signal through MYD88 but activate the IRF7 pathway leading to the production of IFN α (7; 40).

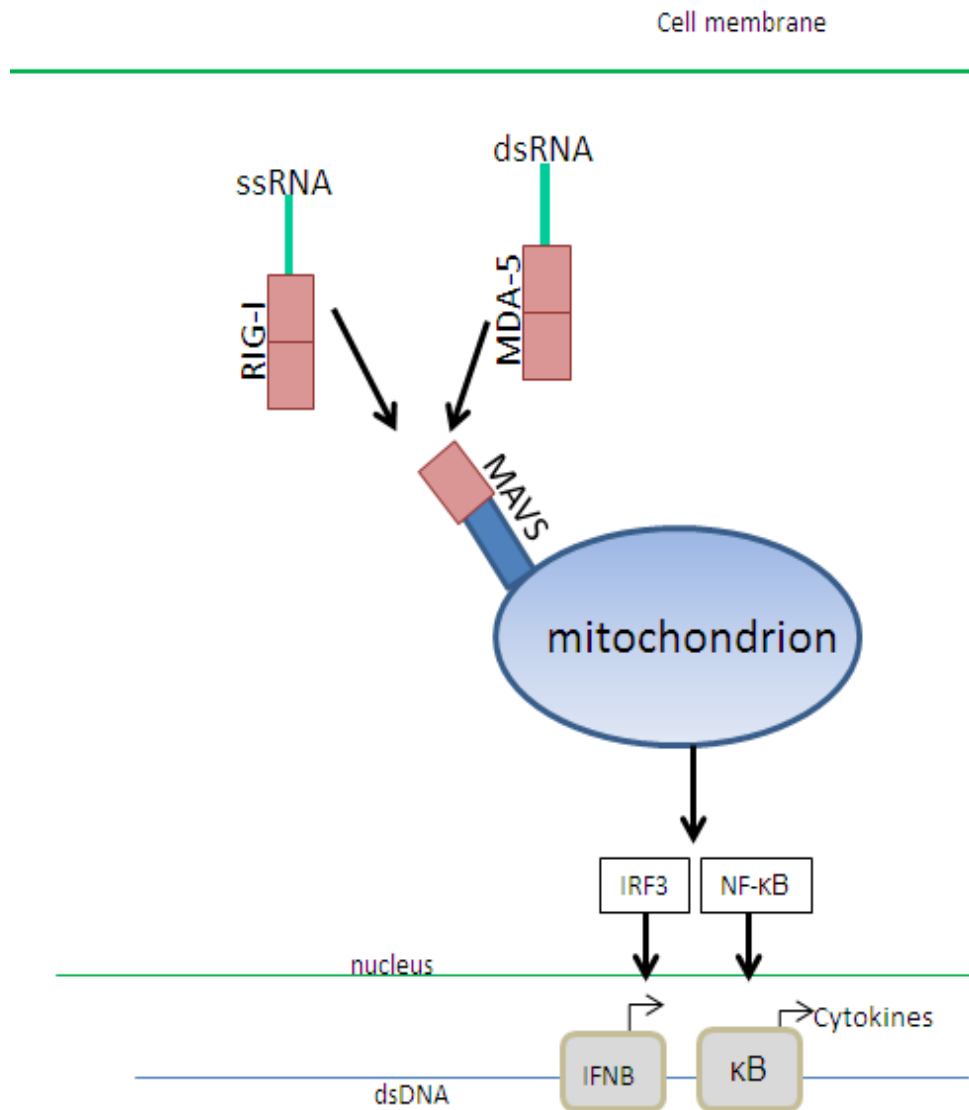


Figure 2: RLR signaling

RIG-I and MDA-5 are cytoplasmic PRR that recognize ssRNA and dsRNA, respectively. Both of these RLRs contain 2 CARD domains (red rectangles). These CARD domains interact with the CARD domain of mitochondrial associated MAVS. A signalosome is formed and through various adaptor proteins either the IRF or NF- κ B pathways are activated. This results in activation of IFNs as well as a myriad of other pro-inflammatory cytokines.

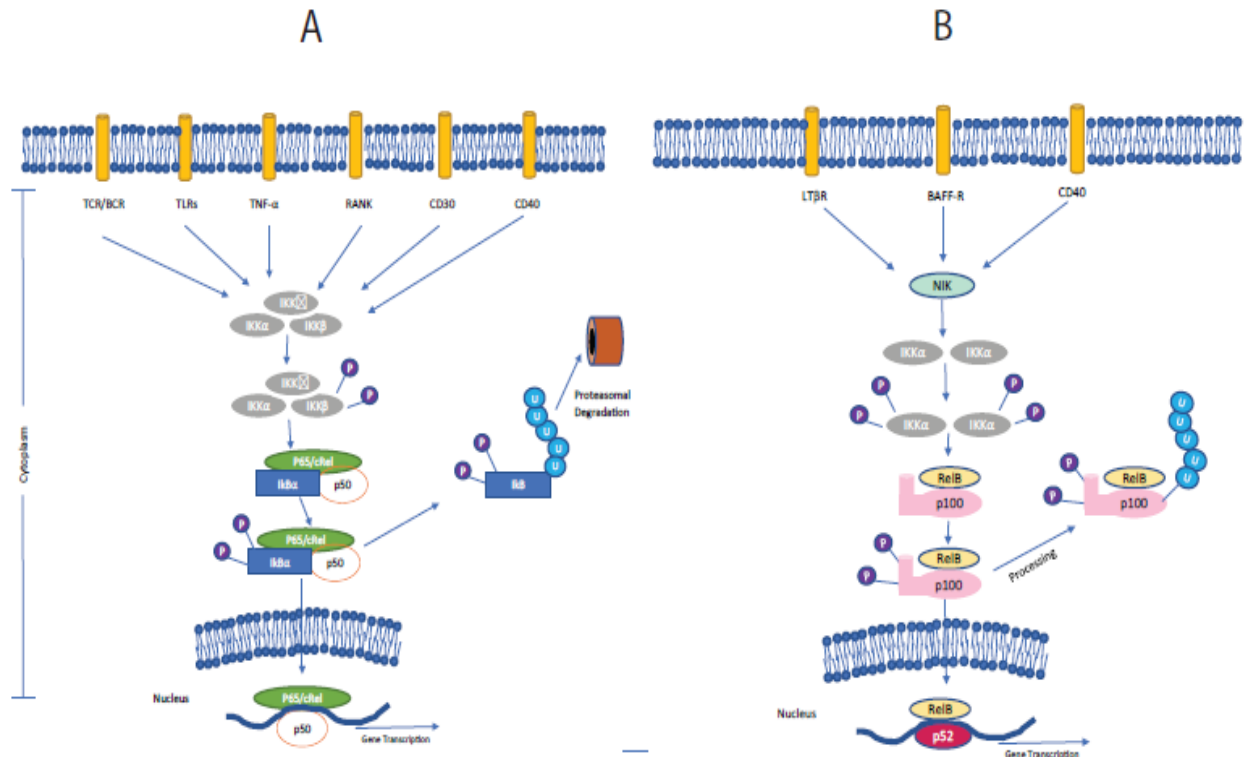


Figure 3: Activation of the canonical and non-canonical NF-κB pathways.

- (A) The canonical NF-κB pathway. Upon receptor stimulation, adaptor proteins are recruited to the cytoplasmic domain of the protein. These signaling adaptors eventually lead to activation of the IKK complex, which is made up of IKKα, IKKβ and NEMO (seen as gray ovals). Activation of IKK is followed by phosphorylation of IκB (blue rectangle) and its ubiquitin mediated proteosomal degradation. This releases the NF-κB subunits (p65 and p50 depicted) so they may translocate to the nucleus of the cell where they act as a transcription factor of pro-inflammatory, apoptotic, and cell cycle genes. (B) The non-canonical NF-κB pathway. Receptor stimulation in this pathway results in activation of NIK (blue oval). Once activated, NIK phosphorylates and activates IKKα (gray oval). IKKα then can phosphorylate p100 (in pink) which like IκB, leads to its proteosomal degradation. This releases the NF-κB dimer (p52/RelB depicted) and it translocates to the nucleus and acts as a transcriptional activator (134).

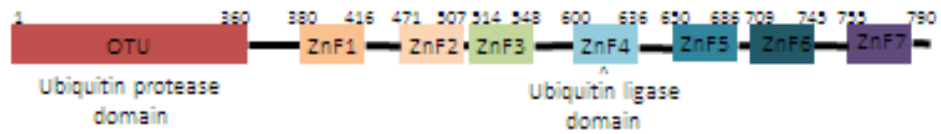


Figure 4: Structural domains of A20

The protein A20 is made up of 8 distinct domains. The N-terminus contains an ovarian tumor domain and acts as a deubiquitinase. The c-terminal domains contain 7 zinc fingers (ZnF). These ZnFs allow A20 to form complexes with other proteins. The fourth zinc finger is a ubiquitin ligase and thus an important catalytic domain (213).

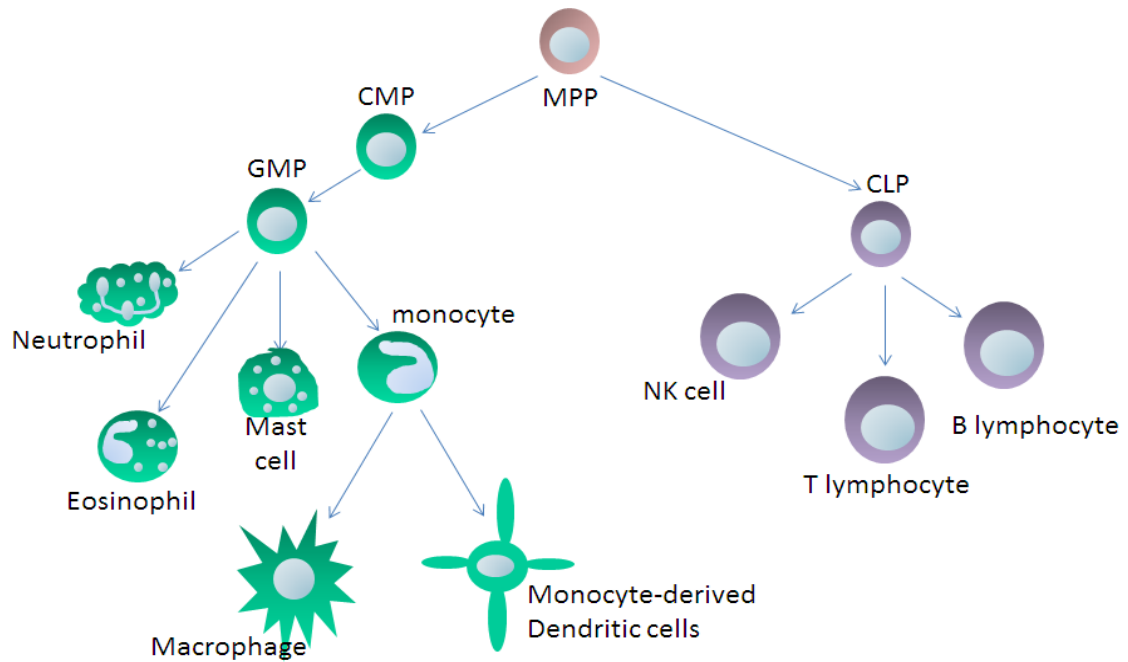


Figure 5: Multipotent progenitor differentiation

Multipotent progenitor cells (MPP) give rise to common lymphoid progenitors (CLP) and common myeloid progenitors (CMP). CLP can then give rise to lymphocytes like NK cells, T-lymphocytes, and B-lymphocytes. CMP can give rise to the progenitor responsible for red blood cell production (not shown) as well as granulocyte-monocyte progenitors (GMP). The specialized myeloid cells are differentiated from the GMP and include neutrophils, eosinophils, mast cells, and monocytes. Monocytes are able to differentiate into macrophages and monocyte-derived dendritic cells (223).

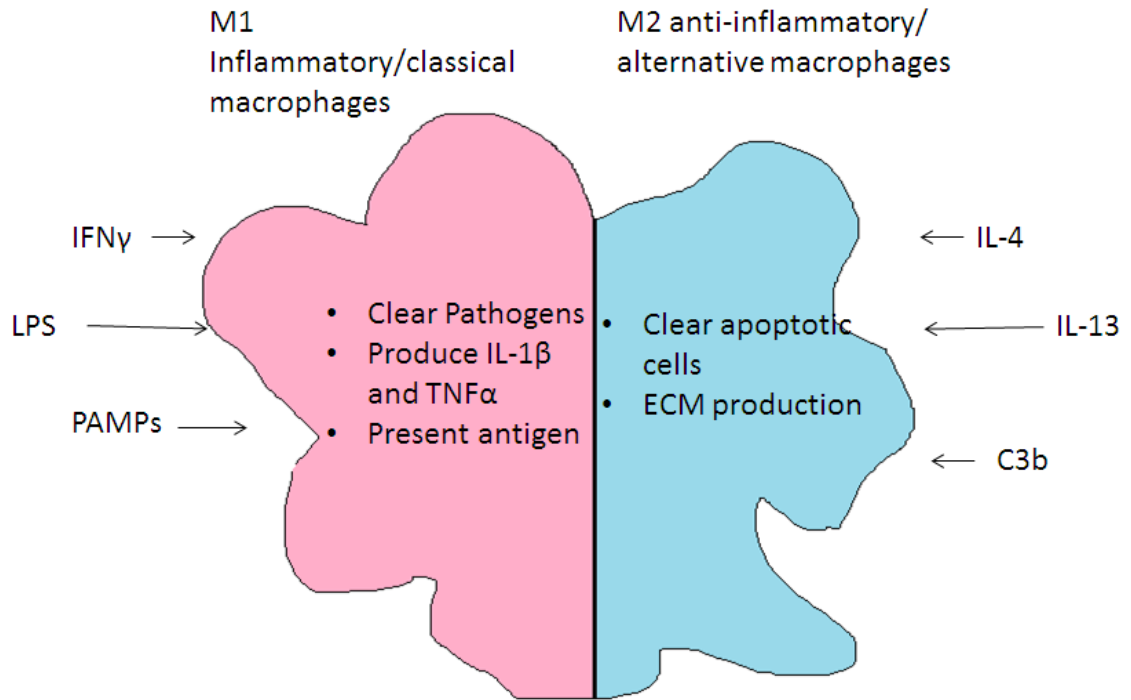


Figure 6: M1 versus M2 macrophages

Macrophages can be grouped as M1 or M2. M1 macrophages are inflammatory or classically activated macrophages. Their activation occurs when a monocyte is exposed to PAMPs like LPS and to IFN γ production. On the other hand, M2 or alternatively activated macrophages, have responded to cytokines like IL-4, or IL-13. Once activated, M1 macrophages are involved in clearing infections, contributing to the inflammatory response by producing cytokines like IL-1 β and TNF α , and presenting antigen to T-cells. M2 macrophages, however, are involved in clearing apoptotic cells and in wound healing by contributing to extracellular matrix production (27; 155).

CHAPTER 2: MATERIALS AND METHODS

AIM 1

Cell line culture

HEK 293T cells and immortalized raf/myc macrophages were maintained in DMEM (Dulbecco's Modified Eagle Medium (Gibco Laboratories; Gaithersburg, MD, USA) supplemented 10% HyClone[™] Cosmic Calf[™] Serum (CCS) (Thermo Fisher Scientific), 0.5% Penicillin/streptomycin (Gibco), and 1% L-glutamine (Thermo Fisher Scientific) to make complete cell culture media (DMEM-10). Cells were maintained in incubators at 37°C with 5% CO₂.

Construction of pWPT1-huHox11

WPRES of pWPT-GFP (gift from Dr. Didier Trono) was amplified using 5'-GGTACCGAGCTCTG CAGAATTA-3' and 5'-CTAGTCGACACTCATTAGCGGCCGCATCTTCAGATGAGATCTCCGATAATCAACCTCTGGATTACAAA-3' followed by 5'-GAAGGATCCTTAACCGGTAAGTGTGTTTAACTTAATTAACAGGCCGGCCTAGTCGACACTCATAGCGGCCGCATCTT-3' and 5'-GAAGGTACCAGAATTAATTCTGAAGCTTGAGCGAAT-3'. Using BamHI and KpnI, the resulting WPRES PCR product was subcloned into pWPT-GFP which resulted in pWPT1. pWPT1 was cut with PmeI and BamHI and pCMV6-human cDNA Hox11 (Origene RC224215) was cut with EcoRV and BamHI. The resulting linearized vector and huHox11 cDNA was ligated and resulted in pWPT1-huHox11.

Production of pWPT1-vRaf-T2A-v-Myc

vRaf was amplified from the original J2 plasmid using 5'-GGAGGTACCAGATC TCCACCATGCCCCCTCCGCACAGGAGGAAA-3 and 5'-GGAAAGCTTAAAAACAG GCAGTCTTGTGGATGT-3'. The resulting PCR product was subcloned into pBS-T2A using KpnI and HindIII to make pBS-vRaf-T2a. v-Myc was amplified from the original J2 plasmid using 5'-GAAGGATCCATGCCGCTCAGCGTCAGCCTCCCCAGCAAGA A-3' and 5'-GGTTCTAGATATCTTCCTATGCACGAGAGTTCCTTA-3 and cloned into pCRTM4-TOPO[®] TA vector (Thermo Fisher Scientific). The v-Myc fragment was cut from the topo vector using XbaI and BamHI and cloned into pBS-vRaf-T2A cut with the same enzymes. This resulted in pBS-vRaf-T2a-v-Myc. The vRaf-T2A-vMyc fragment was cut from the pBS backbone using EcoRV, BglII, and ScaI and then subcloned into pWPT1 linearized with BamHI and PmeI.

Production of retroviruses

6 well plates were coated with 100 µg/mL of poly-D-lysine (Sigma). HEK cells were seeded at 4×10^5 24 hours before transfection. pWPT1 with oncogenic insert was used in combination with the Lentiviral packaging plasmids pMD2.G and pCMVR8.74 (gifts from Dr. Didier Trono) were co transfected by modified calcium-phosphate DNA precipitate formation as previously described (106). Supernatants were harvested 24 and 48 hours post-transfection. Viruses were titered by p24 ELISA (Clontech) as described in the manufacturer protocol.

Primary cell isolation and initial culture

All primary white blood cell cultures were maintained in non-tissue culture treated plates or in bacterial petri dishes. C57BL/6J mice were first killed by cervical

dislocation and soaked in 75% ethanol. When multiple mice were used, cells were pooled. Cells were incubated at 37°C in 5% CO₂.

Bone marrow

Muscle was removed from femurs and the ends were clipped off. Marrow was then flushed from bones and tips with PBS using a 25.5 gauge needle. Cell suspension was then passed through a cell strainer. Cells were pelleted and then run on lymphocyte separation medium (LSM; Corning) as described by the manufacturer. The white blood cell (WBC) layer was removed and washed 2 times in PBS. Resulting cells were cultured in DMEM-10 supplemented with GM-CSF from a cell line supernatant diluted to 1X or in Iscove's Modified Delbucco's Medium with 10 ng/mL of IL-3 (R&D systems) and 100 ng/mL stem cell factor (R&D systems), and 2 ng/mL IL-6 (R&D systems) until transduction. *P. alecto* bone marrow was obtained from CSIRO Canberra, Australia and was cultured in DMEM-10 supplemented with 1x GM-CSF or 1x M-CSF (both from supernatant from producer cell lines) for 7 days.

Spleen

Spleens were removed and cut into small pieces with sterile dissection scissors. The pieces were incubated in DMEM-10 supplemented with 1 mg/mL of collagenase D (Worthington) at 37°C for 30 minutes. Tissue was then passed through a cell strainer, pelleted, and run on LSM as above. WBC layer was isolated and washed 2 times and then cultured in DMEM-10 supplemented with 1X GM-CSF.

Blood

Blood was obtained by a terminal cardiac puncture and layered directly onto LSM. WBC layer was isolated and washed two times and then cultured in DMEM-10 supplemented with 1x GM-CSF.

Retroviral transduction.

The night before transductions were to be performed, plates were coated with 50 µg/mL of RetroNectin reagent (Takara) and stored at 4°C. On the day of transduction virus-containing supernatant was added to the coated wells to an MOI (Multiplicity of Infection) ≥ 5 . Plates were spun at 1000×g for 90 minutes at 32°C. Plates were incubated for an additional 2.5 hours at 32°C. Virus supernatant was then removed and 2-4 day *ex vivo* cell cultures were added directly to virus coated plates. Original culture conditions were maintained. Cells were incubated in these plates for 48 hours at 37°C with 5%CO₂.

Post-transduction primary cell culture

Cells that were transduced with pWPT1-huHox11 were maintained indefinitely in IMDM media with IL-3 at 2.5×10^5 cells/mL. Cells transduced with a raf/myc virus were cultured in DMEM-10 with 1x GM-CSF for an additional 7-8 days at which point the media was removed and replaced with DMEM-10 without GM-CSF. After an additional 2 weeks of culture, CytodexTM 1 beads (GE Health Sciences) prepared according to the manufacturer's protocol were added to a final concentration of 200-400 µg/mL.

Phagocytosis Assay with pHrodo *S. aureus* BioParticles

Pteropus alecto bone marrow derived phagocytic were grown on coverslips in 6 well plates for 7 days in media supplemented with M-CSF at 1X from a producer cell

line. They were either pretreated with treated with 10 μ M cytochalasin D or mock treated for 30 mins. After, 1mg/mL of pHrodo *S. aureus* Bio-Particles in DMEM-10 was added directly to the coverslips for 1 hour at 37°C. The cells were fixed in the plates using 4% paraformaldehyde and mounted in Fluoromount-G Mounting medium (SouthernBiotech).

Murine macrophages were seeded on coverslips at 2.5×10^5 . 24 hours later cells were pre-treated with 10 μ M cytochalasin D (Bio-Mol, Cat# T-109) or mock pre-treated with DMSO in DMEM-10. Media was then was removed and pHrodo *S. aureus* Bio-Particles at 1 mg/mL in DMEM-10 were added directly on top of the coverslips. Cells were incubated at 37°C for one hour and then fixed in 4% paraformaldehyde and mounted in Fluoromount-G Mounting Medium (SouthernBiotech).

Interferon Stimulation

Raf/myc immortalized macrophages were seeded in 6 well plates at 2.5×10^5 . Adherent cells were then treated for indicated times with recombinant murine IFN γ (R&D Systems) at 100 U/mL and then prepared for flow cytometry as described below. Anti-I-A/I-E (2G9) was used for staining.

Flow Cytometry

For surface receptor characterization, Fc receptors were first blocked for 10 minutes with mAb 2.4G2 (hybridoma supernatant produced in the Schaefer lab) in PBS with 1% CCS (Thermo Fisher Scientific). Antibody cocktails and fluorescent minus one (FMO) controls were then added to the blocked cells and incubated on ice for 30 minutes. Cells were then washed and fixed and then stored in PBS with 1%FBS. Flow Cytometry was performed using a BD LSRII flow-cytometer. Mouse antibodies used in this study were CD11b (M1/70), CD19 (1D3), CD3 (17A2), Ter119 (TER-119), c-kit with

huHox11 immortalized cells (2B8), sca-1 (D7), Flt3 (H1.2F3), Siglec-F(E50-2440), CD86 (GL1), CD34 (RAM34), CD16 (2.4G2), Ly6C (AL-21), CD64 (10.1), Ly6G (1A8) all from BD Biosciences; CD11c (N418; Biolegend); c-kit (ACK2), CD80 (16-10A1) both from ebiosciences; and F4/80 (CI:A3-1; Serotec). Compensation was performed with UltraComp ebeads (eBiosciences) and unstained cells from an appropriate source. FACS data were analyzed in Flowjo (TreeStar). In cocktails containing over 4 antibodies negative cutoffs were based on FMO controls. For all other experiments, negative cutoffs were based on unstained controls.

Light microscopy

pWPT1-vRaf-T2A-v-Myc transduced macrophages were imaged 34 days post-transduction in a 24 well plate. Images were captured with a Zeiss Axio Observer A1 inverted microscope under the 5× or 10x objective.

Vector graphics

All graphics depicting vectors were created using Accelrys Gene 2.5.

AIM 2

Cell culture

Black Flying Fox Kidney (PaKiT) and non-transformed PaKi cells have been described previously (46). MRC-5, and (HEK) 293T (ATCC® CRL-3216TM) were obtained from the American Type Culture Collection (ATCC). 293 and 293 A20^{-/-} (160) were obtained Dr Ling Shao by Material Transfer Agreement with University of Southern California. PaKiT, PaKi, MRC-5, and HEK cells were maintained in DMEM (Dulbecco's Modified Eagle Medium (Gibco Laboratories) supplemented 10% HyCloneTM Cosmic CalfTM Serum

(CCS) (Thermo Fisher Scientific), 0.5% Penicillin/streptomycin (Gibco and 1% L-glutamine (Thermo Fisher Scientific) complete cell culture media (DMEM-10). Jurkat and Jurkat CRISPRexon3A20KO T-cells were obtained from Dr Daniel Krappman (Helmholtz Zentrum München - German Research Center for Environmental Health, Neuherberg, Germany) and maintained in Roswell Park Memorial Institute 1640 (RPMI 1640) supplemented with reagents as listed in above in DMEM-10. All cell cultures were maintained at 37°C in 5% CO₂.

Plasmids

pCMV-CedarP (118) was sub-cloned into MSCV-3x-HA. Subsequently, the resulting MSCV-3xHA-CedarP was cut with AgeI-HF/NotI-HF/FspI and subcloned into a pcDNA3 construct cut with AgeI-HF and NotI-HF which resulted in production of pcDNA3-CedarP-3x-HA. MSCV-Thy1.1 and MSCV-dnIkB-IRES-Thy1.1 were gifts from Thomas C. Mitchell Ph.D at University of Louisville. pBluescript is abbreviated pBS (Stratagene).

Cedar virus infections

All adherent cell lines were seeded 24 hours before infection. The next day cells were infected with the rCedV-GFP (118) at the indicated Multiplicity of Infection (MOI). Jurkat cells were infected upon seeding at 5×10^5 /mL. Alternatively, Recombinant Equine and Human TNF α at 15 or 30 ng/mL (R&D Systems) was added to DMEM-10. rCedarV-GFP titers were determined by serially diluting supernatants from infections. Dilutions were then incubated with HEK 293T cells and 48 hpi (hours post-infection) GFP expression from rCedV-GFP was used to count the number of fluorescent virus plaques.

Western blots

To examine protein expression, whole cell lysates were obtained using Pierce RIPA buffer (Thermo Fisher Scientific) supplemented with cOmplete™ protease inhibitor cocktail (Sigma-Aldrich) and phosphatase inhibitor cocktail [Cell Signaling Technology (CST)] at indicated hours post-infection (hpi) or post-treatment. Lysates were loaded onto 4–12% Bis-Tris protein gel (Invitrogen; Carlsbad, CA, USA); protein gels were transferred to PVDF membranes (Bio-Rad; Hercules, CA, USA; 350 mA, 1.5 h) for standard western blotting techniques. Antibodies used for immunoblots included: polyclonal terminal bleed rabbit serum from anti-cedar P and N produced in *E.coli* and were obtained from Jennifer Barr and Gary Crameri (AAHL/CSIRO), anti-Turbo GFP polyclonal antibody (Evrogen), anti- β -actin polyclonal antibody (Abcam), anti- β -tubulin polyclonal antibody (Abcam, anti-phospho-NF- κ B P65 (Ser536) monoclonal mAb clone 93H1 (CST). Anti-I κ B α amino-terminal antigen mAb clone L35A5 (CST). Anti-I κ B α carboxy-terminal antigen mAb clone 112B2 (CST), Anti-HA mAb clone F-7 (Santa Cruz Biotechnology). Protein levels were quantified using Image J software (NIH). Three A20 antibodies were used for western blot) and induced: anti-A20 monoclonal (mAb) antibody clone D13H3 (CST), anti-A20 c-terminus epitope mAb clone 59A426 (Santa Cruz Biotechnology), anti-A20 mAb clone A12 (Santa Cruz Biotechnology). Except where indicated, clone D13H3 was used.

Flow Cytometry

If no surface staining was required, cells were fixed in paraformaldehyde and stored in PBS supplemented with 1% CCS (Thermo Fisher). To examine Thy1.1 surface expression, cells were stained with anti-Thy1.1 clone ox-7 (Biolegend) before fixation.

Flow Cytometry was performed using a BD LSRII flow-cytometer. Data were analyzed using Flowjo software (FlowJo LLC).

Transfection experiments

When being prepared for a transfection, all cells were seeded in 100 µg/mL Poly-D-Lysine (Sigma) coated tissue culture plates or flasks. 24 hours later, transfections were performed using Lipofectamine 2000™ or *ltx* (Thermo Fischer Scientific) at a ratio of 3 µL transfection reagent to 1 µg DNA in OPTI-MEM (Gibco), as described in manufacturer protocol.

NF-κB and Cedar P

For luciferase assays, 1.5×10^5 HEK 293T cells were plated per well in a 24-well plate. Indicated amounts of pcDNA-CedarP-3xHA and MSCV-dnIκB-IRES-thy1.1, 15 ng of the NF-κB-responsive luciferase reporter pBVI-NF-κB-Luc were transfected into cells. Carrier plasmid, pBS was used to standardize total DNA concentrations across conditions. At 24-48 hours post-transfection, appropriate wells were treated with 30 ng/mL of recombinant human TNFα for 3 hours (R&D Systems). One half of the cells were lysed in Reporter Lysis Buffer. Lysates were then used to perform luciferase assays using the Luciferase Assay System (Promega) according to the instructions of the manufacturer. The other half of the cells were prepared for western blot and run as described above.

NF-κB blockade and Cedar virus

HEK 293T cells were seeded at 2.5×10^5 in 6 well plates and transfected 24 hours later with 60 ng of 5xNF-κB-Luc and 1.5 µg pf MSCV-dnIκB-thy1.1 or 1.5 µg pBS. Forty-eight hours post-transfection, cells were either left uninfected/untreated or infected

with rCedV-GFP at an MOI of 0.5. The following day, appropriate wells were treated with 30 ng/mL of recombinant human TNF α (R&D Systems) for the indicated times. Twenty-four hours post infection or at the indicated time post TNF treatment, supernatants were collected for virus titer. Titers were determined as described above. Half of cells were processed for flow cytometry as described above while the other cells were lysed in Reporter Lysis Buffer (Promega) for a standard luciferase assay. Lysates were then used to perform luciferase assays using the Luciferase Assay System (Promega) according to the instructions of the manufacturer.

Confocal Microscopy

Coverslips were pretreated with 100 μ g/mL of poly-D-lysine (Sigma) and placed in 6-well plates. HEK 293T cells were seeded at 1.25×10^5 . Twenty-four hours later, cells were infected with an MOI of 0.5 or transfected with 250 ng of pcDNA-3xHA-CedarP for 24 hours. Cells were then fixed in 4% paraformaldehyde (Sigma). Subsequently, staining was performed with anti-A20 mAb clone 59A426 (Abcam) followed by anti-mouse IgG1 AlexaFluor 647 (Thermo Fisher) for cells infected with rCedV-GFP or anti-HA mAb clone C12A5 (Santa Cruz) followed by anti-mouse IgG_{2b} Alexafluor 647 (Thermo Fisher Scientific) for cells transfected with pcDNA-CedarP-3xHA. DNA staining was performed using DAPI dye (Molecular Probes). Coverslips were mounted in Fluoromount-G Mounting Medium (SouthernBiotech). Cells were visualized with a 40 $\times$ or 63 $\times$ oil objective, on a Zeiss 710 or Zeiss 700 microscope. Zen software was used to analyze images.

RNA extraction, cDNA production and PCR amplification of A20 transcripts

Total RNA was extracted from Jurkat or HEK 293T cells left uninfected or infected for 24 hours with rCedVp-GFP at an MOI of 0.1 or 1.0. Total RNA extraction was performed using RNazol® RT (Sigma) according to the manufacturer's protocol. Following this, poly(A) RNA was purified from the sample using Poly(A)Purist™ MAG kit (Thermo Fisher) and was resuspended in 15 µL of ultra-pure water (Quality Biologicals). For cDNA preparation, 9 µL of mRNA was used with High-Capacity RNA-to-cDNA™ kit (Thermo Fisher). Conventional PCR to amplify cDNA was then performed using AccuPrime Pfx SuperMix™ per the manufacturers protocol and primers, as described (TABLE 1). The resulting cDNA was cloned into pBS using KpnI and XhoI restriction sites added during PCR, transformed into XL1-Blue *E.coli* (Agilent; Santa Clara, CA, USA) , and screened for inserts. Plasmids were then sequenced using M13 -40 Forward or M13 -48 Reverse. All vector. PCR, and sequencing graphics were made using Accelreys Gene 2.5.

Generation of PaKi-5 ×NF-κB-Gluc-NeoR

MSCVsin-5 ×NF-κB-Gluc-NeoR was cut with ScaI and SmaI. 0.5 µg of agarose gel-purified 5 ×NF-κB-Gluc-NeoR fragment (Zymo Research) was mixed with 1.5 µl of lipofectamine LTX (Invitrogen) and 2.5 ul of PLUS reagent and incubated at room temperature for 30 minutes. The resulting mixture was then used to transfect 2.5×10^5 PaKi cells. 24 hours later G418 (Gold Biotechnology; St. Louis, MO, USA) at 0.6 mg/mL was added to cell culture and was included in subsequent passages for 2 weeks at which point drug was removed. A clonal cell line (PaKiGluc) was then made using limiting dilutions.

Gaussia Luciferase Assay

PaKiGluc were seeded in 12 well plates at 1.25×10^5 . Twenty-four hours later media was removed and replaced with DMEM-10 or DMEM-10 supplemented with 15 ng/mL of recombinant equine TNF α (KingFisher Biotechnology). Supernatant was collected at indicated time points. Luciferase activity in the supernatant was measured with a Gaussia Luciferase kit (Targeting Systems). Luciferase activity at each time point was reported as a fold increase as compared to the luciferase activity of the untreated cells equivalent time point.

Statistical Analysis

Experiments that aimed to address possible differences in percent of infected cells had a randomized block design. The P values were determined using a paired Student's t test. Experiments to address possible differences in titer were not paired. An unpaired Student's t test was used to determine the P value. All statistical analyses were performed using GraphPad Prism software, version 5 (GraphPad Software, Inc.).

Primer name	exon(Ex) and Restriction site	Primer sequence
B1225	huA20-Ex1-Se:Xho	GAAGTCCGAGCTTGGGACTTTGCCGAAAGGAT
B1226	huA20-Ex2-Anti:Hind	CGAAAGCTTCTCCCGTATCTTCACAGCTTT
B1227	huA20-Ex2-Se:Xho	GAAGTCCGAGCTCAACTGGTGTGCGAGAAGT
B1228	A20-Ex3-Anti(2):Kpn	GAAGGTACCCACATGTACTGAGAAGT
B1229	huA20-Ex4-Anti:Kpn	GAAGGTACCTTCCAGTGAGTTGTACTGAA
B1230	huA20-Ex5-Anti:Kpn	GAAGGTACCTGATTCCAAACTTCTTAGCATT
B1231	huA20-Ex6-Anti:Kpn	GAAGGTACCCGGTCTCTGTTAACAAGT
B1232	huA20-Ex7-Anti:Kpn	GAAGGTACCACTCATGCTGAACAAGTTCAAA
B1233	huA20-Ex8-Anti:Kpn	GAAGGTACCGACGCTGTGGGACTGACTTT
B1234	huA20-Ex9-Anti:Kpn	GAAGGTACCGGGCCTTGAGGTGCTTTGT

Table 1. Primers used for amplification of A20 cDNA.

mRNA from uninfected or infected cell lines containing bilallelic knockouts of exon3 of A20 were used to make complimentary cDNA. This cDNA was used with the above primers to amplify varying regions of human A20. The exon to which the primer maps is listed in the middle column, along with the restriction enzyme added to the PCR fragment.

CHAPTER 3: RESULTS

***Pteropus alecto* bone marrow derived myeloid cells are phagocytic.**

Currently, no bat immune cell lines exist. We aimed to develop a macrophage cell line from *Pteropus alecto* bats. To do this, we first identified culture conditions for deriving myeloid like cells from *P. alecto* bone marrow. Viability of immune cells are highly dependent on cytokines and other growth factors. However, *P. alecto* specific cytokines are not commercially available. We evaluated the ability of culture media supplemented with murine granulocyte-macrophage colony-stimulating factor (GM-CSF) or murine macrophage colony-stimulating factor (M-CSF) to sustain a *P. alecto* culture. Bone marrow was cultured for 7 days using GM-CSF and M-CSF supernatants, collected from a feeder cell line, to a final concentration of $1\times$. We were unable to culture viable cells in media supplemented with M-CSF (data not shown). Bone marrow cultured in media supplemented with GM-CSF for 7 days yielded cells with myeloid-like morphology (Figure 7A). To further characterize these cells, we performed a phagocytosis assay in which cells were mock pre-treated or treated with the actin polymerization inhibitor, cytochalasin D. After 30 minutes, cells were incubated for one hour with pHRODOTM Red conjugated to *Staphylococcus aureus*. This dye fluoresces under acidic conditions, such as those found in lysosomes. When we analyzed these pHRODO-conjugated cells by confocal microscopy, we detected fluorescence in the *P. alecto* bone marrow treated with pHRodo Red *S. aureus* but not when the cells were pre-treated with cytochalasin D (Figure 7B). This, combined with the responsiveness of the cells to GM-CSF and cell type morphology, suggest that myeloid-like cells can be cultured from *P. alecto* bone marrow using a mouse derived growth factor.

Mouse bone marrow immortalized with human Hox11 produces early progenitor cells

P. alecto bone marrow harvest is a terminal procedure and thus, sample is limited. To conserve difficult to obtain bat samples, we used mice to optimize a protocol for myeloid cell immortalization. We modified an existing replication deficient lentiviral construct (pWPT-GFP) such that the GFP was removed and a polylinker was added. Hox11 was inserted into this construct to make pWPT1-huHox11 (Figure 8A). This plasmid was used in combination with lentiviral packaging helper plasmids to create a human Hox11 (also known as TLX1) expressing lentivirus. Hox11 has been shown to immortalize embryonic stem cells in mice (85). We hypothesized Hox11 immortalization may also work with stem cells found in bone marrow. To immortalize myeloid cells with this virus, we harvested bone marrow from the femurs of C57BL/6 mice and cultured the isolated PBMC (peripheral blood mononuclear cells) in IMDM supplemented with murine IL-3, IL-6, and stem cell factor (SCF) for 48 hours. We used RetroNectin-coated plates to promote infection of the bone marrow-derived cells at a MOI of 5 for 48 hours. After this 48 hour transduction period, cells were removed from the plates containing virus and transferred into media that no longer contained virus, IL-6, or SCF, but still contained IL-3. Cells were continually passaged and replication kinetics increased with time, presumably as transduced cells increased in numbers (Figure 8B). The cell morphology was round and cells grew in suspension (data not shown). After 75 days *ex vivo*, cells were deemed immortalized.

We analyzed the Hox11 immortalized cells by flow cytometry to characterize their surface marker expression. Hox11 immortalized bone marrow was stained with antibodies for CD11b, a pan-myeloid marker, and c-kit, found on early stem cell

progenitors (62). This analysis proved bone marrow cells immortalized by human Hox11 do not express CD11b, but express hi levels of c-kit (Figure 9). This expression pattern, combined with the cell morphology, suggested Hox11 mediated immortalization was not ideal for making a macrophage-like cell line. This is consistent with what others found in Hox11 immortalized myeloid cells (181). We also attempted immortalization with Hoxa9 and Myb and observed the same phenotype (data not shown).

Construction of a v-Raf/v-Myc retrovirus

To address the issues of the differentiation block we observed in Hox11 transduced cells, we constructed a replication deficient lentivirus containing two oncogenes, vRaf and v-Myc. Previously, a group constructed a virus, J2, which contained these two oncogenes. For our purposes, problems with the design of this vector included that it could only infect mouse cells, the virus was replication competent and the splicing mechanism yielding an mRNA competent for translation of v-Myc was not clear. Still, these investigators were able to immortalize macrophages from bone marrow. These macrophages were mature, in that the surface receptors CD11b and F4/80 were expressed. The transformed cells also grew without cytokine supplementation of the media (25). This cytokine independence was in contrast to what we observed in our Hox11 transduced cells, which required IL-3 indefinitely. We thus used the v-Raf and v-Myc oncogenes from J2 to create an extensively modified viral vector. Specifically, we cloned the v-Raf and v-Myc oncogenes from the original J2 virus into a vector containing the self-cleaving peptide, T2A. v-Raf-T2A-v-Myc was then moved into pWPT1 to make pWPT1-v-Raf-T2A-v-Myc and replication deficient lentiviral supernatant was made as described in the Hox11 experiments (Figure 10A).

Cell immortalization using the v-Raf/v-Myc virus

Harvesting bone marrow is an invasive procedure, and marrow is thus not an ideal starting material for production of macrophage cell lines from non-laboratory animals. We hypothesized that the v-Raf/v-Myc protocol may work with blood where sample is more easily acquired and mature myeloid cells are also prevalent. Tissue from spleen also contains abundant mature myeloid cells. We thus harvested blood and spleen, alongside bone marrow from mice. PBMC from all three tissue types were then cultured in media supplemented with GM-CSF for 4 days. Next, the cells were transduced using RetroNectin with the v-Raf/v-Myc virus at an MOI of 3-5. After 2-3 days in the presence of virus, transduced cells were moved to fresh media, still with GM-CSF supplementation. By 14 days ex vivo, cell proliferation had ceased, cells had adhered to the bottom of the culture plates, with morphology consistent with myeloid cells (data not shown). Media was removed and replaced with media that did not contain GM-CSF (Figure 10B). This apparent cell cycle arrest was also observed during J2 virus infection of bone marrow, but could be reversed with the addition of CytodexTM 1 beads (25). To address whether we could reverse the cell cycle arrest seen with transduction of our v-Raf/v-Myc virus, we added 200-400 ng/mL of CytodexTM 1 beads at 28 days ex vivo (Figure 10B). Several days later, it became apparent that cells harvested from spleen or blood and then transduced with v-Raf/v-Myc were adhering to the CytodexTM 1 beads and were increasing in number. This proliferation was not observed in bone marrow derived cultures. At 35 days ex vivo, we imaged the transformed cells derived from blood. Cells were found growing on the beads as well as adhering to the culture plate. Their morphology was consistent with monocytes and macrophages (11A-11C). At this point, if beads were removed, cell replication ceased. With time, however, we were able

to slowly decrease the concentration of beads to yield a bead-independent cell line (data not shown). Taken together, we were able to immortalize cells from spleen and blood (but not bone marrow) with a v-Raf/v-Myc lentivirus. These cells were growth factor and cytokine independent and transiently required CytodexTM 1 beads for replication.

v-Raf/v-Myc transformed cells do not express surface receptors characteristic of early progenitors.

The J2 virus that transformed bone marrow produced cell lines that expressed markers of mature macrophages (25). To determine if the blood derived cells we immortalized with v-Raf/v-Myc had characteristics consistent with mature macrophages, instead of progenitor cells (as was seen during Hox11 immortalization), we analyzed cell surface receptors by flow cytometry. We hypothesized that early progenitor markers would be absent in our blood derived cell line. We stained this cell line with a panel of early progenitor markers alongside fresh bone marrow which contains various subsets of early progenitors. We used a gating strategy that eliminated dead cells and cells that expressed Ter119 (Red blood cells), CD3 (T-cells), and CD19 (B-cells) (Figure 11A). In bone marrow, 63% of the Ter119-/CD3-/CD19- population was CD11b+ (Figure 11B). In contrast, 92% of these cells were CD11b positive in v-Raf/v-Myc transduced blood-derived cells (Figure 11B). We eliminated the CD11b- cells for our evaluation of early progenitor markers. CD11b+ cells from bone marrow had small subsets of cells that expressed all of the early progenitor markers we analyzed; CD34, c-kit, sca-1, and flt3 (Figure 11B). In other words, bone marrow contains early myeloid progenitors. Our blood derived cell line, on the other hand, did not express early progenitor markers (Figure 12B). A small population of CD16+ cells was present in our cell line however; this marker is not limited to early progenitors. In fact, it is important for the antibody

dependent cytotoxicity activity of monocytes (227). Overall, this analysis showed that our cells are in the myeloid lineage, but are not early myeloid progenitors.

v-Raf/v-Myc transformed cells express surface receptors characteristic of mature macrophages.

We performed additional flow cytometric analysis, staining for markers associated with more mature myeloid subsets. GM-CSF was used in the initial culture off these cell lines. GM-CSF can induce differentiation of cells to granulocytic cells like neutrophils and eosinophils (199). To determine if v-Raf/v-Myc transformed cells belong to the granulocyte family of myeloid cells, we stained our blood derived cells with Siglec-F, Ly6C, and Ly6G. We determined, through the use of fluorescence minus one (FMO) controls and additional analysis not shown, that these cells do not express these granulocyte surface receptors (Figures 13A and 13C-D). Some subsets of macrophages and most dendritic cells express CD11c. v-Raf/v-Myc transformed cells however, are CD11c- (Figure 13B). F4/80 is a mouse specific marker of mature macrophages (131). This surface marker was prevalent in both blood and spleen derived cell lines (Figure 13F, Figure 13). Blood derived, v-Raf/v-Myc transformed cell lines also express CD64 (FcγRI), a marker associated with pro-inflammatory macrophages (Figure 13E) (6). Additional analysis on spleen derived, v-Raf/v-Myc transformed cell lines revealed macrophage markers CD80 and CD86 are also expressed on these cells (Figure 14). The receptor profile, thus, indicates that v-Raf/v-Myc immortalizes a cell type consistent with a mature macrophage.

v-Raf/v-Myc transduced cells lines have functional macrophage characteristics.

The cell lines immortalized with the v-Raf/v-Myc virus have surface receptors which are indicative of macrophages. To determine whether these macrophages were functional, we examined their ability to upregulate major histocompatibility complex II (MHCII) and to phagocytose. MHCII can be expressed on macrophages cultured in GM-CSF (87). To determine if blood derived v-Raf/v-Myc cell lines expressed MHCII we performed flow cytometry on cells stained with anti-MHCII. We found that under regular culture conditions, the macrophages were MHCII- (Figure 15A). To determine whether we could induce the macrophages to express MHCII, we treated them with 100 U/mL of IFN γ for 24 or 48 hours and then stained for MHCII expression. Flow cytometric analysis showed that at 24 and 48 hours post-IFN γ treatment, a large population of MHCII+ cells was present (Figure 15).

One of the main roles of macrophages is to phagocytose foreign material. To determine if blood derived v-Raf/v-Myc transformed cells were capable of this vital function, we performed a phagocytosis assay. This assay was done as was described in the bat bone marrow experiments, where we treated the cells with cytochalasin D or mock treated them and then exposed them to pHRODO red conjugated *S. aureus* for 1 hour. We examined the macrophages by confocal microscopy. The analysis showed that *S. aureus* was entering in the macrophages as determined by the pH sensitive red fluorescence observed in the cell and that this internalization was actin polymerization dependent (Figure 16). Thus, v-Raf/v-Myc transduced cells exhibit IFN γ responsiveness and are phagocytic. These functional properties along with the cytokine independence of transformed cells and the ability to transform cells from a small volume of blood, make the v-Raf/v-Myc virus ideal for application to less studied species. This, combined with

the data that demonstrate we can derive phagocytic cells from *P. alecto* using GM-CSF, suggests v-Raf/v-Myc transduction may allow for the production of a *P. alecto* macrophage cell line.

Cedar virus activates the NF- κ B pathway

In a large transcriptomic to proteomic study, one group demonstrated that transcripts and proteins involved in the NF- κ B pathway were significantly upregulated in a *Pteropus alecto* cell line infected with Hendra virus (HeV) (226). Although this finding suggests the NF- κ B pathway is important to study, because most henipaviruses are Biosafety level 4 (BSL-4) pathogens, progress in this field has been slow. In order to advance studies on henipavirus-mediated innate immune responses at a lower BSL, we aimed to determine if the non-pathogenic henipavirus, Cedar virus (CedV), could also activate the NF- κ B pathway. Therefore, we infected HEK293T cells at an MOI of 1.0 with a recombinant CedV containing a GFP open reading frame (rCedVGFP) (118). We prepared lysates from these cells at various time points between 3 and 24 hours post-infection (hpi). Alternatively, these cells were treated for 2 hours with TNF α .

Phosphorylation of P65 is required for activation of NF- κ B and is thus used often as an indication of NF- κ B pathway activation (127). We found that at all time points examined during infection, pP65 levels were increased, but that they were most profoundly elevated at 9 and 12 hpi (Figure 17 A-B). CedV nucleoprotein (N) and phosphoprotein (P) were also detected as early as 3 hpi, however GFP was not detected until 24 hpi (Figure 17A). Interestingly, we found that although we detected more pP65 in TNF α treated cells than in rCedVGFP cells, A20, a negative regulator of the NF- κ B pathway (224), was strongly

expressed at 24 hpi with CedV but was not at detectable levels in HEK cells treated with TNF α (Figure 17A).

To confirm the robust expression of A20 found during CedV infection, we infected HEK 293T cells with rCedVGFP for 24 hours and examined these cells by western blot or confocal microscopy. Western blot analysis showed a robust activation of A20 at 24 hours post rCedVGFP infection (Figure 18A). We stained the cells with an anti-A20 antibody and detected an increase in A20 expression in rCedVGFP infected cells when compared to the uninfected control (Figure 18B). Together, the results of these experiments demonstrate that rCedVGFP can induce the NF- κ B response and thus can be used as a model henipavirus in NF- κ B studies.

CedV infected fibroblasts from humans and *P. alecto* do not share the same NF- κ B profile.

To compare the NF- κ B response in the reservoir of henipaviruses to what is found in the human host, we first addressed the ability of *P. alecto* cell lines to activate the NF- κ B pathway. To achieve this, we treated the *P. alecto* cell lines, PaKi and PaKiT with equine TNF- α and harvested cell lysates between 30 mins and 4 hours post-TNF α treatment. We found an increase in pP65 in response to TNF α treatment in both cell lines (Figure 19A-B). PaKiT cells were also probed with an antibody recognizing I κ B. The degradation of this protein is required for canonical NF- κ B activation (98). We found that at 30 and 60 minutes post TNF α treatment, I κ B had been significantly degraded in PaKiT cells (Figure 19A). To verify that activation of the NF- κ B pathway yielded κ B mediated transcription, we constructed a luciferase reporter cell line in PaKi cells. This cell line has a NF- κ B driven promoter which, when activated, leads to the production of a secreted Gaussia luciferase. We treated these cells with eqTNF α and collected an aliquot

of supernatant at 2, 4, and 6 hours post-treatment. At the same time points, we collected an aliquot of the supernatant from untreated cells. Gaussia luciferase activity was measured from these aliquots at each time point. The TNF α -mediated increases in NF- κ B were reported as a fold increase in luciferase activity as compared to activity from the untreated control supernatant at the same time point. Consistent with western blot data, we found that NF- κ B activity was increased at 2 and 4 hours (Figure 19C). This cell line was able to maintain TNF α mediated NF- κ B activation through 6 hours (Figure 19C). In all, we demonstrated that two cell lines from the henipavirus reservoir *P. alecto*, are able to activate the NF- κ B pathway in response to TNF α .

We then proceeded to examine the NF- κ B responses in two comparable non-transformed cell lines; one from human and one from bat. MRC-5 cells are human lung fibroblasts immortalized by serial passaging (ATCC provided information). PaKi cells are kidney derived fibroblasts but were immortalized similarly. We infected both of these cell lines with rCedVGFP at an MOI of 0.1 or 1.0 for 24 hours. After infection, cells were lysed and protein expression was analyzed by western blot. We found that while the human MRC-5 cells had an increase in pP65 at both MOIs 24 hpi, there was no increase in pP65 in PaKi cells at the same time point (Figure 20A-B). This is in contrast to the increase in pP65 in PaKi cells that was seen in response to TNF α treatment (Figure 19B). These data also showed that rCedVGFP infected PaKi cells have more than twice as much GFP protein expression than do infected MRC-5 cells (Figure 20A and 20C). To verify this increase, we also examined MRC-5 and PaKi infected cells by flow cytometry. We saw significantly more PaKi cells with detectable GFP than we did in infected MRC-5 cells (Figure 20D). We also probed the western blot membranes from

above, with anti-A20. A20 is a negative regulator of NF- κ B and it is activated by the pathway itself (224). Interestingly, we found a robust induction of A20 expression 24 hpi with rCedVGFP regardless of whether the NF- κ B pathway was (MRC-5) or was not (PaKi) activated (Figure 20A). Thus, although the bat and human cell lines both induce A20, at 24 hpi the activation of NF- κ B are contrasting (Figure 20A-B).

To determine if the activation of NF- κ B in *P. alecto* cells was occurring in earlier hpi, we performed a 24 hour time course. We infected PaKiT cells with rCedVGFP and collected cell lysates at varying time points between 3 and 24 hpi. We found the largest induction of pP65 was twofold above uninfected activity and that this occurred 3 hpi (figure 21A-B). The increase in pP65 also returns to that of baseline early during infection (Figure 21A-B). In all, these data support the hypothesis that bats have an overall less robust cell intrinsic innate immune response to henipaviruses. They also suggest the kinetics of their immune response may be different than what is found in humans.

Effect of NF- κ B on CedV infection in HEK293T cells

Though the NF- κ B response is mounted by the host as an antiviral response, viruses have also evolved mechanisms that allow them to use activation of NF- κ B to their benefit (90). This fact, in addition to our observation that pP65 was inversely correlated with the amount of virus protein observation in MRC-5 and PaKi cells, prompted us to examine whether activation of the NF- κ B pathway plays a role in controlling viral load (Figure 20). To explore if the NF- κ B pathway helps restrict virus reproduction, we transfected HEK293T cells with a NF- κ B dependent luciferase reporter plasmid (5xNF- κ B-Luc) and either a dominant negative I κ B (dnI κ B) plasmid that prevents activation of

NF- κ B, or an empty vector. The dnI κ B plasmid also encodes for expression of the surface receptor Thy1.1 which can be detected via flow cytometry analysis. We infected the HEK293T cells with rCedVGFP at an MOI of 0.5, 24 hours post-transfection. Twenty-four hours later, supernatant from infected cells was used to determine a viral titer and cells were processed for flow cytometry and luciferase assays. Luciferase assays showed that rCedVGFP induces a 6-fold increase above baseline activation of the NF- κ B pathway and that this activation is blocked in the presence of dnI κ B. (Figure 22C). At the same time, cells infected with rCedVGFP and transfected with dnI κ B produced significantly more virus than those transfected with an empty vector, as determined by a titer of their supernatant (Figure 22D). Similarly, when examined by flow cytometry, we found that transfection of dnI κ B significantly increased the amount of detectably infected cells present 24 hpi, when compared to cells that were transfected with an empty vector. This observation implies a higher viral load within the cell (Figure 22A-B). Overall, these data suggest that activation of the NF- κ B pathway helps restrict virus replication.

Cedar P is sufficient to induce A20 and does so independent of NF- κ B activation.

We observed a robust A20 induction in response to rCedVGFP in all cell lines we examined (Figures 17-19 and 16-17). It has previously been reported that the phosphoprotein of MeV was sufficient to induce A20 in some cell types (230). We hypothesized that CedV P may also induce A20 expression. To address this hypothesis, we transfected increasing amounts of pCMV-CedarP or pBS into HEK 293T cells such that each condition received a total of 3 μ g of DNA. Twenty-four hours post-transfection, lysates from the untransfected and transfected cells were prepared. Protein expression was examined by western blot using anti-Cedar P and anti-A20 antibodies.

Cedar P transfection alone was sufficient to induce A20 protein expression. This expression was detected at all concentrations of Cedar P transfected (Figure 23A). A20 expression, relative to β -actin, increased as the amount of transfected Cedar P increased (Figure 23B). These data demonstrate that Cedar P is sufficient for induction of A20 and that this induction is proportional to the dose of Cedar P.

Typically, A20 expression is induced by activation of the NF- κ B pathway (98). Our next aim was to address whether Cedar P induces A20 expression through activation of the NF- κ B pathway. We co-transfected a dominant negative mutant of I κ B (dnI κ B) with pCMV-CedarP to determine if NF- κ B signaling is required for Cedar P mediated A20 induction. An NF- κ B responsive luciferase plasmid (5xNF- κ B-Luc) was co-transfected with each condition. After 24 hours of transfection, cells were either untreated or treated with TNF α for 3 hours. Cells from each condition were divided, with one portion lysed for western blot to examine A20 protein expression, and the remainder lysed for a luciferase assay. Transfection of Cedar P, without dnI κ B, led to a decrease in NF- κ B activation as demonstrated as by a decrease in luciferase activity below that recorded in cells transfected with 5xNF- κ B-Luc alone (Figure 24, lanes 1-2). As expected, HEK293T cells transfected with the luciferase reporter alone and then treated with TNF- α , had increased luciferase activity when compared to the untreated control (Figure 24, lanes 1 and 5). In contrast, no increase in luciferase activity was recorded in TNF α treated HEK 293T cells pre-transfected with Cedar P. Instead, the luciferase activity recorded was again below the levels recorded in untreated HEK293T cells transfected with 5xNF- κ B-Luc alone (Figure 24, lane 1 and 7). When Cedar P was transfected with dnI κ B, luciferase activity was undetectable (Figure 24, Lane 3). A20,

however, was detected in the lysates prepared from the same cells but analyzed by western blot (Figure 24 Lane 3). Presence of the truncated dnI κ B protein was confirmed as well (Figure 24 lane 3). Together, these data show that CedV P is sufficient for A20 expression and that the expression does not require activation of the NF- κ B pathway.

CedV and CedV P induce an alternate A20 protein in biallelic A20 exon 3 knockout cell lines.

The observation that A20 can be expressed independent of NF- κ B, is to our knowledge, an unprecedented observation. We thus we sought to examine the effects of A20 expression during rCedVGFP infection. We obtained a Jurkat cell line with a CRISPR mediated mutation in Exon 3 of A20. This mutation is predicted to cause frameshift and/or a large deletion within Exon 3 on both chromosomal copies, leading to nonsense-mediated decay of the A20 mRNA. This cell line has been validated as failing to produce A20 protein in response to typical A20-inducing stimuli, including TNF α and PMA+Ionomycin stimulation. These cells, as well as a wild type Jurkat cell line, were infected with rCedVGFP at an MOI of 0.1 or 1.0 for 24 hours or treated with 15 ng/mL of TNF- α for 2 or 4 hours. The cells were lysed and protein expression was analyzed by western blot. We detected CedV P in both the WT and KO cell lines infected with rCedVGFP (Figure 25A). We also found, as expected, that TNF α treatment led to an increase in pP65 and that this increase was much stronger in the KO cell line than in the WT cell line (Figure 25A). The most striking observation, however, was that A20 expression was detected in both the WT and KO cell lines infected with rCedVGFP (Figure 25A). This was in contrast to the TNF α treatment where we observed A20

expression only in the WT Jurkat cell line. The A20 detected only in WT Jurkat cells was slightly smaller in size than the A20 induced in response to rCedVGFP (Figure 25A).

We hypothesized that rCedVGFP was inducing a form of A20 that does not include exon 3 and thus was not encoding the frame shift mutation. This form of A20 will here forward be referred to as *A20, for clarity. To test our hypothesis and verify that our observation was not a cell line artifact, we obtained another biallelic exon 3 KO cell line, which was made using TALEN (Transcription activator-like effector nucleases) deletion of a 34-bp region within A20 exon 3 in 293 cells. We infected these 293 cells, along with a WT 293, with rCedVGFP at an MOI of 1.0. We also treated different samples of 293 cells with 30 ng/mL of TNF α for 2 or 4 hours. These cells were lysed and analyzed by western blot. We found that our observations from the infected Jurkat cell lines were recapitulated in the 293 cells. More specifically, we detected *A20 in both the WT and A20 KO 293 cells infected with rCedVGFP (Figure 25B). In order to detect A20 during TNF α treatment, we cut the blot in half and increased the exposure time. This permitted us to evaluate A20 expression and confirm that like in Jurkat cells, A20 is slightly smaller in size than *A20 in 293 cells (Figure 25B).

We detected robust expression of an A20 protein during CedV P transfection (Figure 23A) and so we hypothesized CedV P induces *A20. We transfected pCMV-CedarP into WT and KO A20 293 cells. After 24 hours, we lysed these cells and analyzed the lysates by western blot. We found that *A20 was expressed in both the WT and KO 293 cells transfected with Cedar P (Figure 26). Together, these data show that CedV and CedV P can induce the expression of a unique A20 (*A20) that does not contain exon 3.

***A20 contains carboxy and amino terminal A20 epitopes.**

*A20 is produced in cell lines engineered to cause a missense mutation early in the mRNA, in Exon 3 (amino acids 99 – 162 out of a total of 790). However, *A20 is larger than A20, meaning that the apparently absent exon 3 is most likely substituted by one or more larger, unknown exon(s). To investigate what other portions of A20 are encoded in the mRNA of *A20, we ran western blots from the lysates of HEK293 WT and A20 KO cells infected with rCedVGFP. In all previous figures with western blots including A20, we used an antibody clone (D13H3) that recognizes a region in the N terminus of A20. We probed the 293 rCedVGFP infected lysates again with A20 antibody clones that map specifically to amino acids 1-100 of A20 or to amino acids 440-790. We detected *A20 using both antibodies (Figure 27). These results provide evidence that portions of both the carboxy and amino terminus of the A20 protein are present and that *A20 is neither a truncated version of A20 nor a cross-reactive protein detected by a single antibody against A20.

Cedar P localizes to the nucleus.

CedV P protein is sufficient for expression of *A20 (Figure 26). Additionally, the apparent loss of the missense mutation in exon 3 demonstrated by the presence of the C-terminus of A20 (Figure 27B) suggests that transcription is being altered. To address whether CedV P may be acting as a transcription factor or a splicing factor, we investigated its ability to localize to the nucleus. For this experiment, we first made a construct with Cedar P containing a 3xHA tag fused to the N-terminus of the protein (Figure 28A). We transfected the resulting pcDNA3-3xHA-CedarP into HEK293T cells and lysed the cells for western blot, 24 hours later. We probed this western blot with

anti-HA and detected a protein at the molecular weight of CedV P (approximately 85 kDa Figure 26B). Following this, we transfected the plasmid into HEK293T cells grown on coverslips. After 24 hours, we stained these cells with anti-HA and an IgG2b specific secondary antibody conjugated to AlexaFluor 555. The cells were analyzed by confocal microscopy. The images show that Cedar P can be found in the cytoplasm, but that its presence is most predominant in the nucleus (Figure 29B). This supports our hypothesis that CedV P may be a transcription factor or a splicing factor and confirms its ability to localize to the nucleus.

Attempts to amplify cDNA of *A20

Our data thus far led us to hypothesize that *A20 mRNA contains nucleotide sequences that are alternatively spliced from the A20 gene. To attempt to identify aspects of the mRNA splicing pattern unique to *A20, we designed primers that span the length of A20 cDNA open reading frame (Figure 30). Total RNA was extracted and mRNA was purified from both Jurkat and 293 WT and A20 KO cells. Next, we made cDNA which was used as template with each of the primer sets (Figure 30, inset table). The resulting PCR products were run on an agarose gel (gels from Jurkat cells not shown). A20 cDNA was detected in both uninfected and infected WT and KO A20 293 cells (Figure 31). We excised DNA from these gels and they were sequenced. Sequences from Jurkat cells, as expected, did not contain cDNA mapping to exon 3. Instead, an approximately 150 base pair insertion that maps to chromosome 11 was found between exon 2 and exon 4 (Figure 32A). This was recapitulated using several unique primer pairs. This insertion contains a missense mutation and would yield a truncated protein and cannot, therefore, be the sequence that corresponds to *A20. Additionally,

we found cDNA sequences from rCedVGFP-infected A20 KO Jurkat cells that did not contain the chromosome 11 insertion. These sequences mapped to exon 2 and were directly followed by exon 4 (Figure 32B). Like the chromosome 11 insertion sequences however, this splicing pattern causes the exon2-exon 4 junction to change the reading frame, rapidly leading to encounter of a stop codon. This splicing pattern thus also cannot be the sequence that corresponds to *A20. Sequences from rCedVGFP infected A20 KO 293 cells yielded similar results. Specifically, Exon 3 was absent from the sequences, and early stop codons were present. For example, in sequencing using primers to amplify exons 2 to 8 we found an intron 4 insertion after exon 2. This intron contained a stop codon (Figure 32C). To date, our PCR amplification strategy has not identified a splicing pattern that would translate a protein consistent with known features of *A20.

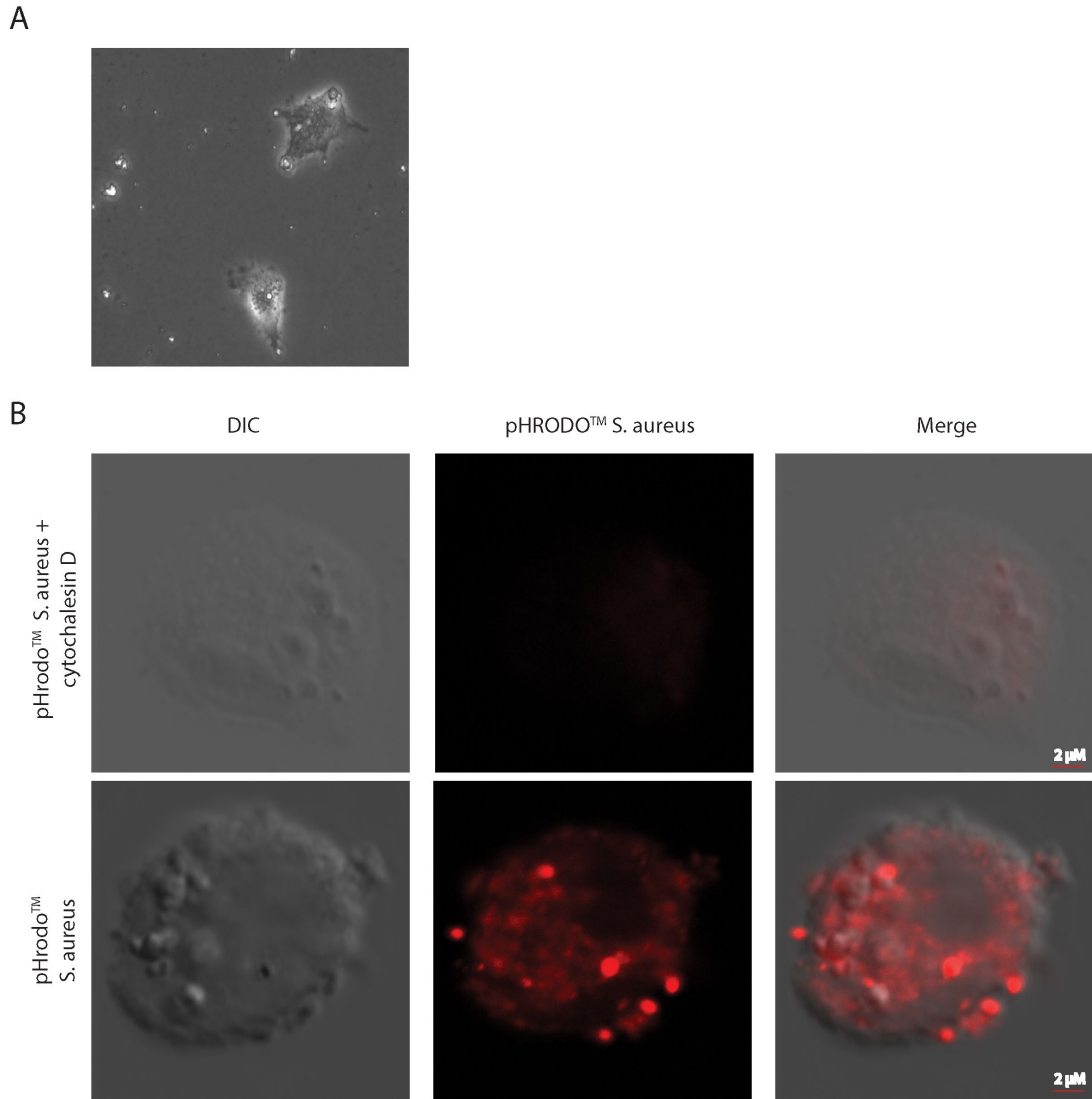


Figure 7: Bone marrow cells from *Pteropus alecto* are phagocytic.

(A) *P. alecto* bone marrow cultured for 7 days in murine GM-CSF was imaged with widefield light microscopy. (B) Bat bone marrow was cultured in GM-CSF for 7 days on coverslips. On day 7, the cells were either mock pre-treated or treated with 10 μ M cytochalasin D for 30 minutes. Cells were then treated with pHrodo™ red *S. aureus* BioParticles for one hour and then prepared for confocal microscopy. Red color indicates internalized pHrodo *S. aureus* BioParticles.

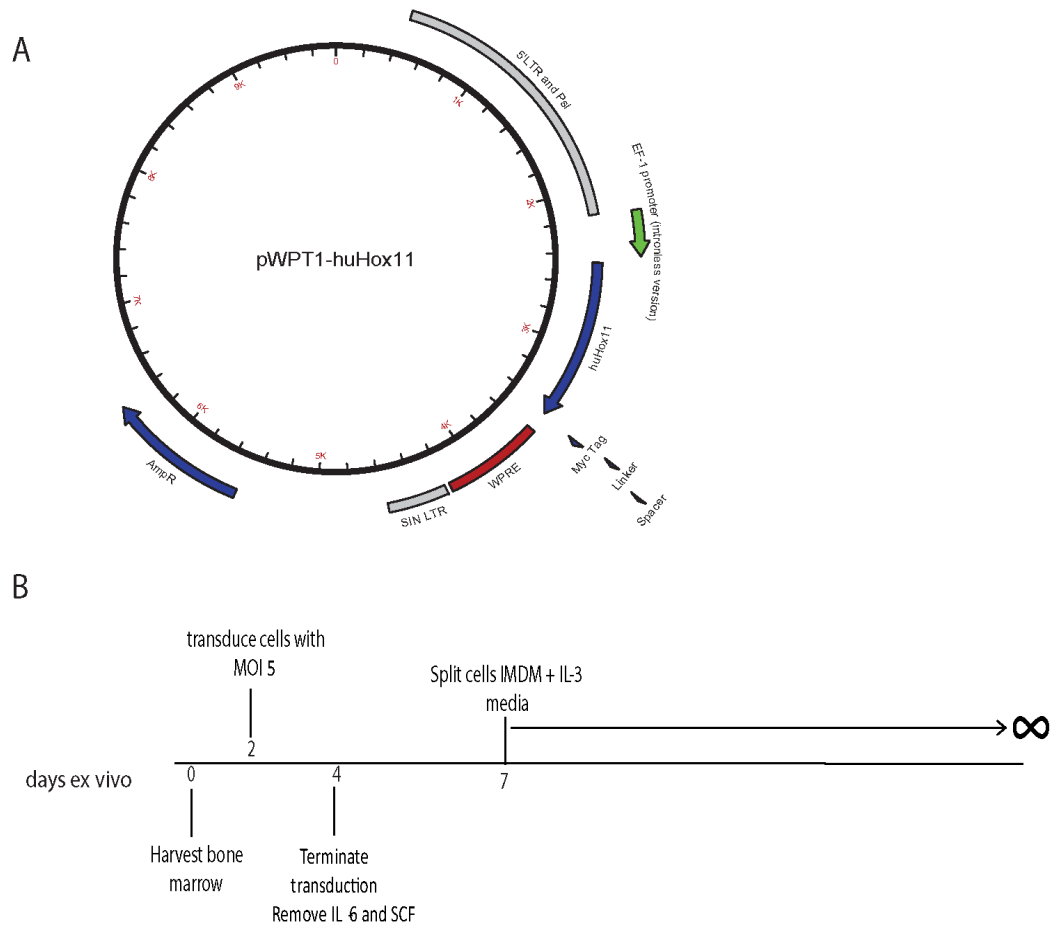


Figure 8: Construction of pWPT1-huHox11 and production of huHox11 transformed cell line

(A) pCMV6-humancDNAHox11 was cut with BamHI and EcoRV. The human Hox11 fragment was cloned into pWPT1 that had been linearized using BamHI and PmeI. (B) Timeline for production of Hox11 immortalized cells. Bone marrow is harvested and cultured in IMDM with IL-6 (10 ng/mL), IL-3 (6 ng/mL), SCF (100 ng/mL). 2 days later cells are transduced with Hox11 lentivirus. After an additional 2 days, the cells are moved to wells without virus and IL-6 and SCF are removed from the media. At one week ex vivo, cells are split 1:1 and are continually passaged.

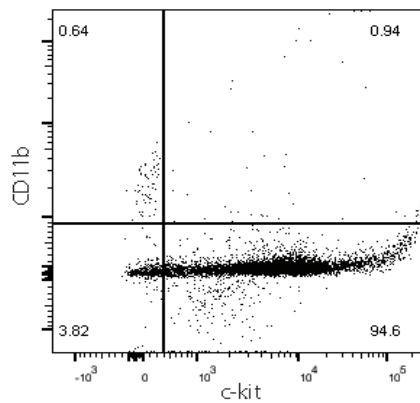


Figure 9: Hox11 transduced bone marrow cells are early progenitors.

Bone marrow was harvested from B57BL/6J mice and cultured in IMDM with IL-3, IL-6 and SCF. 48 hours later cells were transduced with a huHox11 lentivirus. After 75 days ex vivo, cells were stained with anti-c-kit and anti-CD11b. Gates were drawn using unstained controls.

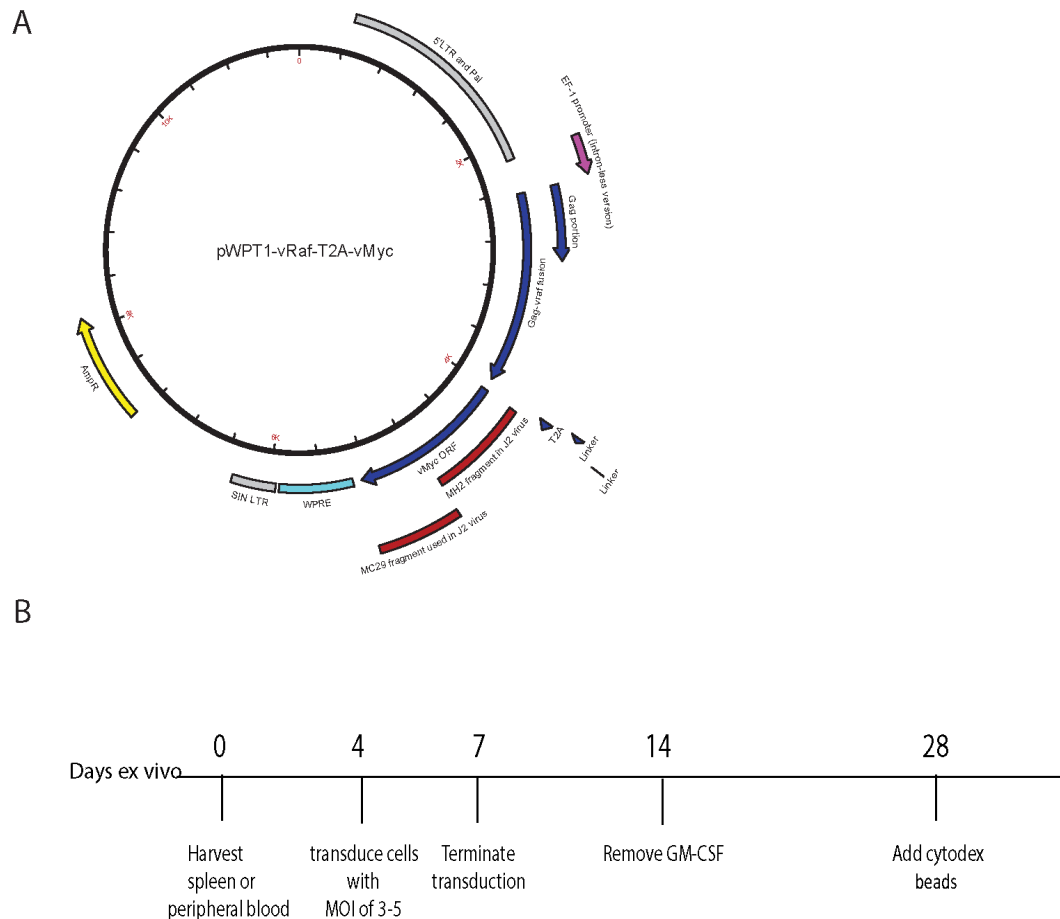


Figure 10: Construction of pWPT1-v-Raf-T2A-v-Myc and production of v-Raf/v-Myc transduced cell line.

(A) v-Raf and v-Myc were amplified from the retroviral vector J2. The v-Raf transcript was moved into pBS-T2A. Subsequently, v-Raf was ligated into this vector resulting in pBS-v-Raf-T2a-v-Myc. v-Raf-T2A-v-Myc was then moved as one piece, into pWPT1. This resulted in pWPT1-v-Raf-T2A-v-Myc. (B) Time line for production of v-Raf/v-Myc immortalized cells. Splenocytes or blood are harvested and cultured in GM-CSF. After 4 days, cells are transduced with v-Raf/v-Myc. After an additional 2-3 days, cells are moved to fresh GM-CSF supplemented media. After two weeks off *ex vivo* culture, GM-CSF supplement can be removed from the media. Cultures receive CytodexTM 1 beads at 200-400 µg/mL at 28 days ex vivo. Cells that expand are immortalized and can be maintained in culture, indefinitely.

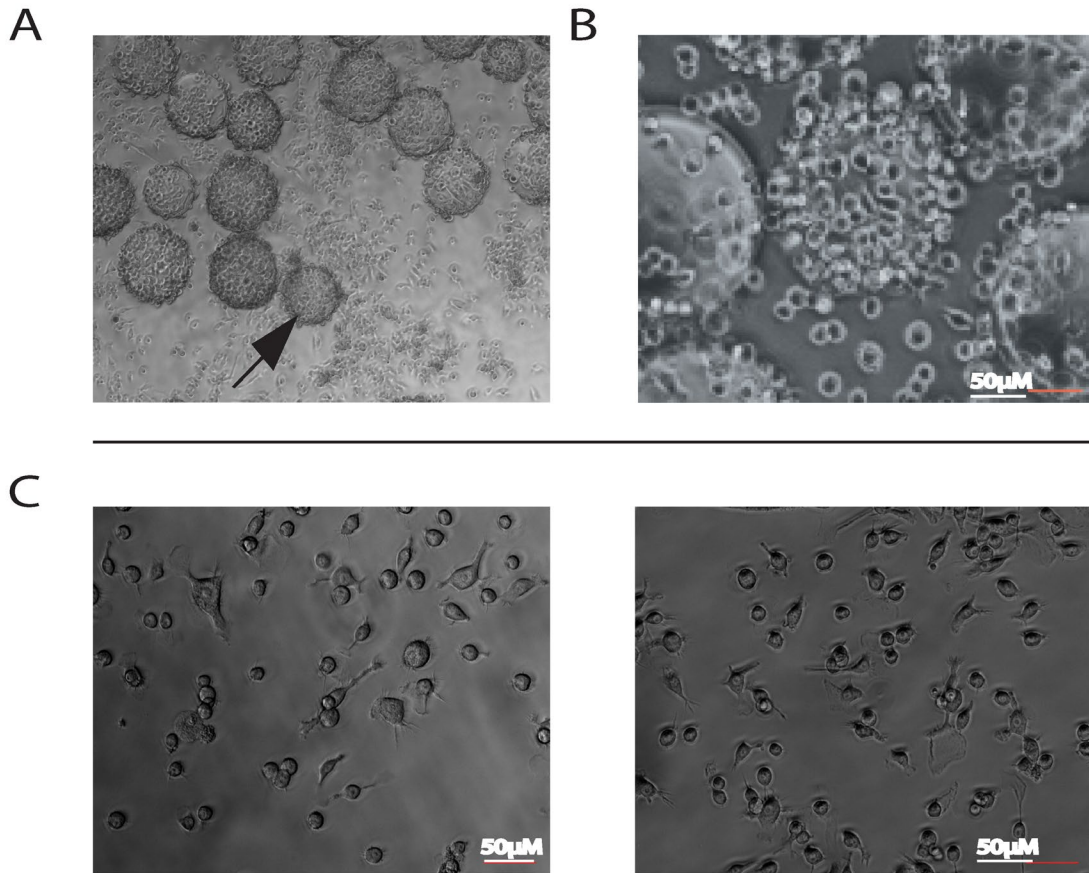


Figure 11: v-Raf/v-Myc immortalized macrophages exhibit macrophage/monocyte-like morphology.

Macrophages 31 days post-transduction with the v-Raf/v-Myc virus. (A) Macrophages growing on CytodexTM 1 microcarrier beads. Arrow is pointing to a microcarrier bead imaged with a 4x objective. (B) Individual cells can be seen growing on the microcarrier beads. (C) Macrophages growing in the same well as the images above, can be seen adherent on the plate.

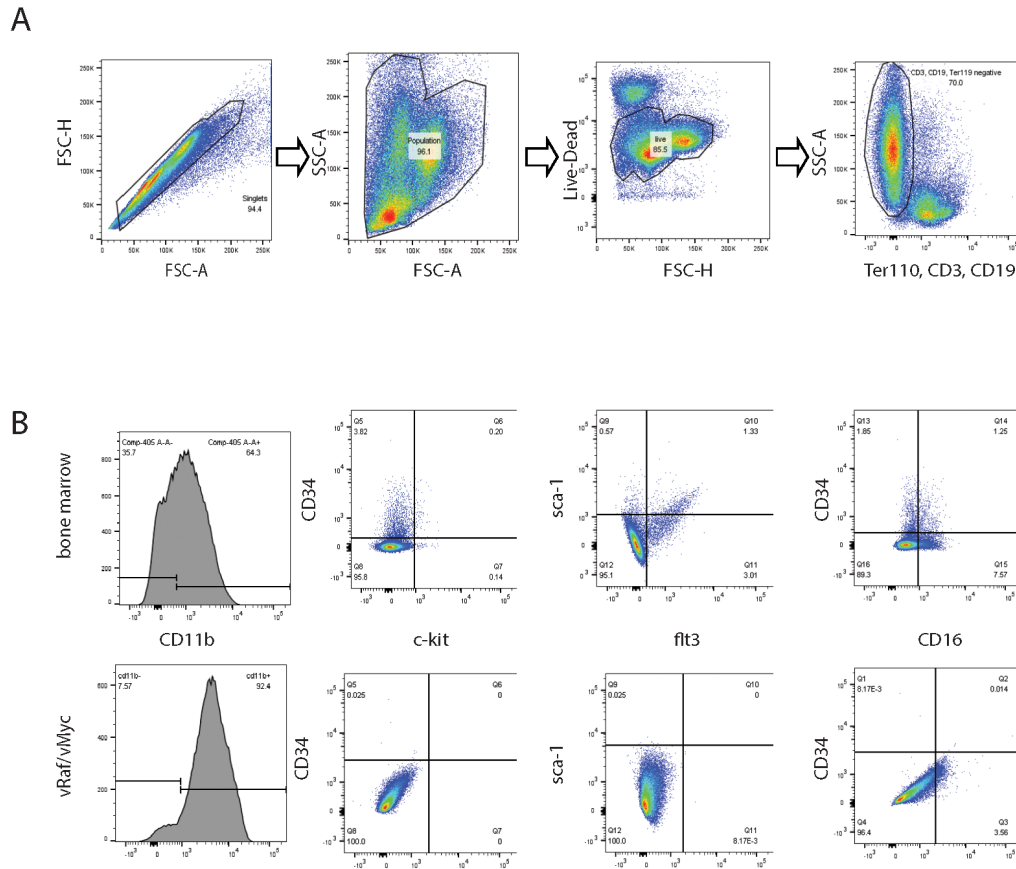


Figure 12: Early myeloid progenitor markers

(A) Bone marrow and polyclonal v-Raf/v-Myc transformed blood were gated using the same strategy. Bone marrow is shown as an example. Non-singlets were excluded. The population of cells was then gated to exclude dead cells. Following this, lymphocytes and red blood cells were excluded. The final population was live, CD3-, CD19-, Ter119-. (B) The CD11b expression profile of live, CD3-, CD19-, Ter119- cells from bone marrow or v-Raf/v-Myc transformed blood is shown. Live, CD3-, CD19-, Ter119- CD11b + cells were then examined for CD34, sca-1, c-kit, CD16, and flt-3 expression. Negative gates were drawn based on FMO controls for CD11b, sca-1, flt-3, CD34, CD16, c-kit, and lineage dump gate.

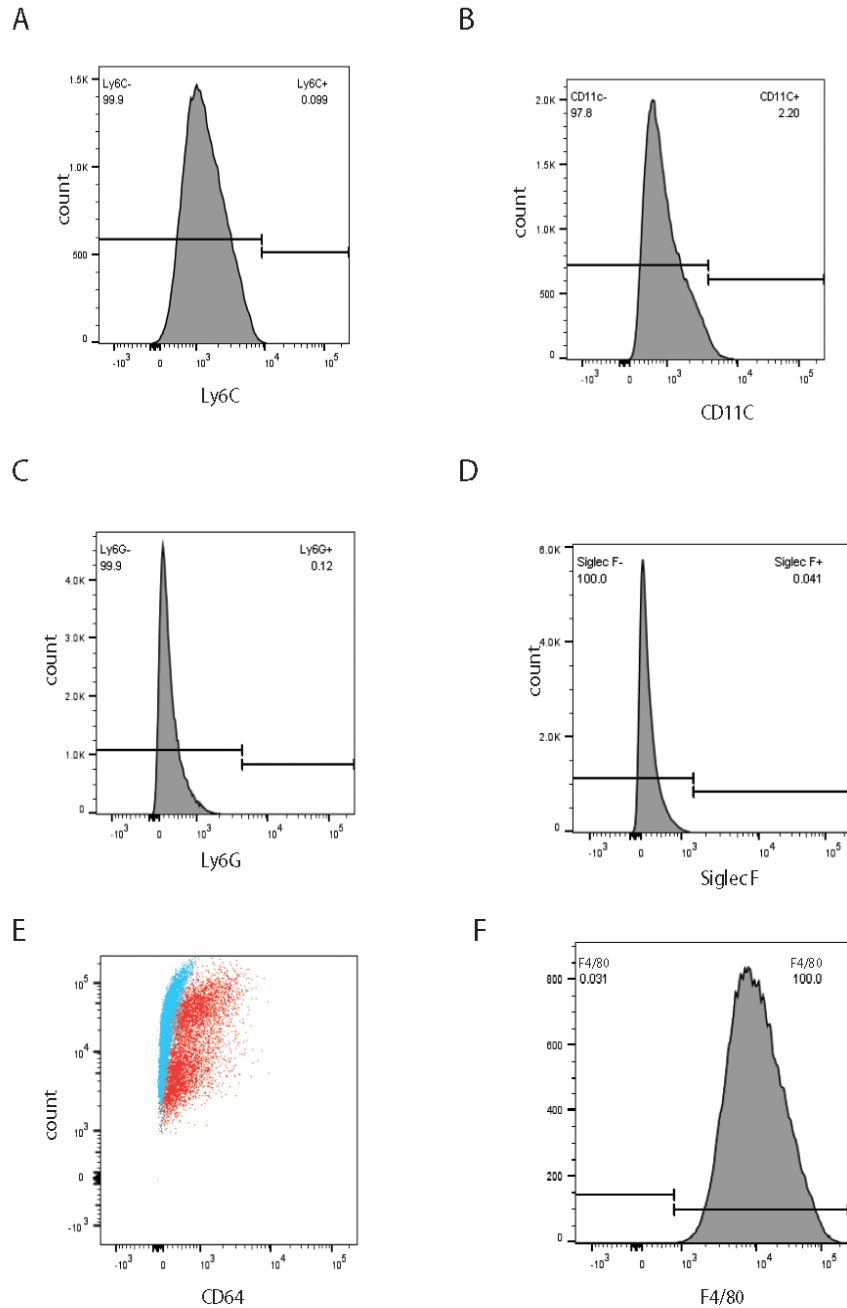


Figure 13: Mature myeloid cell differentiation markers

Blood derived v-Raf/v-Myc immortalized cells were stained with anti-CD11b, CD11c, Ly6G, Ly6C, Siglec F, and CD64. CD11b+ cells were then examined for expression of (A) CD11c, (B) Ly6G, (C) Ly6C, and (D) Siglec F. FMO controls were used to set negative staining cutoffs shown in histograms. (E) CD11b+ cells stained with CD64 (red) and the corresponding FMO control (blue). (F) Blood derived v-Raf/v-Myc immortalized cells were stained with anti-F4/80. Unstained cells were used to set negative staining cut off.

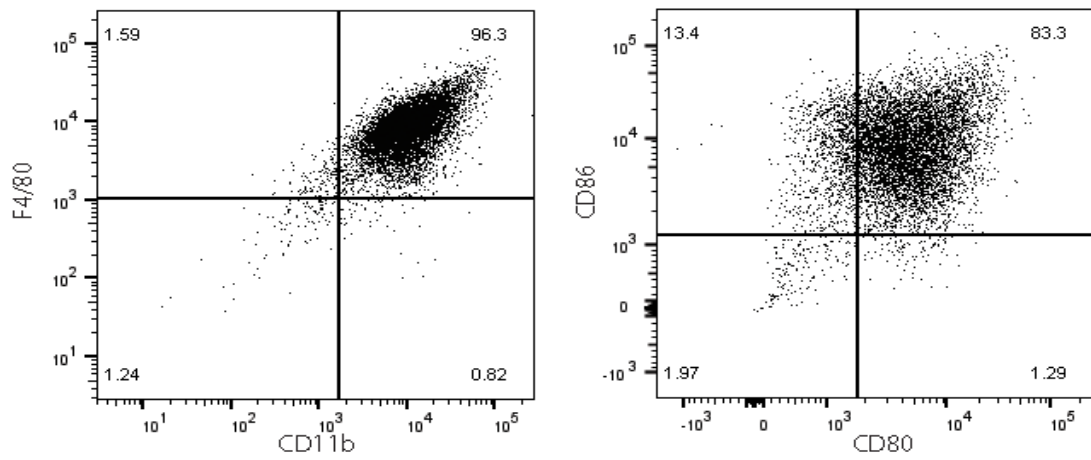


Figure 14: Spleen derived v-Raf/v-Myc immortalized cells express mature myeloid markers.

Spleen derived v-Raf/v-Myc immortalized cells were stained with anti-CD11b, F4/80, CD86, and CD80. Negative gates were based on unstained controls

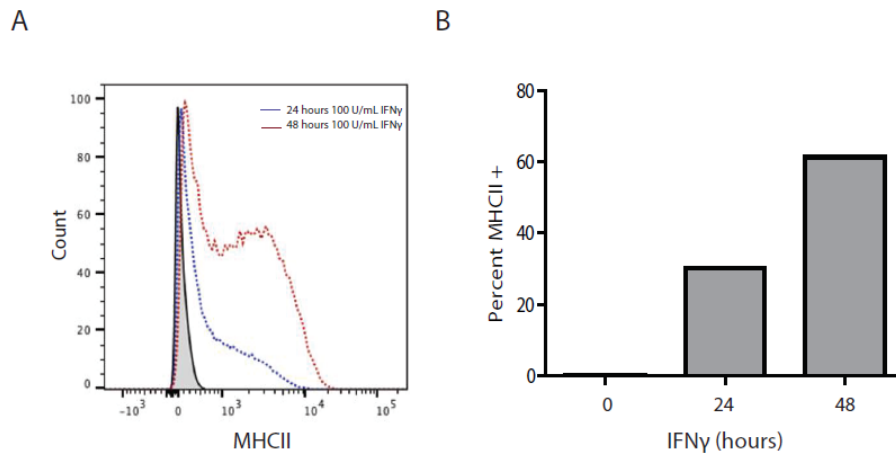


Figure 15: v-Raf/v-Myc immortalized peripheral blood expresses MHCII in response to IFN γ .

Blood immortalized with v-Raf/v-Myc were unstimulated or stimulated for 24 and 48 hours with 100 U/mL IFN γ . Cells were stained for flow cytometry with an anti-MHCII antibody. (A) The gray peak represents unstimulated cells. The dotted lines peaks are 24 hours IFN γ stimulation (blue) or 48 hours of IFN γ stimulation (red). (B) Percentage of MHCII + cells at 0, 24, or 48 hours post-IFN γ stimulation.

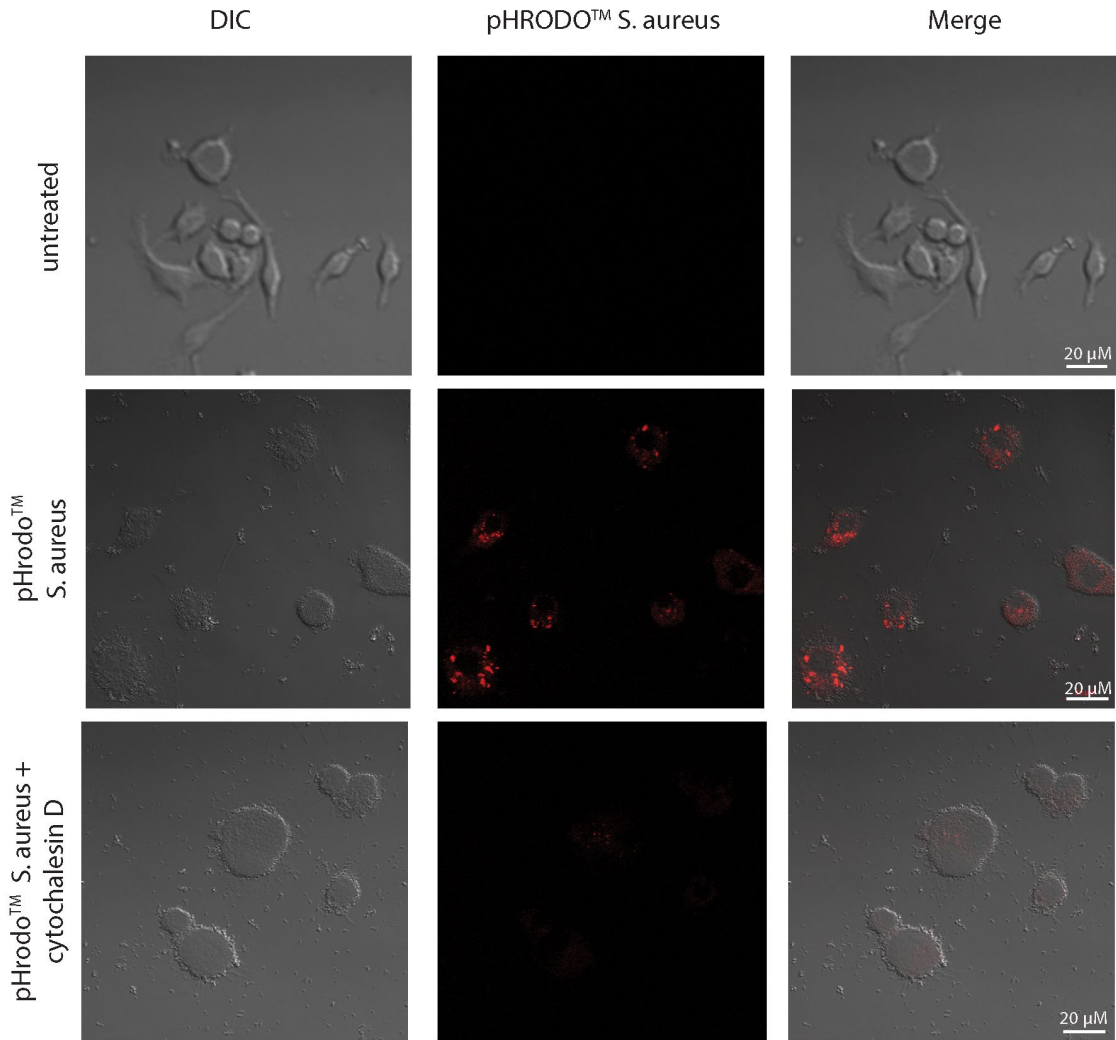


Figure 16: v-Raf/v-Myc immortalized macrophages are phagocytic

Macrophages growing on coverslips were either mock pre-treated or pre-treated with 10 μ M cytochalasin D for 30 minutes. Cells were then treated with pHrodo™ Red *S. aureus* BioParticles for one hour and then prepared for confocal microscopy. Images were taken with a 40X objective. Red color indicates internalized pHrodo™ *S. aureus* BioParticles.

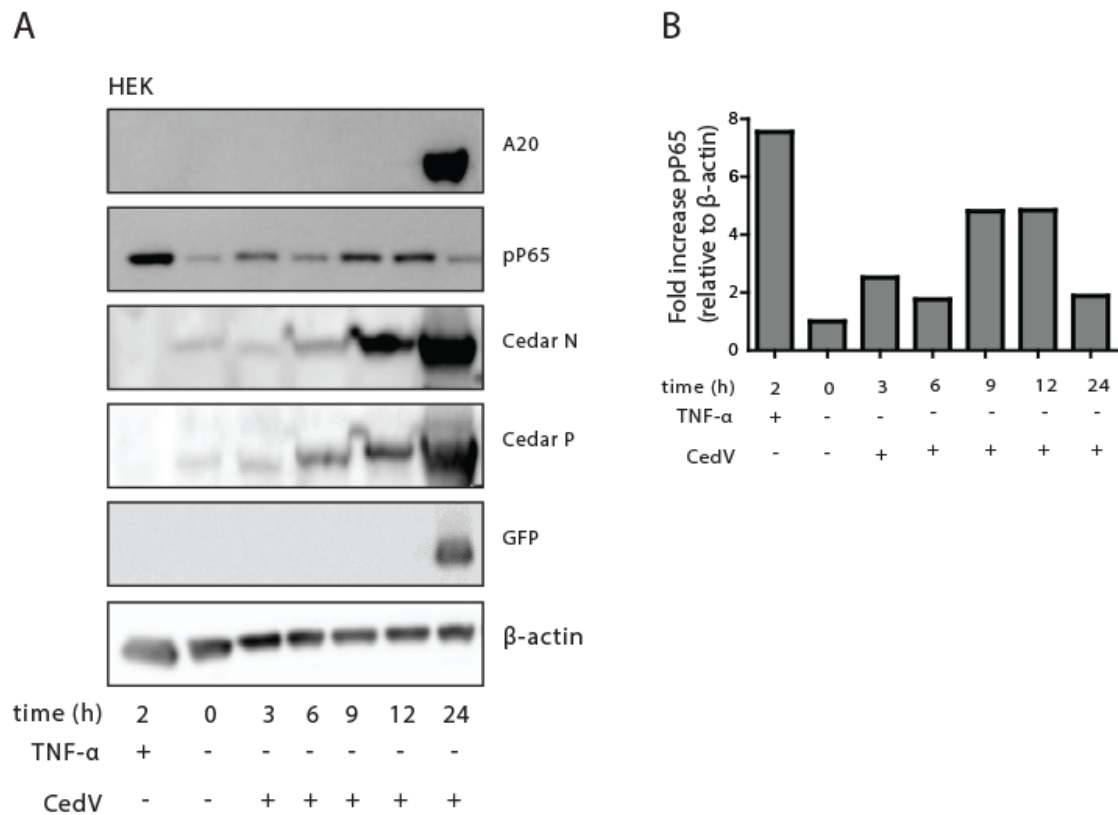


Figure 17: CedV activates the NF-κB pathway in HEK 293T cells.

(A) HEK 293T cells were infected with rCedVGFP at an MOI of 1.0. Lysates were collected at indicated times post infection. Alternatively, cells were treated with 15 ng/mL of rhTNFα. Lysates were then analyzed by western blot. (B) Expression of pP65 was quantified using ImageJ and normalized to β-actin. These values were used to determine the fold increase of pP65 as compared to the uninfected control.

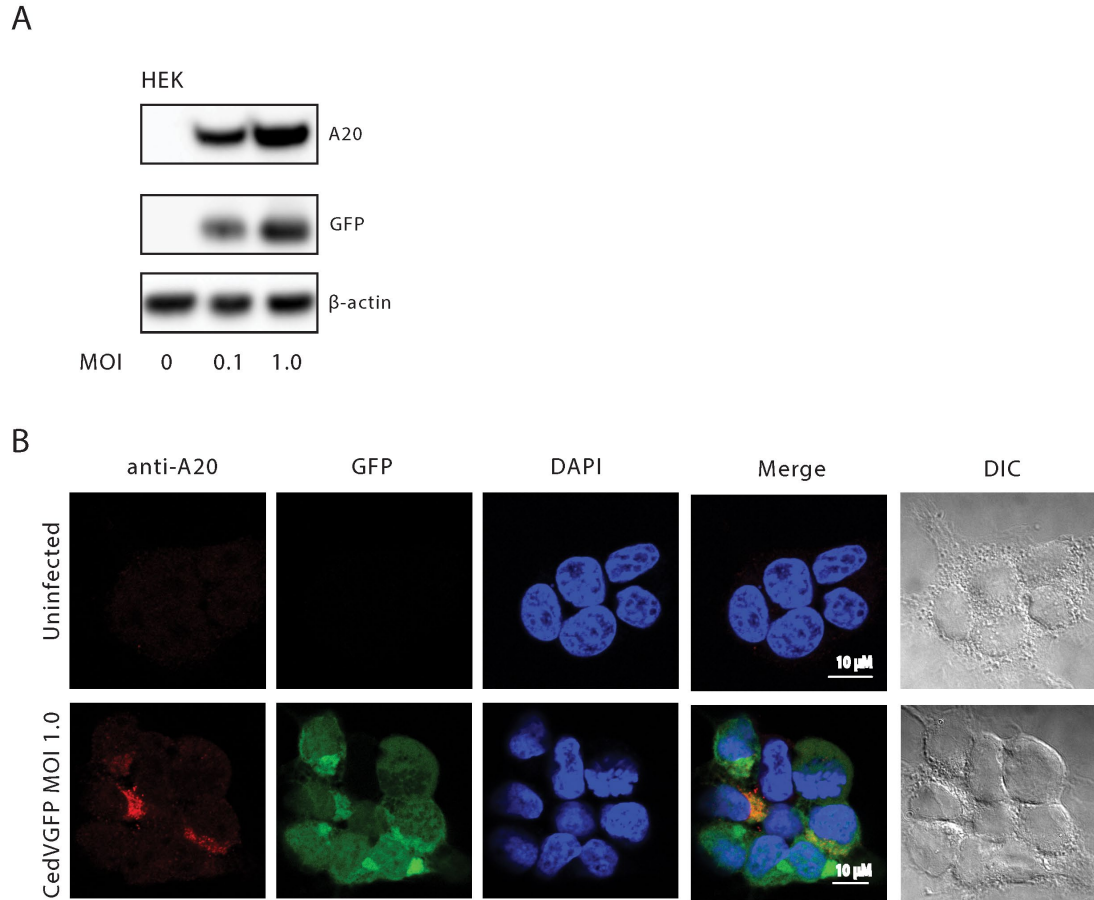


Figure 18: CedV infection induces A20 expression

(A) Western blot of HEK lysates infected for 24 hours with rCedV-GFP at the indicated MOI. The anti-A20 antibody used is clone D13H3 (B) HEK cells 24 hpi with rCedVGFP at a MOI of 0.5. Cells were fixed and stained with anti-A20 (clone 59A426) followed by an AlexaFluor 647 conjugated goat anti-mouse IgG1 secondary (red). Cellular nuclei were stained with DAPI. Images were taken with a 63x oil objective.

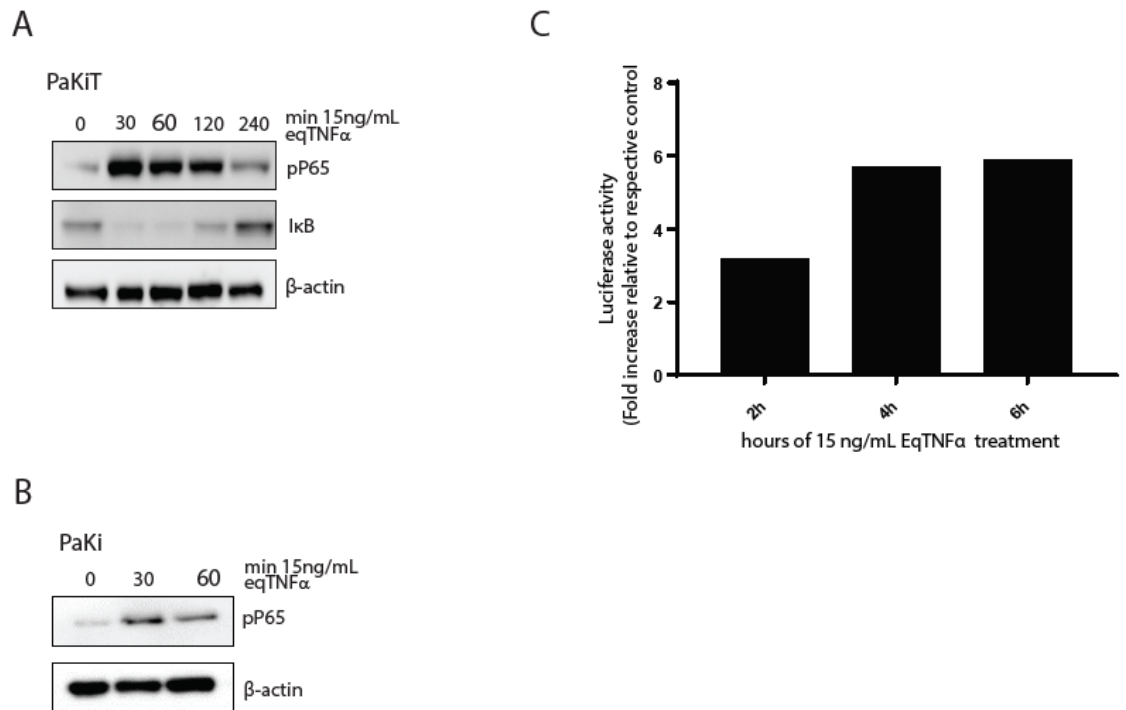


Figure 19: PaKiT and PaKi cells can be induced to activate the canonical NF- κ B pathway

(A) Western Blot images of cell lysates from (A) PaKiT or (B) PaKi cells treated with 15 ng/mL of rEqTNF α for indicated times. (C) PaKi cells were stably transfected with MSCVsin-5xNF- κ B-Gluc-NeoR, selected with hygromycin treatment, and cloned. The resulting cell line was treated with 15 ng/mL of rEqTNF α . Supernatants were collected from the treated cells and their respective untreated time controls, and luciferase activity was measured. Luciferase activity is reported as a fold increase with respect to the activity measured in the supernatant from cells untreated for an equivalent amount of time.

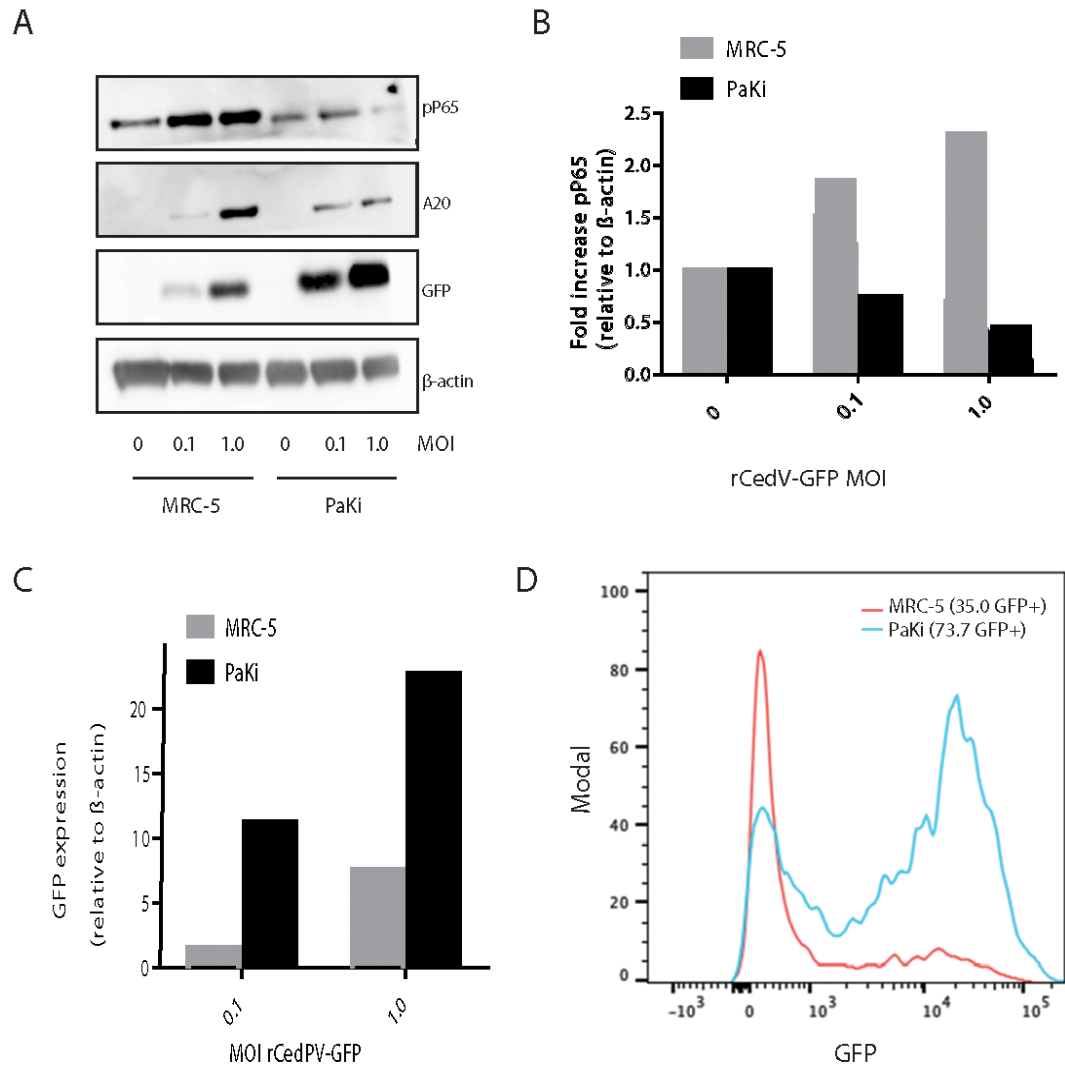


Figure 20: MRC-5 but not PaKi cells induce NF- κ B at 24 post CedV infection

(A) Western blot image of lysates from human (MRC-5) and bat (PaKi) mock infected or infected with rCedVGFP for 24 hours at the indicated MOI. Image is a representative of 4 independent experiments. (B) Fold increase in pP65 as compared to each cell line's uninfected control. pP65 raw value was calculated with ImageJ and is normalized to each sample's β -actin loading control. (C) GFP band intensity was quantified with ImageJ and normalized to the corresponding β -actin loading control. (D) MRC-5 and PaKi cells were infected with rCedVGFP at an MOI of 1.0 for 24 hours and were then analyzed by flow cytometry. Histogram is representative of 3 independent experiments.

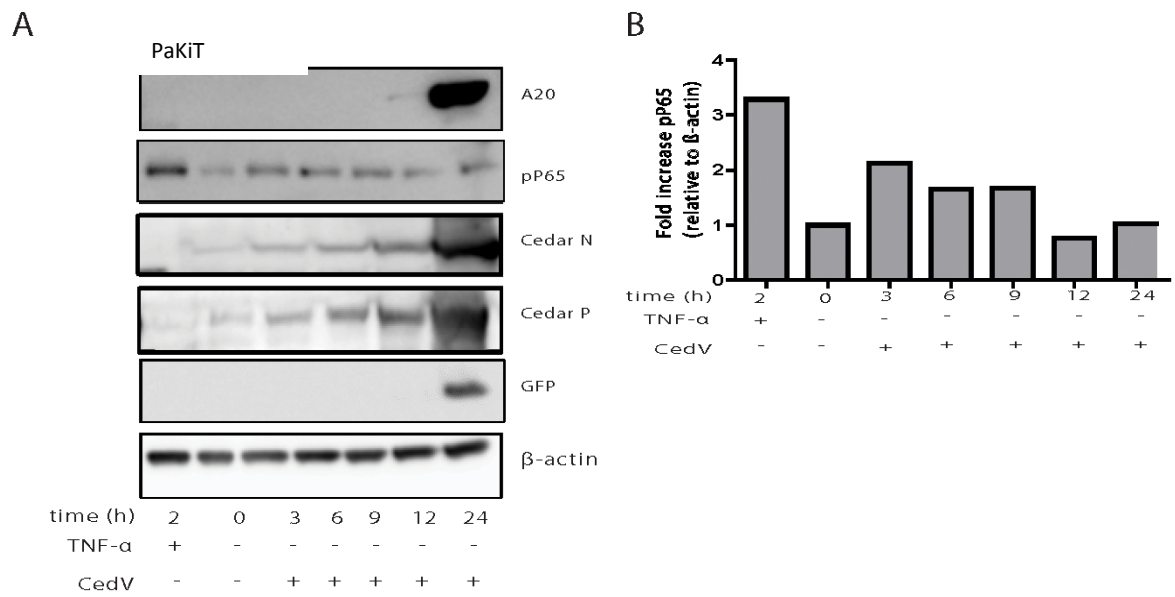


Figure 21: *P. alecto* cells mount an early but modest NF-κB response.

(A) PaKiT cells were infected with CedV at an MOI of 1.0. Lysates were collected at indicated times post infection. Alternatively, cells were treated with 15 ng/mL of rEqTNFα. (B) Expression of pP65 was quantified using ImageJ and normalized to β-actin. These values were used to determine the fold increase of pP65 as compared to the uninfected control.

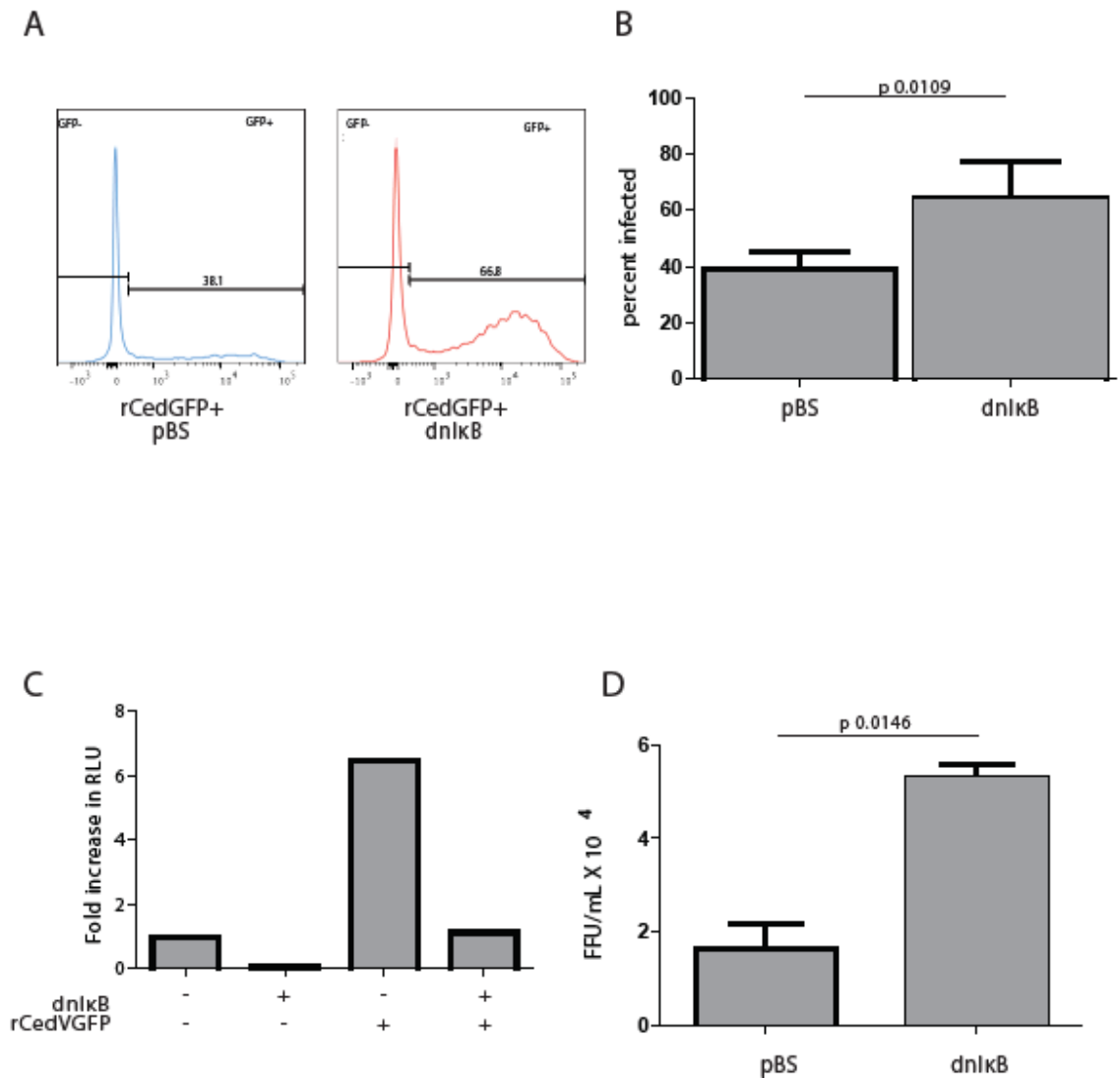


Figure 22: NF-κB signaling dampens CedV replication in HEK 293T cells.

HEK 293T cells were transfected with pBS or pMSCV-dnIkB-IRES-Thy1.1. 48 hours post transfection cells were infected or mock infected with rCedVGFP at an MOI of 0.5 and 24 hpi cells were analyzed (A) Cells were stained for thy1.1 and flow cytometry was performed. Histogram is gated on thy1.1 positive cells when applicable and is representative of three independent experiments. (B) Percentage of GFP positive cells from three independent experiments. Error bars represent standard deviation (SD). p value was determined using a paired Student's t test (C) Titers calculated from supernatants of mock transfected or transfected cells infected with rCedVGFP as described above. p was determined using an unpaired two-tailed Student's t test where n=2-3. (D) Luciferase assay on lysates of cells transfected and infected as described. Values are recorded as fold change in relative luciferase units (RLU) as compared to the RLU of mock infected, untransfected cells

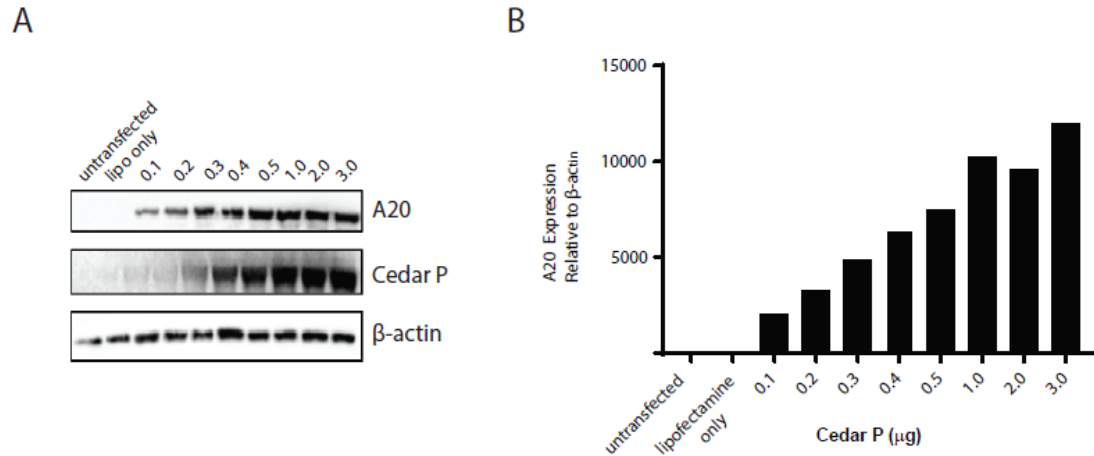


Figure 23: CedV P induces A20.

(A) HEK 293T cells were transfected with 0.1-3.0 μg of pCMV-CedV-P or pBS, pBS was used as filler so all conditions received a total of 3 μg of DNA. At 24 hours post transfection, cells were lysed and analyzed by western blot. (B) Western blot shown was used to quantify protein expression using ImageJ. A20 (stained with clone D13H3) expression is reported as relative to each lysate's β -actin control. Images are representative of 2 independent experiments.

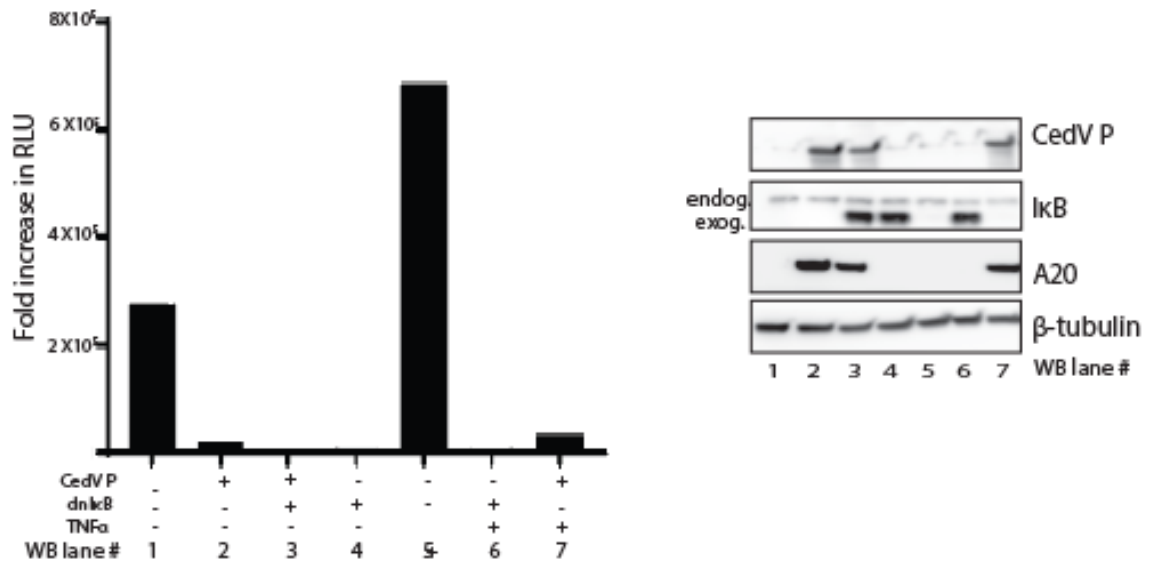


Figure 24: CedV P induces A20 expression independent of the NF-κB pathway.

HEK 293T cells were either untransfected or transfected with variations of pCMV-CedarP, and MSCV-dnIκB-IRES-thy1.1. 24 hours post-transfection, cells were either left untreated or treated for 3 hours with rhTNFα. Cells were then split in half and were either lysed for luciferase assay or lysed for western blot. On the left are the relative luciferase readings. On the right is the protein expression of each condition. The higher molecular weight band seen when the membrane was probed with anti-IκB, corresponds to endogenous dnIκB expression. The smaller size protein is the truncated dnIκB protein made from MSCV-dnIκB-IRES-thy1.1 transfections. Lanes are labeled 1-7 in both the left and right images. Matching numbers indicate that the sample (luciferase or protein lysate) was prepared from the same transfection condition.

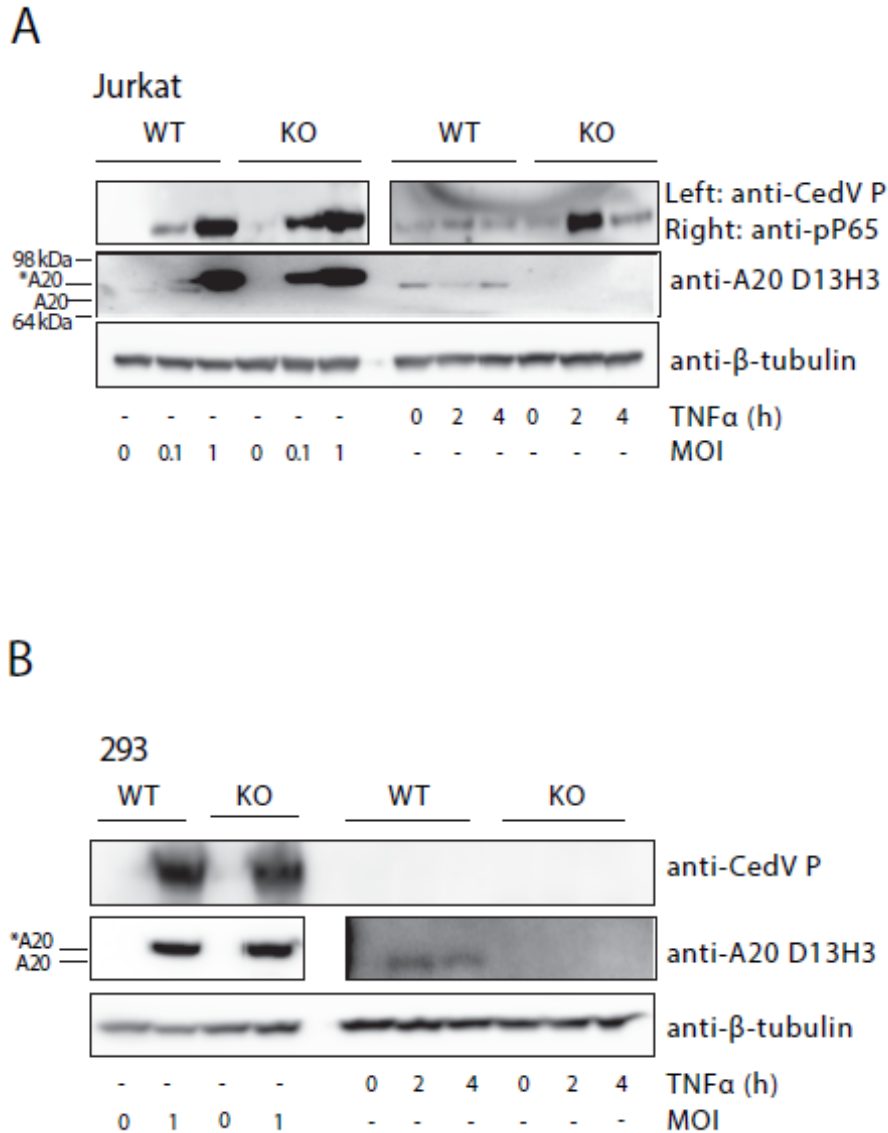


Figure 25: A20 protein expression is detected in CedV infected A20 exon 3 biallelic knockout cell lines

(A) WT Jurkat cells or Jurkat cells with a biallelic CRISPR knock out of exon 3 of A20 were infected with rCedVGFP for 24 hours at an MOI of 0.1 or 1.0 or treated with 15 ng/mL of rhTNF- α . Lysates were prepared for western blot. Lysates from infected Jurkat cells were probed with anti-Cedar P while those treated with TNF- α were probed with anti-pP65. (B) WTHEK293 cells or HEK293 cells with a biallelic TALEN knock out of exon 3 of A20 were infected with rCedVGFP for 24 hours at an MOI of 1.0 or treated with 30 ng/mL of rhTNF α . Lanes with Cedar-infected lysates were separated from lanes with TNF-treated lysates by cutting of the membrane, allowing for a longer exposure of the TNF α treated lanes. Asterisk indicates alternative A20. anti-A20 antibody is clone D13H3.

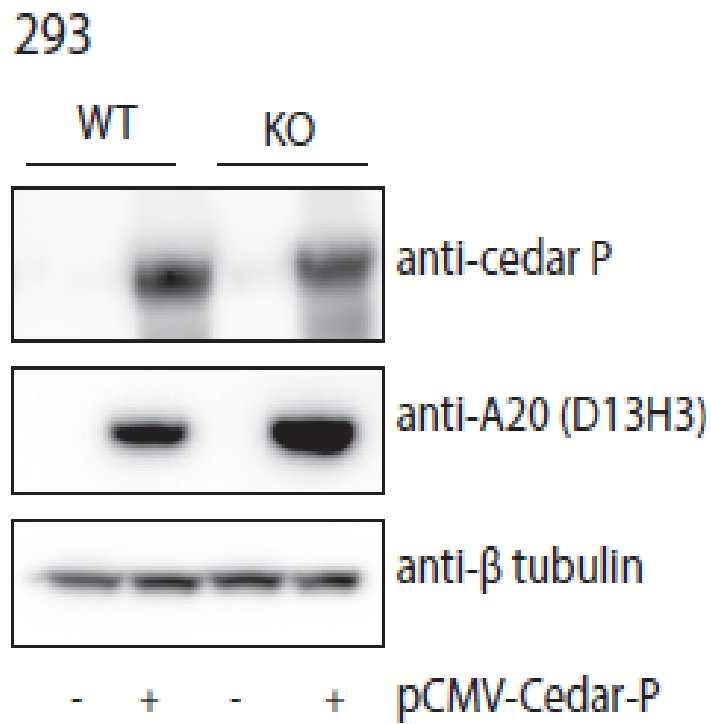


Figure 26: Cedar P is sufficient for A20 protein expression detected in HEK293 A20 exon3 KO cells.

Western blot analysis of lysates prepared from HEK293 biallelic exon3 KO cells transfected for 24 hours with pCMV-Cedar-P. Anti-A20 is clone D13H3.

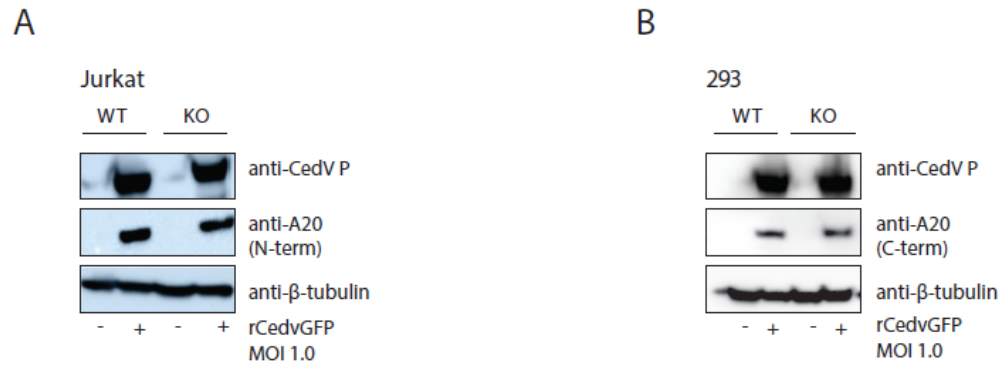


Figure 27: A20 C and N terminus epitopes are present in alternative A20.

(A) WT Jurkat cells or Jurkat cells with a biallelic CRISPR knock out of exon 3 of A20 were infected with rCedVGFP for 24 hours at an MOI of 1.0 or treated with 15 ng/mL of rhTNF- α . Lysates were prepared for western blot. A20 mAb clone A12 was used to detect *A20 protein expression (B) WTHEK293 cells or HEK293 cells with a biallelic TALEN knock out of exon 3 of A20 were infected with rCedVGFP for 24 hours at an MOI of 1.0. A20 mAb clone 59A426 was used to detect c-terminus epitope.

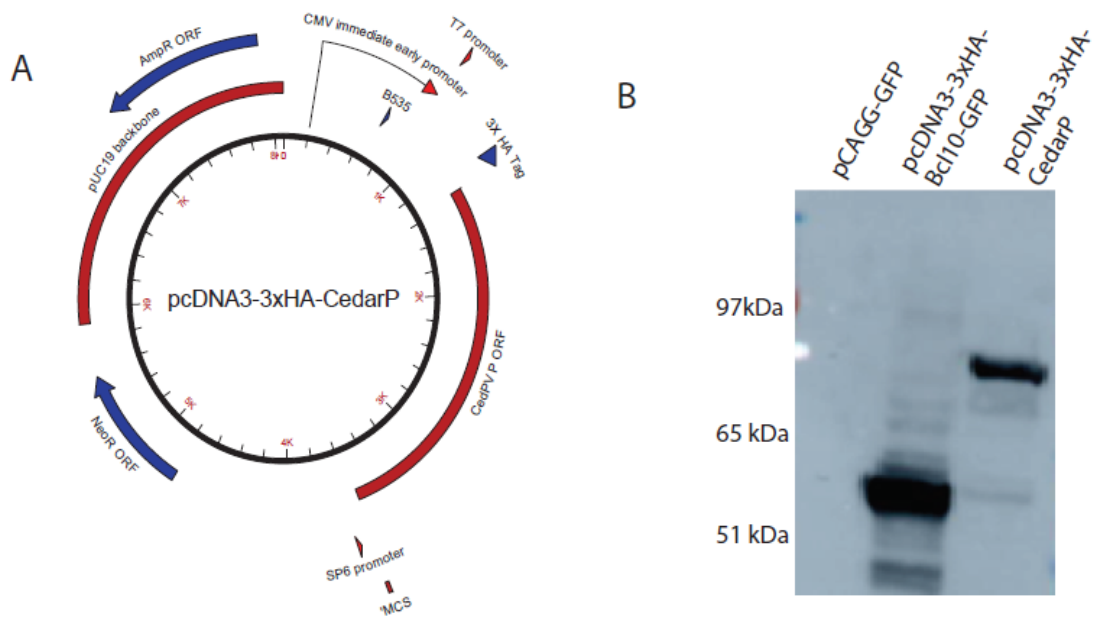


Figure 28: Construction of pcDNA3-3xHA-CedarP

(A) Final DNA construct of pcDNA3-3xHA-CedarP cloned from pCMV-3xHA and pCMV-CedarP. 3xHA tag is located at the N-terminus of Cedar P and is indicated by a blue triangle. (B) HEKHEK293T cells were transfected for 24 hours with 2.5 μ g of pCAGG-GFP, pcDNA3-3xHA-Bcl10-GFP, or pcDNA3-3xHA-CedarP. Lysates were made and used in western blot analysis. Membrane is probed with anti-HA clone F7. CedV P is tag is 84 kDa without HA

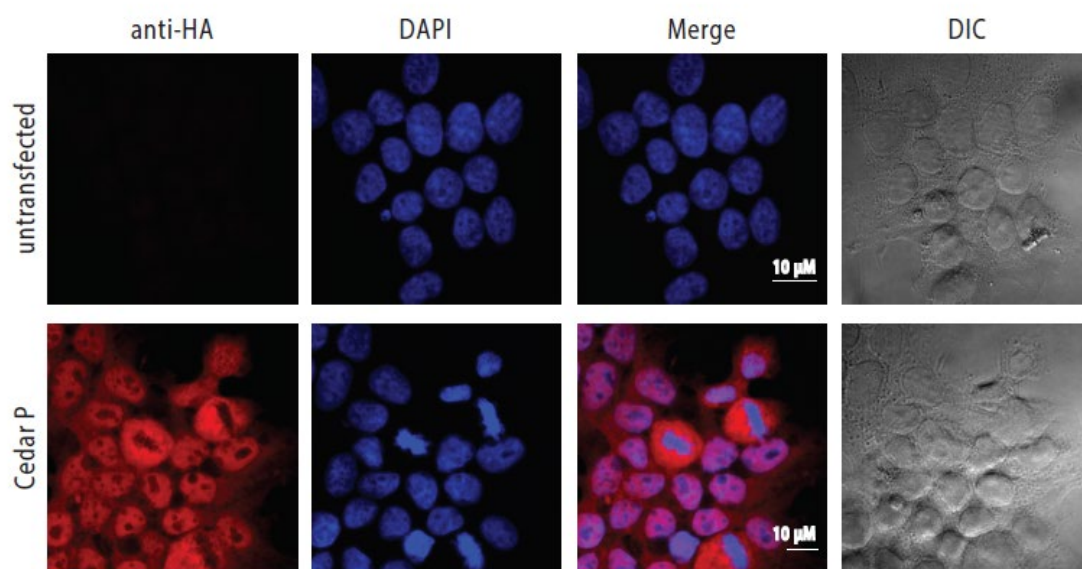


Figure 29: Cedar P localizes to the nucleus.

Cells were mock transfected or transfected with 250 ng of pcDNA3.1-3xHA-CedVP. 24 hours post transfection cells were fixed and analyzed with anti-HA followed by an AlexaFluor555 conjugated goat anti-mouse IgG2b secondary (red). Cellular nuclei were stained with DAPI.

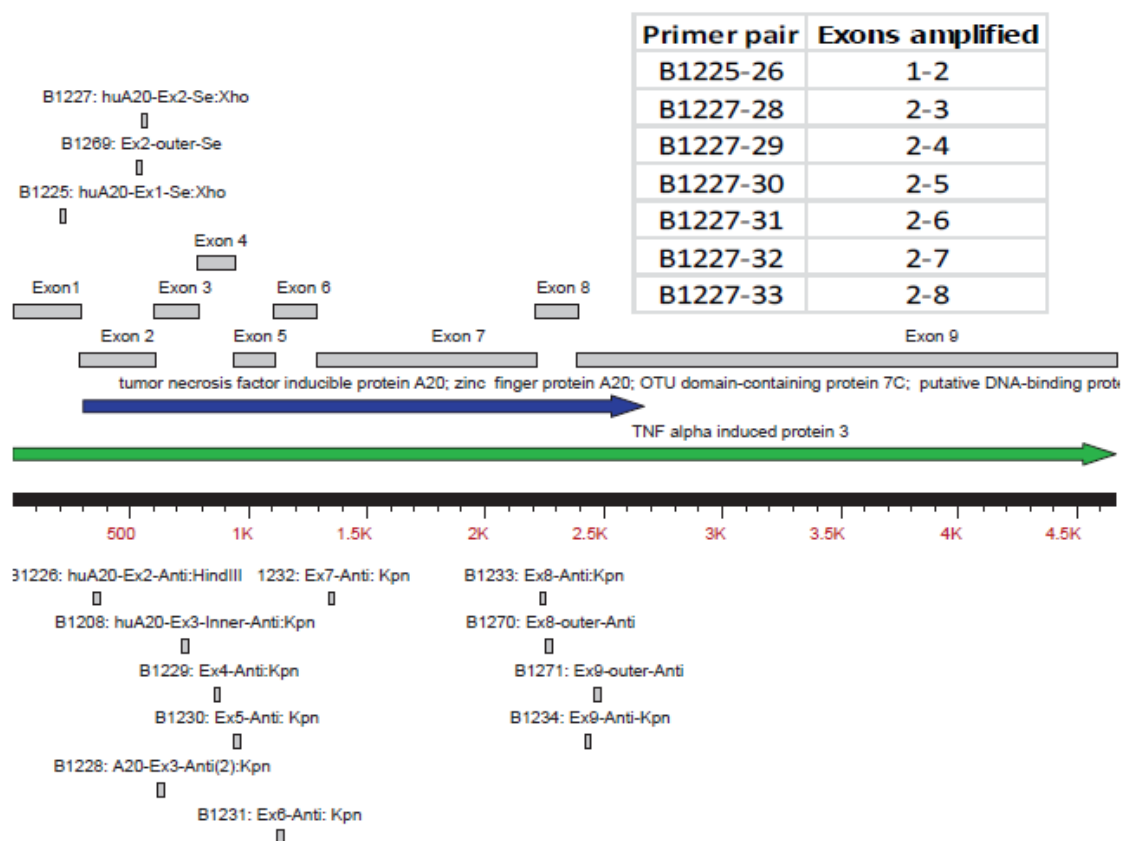


Figure 30. Map of A20 exons and primers used to amplify A20 cDNA.

The large blue arrow indicates the A20 open reading frame. The arrangement of the exons that make up human A20 mRNA are shown above the open reading frame. The locations of the sense primers used to amplify various fragments of the A20 cDNA are depicted above the exons. Below the scale bar, are the locations of the anti-sense primers used in conjunction with the above. In the inset table, are the combinations of primers used to amplify the A20 cDNA.

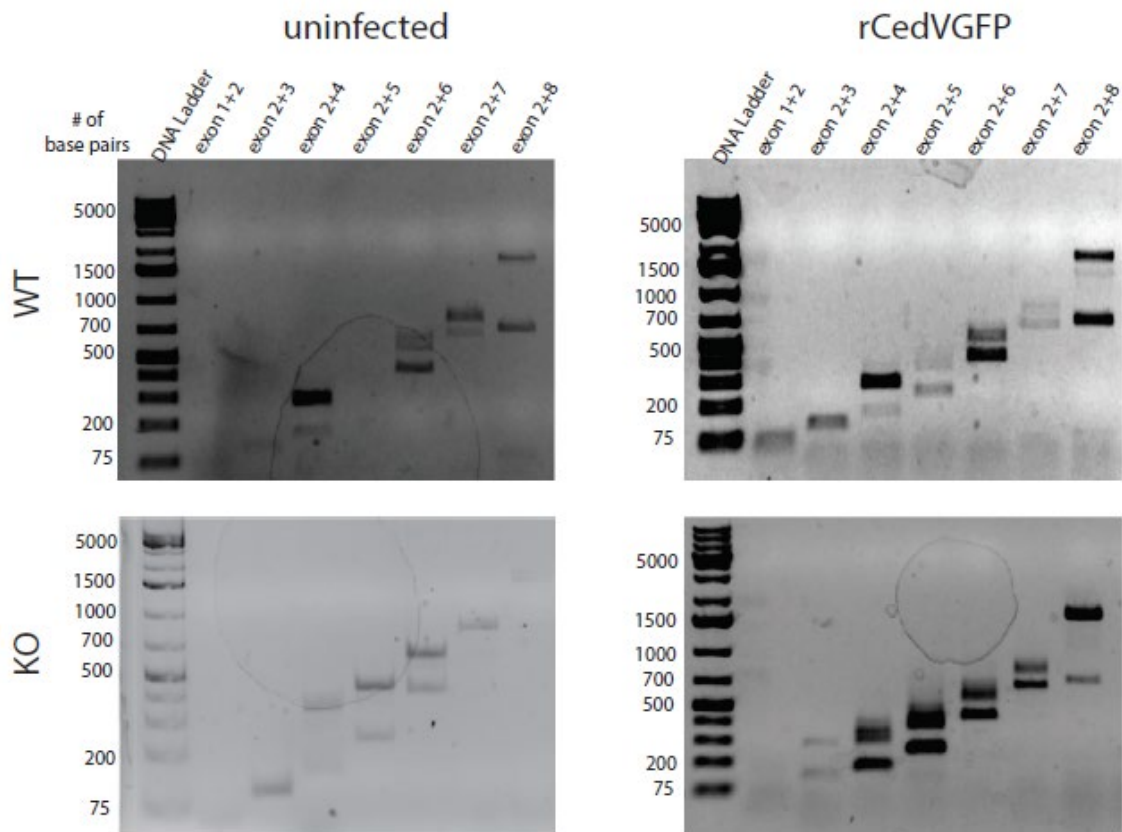


Figure 31. PCR products amplified from primers mapping to 8 exons.

HEK293 WT or biallelic exon3 KO cells were either uninfected or infected with rCedVGFP at an MOI of 1.0. mRNA was isolated and cDNA was made. PCRs using primers to amplify exon1 to 2 and 2 to each of the subsequent 6 exons were used to amplify A20 cDNA. PCR products were run on a 1.2% gel.

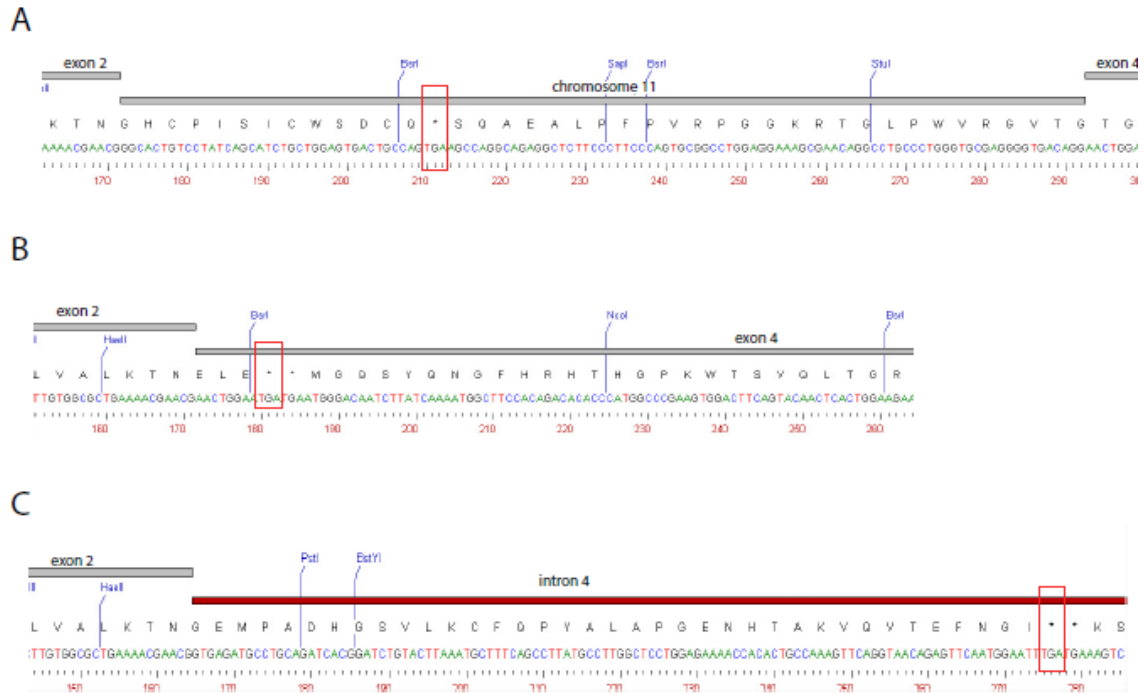


Figure 32: Sequence results from infected A20 KO cell lines

Jurkat biallelic exon 3 CRISPR KO cells were infected with rCedVGFP for 24 hours at an MOI of 1.0. mRNA was isolated and cDNA was made. (A) Primers B1227 and B1229 or (B) primers B1227 and B1230 were used to amplify the sequences. (C) HEK293 biallelic exon 3 TALEN KO cells were infected as in A and B. mRNA was isolated and used to make cDNA. Primers B1227 and B1233 were used to amplify the sequence shown. Red rectangles indicate the site of the early stop codon.

CHAPTER 4: DISCUSSION

SUMMARY OF FINDINGS

In the work presented above, we have designed a new method for the immortalization of macrophages and investigated the NF- κ B response to infection by an authentic henipavirus. This investigation provides the scientific community with new insight on bat immunology, specifically in the important viral reservoir *P. alecto*. It also provides a promising lead for the creation of an immortalized macrophage cell line from bats and/or other types of mammalian reservoirs. The data we collected through the course of the above experiments have afforded the following conclusions:

1. Transduction of murine bone marrow with human Hox11 gives rise to an immortalized cell line. This cell line is CD11b- and c-kit+. These markers and the cells' morphology are consistent with early progenitor cell populations.
2. A new v-Raf/v-Myc lentivirus was designed and constructed that can immortalize cells from both murine blood and spleen. These cells require, at least initially, Cytodex 1 beads for replication. The surface receptor profile and morphological characteristics of v-Raf/v-Myc immortalized cells are indicative of a mature macrophage.
3. Cells immortalized by v-Raf/v-Myc virus can perform important macrophage functions. They can upregulate MHCII in response to IFN γ and can phagocytose *S. aureus*.
4. *Pteropus alecto* cell lines can activate the NF- κ B pathway in response to TNF α . The TNF α response leads to induction of phosphorylation of the NF-

κ B p65 subunit, degradation of I κ B α and results in activation of a NF- κ B responsive promoter.

5. Cedar virus (CedV) can infect *P. alecto* cell lines. More virus protein is detected following infection of PaKi cells as compared to infection of the comparable human cell line, MRC-5. We have also shown CedV can infect a human T-cell line.
6. CedV induces the canonical NF- κ B pathway, but this activation response is different in human cell lines in comparison to *P. alecto* cells lines immortalized similarly. Overall, a stronger NF- κ B response with lower levels of viral protein is observed in human cell lines, while bats have a weaker NF- κ B response and higher levels of detectable virus protein. The initiation of the NF- κ B response starts and ends earlier after infection in PaKiT cells than in HEK293T cells. In HEK293T cells, the NF- κ B pathway plays a role in restricting virus replication.
7. CedV infection activates a robust A20 response in bat and human cell lines. CedV P is sufficient for induction of A20 expression but does not require activation of the NF- κ B pathway to do so. In fact, CedV P is able to block TNF α mediated activation of NF- κ B in HEK293T cells. Upon transfection, Cedar P localizes to the nucleus of HEK293T cells.
8. The A20 protein that is induced during CedV infection or by heterologous expression of CedV P can be expressed in A20 exon 3 KO cell lines and thus does not contain exon 3. This type of A20 (*A20) has not been described previously. *A20 could be detected with all three monoclonal A20 antibodies

tested, which mapped to both C and N terminal epitopes. To date, we have been unable to elucidate the splicing pattern that yields the *A20 mRNA and translated protein.

INTERPRETATION AND SIGNIFICANCE OF FINDINGS

We launched our investigations with plans to construct a cell line that has the potential to immortalize many species. Past and current emerging viral diseases have confirmed the importance of studying reservoirs. Understanding how a reservoir's immune system tolerates deadly pathogens can provide invaluable information as to how to treat the disease we see in humans.

Limitation of current *P. alecto* sample preparation.

To generate a protocol for immortalization of a bat macrophage cell line, we first needed to verify that we could derive bat macrophage-like cells. We used murine GM-CSF to do this. Though we were able to derive macrophages from *P. alecto* bone marrow that we had acquired from collaborators in Australia, the total number of viable cells was less than ideal. Many cells that we put into culture died within the first day. This observation is perhaps unsurprising as others have reported that cryopreservation can affect immune cell function and our cells had been stored under these conditions for several years (32; 36). Nevertheless, the fact that we can derive phagocytic cells with a poor starting sample suggests that we will likely have better success, if a healthier starting sample is obtained. This is one of the many reasons that starting with blood, which can be harvested and processed much more quickly than can bone marrow, is more ideal. The next goal then, was to optimize a system that we could apply to a healthy *P. alecto* macrophage culture.

Limitations in Hox11 transduction

The molecular cloning of Hox11 was simple in that there was one cDNA that could be transferred into a modified lentiviral vector in a single step. Unfortunately, however, in addition to requiring stem cells, fetal liver (as reported by another group) or bone marrow, the resulting cell line is locked in an early progenitor state (85). Hematopoietic stem cells (HSC) that exist in the bone marrow, give rise to early progenitors. These stem cells have the potential to become either a myeloid or lymphoid progenitor, and eventually to become one of many mature subsets of white blood cells (130). This process of changing from an early progenitor to a mature, functional cell, is known as cell differentiation. During differentiation, cytokines and growth factors drive the fate of the cell. This occurs concurrently with changes in surface receptors (103; 130; 220). Our Hox11 immortalized cells are CD11b- and c-kit+. C-kit is a marker found on many subsets of early progenitors. Combined with the fact that these cells were exposed to IL-6 and IL-3 upon *ex vivo* culture, we likely immortalized a myeloid progenitor cell line (103). Hox11 has been shown to immortalize a myriad of progenitors, however, so the definitive cell type is not clear without additional experimentation (168). What is clear, based on the literature, is that Hox11 immortalized cells are in a differentiation block (181). The TLX1 overexpression keeps the cells from being able to mature to more functional cell types, which are important for infectious disease studies (85). What is novel from our data, however, is that while the previous protocol available uses a virus cell line for infection and methylcellulose culture conditions, our protocol uses RetroNectin transduction and can be completed using standard cell suspension culture techniques (85). The most relevant observation for our purposes however, is that we have provided an example by which a human oncogene works in a non-human host to

produce a blood cell line. This is evidence that other related oncogenes may work across species.

Characteristics of cell lines resulting from v-Raf/v-Myc and their potential value in comparative immunology applications.

In looking for better alternatives to Hox11, we investigated the possibility of making a cell line that was mature, cytokine independent (Hox11 cells require IL-3 indefinitely), and could be made from peripheral blood. The J2 virus met the first two requirements and we hypothesized that it could meet the third (26). Because the J2 virus was replication competent and relied on a messy producer cell co-culture system, we aimed to design an improved virus. Additionally, the J2 virus encodes for two oncogenes, but how they are both expressed in the context of the J2 transcriptional unit is not clear. We constructed a replication deficient lentivirus that contains a T2A self-cleaving peptide between the two oncogenes. This eliminates the need for “messy co-culture,” is safer, and the mechanism by which each oncogene is translated is clear. We adapted our protocol accordingly and used RetroNectin for our transduction. This alteration likely increases efficiency compared to the previous system used (124). The original J2 virus immortalized spleen, fetal liver, bone marrow, and brain tissue derived microglia (24; 26; 76; 150; 182). Additionally, alveolar macrophage cultures were immortalized by J2 (237). We demonstrated that our improved v-Raf/v-Myc virus can be used to immortalize blood. This is the first time a tissue or cell type that can be obtained non-invasively, has been used with a v-Raf/v-Myc based virus. This is ideal when considering applications for emerging virus reservoirs. Many bats are protected species and so sample acquisition is extremely limited. Equally important is that this protocol does not require a large volume of blood to be successful. The blood volume of many

reservoir species is quite small but we produced cell lines when we started with as few as 100,000 PBMC.

Our cell line's IL-3 independence is another factor that contributes to its candidacy for use in reservoir cell lines. Since our undertaking of the task of immortalizing bat macrophages, another group has shown they can successfully culture primary macrophages and dendritic cells from *P. alecto*. This required the production of several *P. alecto* cytokines to supplement the growth media (238). This is not logistically realistic for long term cultures. We found a short term treatment with murine GM-CSF was able to support *P. alecto* bone marrow. Our v-Raf/v-Myc protocol requires GM-CSF for only 14 days. Should murine GM-CSF not work with the v-Raf/v-Myc immortalization protocol, 14 days of engineered *P. alecto* GM-CSF is much more feasible. This is especially true when one considers that during the first 14 days of transduction, the culture size need only be one well of a 24 well plate.

The v-Raf/v-Myc cell lines that were immortalized from peripheral blood did not express markers of early progenitors, but did express F4/80 and CD11b. F4/80 is a mouse specific marker of mature macrophages, which is essentially exclusive to that cell lineage (12). This marker was also found on splenic derived cell lines and in many of the other J2 produced cell lines (26; 237). Confirming the functional abilities of the macrophages immortalized with v-Raf/v-Myc expands the applications they can have. For example, if we consider that these cells can respond to IFN γ and upregulate MHCII there are some interesting functional studies that could be investigated in new species. Perhaps bats are less sensitive to interferon signaling and are less likely to upregulate MHCII during infections. This could be directly tested if a human and bat macrophage

cell line existed. Dissecting the signaling pathway that leads to MHCII expression would be possible too. Additionally, some viruses, like Influenza, can infect macrophages. A macrophage cell line could be made from a given influenza strain's reservoir species. Following this, investigators could examine what occurs when a virus enters the cell and compare it to what happens in the spillover host. Importantly, cell lines made with vRaf/v-Myc would allow for more direct comparisons to be made. Current methods mostly rely on leukemias isolated from species of interest. There is significant variability in cell types immortalized during leukemias so any myeloid leukemia isolated from species A, may be quite different from species B. One potential problem when investigating immune cells from non-traditional species is the difficulty in obtaining antibodies that cross react with the species of interest. In other words, deciphering whether or not you have the same cell subset from species A and species B is limited by reagents. With the potential use of this v-Raf/v-Myc virus, there would be specific expectations regarding the phenotype of the resulting immortalized cell line, even if extensive characterization was not possible. With human or mouse samples, extensive characterization will be possible. Those characteristics identified in human/mouse could give investigators an idea about their non-human/mouse cell line's phenotype. Though this still is not a perfect solution, it would certainly leave investigators less blinded regarding the cell types they are attempting to compare. The development of the v-Raf/v-Myc lentivirus that immortalizes peripheral blood provides a step forward and the opportunity to collect a lot of previously unattainable data about reservoir species.

Future directions for the v-Raf/v-Myc virus

At this point, it is appropriate to initiate translational research. Perhaps easiest, would be to next work with an animal for which reagents exist. For example, creating the comparable human cell line would be the most widely used application and all the necessary reagents to immortalize and phenotype the resulting cell line already exist. Additionally, human blood is easy to obtain. Still, with many of the emerging outbreaks arising from bats, beginning attempts at immortalization of macrophages from a bat species is important. Experiments obtaining fresh, never cryopreserved samples should be the priority moving forward with attempts at immortalization using v-Raf/v-Myc. In the past, several groups have used *in vivo* infections with v-Raf/v-Myc retrovirus (i.e., J2 virus) to immortalize cells *in vivo* (3; 25). *In vivo* v-Raf/v-Myc lentivirus infections in a bat-mouse (discussed earlier), may provide a way to immortalize bat macrophage cell lines (232). Alternatively, the bat-mouse could serve as a source for more attainable and healthy bat immune cells that could then be used with standard *ex vivo* v-Raf/v-Myc transductions.

The J2 virus immortalizations discussed have always yielded macrophage-like cells. However, *in vivo* evidence exists that B-cells can also be transformed. For example, one group reported that when they infected pristine-treated mice *in vivo*, they had equal numbers of monocytes and B cell tumors (114). It is therefore quite possible that a B-cell line could be made using the J2 virus with the correct culture conditions. If this too could be adapted to bats, interesting antibody studies could be designed.

Considering the potential to immortalize other types of immune cells using v-Raf/v-Myc, it would be helpful to make a tagged v-Raf/v-Myc. Adding a surface expression tag that can be stained will allow for detection of viral transduction using flow

cytometry or microscopy. Staining for the tag, in combination with surface markers, would allow investigators to see what type of cells are being transduced with the virus, and which types are growing out post-transduction. Under B-cell culture conditions, or any other immune cell category, we can first verify if 1) we can successfully transduce that cell type and then 2) determine if there is any outward growth of cells of that particular lineage. This tagged virus will also provide us with more information about the macrophages transduced in our experiments. With the tag present in infected cells, we will be able to see what markers are on the cells upon transduction and what markers are expressed after immortalization. In other words, is it: 1) mature macrophages that are being transduced and then expanding due to the oncogenes dysregulation of cell cycle, or 2) more naïve monocytic cells that are transduced and undergo some type of differentiation post-transduction?

Outside of emerging infectious disease, there are other potential applications of our improved v-Raf/v-Myc virus. If this protocol can be applied to humans, it will allow investigators to create immortalized cell lines from people with rare myeloid dysfunction diseases. This equips scientists with a way to increase their data output by increasing the timeline a sample with a particular mutation of interest is available to study. It also removes the limitation on starting material that is inherent when studying primary cells from blood draws. Still, in mice we have been able to immortalize a macrophage cell line from KO mice of interest. Deriving macrophages from bone marrow takes several days. The days of waiting to begin an experiment are eliminated when preliminary data can be collected from a cell line that is ready to use on demand.

NF-κB

CedV infections and NF- κ B signaling in bats and humans

Whilst identifying a candidate virus for macrophage immortalization, we were able to make progress comparing the immune response to CedV between *P. alecto* and humans with the tools we already had available. We found some intriguing differences between the comparable PaKi and MRC-5 cell lines. By both western blot and flow cytometry, PaKi cells had more detectable CedV protein than MRC-5 cells did. This is at first counter-intuitive. One could hypothesize that bats have very low levels of infection and thus a very minor immune response. The low immune response then, means that immunopathology would likely be not existent and so no disease is seen. There are other plausible hypotheses for our observations too. For example, early during infection increased replication of virus could be beneficial. A higher viral burden may promote apoptosis of the cells. This would be in line with what was reported in the transcriptomic study with PaKiT cells infected with HeV. They saw that PaKiT cells had a robust immune response to HeV and that this was followed by an upregulation in apoptosis pathways, and ultimately in apoptosis. This occurred at between 48 and 72 hours (226). A low NF- κ B response early in infection, as was seen in our CedV infected PaKi and PaKiT cells, may mean the virus replicates to higher levels but that higher titer induces apoptosis that would not occur with less viral stimulation. It will be interesting to complete our CedV infections with time points that extend out to 48 and 72 hours. Viral protein and apoptotic markers can be evaluated at these time points. Virus titers should also be examined at all 3 time points. This will allow us to gather data that could potentially support the hypothesis that an overall higher viral load early after infection yields a more rapid decrease at later time points, and that this is the result of apoptosis of infected cells.

MRC-5 cells can be transfected so it would be interesting to transfect the dnI κ B plasmid into these cells and then infect them with CedV. In this way we would be able to demonstrate if a decrease in NF- κ B activity, also leads to an increase in virus protein detection, as was seen in HEK293T cells.

We saw little NF- κ B response in the bat cells but found that a decrease in this same response led to increased virus replication in human HEK293T cells. This would also be in line with our above hypothesis. If (1) a loss of NF- κ B activation means more virus is present, and (2) increased viral replication early on mediates a pro-host induction of apoptosis, then (3) down-regulation of the NF- κ B response would be beneficial. This is one of many mechanisms that would serve as a possible explanation for why a reservoir cell line might have more detectable virus than the comparable human cell line. Last, MRC-5 (data not shown) and PaKi Gaussia luciferase cell lines have been constructed. It will be important to infect these cell lines to confirm NF- κ B pathway activation carries through to transcriptional activation of κ B promoters.

***A20 and its possible roles and effects on infections**

In contrast to the differences in NF- κ B signaling we observed in PaKi and MRC-5 cells, we found one striking commonality. Across all of the cell lines we tested, both *P. alecto* and human, a robust induction of A20 was observed. We determined that it is a unique A20 (*A20) and that its expression far surpassed that of A20 induced during TNF α treatment. When loading the same total amount of protein from TNF α treatments and CedV infections, it was necessary to cut the protein membranes stained with anti-A20 to separate the conditions. The expression of *A20 post-CedV infection was so much stronger than A20 post TNF α treatment that when blots were developed together,

the exposure of *A20 obscured the TNF α controls. It is possible that this is attributable not to the ratio of A20:*A20 produced, but rather to the differences in the stability of each form of A20.

If *A20 is pro-viral then there may be reason to induce a more stable form of A20 protein. There is some anecdotal evidence for this. Western blot experiments in our laboratory involving non-CedV conditions such as TNF α , show many degradation products. In contrast, when we probe the entire western blot membrane containing lysates from CedV infected cells, no degradation products are detected. It is reasonable to hypothesize that because *A20 still retains important catalytic domains, that it this isotype is produced not for a different function but as a more stable alternative to A20.

Definitively testing whether *A20 is more stable than A20 *in vitro* could prove complicated without more information. To start, it may be informative to know what binding partners *A20 has. Co-precipitation followed by mass spectrometry could help determine some of these partners. Following this, promising candidates could be knocked down and the presence of degradation products of *A20 measured by western blot. This approach could address how *A20 is stabilized but not whether it is a more stable form of A20. Staining cells infected with CedV with anti-A20 and fluorescently labeled ubiquitin would allow us to determine if one of these two A20 isotypes is more ubiquitinated than another. Since certain ubiquitination leads to proteosomal degradation, these data could also suggest something about the protein's stability. Human T and B cell receptor engagement leads to MALT1 mediated cleavage of A20 after arginine 439 (65; 145). More sequence data about *A20 would also allow us to

determine if A20 cleavage sites are present as well as inform on possible protein binding domains.

Evidence exists that A20 protects cells against TRAIL (tumor necrosis factor-related apoptosis-inducing ligand)-induced apoptosis (23; 60; 235). *P. alecto* cells are sensitized to TRAIL-induced apoptosis during HeV infection and A20 is also induced (226). Earlier, we hypothesized that apoptosis may be upregulated in bats as a protective mechanism by which infected cells are killed to cease virus replication. This apoptosis may be supported by a lower NF- κ B activation which our data show correlates with more virus in the first 24 hours of CedV infection. A20 (or *A20) then, could serve as a virus mediated mechanism to block TRAIL mediated apoptosis. By doing so, infected cells would be less likely to apoptose and virus would have more time to replicate and infect additional cells. This is one explanation for a robust *A20 induction.

Another possible role that *A20 may play is blocking the IFN response during infection. IFN blockade occurs during IAV infection and results in increased viral replication (72). Evidence exists that A20 can inhibit IRF3 signaling and IFN β promoter transcription during Newcastle disease virus infection of HEK293 cells (186). What upstream signaling molecules are involved in this IFN block is not yet completely clear (132; 170). It will be interesting to determine if any other viruses induce *A20. With the sizes of A20 and *A20 being so similar, there is a good possibility that during other virus infections *A20 was misidentified as A20. This will be especially important to investigate in other Henipaviruses. As mentioned, group who performed HeV infections with PaKiT cells noted a significant induction of A20 mRNA (226). This was followed up with western blot studies from PaKiT cells infected with HeV. Upon protein analysis,

they also found a robust induction of some form of A20. This suggests this phenomenon may be conserved across Henipavirus species and will be an interesting observation to follow up in the future. This follow up should include both an investigation as to whether the A20 observed during HeV infection is *A20, as well as whether *A20 and not A20 is induced in CedV infected *P. alecto* cells as well.

It is certainly going to be necessary to make an A20 KO cell line that targets additional exons as it is not possible to dissect the role of *A20 in the exon 3 KO cell lines (which still produce *A20). We could create a KO that introduces a missense mutation early in exon 2, which contains the epitope for the N-terminal anti-A20 antibodies that detect *A20. Perhaps even better would be to make a cell line with a full A20 deletion. Once a cell line that is unable to make *A20 is constructed, we can address many of the above proposed hypotheses. For example, we can infect WT and true A20 KO cell lines with CedV and examine whether a change of titer, interferon production, and apoptosis occur.

Future experiments for determining *A20 sequence during henipavirus infections

In order to make progress on any KO vs WT A20 experiments, it is going to be vital to determine the sequence of the *A20 transcript. Our current methods have been unsuccessful. One plausible explanation is that our primers are not optimal. The forward primer for all but one of the PCR pairs, maps near the 3' end of exon 2. If that region of exon 2 is not present in *A20, then no forward priming would occur on the cDNA of the *A20 transcript. Instead, we are amplifying transcripts that are translated but don't yield a full length protein. Specifically, in the case of the Jurkat A20 KO cells, it appears that at least one allele may have incorporated a portion of chromosome 11 with a frame shift

mutation. Multiple primer pairs result in A20 sequences with a chromosome 11 insertion but it does not appear in any of our sequences of infected 293 A20 KO cells. Though the out of frame insertion of chromosome 11 sequences into Exon 3 of A20 was not the intended mechanism for the A20 KO, if this hypothesis is correct, it was effective and so the cell line tested positive for an A20 exon3 deletion.

To address the issues with amplifying *A20 cDNA, the next experiment that should be executed is a RACE (Rapid Amplification of cDNA ends) PCR using mRNA from CedV infected A20 KO cells. This method allows amplification of cDNA for which only a portion of the mRNA sequence is known (191). Once we have successfully determined *A20's sequence we can move forward with determining if HeV induces *A20 mRNA and if CedV induces *A20 mRNA in *P. alecto* cell lines.

Determining if *A20 is present during HeV infection is fairly straightforward. Experiments could be performed as they were during CedV infections. mRNA from infected cells could be isolated, cDNA made, and with proper primers designed based on RACE PCR sequence, *A20 amplified. It is good idea to first have the sequence of *A20 so trial and error based methods are not used on samples that are difficult to obtain. Another important experiment will be demonstrating that *A20 is produced *in vivo*. This could be done by extracting mRNA from tissue samples of a HeV infected animal and amplifying *A20 from the cDNA.

Like the HeV experiment described above, amplifying cDNA of *A20 in *P. alecto* should also be straightforward. Once we know the domains or exons present in *A20, we can design primers that map to those domains of A20 in the *P. alecto* sequence.

The A20 sequence from *P. alecto* is available in Genebank. Alternatively, RACE PCR could be used to amplify *P. alecto* cDNA as well.

Henipavirus Phosphoproteins as activators of *A20

Our data demonstrated that CedV P is sufficient for A20 induction. It is important to recognize that CedV P differs from other paramyxovirus P in an important way. In order for proper replication of paramyxovirus genomes to occur, total genome length must be a multiple of 6 (113). Each group of 6 bases is known as a hexamer phase. Paramyxoviruses contain stutter sites at a particular hexamer (the sequence is conserved but location varies). At these stutter sites, 1 to 6 additional guanines are added (112). These additions result in a frame shift mutation that ultimately results in production of a different protein (113). These accessory proteins are often involved in antiviral immune evasion and contribute to pathogenicity (11; 100). Cedar virus, however, is unable edit its P protein and this is thought to contribute to its lack of pathogenicity (129; 148). This phenotype means that we can be sure that the A20 induction is CedV P and not an edited accessory protein. We hypothesize that other paramyxoviruses may use their P proteins in their traditional isoform, to induce *A20 but it will be important to assess their accessory proteins as well.

In our work, we used different antibody clones to determine what domains of A20 are present in *A20. We noted the presence of c-terminal A20 as determined by western blot analysis with a monoclonal A20 antibody that maps to amino acids 440-790. This portion of A20 is where the important ubiquitin ligase domain exists. This domain is responsible for the addition of K48 linked ubiquitin which targets a protein for degradation. In the case of NF- κ B activation this is RIP1 (196). This is consistent with

our data that demonstrates CedV P's ability to block the NF- κ B pathway. To be clear, even though *A20 is missing some portion of A20, we are suggesting it retains the functional capabilities that the C-terminus of A20 has. This retention of catalytic activity could mean when P is transfected and thus NF- κ B independent *A20 induced, *A20 still has the effect of ubiquitinating RIP1 and thus decreasing the NF- κ B signal.

The evidence that a single viral protein can induce human cells to produce a unique form of a protein that excludes at least one exon of A20, suggests the mechanism occurs at or before transcription. Our data also demonstrate that Cedar P localizes to the nucleus of the cell during transfection. We hypothesize then, that CedV may induce alternative splicing of A20 during infection. Cedar P may do this directly, as our data show that NF- κ B is not the major transcription factor responsible for the induction of *A20.

Alternative splicing as a mechanism for *A20 production

After transcription occurs, pre-cursor mRNA must be spliced to remove the non-coding introns and join together the exons. This is mediated by the spliceosome complex. Alternative splicing (AS) is a way by which one gene can encode for more than one mRNA. These unique mRNAs then lead to a unique sequence of amino acids and therefore an edited protein. These edits can occur by inclusion of one or more introns (alternative exons) or exclusion of one or more exons (195). These edits are usually small but can affect the resulting protein in many ways, including changes in binding affinity, localization, and enzymatic properties (35)

Exonic silencing splicers (ESS) are short stretches of exons that inhibit that exon from being spliced. This is done by recruiting proteins that will affect the splicing

machinery and prevent it from being able to include the exon in the mRNA. Other proteins are recruited to RNA sequence elements and stabilize and thus promote inclusion of the exon. A change in the availability of these proteins thus affects splicing patterns (110). Many other RNA binding proteins can also affect the ability of the spliceosome to access certain splice sites and some proteins that regulate splicing are cell type specific (49).

Although it has been known for quite some time that RNA secondary structure can affect splicing, recently, more evidence for the role of RNA in regulating AS has become available (33; 126). Long non-coding RNAs (lncRNAs) have been shown to affect AS by mechanisms which include direct interactions with splice sites and chromatin remodeling. Negative sense lncRNA can also form RNA-RNA duplexes to affect splicing patterns (184).

Viruses too can cause cellular AS, although not many specific mechanisms exist to explain the process (29; 83). In some cases, the virus likely affects an intermediary factor which then goes on to affect AS. In other cases, however, specific virus proteins are able to alter splicing (30). Dengue virus's NS5 protein, for example, is able to incorporate itself into the spliceosome to alter its efficiency and splicing pattern (54). A lot remains to be explored if we are to understand what role viruses can play on this cellular process. If our hypothesis for CedV P acting to induce alternative splicing is true, as is supported by its localization in the nucleus, then additional experiments may serve to expand our understanding of this process.

One way to begin to consider the possibility of CedV P mediating alternative A20 splicing is to perform microscopy on cells infected with CedV. Observing localization of

CedV P with the spliceosome would be evidence that favors this hypothesis.

Unfortunately, direct interaction with the spliceosome may not be the mechanism by which the P protein mediates alternative splicing. Sequence data of *A20 will help inform a better way to definitively say if alternative splicing is the mechanism by which *A20 is made. These sequence data will tell us what is and is not incorporated into mRNA. Once we know where in the transcript the novel splicing is of *A20 occurring, it may be possible to use CRISPR to edit this/those splice sites. If the hypothesis that *A20 is the result of AS is correct, the resulting cell line should be unable to make *A20.

Long-term future directions

When we take a step back to consider both arms of this project, it would be interesting to use human and *P. alecto* v-Raf/v-Myc immortalized cell lines to perform our comparative immunology studies. The group that demonstrated MeV P induces a strong increase in A20 expression found this only occurred in the myeloid cell lines they studied (230). The influenza immunopathology studies involving A20 that were covered in the introduction were also performed in macrophages (142). Generally speaking, A20 is described a central regulator of immunopathology (212). It makes sense then, to study comparative immunology of CedV mediated A20 expression in a myeloid cell line. Should successful human and *P. alecto* macrophage cell lines be made, the most vital experiments considering the role of *A20 production and NF- κ B activation could be repeated in a perhaps, more relevant cell type. This could result in a very well controlled and informative comparative immunology study.

GENERAL CONCLUSIONS

In general, we can conclude that our improved v-Raf/v-Myc lentivirus can immortalize macrophages from murine blood and spleen. The resulting cell lines express markers associated with mature myeloid cells, are responsive to IFN γ and can phagocytose. Importantly, they grow independent of IL-3 or other supplemental cytokines. Together, the above observations demonstrate that we have identified a promising candidate for cross-species macrophage immortalization and developed a safer and more direct method for immortalizing macrophages from mice.

Our studies into the innate immune response of CedV have also resulted in some interesting conclusions. First, we can conclude that *P. alecto* kidney cell lines activate the NF- κ B pathway in response to TNF α . To our knowledge, this is the first time NF- κ B pathway activation in *P. alecto* has been confirmed at the protein level. This confirmation means that a positive control for NF- κ B activation in bats now exists. We have not yet confirmed κ B mediated transcription in a *P. alecto* cell line during CedV infection. Still, there are differences in activation of the NF- κ B pathway at least as far downstream as phosphorylation of P65. Both intensity and the kinetics of phosphorylation differ between comparable bat and human cell lines. We can conclude however, that the human HEK 293T cell line activates NF- κ B in response to CedV, and that the inability to mount this response is associated with a higher percent of detectably infected cells and results in less progeny virus produced.

The data are clear that CedV induces a robust A20 response and that Cedar P is sufficient for this phenotype. What is more, we provide conclusive evidence that this occurs without the activation of the NF- κ B pathway. To our knowledge, no one has reported an alternative mechanism for activation of A20. We can also conclude that this

new *A20 must be different from A20 in some way as it expresses N- and C-terminal regions of A20, despite clearly lacking exon 3, and is larger in size. We cannot yet describe the sequence, nor the role it plays, but we have shown that it is virus induced and mediated by at least, the P protein. In all, we can conclude from the CedV work that the NF- κ B pathway plays a role during infection and is worth continuing to investigate, most importantly with regards to its negative regulator, *A20.

REAL WORLD IMPLICATIONS AND NOVELTY

Prior to this work, there were no clear leads on immortalizing macrophages from bats, and the standard hTERT and SV40 antigen attempts were unsuccessful (46). Here we provide evidence for a practical protocol for macrophage immortalization with true promise for cross-species adaptability. With some effort to translate this research into other species, we could open the doors to many new investigations in viral reservoirs. The ability to immortalize macrophages from many species using the same mechanism means the field of comparative immunology could make some rapid and vital progress.

Evidence of global circulation of Henipaviruses is strong (15; 61; 175; 216). There is no reason not to expect that in time, another spillover will occur. Our data on NF- κ B and A20 in the non-pathogenic henipavirus, CedV, means we are making progress towards understanding these diseases better. In the past, the disease observed during emerging virus outbreaks has been associated with immunopathology. Studying regulation of the pathways involved in inflammation during viral infections is therefore, a priority. Here, we have set a foundation for this. We have established NF- κ B controls that work in bats as well a Gaussia luciferase cell line, identified more *P. alecto* cross reactive antibodies, and provided evidence for some ways *P. alecto* henipavirus immune

responses contrast with humans'. This provides a jumping off point for more Henipavirus work.

Last, our experiments with CedV P and *A20 have demonstrated there are mechanisms by which viruses alter our cells that we have not yet explored. Little evidence of virus mediated alteration of host mRNA splicing exists. With only a few additional experiments, we may provide some strong evidence of a new mechanism. There are many paramyxoviruses that cause disease in humans and there is a good possibility this P mediated induction of an alternate *A20 occurs during infection with other species. This possibility is especially likely when we consider those data published previously on A20 in MeV infections (230; 240). It is not unreasonable to speculate that this *A20 may result in an increase in viral fitness. If that is true, *A20 induction is a novel antiviral target.

In closing, we have provided a new method for immortalizing macrophages from murine blood and shown that this method has promise for cross-species applicability. This new tool would help advance the field of comparative immunology. Alongside this, we have provided observations in comparative immunology during henipavirus infection in bats and discovered a new type of the A20 protein. We can draw some interesting conclusions but also importantly, have provided data that though not conclusive, has the potential to have broad applications and true novelty. These data come at a time when our world is facing a record-breaking pandemic. It is perhaps more important than ever to focus our efforts on understanding emerging viruses. The above work does just that.

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