Dynamics of bat-coronavirus interactions: role of innate antiviral responses

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Abstract

Bats are speculated to be reservoirs of several emerging viruses including coronaviruses (CoVs) that cause severe acute respiratory syndrome (SARS), Middle-East respiratory syndrome (MERS), porcine epidemic diarrhea and swine acute diarrhea syndrome. These viruses cause significant disease in humans and agricultural animals. MERS-CoV causes serious disease in humans with a thirty-five percent mortality and has evolved proteins that can effectively suppress an innate antiviral response in human cells. Bats that are naturally or experimentally infected with these or similar viruses do not show apparent signs of disease and the molecular mechanisms of protection are not yet known. My doctoral thesis tested the hypothesis that big brown bat cells have unique adaptations in innate antiviral signaling pathways involved in the control of virus replication and coronavirus-induced inflammatory cytokines. To test this hypothesis, we generated the first commercially available North American bat (*Eptesicus fuscus*; big brown bat) kidney epithelial cell line. Using this cell line, we were able to demonstrate that big brown bat cells have evolved a unique repressor molecule, c-Rel that can effectively suppress double-stranded RNA (poly(I:C)) mediated expression of a key inflammatory cytokine, tumor necrosis factor alpha (TNFα). MERS-CoV is thought to have evolved in insectivorous bats before spilling over to camels and eventually to humans. To further our understanding about bat-coronavirus interactions, we demonstrated that big brown bat cells are resistant to MERS-CoV-mediated subversion of antiviral responses. We determined that interferon regulatory factor 3 (IRF3) plays a critical role in controlling MERS-CoV propagation in big brown bat epithelial cells. Indeed, my doctoral thesis has identified two unique adaptations in big brown bat cells that might allow these bats, and probably other species of bats to successfully co-exist with coronaviruses. My thesis supports the hypothesis that bats function as global reservoirs for

emerging coronaviruses by providing definitive examples of adaptations that would allow bats to co-exist with these viruses. Future work from my thesis will focus on adapting some of these antiviral strategies in human cells to control coronavirus-mediated disease in humans.

Acknowledgements

Looking back in time, work in my thesis would not have been possible without the tremendous guidance and mentorship that I received from my PhD supervisor, Dr. Vikram Misra. He has always gone over and beyond his role as a supervisor. I take pride in being Vikram's student and it is an absolute honor to work with him. I cannot thank you enough 'V' and I look forward to our continued collaboration in the future.

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Thank you.

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List of abbreviations

ACE2	Angiotensin-converting enzyme 2
APC	Antigen presenting cell
BHK-21	Baby Syrian hamster kidney cells 21
CAT	Chloramphenicol acetyltransferase
cDNA	complementary DNA
ChIP	Chromatin immunoprecipitation
CHPV	Chandipura virus
CoV	Coronavirus
CPE	Cytopathic effect
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
Ct	Cycle threshold
CWHC	Canadian Wildlife Health Cooperative
DC	Dendritic cell
DMEM	Dulbecco's minimum essential medium
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's phosphate-buffered saline
DPP4	Dipeptidyl peptidase 4
DsiRNA	Dicer-ready small interfering RNA
dsRNA	double-stranded RNA
EDTA	Ethylenediaminetetraacetic acid
Efk3	*Eptesicus fuscus* kidney cells - 3
EPHB2	Ephrin type-B receptor 2
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde-3-phosphate
GBP1	Guanylate-binding protein 1 precursor
HA	Hemagglutinin
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hpi	hours post infection
HSV	Herpes simplex virus
hTERT	human Telomerase reverse transcriptase
IFI27	Interferon alpha-inducible protein 27
IFI6	Interferon alpha-inducible protein 6
IFN	Interferon
IFNAR2	Interferon alpha receptor 2
IFNβ	Interferon beta
IKKε	Inhibitor of nuclear factor kappa-B subunit epsilon

IL 12A	Intereukin 12A
IL 12B	Intereukin 12B
IL-1	Interleukin-1
IL-8	Interleukin 8
IL18	Interleukin 18
IL1β	Interleukin 1β
IPS-1	Interferon beta promoter stimulator-1
IRF3	Interferon regulatory factor 3
IRF7	Interferon regulatory factor 7
ISG	Interferon stimulated gene
ISG20	Interferon inducible gene 20kDa protein
ISGF3	Interferon stimulated gene factor 3
JAK1	Janus kinase 1
LPS	Lipopolysaccharide
LRR	Leucine-rich repeat
MAPK	Mitogen-activated protein kinase
MDA5	Melanoma differentiation-associated protein 5
MERS	Middle East respiratory syndrome
MHC-I	Major histocompatibility complex I
MyPvTag	Myotis polyomavirus large tumor antigen
NCBI	National Centre for Biotechnology Information
ncsiRNA	negative control siRNA (scrambled siRNA)
NEAA	Non-essential amino acids
NFkB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NGS	Next generation sequencing
NLR	NOD-like receptors
NOD	nucleotide-binding oligomerization domain
NPC1	Neimann-Pick type C1
OAS1	2'-5'-oligoadenylate synthetase
PAMP	Pathogen associated molecular pattern
PCR	Polymerase chain reaction
PEDV	Porcine epidemic diarrhea virus
pIRF3	phosphorylated interferon regulatory factor 3
poly(I:C)	Polyinosinic-polycytidylic acid
PRR	Pattern recognition receptor
qRT-PCR	quantitative real-time polymerase chain reaction
RHD	REL homology domain
RIG-I	Retinoic acid-inducible gene I
RLR	RIG-I-like receptor

RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute 1640
RSAD2	Radical S-adenosyl methionine domain-containing 2
SADS	Swine acute diarrhea syndrome
SARS	Severe acute respiratory syndrome
SD	Standard deviation
siRNA	Small interfering RNA
ssRNA	single-stranded RNA
STAT1	Signal transducer and activator of transcription 1
STAT2	Signal transducer and activator of transcription 2
STING	Stimulator of interferon gene
SV40	Simian virus 40
SV40Tag	Simian virus 40 large tumor antigen
T-antigen	Tumor antigen
TBKI	TANK-binding kinase I
TCID50	Tissue culture infectious dose 50
TIR	Toll/interleukin-1 receptor
TLR	Toll-like receptor
TNFα	Tumor necrosis factor alpha
TRAF3	Tumor necrosis factor receptor-associated factor 3
TRIF	TIR domain containing adapter-inducing interferon beta
TYK2	Tyrosine kinase 2
VSV	Vesicular stomatitis virus
WHO	World Health Organization
WNS	White-nose syndrome

Chapter 1: Introduction

Bats belong to the order Chiroptera and constitute more than 1200 species (1). Bats are an ancient and diverse group of ecologically important mammals, making up almost a quarter of all mammalian diversity and inhabiting all continents, other than Antarctica. Phylogenetic analyses based on molecular data classified bats into the suborders Yinpterochiroptera and Yangochiroptera (2, 3). The Yinpterochiroptera suborder includes the non-echolocating Pteropodidae family and the echolocating Rhinolophoidea family. The Yangochiroptera consist of the remaining echolocating microbat families. The two suborders diverged over 50 million years ago (4-6).

Since the severe acute respiratory syndrome (SARS) outbreak of 2003, over two hundred different viruses have been detected in bats with new viruses frequently being identified (1, 7).

Bats have also been implicated as reservoirs of several viruses that cause serious disease in human and agricultural animals. These viruses include filoviruses such as Ebola and Marburg virus, paramyxoviruses such as Nipah and Hendra virus and coronaviruses (CoVs) that cause SARS, Middle-East respiratory syndrome (MERS), porcine epidemic diarrhea and swine acute diarrhea syndrome (1, 7, 8). However, bats experimentally infected (9-11) with these viruses or naturally infected with similar viruses do not demonstrate clinical signs of infectious disease observed in humans and other agricultural animals.

Lack of disease following infection with these viruses has led to speculations about the unique virus-bat molecular interactions that allow bats to remain persistently infected with these viruses (7, 12, 13). Several hypotheses have emerged about this unique ability of bats to resist virus-induced disease. Speculations include co-evolutionary adaptations between bats and their viruses and bats' ability to fly (1, 14). Bats are the only mammal capable of true flight. During flight, the body temperature of bats rises to over 40 degrees Celsius and the rate of metabolism increases several fold (15). High metabolic rates associated with flight produce reactive oxygen radicals, which can damage cellular DNA (16). There is also evidence that certain genes involved in antiviral responses in bats have undergone evolutionary adaptive changes due to their role in DNA repair (14).

A recent article speculates that since bats are primed to resist viral infections due to activated antiviral response in their cells, their viruses have co-evolved to make increasing amounts of viral proteins that can modulate these immune responses in bats (13). When humans are infected with these viruses, the viruses continue to make high levels of these immune response modulating proteins. However, authors speculate that unlike bat cells, human cells do not constitutively express high levels of antiviral molecules and therefore, immune responses mounted by human cells may be easily overwhelmed by these viral proteins. This theory needs to be validated by experimental work.

In 2009, Misra *et al.* detected novel polyoma and coronaviruses in Canadian brown bats (17). They were also able to perform basic innate immunology studies on samples collected from white-nose syndrome fungus, *Pseudogymnoascus destructans,* infected bats (18, 19). However,

there was a need to develop a cell line permissive to virus replication to define the cellular innate and intrinsic antiviral responses in bats.

During my PhD in Dr. Misra's laboratory (Bat Zoonoses Lab), I, with the help of my colleagues and collaborators generated an *in vitro* tool from a Canadian hibernating bat species, *Eptesicus fuscus* (big brown bat). We characterized the cellular innate responses in kidney cells derived from this bat. We demonstrated that big brown bats and possibly other insectivorous bats have a unique mechanism to suppress virus induced over-inflammation. We further characterized the role of a key molecule, interferon regulatory factor 3 (IRF3) in regulating interferon beta (IFNβ) signaling in bat cells. Finally, we were able to show that bat cells have evolved a mechanism to resist coronavirus mediated suppression of innate immune responses.

To summarize, my doctoral thesis provides answers to some intriguing questions about how bats have evolved different strategies to survive viral infections. My thesis also raises several questions that will allow other researchers, including myself, to develop future plans to better understand this intriguing viral reservoir.

Chapter 2: Literature review

2.1. Bats as global reservoirs of emerging viral pathogens

Chiroptera is a very diverse order consisting of over 1200 species of bats (1). Over 200 different viruses have been detected in bats and new viruses are detected on a regular basis (1, 7, 20). Bats that are naturally or experimentally infected with some of these viruses do not show typical signs of disease (9, 10, 21) and this has led to growing speculations about unique adaptations in the immune system of this intriguing mammalian viral reservoir (7, 22-24). There were few studies looking at viruses and immunological responses in bats before the 2003 severe acute respiratory syndrome (SARS) outbreak (25-27). Research on bats has since increased and researchers are trying to decipher the unique immune responses in bats that allow them to co-exist with some of these viruses.

Of the several viruses that bats are speculated to be reservoirs for, my PhD thesis focuses on coronaviruses (CoVs). Bats have been recognized as global reservoirs of emerging coronaviruses (28) and several questions remain about how bats maintain these viruses without developing disease. We also do not understand how these CoVs occasionally jump (spill over) from bats to other species. There are some computer simulations or models that predict several factors that may play a role in zoonotic transmission of viruses from bats. These include factors that affect virus maintenance in bats, susceptibility of bats to virus infections and inter- and intra- species transmission of viruses within bat populations (29, 30). All of these factors need to come together to create an ideal scenario that will allow a virus to 'jump' from wildlife to humans (31).

2.1.1. Coronaviruses that cause SARS and MERS

Coronaviruses are the largest enveloped ribonucleic acid (RNA) viruses with a genome size of approximately twenty-six to thirty-two kilobases. They contain a positive sense, single-stranded RNA genome that encodes several structural, non-structural and accessory proteins (32). These include proteins that help in virus replication and modulation of host immune responses against the virus (Fig 2.1). The latter is discussed in section 2.3.

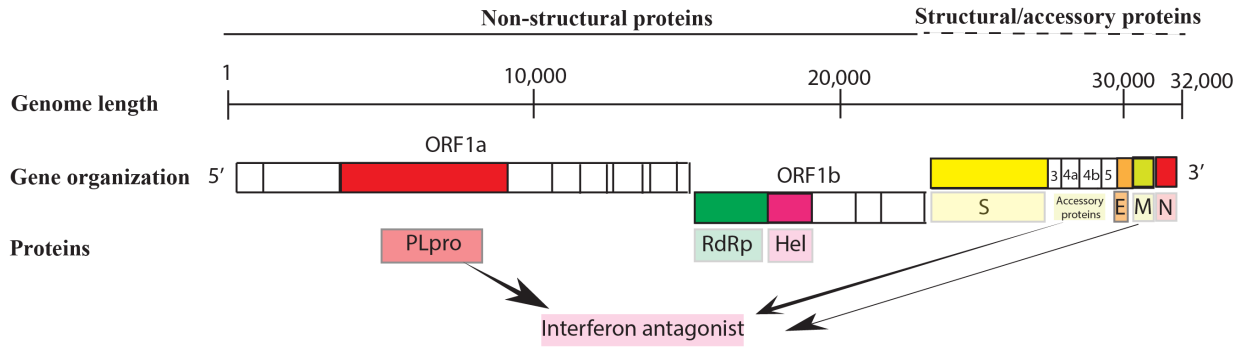

Figure 2. 1. MERS-CoV genome codes for several proteins that can modulate the host interferon responses. MERS-CoV encodes non-structural proteins that help in virus replication and structural proteins that become part of the progeny virus. Although dispensable for replication, accessory proteins play important roles in pathogenesis and host defence antagonism. Several MERS-CoV proteins, such as matrix (M), PLpro, ORF 4a, ORF 4b and ORF 5 have been shown to antagonize host antiviral interferon responses.

Accessory proteins differ amongst coronaviruses that belong to different groups. For example, SARS-CoV and MERS-CoV differ in the number of accessory genes (33-35). Accessory proteins are dispensable for coronavirus replication but they play an important role in pathogenesis and virus fitness under the natural environment of a host (34, 35). Coronavirus accessory genes have co-evolved with their natural host for optimum functionality (34) and thus it is important to identify the role of accessory proteins in both their natural and spill over hosts.

We need further functional studies to study the role of bat CoV accessory proteins in modulating antiviral defence responses in bats and spill over hosts such as humans. This might allow us to identify a potential future pandemic strain that might be circulating in the wild or the possibility of the emergence of such a strain through genetic recombination of coronavirus genomes.

Two important coronaviruses that are highly pathogenic in humans are the SARS and MERS coronaviruses. SARS-CoV caused a pandemic in 2003 and MERS-CoV continues to cause outbreaks in the Kingdom of Saudi Arabia, with occasional travel associated cases in other countries. In 2003, the SARS outbreak infected a total of 8,098 people worldwide. Of these, 774 died (36). Since September 2012, the World Health Organization has been notified of 2,090 laboratory-confirmed cases of MERS, with at least 730 deaths (37). In coronavirus infections, disease is driven by lower respiratory tract infection, a weakened immune response and an exaggerated inflammatory response (38-40). Currently, only supportive care is available for patients suffering from SARS and MERS.

SARS-CoV is thought to have spread from caged palm civet cats to humans. However, it was clear that civet cats were amplifying hosts and not the reservoirs for SARS-CoV (41). SARS-CoV and SARS-CoV-like viruses have since been detected in Chinese Horseshoe bats (41-44), further highlighting the important role of bats as reservoirs of coronaviruses. Bats have also been speculated as the ancestral hosts of MERS-CoV (45-47). MERS-CoV is spread primarily from camels-to-humans and human-to-humans. However, it is unclear how camels acquired this virus. Since similar viruses have been detected in bats and camels (45, 46, 48), it is speculated that bats

originally transmitted this virus to camels (Fig 2.2). We do not know if bats continue to transmit this virus to camels.

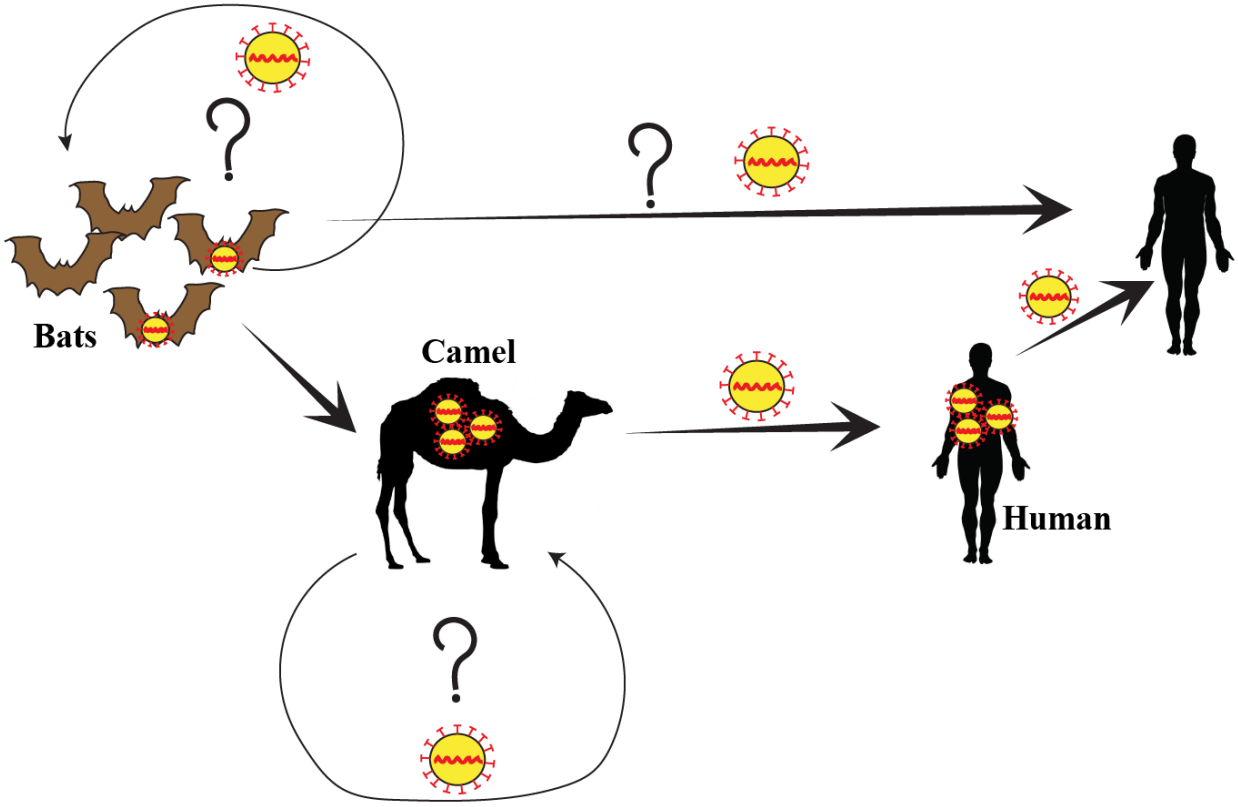

Figure 2. 2. Insectivorous bats are speculated as the ancestral host of MERS-CoV. Several studies have speculated that MERS-CoV evolved in bats and spilled over to camels some time in the past, likely more than 30 years ago. A large percentage of camels in the Middle East, eastern and central Africa are seropositive for MERS-CoV (49). Although direct camel to human transmission has not been studied, similar strains have been observed in both camels and humans, hinting at a camel to human transmission cycle. MERS-CoV can also be transmitted from human-to-human. Inter- and intra-species transmission of MERS-CoV or MERS-like-CoVs in bats or camels is yet to be documented. The direct transmission of MERS-CoV from bats to humans has also not been documented.

In 2009, Misra *et al.* detected novel polyoma and coronaviruses in Canadian brown bats (17). Our research group has recently shown that Canadian little brown bats are persistently infected with a bat coronavirus (50). With bats being speculated as reservoirs or ancestral hosts for several coronaviruses including SARS and MERS CoVs, and because bats infected with these

viruses do not display any apparent symptoms, my thesis aimed to characterize the immune responses and unique adaptations, if any, in big brown bat cells towards these viruses.

2.1.2. Other viruses in bats

Several other viruses have also been detected in bats. A comprehensive list of viruses detected in bats have been reviewed by Moratelli *et al.* and Calisher *et al.* (1, 7). Some notable examples of viruses that have caused significant outbreaks in humans and agricultural animals include filoviruses such as Ebola and Marburg virus, paramyxoviruses such as Nipah and Hendra, coronaviruses such as those causing SARS, MERS, porcine epidemic diarrhea, swine acute diarrhea syndrome and lyssavirus such as rabies virus. These viruses or viruses similar to them have been detected in different species of bats.

Of the several viruses that have been detected in bats, Nipah and Hendra viruses are well studied. Both Nipah and Hendra viruses have been extensively studied in their natural host i.e. bats. Nipah virus continues to cause periodic outbreaks in Bangladesh. The transmission cycle of Nipah virus from bat-to-human and human-to-human has been reviewed by Gurley and Luby *et al.* (51, 52). To summarize, fruit bats contaminate harvested date palm sap while drinking out of earthen pitchers on trees. The farmers then go on to sell this sweet drink that is traditionally consumed raw and unpasteurized. The unpasteurized drink, if contaminated with Nipah virus, subsequently causes outbreaks of Nipah encephalitis. Fruit bats or flying foxes have been successfully identified as the reservoirs of Nipah virus (53-56). Pteropid (fruit) bats experimentally infected with Nipah virus do not show visible signs of disease. Although infected bats show major histopathological changes and a separate study also showed that henipaviruses

can inhibit antiviral interferon responses in fruit bat cells (57), experimentally infected fruit bats only developed a sub-clinical infection with low levels of virus secretion in their urine. The authors speculate that the low level of virus secretion would be enough to sustain Nipah virus in a wild bat population (10). There is indeed a need to further dissect how bats maintain these viruses and reasons that contribute to the emergence and spill over of these viruses.

Hendra virus is another Henipavirus that has been well studied in its natural host i.e. Pteropid fruit bats (55, 56). The transmission cycle of Hendra virus has been reviewed by Plowright *et al.* and Enchery *et al.* (29, 56). In short, Hendra virus spills over from bats to horses and eventually from horses to humans. Both horses and humans are known to be dead-end hosts that develop significant disease and display high mortality when infected with Hendra virus. Similar to Nipah virus, experimental infection of Pteropid fruit bats with Hendra virus does not produce any obvious signs of disease (55). Controlled *in vivo* studies and wildlife surveillance studies have also led to the prediction of several virus maintenance and spill over models for Hendra virus in bats (29-31). Reproduction and nutritional stress have also been identified as risk factors for Hendra virus infection in little red flying foxes (*Pteropus scapulatus*) (58). Wildlife surveillance and ecological studies will continue to inform public policies to prevent future outbreaks. Monitoring wild bat populations for circulating viruses will also allow us to predict the next high impact bat-borne pathogen that could potentially jump from bats to humans or agricultural animals with devastating outcomes.

The lack of visible disease in bats that are infected with some of these viruses is very intriguing. However, there are some examples where viruses have been speculated to cause disease in bats.

A filovirus, Lloviu virus was detected in dead insectivorous bats in Europe (59, 60). Speculations remain about its ability to cause disease in bats as controlled laboratory infection studies in bats is yet to be performed. Tacaribe virus, an arenavirus has been demonstrated to cause disease in Jamaican fruit bats at high infectious doses (61). However, low-dose infections in Jamaican fruit bats resulted in asymptomatic and apathogenic infection with virus clearance. Cogswell-Hawkinson *et al.* also observed that un-infected bats that were housed in the same cage did not seroconvert and viral RNA could not be detected in these bats, suggesting that transmission did not occur. Based on disease symptoms and the nature of disease progression, the authors speculate that bats may not be the original reservoir for Tacaribe virus. As of writing this thesis, with the exception of rabies virus, Tacaribe virus is the only virus that we currently know of that can potentially cause lethal disease in bats.

2.2. Innate immune response and bats

Innate immune response is the first line of defence against an invading pathogen. It is an universal and ancient form of host defence against infection. The innate immune system consists of evolutionary conserved cellular receptors or pattern recognition receptors (PRRs) that recognize conserved structures in micro-organisms called pathogen associated molecular patterns (PAMPs) (62). PAMPs include several molecules derived from micro-organisms such as viral RNA/DNA sequences and bacterial and fungal cell wall components. Other molecules such as self-DNA-autoantibody complexes or aberrant localization of molecules such as endosomal DNA can also activate innate immune responses (63, 64).

The recognition of PAMPs by PRRs lead to a cascade of signaling events, which ultimately culminate in the secretion of antimicrobial cytokines and chemokines by the host cells (65). In this literature review for my doctoral thesis, I have reviewed a key antiviral pathway, the Toll-like receptor 3 (TLR3) pathway, that is commonly triggered by RNA viruses such as coronaviruses. I have also elaborated on the downstream signaling events specific to the activation of TLR3 by viral components. Other PRRs that identify different microbial components to initiate several signaling cascades in mammalian cells have been reviewed elsewhere (62, 65-67).

2.2.1. Cellular receptors of innate antiviral signaling and their ligands

The innate immune response relies on the recognition of evolutionarily conserved structures on pathogens (PAMPs), through germ line-encoded PRRs, of which the family of TLRs has been studied extensively. In fact, the discovery of TLRs was an important event for immunology research and was recognized as such with the awarding of the 2011 Nobel Prize in Physiology or Medicine to Jules Hoffmann, Bruce Beutler and Ralph Steinman.

Mammalian cells have evolved to encode several cellular receptors to detect microbial components. These include Toll family of receptors that are present largely on cellular surfaces and in endosomal compartments. Cytoplasmic receptors include RIG-I-like receptors (RLRs) such as retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated protein 5 (MDA5) and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) (Fig 2.3). Several TLRs, RLRs and NLRs have been recognized in humans, each recognizing a particular component of a microorganism (Fig 2.3).

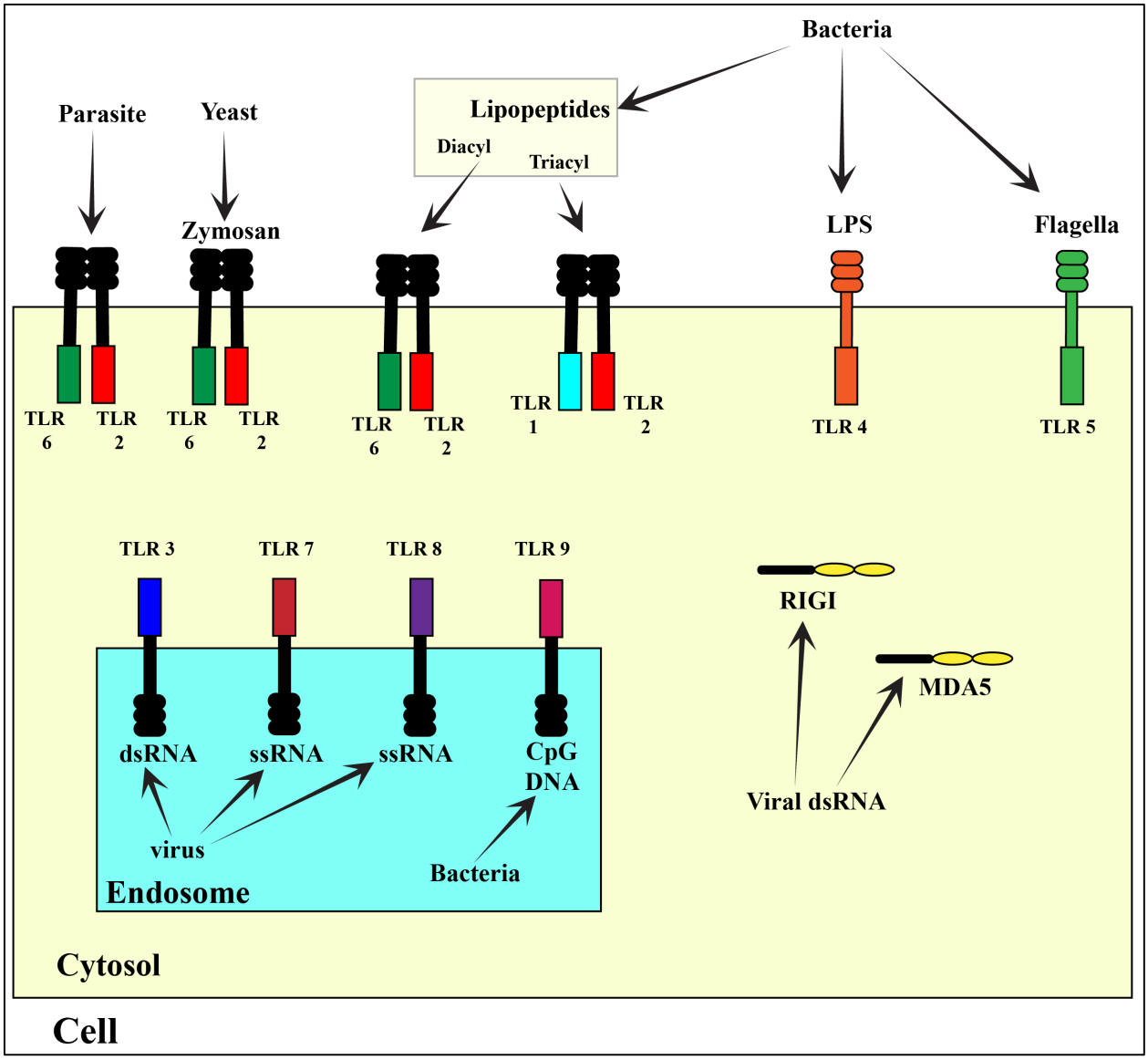

Figure 2. 3. Pattern recognition receptors (PRRs) have evolved to detect conserved structures in invading pathogens and are distributed across different locations in a cell. TLRs 3,7,8 and 9 are located in endosomes and detect viral and bacterial nucleic acids. TLR2/6 detect surface proteins on parasite and yeast. TLR2/6 can also be activated by diacyl-lipopeptides from bacteria. TLR1/2 is activated by triacyl-lipopeptide from bacteria. TLR 4 and 5 are activated by bacterial lipopolysaccharide (LPS) and flagellar protein respectively. Cytosolic receptors such as RIG-I and MDA5 detect viral nucleic acid (double-stranded RNA) in the cytosol.

Once these PRRs recognize a specific foreign molecule in a cell, they initiate signaling cascades that activate the expression of several anti-microbial peptides and proteins such as cytokines and

chemokines. Our knowledge of the mammalian innate immune responses comes mostly from studies done in human and rodent cells. We are yet to fully understand the extensive innate immune signaling pathways in bats.

The role of TLRs, RLRs and NLRs have been reviewed by Mogensen in detail (65). Since my thesis focusses on understanding the differences in innate immune responses to RNA viruses in bat and human cells, I have elaborated on the role and downstream signaling effects of double-stranded RNA recognition by TLR3, RIG-I and MDA5 in mammalian cells.

2.2.2. Toll-like receptor 3 (TLR3), retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated protein 5 (MDA5) signaling pathways

To describe simply, when an RNA virus, such as a coronavirus, infects a mammalian cell, it produces double-stranded (ds) RNA while replicating in the infected cells (68, 69). Mammalian cells do not inherently contain long strands of dsRNA. When virus replication produces dsRNA, cellular PRRs such as TLR3, RIG-I and MDA-5 recognize these molecules as 'foreign' and initiate downstream signaling events (65, 70-72).

TLRs were originally discovered based on their homology to the *Drosophila melanogaster* Toll protein (73). Structurally, TLRs are integral glycoproteins characterized by an extracellular ligand-binding domain containing leucine-rich repeat (LRR) motifs and a cytoplasmic signaling Toll/interleukin-1 (IL-1) receptor homology (TIR) domain (74). Ligand binding to TLRs through PAMP-TLR interaction induces receptor oligomerization, which subsequently triggers intracellular signal transduction. Ten TLRs have been discovered in humans, and they each

recognize distinct PAMPs from several microbial pathogens, including viruses, bacteria, fungi and protozoa.

TLRs can be divided into subgroups based on the PAMPs they recognize. TLRs 1, 2, 4 and 6 recognize lipids, whereas TLRs 3, 7, 8 and 9 recognize nucleic acids. Another way of grouping TLRs is by their location. Some TLRs (TLR1, 2, 4, 5, 6 and 10) are expressed at the cell surface, whereas others (TLR3, 7, 8, and 9) are located almost exclusively in intracellular compartments such as endosomes and lysosomes (65) (Fig 2.3). The most important cell-types that express TLRs are antigen presenting cells (APCs), such as macrophages, dendritic cells (DCs) and B lymphocytes (75). However, in different experimental systems, TLRs have been identified in most cell types, expressed either constitutively or in an inducible manner during an infection (75-77).

After recognition of dsRNA, signal transduction is mediated by a family of adaptor molecules, which in part determines the specificity of the response (74). TLR-induced signaling pathways can be broadly classified based on their utilization of different adaptor molecules, i.e. MyD88 or TIR domain-containing adapter-inducing interferon β (TRIF) (78, 79). Three major signaling pathways are activated when TLRs bind to their ligands, (i) nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB), (ii) mitogen-activated protein kinases (MAPKs), and (iii) interferon (IFN) regulatory factors (IRFs). NFκB and MAPKs play a role in the induction of pro-inflammatory responses, but IRFs play a central role in the production of antiviral interferons (65, 80).

TLR3 recognizes dsRNA produced during virus replication (65, 70, 71). After recognition, TLR3 signals through TRIF to activate a signaling pathway in which tumour necrosis factor receptor-associated factor 3 (TRAF3) bridges the IKK-related kinases TANK binding kinase 1 (TBK1) and inhibitor of nuclear factor kappa-B subunit epsilon (IKKε), which mediate the phosphorylation of interferon regulatory factor 3 (IRF3) and interferon regulatory factor 7 (IRF7) (81, 82). Phosphorylated IRF3 dimerizes and translocates to the nucleus to initiate the expression of interferons (67, 83).

RLRs such as RIG-I and MDA-5 are IFN inducible RNA helicases that sense cytoplasmic RNA (84, 85). Binding of dsRNA to the C-terminal domains of RLRs trigger signaling via CARD-CARD interactions between the helicase and adaptor protein mitochondrial antiviral-signaling protein (MAVS), which results in an antiviral response mediated by type I IFN production (72, 84).

The downstream signaling cascade from TLR3, RIG-I and MDA5 converge on the activation of NFκB and IRF3, which activate the expression of pro-inflammatory cytokines and antiviral IFNs respectively (86).

2.2.3. Interferons

As mentioned above, the downstream effect of PRR activation by a viral PAMP in a cell is the subsequent production of antiviral interferon molecules. There are three interferon (IFN) families. The type I IFN family encodes homologous IFNα subtypes in humans and a single IFNβ gene product. There are other poorly understood gene products as well such as IFNs ε, τ, κ,

ω, δ and ζ. Type II IFN family consists of IFNγ and type III IFN family consists of IFNλ subtypes (87).

IFNs are known to induce an antiviral state in both virus-infected and uninfected neighboring cells by inducing the expression of a series of IFN stimulated genes (ISGs). IFNβ and all subtypes of IFNα bind to, and signal through a heterodimeric transmembrane receptor made up of the subunits IFNAR1 and IFNAR2. The coming together of these receptors activates the receptor-associated protein kinases Janus kinase 1 (JAK1) and tyrosine kinase 2 (TYK2) (88). These molecules then phosphorylate signal transducer and activator of transcription 1 (STAT1) and STAT2 molecules. The STAT molecules then dimerize and translocate to the nucleus by binding to IRF9 to form the ISG factor 3 (ISGF3) complex. This complex binds to ISG promoters and activate transcription of ISGs. Several ISG products then work in coordination to inhibit virus replication in the cell by employing several different mechanisms that are reviewed in detail elsewhere (89).

2.2.4. The bat innate immune response to viruses: current knowledge and speculations

Bats are currently being explored as the reservoirs of several emerging viruses. Several questions remain about their ability to co-exist with these viruses that are often lethal in spill over mammals such as humans and agricultural animals. *Pteropus alecto* (black flying fox), a fruit bat, is being extensively studied to better understand the bat immune system. By studying the immune transcriptome of this bat, Papenfuss *et al.* identified approximately 500 genes (3.5% of *P. alecto* transcribed genes) that encode immune-related proteins (90). Global pathways such as the antiviral interferon pathway have been substantiated in this bat species by studying the

downstream expression of interferons in response to external stimuli. *P. alecto* homologs to human TLR 1-10 have been sequenced and TLR 13 has been described. Specific immune signal mediators such as RIG-I, major histocompatibility complex I (MHC-I), and interferon regulatory factor 7 (IRF7) have also been detected and characterized (91-93).

P. alecto cells have the ability to constitutively express three IFN-alpha genes and their expression is unaffected by viral infection. These cells also mount an effective antiviral response to viral challenge (94-100). The authors have suggested that the ability to constitutively express antiviral IFNs may allow these bats to effectively control virus replication. The molecular mechanisms that enable this rather unique expression pattern of IFNs in this species of bat is unknown.

Bone marrow-derived immune cells (dendritic cells and macrophages) have been established from *P.alecto* using bat-specific cell culture growth factors such as *P. alecto* Interleukin 4, granulocyte-macrophage colony-stimulating factor (GM-CSF), FMS-like tyrosine kinase 3 ligand (FLT3L) and colony-stimulating factor 1 (CSF-1) (101). Major lymphocyte populations have been characterized from this species of bat (102). Recently, the role and importance of IFN-alpha receptor (IFNAR) in antiviral IFN signaling has been demonstrated in *P. alecto* cells by generating IFNAR2 knockout cells using CRISPR/Cas9 technology. IFNAR2 knockout *P. alecto* cells did not respond to IFN-alpha treatment, suggesting that IFNAR2 is indispensable for IFN antiviral signaling in these cells (103).

As mentioned earlier, bats are made up of over 1200 different species and differences may exist in the way the bats respond to their viruses. This has been recently highlighted in a study by Pavlovich *et al.* where the authors did not detect constitutive IFN-alpha expression in cells from *Rousettus aegyptiacus* (Egyptian rousette bat) (104), unlike Zhou *et al.,* who detected constitutive expression of IFN-alpha in *P. alecto* cells. There is indeed a need to test several of these studies and hypotheses in other species of bats, including insectivorous bats – the more relevant reservoir model of emerging coronaviruses.

The ability of bats to co-exist with several different viruses is now being attributed to unique adaptations in PAMP sensing by bat cells. Several differences have been observed between bat and human TLRs. Bat TLR8 sequences hint at differences at the ligand binding domain (105). It is not known if this would affect the way bat TLR8 recognizes single-stranded RNA (ssRNA). However, in big brown bat cells, ssRNA-40, a known stimulator of human TLR8 did not overtly stimulate TLR8 pathways in big brown bat cells (106). It is also possible that human ligands do not bind to bat TLR8. Bats may have evolved different PRRs that recognize specific PAMPs. Bats seem to have also lost the coding sequences for cytosolic inflammasome DNA sensor, AIM2 (107) and recently a point mutation in a cytosolic DNA sensor has been shown to suppress DNA virus mediated antiviral signalling in *P. alecto* cells (108). These adaptations are believed to be due to bats' ability to fly and the requirement to mitigate high metabolism associated DNA damage.

In spite of all the recent advances, caveats still remain about the TLR3 pathway in bats. Although bat cells have been stimulated with polyinosinic-polycytidylic acid [poly (I:C)] and the

production of interferons have been demonstrated (94, 97, 109-111), any underlying differences between bat and human innate pathways are yet to be identified. In my PhD thesis, I have explored the TLR3 pathway in big brown bat cells and demonstrated that IRF3 is a key transcription factor that is involved in TLR3-mediated antiviral signaling in these cells.

2.3. Pathogen-host interactions: how do coronaviruses modulate innate immune responses?

2.3.1. Viruses have evolved mechanisms to suppress the host's anti-viral defenses

As humans and other mammals (i.e. hosts) evolve ways to counteract virus infections, viruses evolve mechanisms to inhibit an effective immune response. Several viruses have evolved unique mechanisms to evade innate immune responses, particularly the type I interferon response. Viruses modulate these cellular pathways by inducing protein degradation or cleavage, or by mediating protein poly-ubiquitination (112). There exists a constant evolutionary race between a virus and its host as they co-evolve.

Coronaviruses have also evolved several different strategies to inhibit a type I interferon response (Fig 2.4). Coronaviruses such as SARS- and MERS-CoVs can effectively inhibit an interferon response in human cells. Although the mechanisms underlying the inactivation of innate response pathways by these viruses in human cells are not entirely clear, some studies have shown that certain proteins from these viruses can effectively suppress IRF3-mediated activation of interferon gene expression (40, 113-117). Studies have identified that MERS-CoV proteins such as ORF 4a, 4b, 5 and M likely inhibit IRF3 activation in human cells by inactivating TBK1, a kinase that

phosphorylates IRF3 (114). Another mechanism that is employed by SARS-CoV is the downregulation of PRRs. SARS-CoV proteins have been shown to downregulate the expression of PRRs, thus leading to little up-regulation of antiviral pathways in the cell (117). Apart from inhibiting interferon production in cells, coronaviruses such as SARS-CoV can also effectively inhibit further downstream interferon signaling. Studies have shown that SARS-CoV can inhibit interferon mediated expression of ISGs (38). In the absence of an effective antiviral response in the infected cells, the virus propagates unchecked and causes severe disease and associated inflammation.

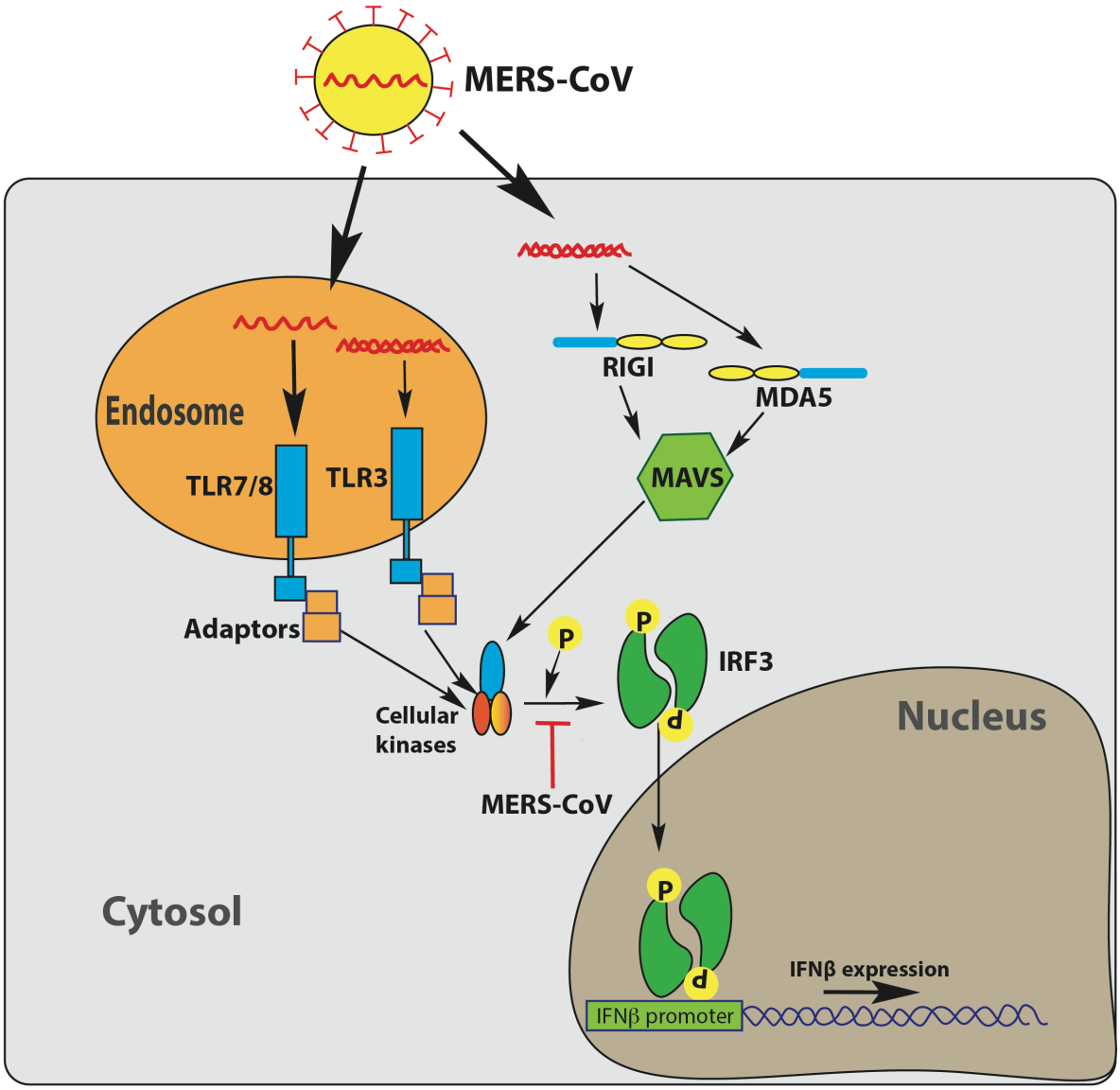

Figure 2. 4. Schematic representation of IRF3 mediated antiviral IFNβ expression and inhibition of IRF3 activation by MERS-CoV. Human cells identify viral single-stranded (ss) and double-stranded (ds) RNA through evolutionary conserved cellular receptors. Endoplasmic TLR3 and cytosolic RIG-I and MDA5 recognize dsRNA. Endoplasmic TLRs 7 and 8 recognize ssRNA. These cellular receptors then signal through various adaptor proteins such as mitochondrial antiviral-signaling protein (MAVS), myeloid differentiation primary response 88 (MyD88) and TIR-domain-containing adapter-inducing interferon-β (TRIF). These signals converge to activate cellular kinases such as TANK binding kinase 1 (TBK1) and inhibitor of nuclear factor kappa B kinase subunit epsilon (IKKε). These kinases phosphorylate IRF3, leading to its dimerization and subsequent localization to the nucleus of the cell to initiate antiviral interferon gene expression. MERS-CoV proteins such as M, ORF 4a, ORF 4b and ORF 5 inhibit IRF3 mediated activation of antiviral interferon responses in human cells (116).

Since bats are speculated to be reservoirs of several emerging viruses, it is of great interest to identify if these viruses can suppress the immune responses in bats as well. There is evidence that some viruses can inhibit an antiviral interferon signaling in bat cells; Henipaviruses propagated in fruit bat cell lines can inhibit interferon production and signaling pathways (57). However, studies looking at systemic immune responses in bats in response to Henipavirus infections are still lacking. The effect of coronavirus infection on the innate antiviral response in bats is not known. A recent study was able to detect IFNβ in Jamaican fruit bats that were inoculated with MERS-CoV, but the mechanism was not studied (9). My doctoral thesis adds further evidence to the observation that bats may indeed have evolved mechanisms to resist coronavirus mediated subversion of innate antiviral responses.

2.4. Tools to study pathogen-host interactions in bats

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This manuscript has been reformatted from the original version for inclusion in this thesis.

Contribution: I approached Dr. Baker with my idea and contacted the other co-authors on this manuscript. I performed the literature review and wrote the first draft. All authors reviewed the final draft.

Although bats have been linked with a number of viruses, identifying wildlife reservoirs can be a challenging process, requiring extensive ecological sampling. Evidence that a pathogen is permanently maintained in a population, often in the absence of disease, is required to confidently identify its reservoir (7, 118). Detection of fragments of viral genomes, combined with serological evidence for viral exposure in multiple individuals of a species are generally the first lines of evidence to associate a host with a pathogen (43, 55, 119, 120). Additional evidence in the form of virus isolation and the ability to link a reservoir to a spillover event is necessary to confirm the association. Examples of bat viruses that are confirmed or speculated to have 'jumped' from bats into humans and agricultural animals include coronaviruses (severe acute respiratory syndrome coronavirus [SARS-CoV], porcine epidemic diarrhea [PEDV] virus), filoviruses (ebolaviruses and Marburg virus), and henipaviruses (Nipah and Hendra viruses) [reviewed by Moratelli *et al.* (1)]. Consistent with the definition of a wildlife reservoir, infection of bats by some of these viruses results in viremia and viral shedding in the absence of clinical disease (21, 55). The spillover of some of these viruses to other susceptible hosts, including humans, can result in severe disease and mortality due to the lack of therapeutics and vaccines.

The emergence of zoonotic diseases is increasing globally and mammals, including bats, are major sources of emerging and re-emerging pathogens (29). The drivers of disease emergence include anthropogenic changes to the environment (e.g., agricultural intensification), climate change and the encroachment of human populations into wildlife habitats (121). The biological characterization of a newly identified virus often begins with its isolation in cultured cells. Although detection of virus-specific antibodies by serology and the use of molecular methods, such as viral sequencing, are often readily achievable, virus isolation from bats has been far more

challenging. For example, bats are implicated as the reservoirs of ebolaviruses based on the detection of viral genome, serological evidence and their confirmed role as a reservoir for the closely related Marburg virus. However, ebolaviruses have yet to be isolated from bats to definitively link them to spillover events (122). Identifying wildlife reservoirs and the viruses they host will require the development of better laboratory tools for virus isolation to study replication kinetics and virus-host interactions.

2.4.1. Current status of cell lines derived from bats

Virus isolation is generally attempted in established immortalized cell lines, such as the type I interferon deficient BHK-21 (baby Syrian hamster kidney cells) (123) or Vero E6 cell lines (African green monkey kidney cells) (124). Different primary cells from bats have been studied and some immortalized cell lines have also been generated (Table 2.1). Established cell lines from non-reservoir host species are convenient tools for virus isolation but do not always support propagation or isolation of viruses from wildlife. For example, a bat herpesvirus isolated from cell lines established from the Schreiber's long-fingered bat, *Miniopterus schreibersii*, failed to replicate in cells from a variety of other species, including cells from the black flying fox, *Pteropus alecto* (125). The henipavirus Cedar virus was isolated from primary cells of its putative natural reservoir, *P. alecto* whereas other cell lines showed no visible cytopathic effect (126). Similarly, the paramyxovirus Menangle virus was isolated using the same *P. alecto* kidney cell line (127).

Table 2. 1. Some cell types established from bats and range of viruses that have been used in studies with these cells.

Bat primary cells or cell line	Origin	Virus/pseudovirus used in the study	Reference
Artibeus jamaicensis primary cells	Embryonic, primary kidney cells	Dengue virus, MERS-CoV	(128); (9)
Carollia perspicillata	Trachea	VSV, Rift Vallet fever virus	(129)
Carollia perspicillata CpKd	Kidney	Bat-associated influenza virus (BIV)	(130)
Eidolon helvum EidNi/41	Kidney	BIV, Ebolavirus, MERS-CoV, Influenza A virus, O'nyong-nyong virus	(130); (131); (132); (133); (134)
Epomops buettikoferi EpoNi/22.1	Kidney	BIV, Influenza A virus	(130); (133)
Eptesicus fuscus Efk3	Kidney	VSV, PED-CoV, herpes simplex virus and MERS-CoV	(110)
Hypsignathus monstrosus hypLu/45.1	Lung	MERS-CoV and Influenza A virus	(132); (133)
Hypsignathus monstrosus hypNi/1.1	Kidney	BIV, MERS-CoV, Influenza A virus, African henipavirus	(130); (132); (133); (135)
Miniopterus schreibersii primary cells	Lymph node, Kidney	Miniopterus schreibersii herpesvirus	(125)
Myotis daubentonii MyDauLu/47.1	Lung	Influenza A virus	(133)
Myotis davidii MdKi	Kidney	Sendai virus	(109)
Myotis myotis	brain, tonsil, peritoneal cavity, nasal, nervous	Rabies virus, European bat lyssavirus	(136)
Myotis velifer incautus (MVI-it) CRL-6012	intercapsular tumor	Novel bat gammaherpesvirus	(137); (138)
Pipistrellus ceylonicus	Embryonic	Chandipura virus, novel adenovirus	(139)
Pipistrellus subflavus ESU-BSL	Lung	MERS-CoV	(132)
Pteropus alecto	Aorta, bone marrow, brain, foetus, foetal membranes, heart, kidney, liver, lymph nodes, lung, muscle, pharynx, placenta, salivary gland, small intestine, skin, spleen, testes, thymus, uterus	Hendra virus, Pulau virus, Sendai virus, Nipah virus	(96); (140); (57)
Pteropus alecto	Kidney	Influenza A virus, Sendai virus, Pteropine orthoreovirus NB, Nipah virus, Hendra virus	(141); (96); (57)
Pteropus alecto	Embryonic	Nipah and hendra virus	(57)
Rhinolophus affinis	Embryonic	VSV	(142)
Rhinolophus sinicus	Splenocytes	VSV	(142)
Rousettus aegyptiacus RO6E, RO5T	Kidney	MERS-CoV, Influenza A virus, Ebola virus, Marburg virus	(132); (133); (143)
Rousettus aegyptiacus RoNi/7.1	Kidney	Bat-associated influenza viruses, MERS-CoV, Influenza A virus	(130); (132); (133)
Tadaria brasiliensis Tb1Lu	Lung	Bovine leukemia virus, Influenza A virus, mammalian reovirus serotype 3 Dearing	(144); (133); (145)
Rousettus aegyptiacus immortalized cells	Foetus	Vaccinia Ankara	(146)

Cell lines have defined characteristics based on their tissue of origin and method of immortalization. For a cell to be suitable for virus propagation, it must bear an appropriate receptor to bind with an incoming virus and it must be permissive. Because viruses differ in their cellular tropisms, working with a few cell lines with epithelial or fibroblastic characteristics limits our ability to isolate or study viruses that may have a tropism towards cell types that are more difficult to immortalize. Current cell lines may lack receptors required by viruses to replicate when cells are infected at low multiplicity of infections. For example, bat cells from various species were resistant to infection with vesicular stomatitis virus (VSV) pseudotypes bearing the SARS-CoV spike protein until the cells were transfected to express the human SARS-CoV receptor, angiotensin-converting enzyme 2 (ACE2) (147). Although the lack of appropriate receptors on cell lines makes it difficult to isolate some viruses, this lack of susceptibility to infection can also help us understand the early events in virus infection. For instance, differences in the susceptibility of cells from a variety of bats to filoviruses also led to the identification of unique amino acid substitutions in the filovirus receptor, Neimann-Pick type C1 (NPC1), providing insight into the host range of ebolaviruses (131). Similarly, Widagdo *et al.* have shown that dipeptidyl peptidase 4 (DPP4, CD26), a cellular receptor for MERS-CoV, is expressed in specific cell types in different tissues which also varies between bat species (148). These studies demonstrate the need to develop cell lines from different organs and bat species and clone them for a variety of cell types as viral receptors may not be equally distributed.

2.4.2. Considerations for development of cell lines

Chiroptera consists of over 1200 bat species and different species may have co-evolved with different viruses. Viruses detected in one species, may not infect cells established from a different bat species. Thus, the choice of bat species for generating cell lines capable of supporting a particular virus should be supported by evidence that the species is susceptible to that virus. For example, SARS-CoV-like coronaviruses have been detected in *Rhinolophus sinicus* (41), and this species is the logical candidate for generating cell lines that can support replication and isolation of these viruses.

Because propagation of viruses in cell lines can select for members of the virus population that replicate most efficiently in the chosen cell type, the choice of cell line should be taken into consideration. Propagation of viruses in cell lines other than those of the natural host may lead to accumulation of adaptive mutations or attenuate the virus (149), which in turn has implications for assessing host-pathogen interactions either *in vivo* or *in vitro*. For example, although wild type Marburg virus causes no disease in mice, it can be adapted to mice by serial passage to cause lethal disease (150). Deep sequencing of Marburg virus after adaptation to mice and following propagation in Vero E6 cells has revealed the sequential mutations associated with each passage as the virus adapts to a new host either *in vivo* or *in vitro* (151). This is also thought to have occurred with Tacaribe virus, which was isolated from artibeus bats and passaged 20 times in newborn mice, resulting in point mutations and deletions (152, 153).

Bats experience a range of body temperatures during different physiological activities (from 4-8°C during hibernation to 41°C during flight) (15). Thus, viruses isolated from bats should be studied at different relevant physiological temperatures. Propagation of viruses at temperatures

other than the nominal of the reservoir species may also lead to the generation of attenuation mutants; a strategy that has been used for vaccine development for decades. There is evidence that arthropod viruses found in mosquitoes and ticks accumulate mutations when grown at different temperatures (154). Thus, propagation of viruses in susceptible cell lines [reviewed by Hare *et al.* (155)] and at optimized temperatures may minimize mutations and produce viruses similar to wildtype virus found in wildlife reservoirs or arthropod vectors [reviewed by Prescott *et al.* (156)].

Controlling for temperature to match temperatures experienced by bats during hibernation is a challenge because *in vitro* cell culture systems are not viable at low temperatures. Although Miller *et al.* were able to grow bat cells at temperatures ranging from 37-41°C (157), our attempts to cultivate big brown bat cells (Efk3) at lower temperatures of 26-30°C were not successful (Banerjee and Misra, unpublished). There is evidence that experimental infection of different reptiles and amphibians with chikungunya virus produce varying amounts of viremia depending on the host's body temperature (158). There is a need to develop a system to test the replication potential of bat-borne viruses at lower temperatures such as those experienced by bats during torpor and hibernation, rather than only propagating them in cells cultured at 37°C.

The virus stock that is used to experimentally infect bats is usually generated by propagation in cells of non-bat origin, such as Vero E6 cells. It is unknown whether propagation of a virus in a monkey cell line alters the genotype of the virus. We have previously infected bats with a virus isolated from bat urine and subsequently propagated in bat cells (Baker *et al*., unpublished). A problem with propagating this virus in bat cells was achieving high virus titre to perform

experimental infection studies in bats, perhaps because bat cells appear to have constitutive type I IFN activation (97). It may be necessary to develop interferon-deficient bat cell lines to propagate bat viruses. Recently, Zhang *et al.* knocked out an antiviral cell signaling receptor, interferon alpha receptor 2 (IFNAR2) in bat cells using CRISPR/Cas9 technology (103). Similarly, this technology can be used to generate interferon-deficient bat cells in culture.

There are several challenges to establishing primary cells from wildlife species. These challenges range from obtaining healthy tissues to determining the ideal cell types. Primary cells maintain genetic integrity and are therefore most appropriate for the study of natural infection of cells. However, primary cells have finite numbers of cell division before undergoing senescence, thus, it can become problematic for long-term studies. Immortalized cells frequently accumulate chromosomal aberrations over time; therefore, caution must be exercised when maintaining cell stocks. Nonetheless, they are convenient and often receptive to genetic manipulations, including transfection of plasmids and targeted changes (e.g. CRISPR), and often the only cells available that are capable of supporting replication. Thus, the ability to conduct experiments in primary or immortalized cells has advantages and disadvantages.

Primary cells can also harbor latent viruses that can become reactivated during *in vitro* cultivation when the cells are outside the host and isolated from other components of the immune system that would otherwise control virus replication. Most mammals, including bats harbor persistent viruses [reviewed by Calisher *et al.* (7)], and viruses have been isolated or identified by next generation sequencing (NGS) from bat cell lines (137). Cell lines from any species of bat should therefore only be handled under controlled biosafety conditions and thoroughly tested

before being used for experimental studies. This is also a limitation when working with wild-caught bats.

2.4.3. Using cell lines to assess the immune response in bats to viral infections

Research in the field of bat immunology steadily increased after bats were implicated as ancestral reservoirs of the coronavirus that caused the SARS outbreak in 2003 (12, 41, 43). Over 200 viruses have been identified in bats and additional new viruses are discovered on a regular basis (1, 159). A few viruses have been documented to cause disease in bats in addition to rabies and related lyssaviruses (160-162). Experimental inoculation of Jamaican fruit bats with high doses of Tacaribe virus caused significant morbidity and mortality similar to natural infection of artibeus bats (163). However, the inoculated bats did not transmit the virus. Based on their observations, Cogswell-Hawkinson *et al.* speculate that Jamaican fruit bats may not be a reservoir host for Tacaribe virus (61). Lloviu virus, a filovirus was isolated from dead bats in Spain (59). These examples appear to be exceptions to the usual resistance to viral disease displayed by most bats. Bats have been hypothesized to have evolved unique aspects of their immune systems, such as a strict control of the inflammatory process (106) and constitutive expression of antiviral interferons (97) to better survive infections with these viruses. Testing this hypothesis has been possible, in part through the establishment of well characterized cell lines (13).

Cell lines from several bat species including members of both the Yinpterochiroptera (*Pteropus alecto, Eidolon helvum* and *Rousettus aegyptiacus)* and Yangochiroptera (*Eptesicus fuscus,* and *Myotis myotis)* suborders, have been established (110, 134, 136, 140, 164) (Table 2.1). These cell

lines have been used to cultivate viruses that are speculated or confirmed to have spilled over from bats. These cell lines have also allowed researchers to study the innate immune responses to viruses. Cytokines produced in response to activation of selected innate immune response pathways in bat cells have led to a better understanding of how bats might respond to viral infections in the wild. Bat cells can respond to viruses or viral antigens through the production of antiviral interferons and interferon stimulated genes (94, 96, 111, 134). The use of bat cell lines has also led to the discovery of viral proteins that counteract the innate immune response in these cells (57). Less work has been performed on the adaptive immune responses of bats using cell lines, in part due to the lack of research on bat T and B cells. However, a recent report used *P. alecto* cell lines to examine the repertoire of self and Hendra virus epitopes presented by MHC class I molecules (165). More research is needed to establish *in vitro* and *ex vivo* cultures of immune cells from representative bat species.

2.4.4. Developing bat specific reagents

Establishing cell lines from different species of bats provides the opportunity to perform comparative studies between bat species and with other mammals. Several cell lines have been developed from bats, including some that support the replication of viruses from different families (Table 2.1). However, to better characterize bat immune responses, we need to develop cell lines or a system where we can continuously establish immune cells *in vitro*. Current wildlife cell lines are composed primarily of epithelial or fibroblast cell types, which limit the responses generated following infection. These cells may lack many of the receptors and cytokine responses of specialized immune cells. Isolating and developing representative immune cell lines will allow the study of both adaptive and innate immune responses of bats. This also involves

developing species and cell-type specific reagents that will allow characterization and culture of immune cells *in vitro*. Recently, immune cells from black flying foxes have been functionally and phenotypically characterized (102), along with the establishment of bone-marrow derived dendritic cells and macrophages using black flying fox reagents developed in the laboratory (101). Systems such as these will further allow exploration of the adaptive immune response in potential reservoir bat species. More efforts are required to generate similar cell types in other bat species. Sharing of resources will play a key role in expanding our understanding of the unique immune response in bats.

Although primary cells are good tools to attempt virus isolation, they have a limited life-span. There are several technologies that can be used to immortalize these primary cells. Immortalized cell lines replicate indefinitely in culture and provide researchers with valuable tools to study viruses and cell-virus interactions. Some of these bat cell lines have been established using existing technology such as simian virus 40 large T-antigen and human hTERT expression systems (166-168), whereas others have been generated by using novel technologies such as a bat polyomavirus large T-antigen to immortalize cells (110) and other bat specific reagents to generate dendritic cells and macrophages (101). Polyomavirus large T-antigen binds to and attenuates the tumor suppressor protein p53 and proteins of the retinoblastoma tumor suppressor family (pRb, p130 and p107) promoting cell cycle progression. Gamma-herpesviruses have also been used to immortalize cells of the immune system, such as B lymphocytes (169). These novel technologies or combination of cell immortalization techniques can be modified for the generation of cell lines from representative cell and tissue types from less studied species of bats and other neglected wildlife reservoirs.

Studying the host-pathogen relationship in wildlife species is often challenging, with *in vivo* experiments difficult and costly to perform. Developing cell lines will allow preliminary research, which can later be validated *in vivo* in bats. These cell lines can be used to study or isolate viruses that spillover from animal reservoirs to humans, and pathogens that cause disease in wildlife. The advantages of developing cell lines do not end at studying the immune responses and isolating viruses. These cell lines can be used to propagate isolated viruses to high titre for the development of diagnostic kits and potential vaccines. Studying pathogen-host interactions may also allow us to identify biomarkers associated with viral infection that could be used to predict and prevent spillover to other susceptible hosts. For example, biochemical or hormonal changes linked to increases in viral replication in wild bat populations may be used to predict periods of increased risk of spillover to other susceptible species.

2.4.5. Using cell lines to study host responses to pathogens that cause disease in bats

In addition to viruses, bats may also be reservoirs, or even dead end hosts, for other pathogens such as bacteria (170), fungi or parasites. Bats do not seem to be refractory to disease when it comes to extracellular pathogens such as fungi (171). The white-nose syndrome (WNS) fungus, *Pseudogymnoascus destructans*, causes significant wing damage at the sites of infection in little brown bats (*Myotis lucifugus*) (19, 171). Big brown bats (*Eptesicus fuscus*) seem to be relatively resistant to the fungus (172). Identifying, isolating and characterizing the interaction of extracellular and intracellular pathogens with their host can be better facilitated by the availability of bat species specific cell lines.

Research to understand bacterial and fungal infections in bats is growing and has led to the identification of potential pathogenic bacterial species in bats (170, 173). Cell lines, as previously shown (174), can be effective tools to dissect the role of these organisms in the overall health of bats. Transcriptomic data from bat cells infected with various pathogens, including bacteria and viruses, will shed light on global pathways that are activated or suppressed in response to these pathogens. These data can be used to develop hypotheses on the overall impact these pathogens might have on bats at an organism level. Recently, a novel *Streptomyces* species with anti-fungal activity was identified in bats (175). WNS has caused the death of millions of little brown bats in North America (171, 176, 177) and this new compound amongst others (178) might help to curb the spread of WNS. Potential toxic effects of this drug can be tested in uninfected bat cell lines before testing them in live bats or other relevant animal models.

2.4.6. Developing *in vivo* bat models

In addition to cell lines, there is a need for laboratory bred bats with known and tested infection status for *ex vivo* and *in vivo* experiments. Establishing a wild-caught bat colony is challenging but achievable with sufficient resources to house and maintain the bats. Although studies using wild-caught outbred bats potentially provide the best representation of what occurs naturally during virus infection, it can be difficult to obtain reproducible data using wild caught animals due to their high genetic diversity. The current infection status and history of viral infections in wild caught bats is also difficult to determine which in turn may affect the outcome and interpretation of experimental infections. There is therefore a need to establish inbred laboratory colonies of bats of known infection status. As much of the knowledge that has been obtained

regarding the host responses of bats has been gained from studies using bat cell lines, experimental infections of captive bats provide an opportunity to test whether observations made *in vitro* also apply *in vivo*. Studies in captive animals are also necessary to confirm the role of bats as 'reservoirs' by carrying out long-term experiments to detect virus shedding and the lack of associated pathology in bats with known infection status (i.e. laboratory bred bats). This was recently attempted with Egyptian rousette bats that were infected with Marburg virus. The bats shed virus between 5 and 19 days post infection (21), but longer term studies might be necessary to further establish bats as reservoirs. Thus there is a need to develop more reliable animal models with known infection status.

2.4.7. Conclusion

Although several different cell lines have been established from different species of bats (Table 2.1), we are still faced with the challenge of isolating several viruses that have been detected in bats. We have detected little brown bat coronavirus sequences (17, 50) and others have detected ebolavirus sequences [reviewed by Olival *et al.* (179)] in bats by polymerase chain reaction (PCR), next generation sequencing (NGS) and serology. As discussed, virus isolation is more challenging than nucleic acid detection but with the availability of additional cell lines from various organs and species and technologies such as CRISPR to develop cell lines deficient in components of the immune system, attempts to isolate and propagate viruses *in vitro* should be more successful.

Chiroptera consists of over 1200 species and much remains unknown about their relationship with their microorganisms. Bats are genetically diverse and results from studies done in one bat

species may not represent other species of bats. Thus, there is a need to study other species of bats to gain deeper insights into bat-microbe co-evolution, bat immune system and ecological interactions of bats. Bats host more zoonotic viruses per mammalian species (180, 181), even more than rodents, the largest group of mammals. Collaboration between laboratories will be required to develop cell lines from different bat tissues, bat specific reagents and to establish laboratory bat colonies to explore the length and breadth of microbe-host interactions in these ecologically important mammals (Fig 2.5).

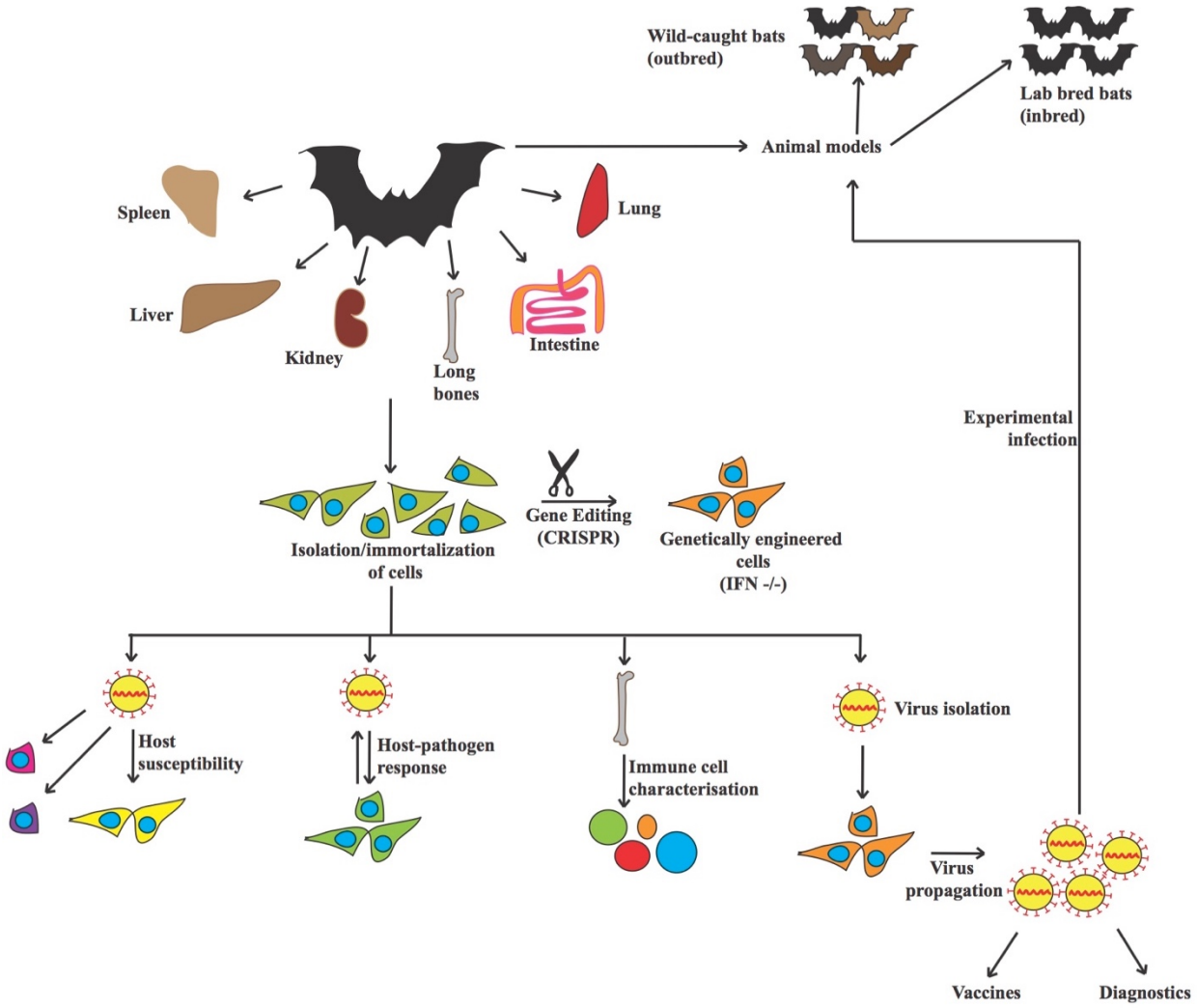

Figure 2. 5. There is a need to develop more model cell lines and animal models to decipher the immune responses of bats to viruses. Viruses display tropism towards different cell types

and there is a need to generate cell lines representative of the multitude of cell types found in bats to increase the chances of isolating the viruses that they carry. Alternatively, genetically modified cell lines can be generated that do not express antiviral interferons from these cell types for virus isolation. These cells may be used to propagate bat-borne viruses to high titres for *ex vivo, in vitro* and *in vivo* experiments. Generating cell lines from a variety of different tissues and bats will allow researchers to explore virus susceptibility and virus-host interactions in these intriguing mammals. Generation of outbred (wild-caught) and inbred (laboratory bred) bat colonies with known infection status will enable systemic immune response studies with relevant viruses isolated from bats. The ability to resist infections with some of these viruses may be an effect of several evolutionary adaptations, which can be better dissected by studying the systemic responses in bats.

Chapter 3: Rationale and Hypotheses

3.1. Rationale to study innate immune signaling and virus-host interaction in bats

As mentioned earlier in Chapter 1 and 2, bats have been speculated as reservoirs of several emerging and re-emerging viruses. The ability of bats to co-exist with viruses that are otherwise lethal in other mammalian species, including humans is very intriguing. Indeed, I am very motivated to study if bat cells have unique innate antiviral adaptations that allow them to control infections with these viruses, unlike human cells.

Little and big brown bats are two of the primary hibernating bats in Western Canada and as previously mentioned in the literature review (Chapter 2), our group has identified novel coronaviruses in these bats. In order to further dissect the host cellular responses to viruses, we needed to establish an *in vitro* model to study bat-virus interactions. At that time, no cell line was commercially available from an insectivorous bat, the likely reservoir of coronaviruses. Thus, we established the first North American immortalized insectivorous bat cell line (Chapter 4), which is now commercially available through Kerafast, Boston, USA. This cell line provided us with an opportunity to test a plethora of questions about virus-bat interactions and bat cellular innate immune responses to invading viruses.

After characterizing the cell line for its ability to respond to TLR ligands such as poly(I:C), I proceeded to identify differences between bat and human cells' response to poly(I:C). At the time, cells from different species of bats (primary and immortalized) had been shown to produce

IFNβ in response to poly (I:C) treatment, however, the differences between the innate immune responses in big brown bat and human cells were largely lacking.

Coronaviruses that cause SARS, MERS and PEDV can very effectively suppress innate antiviral IFN responses in human and pig cells while overwhelming the host with an exaggerated inflammatory response (Chapter 2). This virus-mediated inflammatory response contributes to severe pathology. Since bats naturally or experimentally infected with coronaviruses do not show obvious signs of disease, we tested the hypothesis that big brown bat cells have a mechanism to suppress virus or poly(I:C)-mediated expression of inflammatory cytokines (Hypothesis 1). Poly(I:C), a critical PAMP seemed like an excellent challenge model for us at this point in time because I was primarily interested in the cellular responses, particularly any unique responses in bat cells. Using an infection model with a coronavirus at this time could have complicated my readouts since coronavirus proteins have been shown to suppress innate antiviral responses in human cells (reviewed in Chapter 2). We were not sure how the viral proteins would affect the innate antiviral signaling proteins in bat cells.

After testing my first hypothesis (Chapter 5), I was further interested in testing this hypothesis using an infection model. Since MERS-CoV is speculated to have evolved in bats, I wanted to observe how this virus behaved in bat cells. Since MERS-CoV has been shown to suppress innate antiviral IFN responses in human cells, I was keen on testing if this would hold true in bat cells as well.

In summary, I had two questions in my mind that motivated me to develop my next set of studies, (i) Could big brown bat cells control MERS-CoV-mediated expression of inflammatory cytokines as observed while testing Hypothesis 1 and (ii) could big brown bat cells be resistant to MERS-CoV-mediated suppression of innate antiviral IFN responses? These questions led me to approach Dr. Falzarano at VIDO-InterVac and in collaboration with him, I was able to test my second hypothesis.

3.2. Hypothesis 1: *Big brown bat kidney cells have evolved a unique mechanism to control surrogate virus-mediated inflammatory responses. Refer to Chapter 5.*

To test my first hypothesis, I stimulated human lung and big brown bat kidney cells with known human ligands for TLRs 3, 7, 8, 9, RIG-I and MDA5. Of all the ligands that I used, only poly (I:C) induced the expression of several innate immune response associated genes in both human and bat cells. I checked for the expression of over 30 genes in each cell line using qRT-PCR. This allowed us to focus on genes that were expressed in dramatically different levels in the two cell types. These included genes that are known to be associated with an inflammatory response in humans. I then proceeded to work with a key inflammatory gene, TNFα. Indeed, our work supported our hypothesis that big brown bat cells have evolved a unique mechanism to suppress a poly(I:C)-mediated inflammatory response. The detailed study can be found in Chapter 5 of this thesis.

3.3. Hypothesis 2: *Big brown bat kidney cells are resistant to MERS-CoV-mediated subversion of IFNβ signaling. Refer to Chapter 6.*

To test my second hypothesis, I approached Dr. Darryl Falzarano at VIDO-InterVac for collaboration. Dr. Falzarano is a leading expert in MERS-CoV biology and his expertise was crucial for the success of this project. In this study, I sought to identify if MERS-CoV can suppress IFN responses in big brown bat cells similar to what has been observed in human cells. To our surprise, big brown bat cells mounted a robust IFN response to MERS-CoV infection. I was then able to identify a crucial molecule (i.e. IRF3) in big brown bat cells that regulates the IFN signaling pathway in response to MERS-CoV infection. Indeed, our series of logical experiments support my second hypothesis that big brown bat cells are resistant to MERS-CoV-mediated subversion of innate antiviral responses. The detailed study and results are available in Chapter 6.

Chapter 4: Developing an *in vitro* model to study why bats are refractory to disease caused by viruses that cause serious disease in other mammals such as humans.

4.1. Permission to use

This manuscript has been reformatted from the original version for inclusion in this thesis.

4.2. Citation

Banerjee, A., Rapin, N., Miller, M., Griebel, P., Zhou, Y., Munster, V., and Misra, V. (2016). Generation and Characterization of *Eptesicus fuscus* (Big brown bat) kidney cell lines immortalized using the Myotis polyomavirus large T-antigen. J Virol Methods *237*, 166-173.

4.3. Contribution

Several authors contributed significantly in this study. The cell line was established from a big brown bat kidney. Dr. Misra played a major role in generating the primary cells. Ms. Rapin and I helped him with subsequent passages. Once we had several clones of the immortalized cell line, I tested them for the expression of cell type specific markers. Prior to immortalizing them, I also characterized the ability of the two T-antigens to induce cell growth. I further tested the immortalized cell line for susceptibility to herpes simplex virus (HSV) and vesicular stomatitis

virus (VSV). I tested the ability of the cell lines to respond to poly (I:C) by detecting the expression of interferon beta and also stained the chromosomes. Dr. Zhou's team tested the ability of our cell line to support the replication of porcine epidemic diarrhea virus and Ms. Miller and Dr. Munster tested our cell line for susceptibility to infection with Middle East respiratory syndrome coronavirus. Dr. Griebel's team attempted to characterize the cell line clones by flow-cytometry using cell type specific human monoclonal antibodies.

4.4. Abstract

It is speculated that bats are important reservoir hosts for numerous viruses, with 27 viral families reportedly detected in bats. The majority of these viruses have not been isolated and there is little information regarding their biology in bats. Establishing a well-characterized bat cell line supporting the replication of bat-borne viruses would facilitate the analysis of virus-host interactions in an *in vitro* model. Currently, few bat cell lines have been developed and only Tb1-Lu, derived from *Tadarida brasiliensis* is commercially available. Here we describe a method to establish and immortalize big brown bat (*Eptesicus fuscus*) kidney (Efk3) cells using the *Myotis polyomavirus* T-antigen. Subclones of this cell line expressed both epithelial and fibroblast markers to varying extents. Cell clones expressed interferon beta in response to poly (I:C) stimulation and supported the replication of four different viruses, namely, vesicular stomatitis virus (VSV), porcine epidemic diarrhea coronavirus (PED-CoV), Middle-East respiratory syndrome coronavirus (MERS-CoV) and herpes simplex virus (HSV). To our knowledge, this is the first bat cell line from a northern latitude insectivorous bat developed using a novel technology. The cell line has the potential to be used for isolation of bat viruses and for studying virus-bat interactions in culture.

4.5. Introduction

It is speculated that bats are an important reservoir host for several viruses, such as Ebola virus (family *Filoviridae*, genus *Ebolavirus*), Marburg virus (family *Filoviridae*, genus *Marburgvirus*), severe acute respiratory syndrome coronavirus (SARS-CoV; family *Coronaviridae*, subfamily *Coronavirinae,* genus *Betacoronavirus*), Middle-East respiratory syndrome coronavirus (MERS-CoV; family *Coronaviridae*, subfamily *Coronavirinae,* genus *Betacoronavirus*), porcine epidemic diarrhea coronavirus (PED-CoV; family *Coronaviridae*, subfamily *Coronavirinae,* genus *Alphacoronavirus*) and Hendra and Nipah viruses (family *Paramyxoviridae*, genus *Henipavirus*). There is evidence that many of these viruses have been transmitted from bats to other hosts where they caused serious disease (20, 182, 183). Over 200 different viruses from 27 families have been detected in bats [reviewed by Moratelli *et al.* (1)] but most of these viruses have yet to be isolated and there is scant information regarding the biology of these viruses in bats.

Bats are genetically diverse and are found dispersed across much of the planet. With over 1200 species, bats display major differences in their behavior, feeding habits and the viruses they harbor [reviewed by Moratelli *et al.* (1)]. Very little is known, however, about bat immune responses and if these differ across genera and species. Currently a single bat cell line (Tb1-Lu, ATCC number CCL-88, derived from the lung of *Tadarida brasiliensis*) is available through the American Type Culture Collection. Research groups have established other bat cell lines, from fruit and insectivorous bats using established techniques such as using the *Simian virus 40*

(*SV40*) T-antigen and expressing human telomerase reverse transcriptase (hTERT), but these are not commercially available yet (140, 164, 184).

Bats are the only mammals capable of true flight and as such they may have unique physiological adaptations. For example, they display unique strategies for neutralizing the DNA-damaging by-products of oxidative metabolism produced as a result of increased metabolic activity (16). Zhang *et al.* hypothesize that bats have evolved and accumulated genetic changes as a result of their adaptation to flight. This is to limit collateral damage caused by by-products of an elevated metabolic rate associated with flight (14). These genetic changes may be important in the expansion and contraction of important gene families, including genes involved in the innate response pathway (14).

North American bat species are at risk of drastic population depletion due to white-nose syndrome (176, 177) and conducting terminal *in vivo* experiments might not be entirely possible in future. Establishing stable bat cell lines would provide an alternative for conducting *in vitro* host-pathogen studies. Experiments using cultured bat cells could provide useful preliminary information on bat innate immune defense responses, virus-cell interactions and cellular physiology.

There are several established methods for immortalizing primary cells. The first involves the introduction and stable expression of genes coding for the Simian virus 40 (*SV40*) large T antigen (SV40Tag). The large T antigen binds to and attenuates the tumor suppressor protein p53 and proteins of the retinoblastoma tumor suppressor family (pRb, p130 and p107). This promotes

DNA replication and cell division. This method has been used to immortalize cells from a number of species including human (185), rabbit (186) and rat (187).

The second method involves the introduction and stable expression of the catalytic subunit of the human telomerase reverse transcriptase (hTERT). Ectopic expression of hTERT has been successfully used to immortalize primary cells in a range of mammalian species such as goat mammary epithelial cells (168) and canine Schwann cells (188). This enzyme subunit prevents the shortening of telomeres with repeated cell divisions and thus prevents cellular senescence.

Here we describe a method for establishing and characterizing a kidney cell line (Efk3) from *Eptesicus fuscus* (the N. American Big brown bat) using the *Myotis polyomavirus* T antigen (MyPVTag). We characterized the capacity of MyPVTag to enhance DNA replication in Vero cells and found that it significantly increased their DNA content. We then transfected MyPVTag into primary bat kidney cells and sub-cloned several cell lines. We characterized the lineage of these clones and tested their expression of the interferon beta (IFN beta) gene in response to polyinosinic-polycytidylic acid (poly(I:C)) stimulation. We further tested three cloned kidney cell lines for their ability to support the replication of viruses from the families *Coronaviridae*, *Herpesviridae* and *Rhabdoviridae*. The parental cell line and clones were capable of expressing IFN beta and supported the replication of viruses such as vesicular stomatitis virus (VSV; family *Rhabdoviridae*, genus *Vesiculovirus*), herpes simplex virus (HSV; family *Herpesviridae*, subfamily *Alphaherpesvirinae*, genus *Herpesvirus*), PED-CoV and MERS-CoV. PED-CoV and MERS-CoV are viruses for which transmission from bats, either directly or via an intermediate reservoir, has resulted in high mortality in pigs (189) and humans (32), respectively. VSV and

HSV are members of viral families that have previously been detected in bat species [reviewed by Moratelli *et al.* (1)]. Although *E. fuscus* primary embryonic cells have been described before (190), to our knowledge, this is the first cell line established from a northern latitude insectivorous bat that was transformed by using a viral element (MyPVTag) selected from a known bat virus. Furthermore, the established kidney cell lines were able to support the replication of selected viruses from three different virus families.

4.6. Materials and methods

4.6.1. Ethics statement

All procedures related to the handling and euthanasia of bats were submitted to and approved by the Committee on Animal Care and Supply of the University of Saskatchewan Animal Research Ethics Board (protocol #20090036) and were in accordance with regulations approved by the Canadian Council on Animal Care.

4.6.2. Cell culture

A moribund male *E. fuscus* bat submitted to the laboratory was humanely euthanized. Brain, liver, lungs, spleen and kidney were harvested. Each organ was finely minced, and incubated at room temperature in 0.5% trypsin-EDTA (Gibco, USA) with agitation. Periodically cells were recovered after neutralizing trypsin with fetal bovine serum (FBS; Seradigm, USA) added to 5%. Cells were resuspended in Dulbecco's Minimal Essential Medium (DMEM; Corning, USA) containing penicillin (Gibco, USA), streptomycin (Gibco, USA) and amphotericin B (Sigma, USA), placed in 75 cm^2 flasks (Cellstar, Germany) and incubated at 37^oC in an atmosphere of 5% CO_2. Only kidney cells grew to form a monolayer. These cells were recovered by trypsinization, diluted 1/3 and re-plated. Cell samples at various passages were cryopreserved in

DMEM containing 10% fetal bovine serum (FBS) and 10% dimethyl sulfoxide (EMD Chemicals, USA).

Bat kidney cells were immortalized by using ViaFect (Promega, USA) to transfect cells with either 2.5µg of pcDNA3 (Invitrogen, USA) empty vector or plasmids expressing either *SV40* large T-antigen (SV40Tag) or *Myotis polyomavirus* large T-antigen (MyPVTag). Transfected cells were cultivated in DMEM containing 10% FBS and Geneticin reagent (InvivoGen, USA). Only cells transfected with MyPVTag continued to replicate. Cells were confirmed to be *E. fuscus* cells by amplifying and sequencing a segment of mitochondrial cytochrome b transcripts (191). Human lung fibroblast (MRC5) cells (ATCC CCL-171) were cultured in MEM medium (Corning, USA) supplemented with 10% FBS (Seradigm USA), 1/100 non-essential amino acids (NEAA; Gibco), 1/100 (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; Gibco) and 1/1000 gentamycin (Gibco, USA). Vero cells (Elaine Van Moorlehem, Vaccine and Infectious Disease Organization - International Vaccine Center (VIDO-InterVac)) were cultured in DMEM with Glutagro (Corning, USA) supplemented with 10% FBS (Seradigm, USA) and penicillin/streptomycin. All cell lines were checked and controlled for mycoplasma by a semi-nested PCR (described below).

4.6.3. Chromosome spread

Efk-3B cells were seeded at a concentration of 5 x 10^5 in a T-75 flask. The cells were grown up to 80% confluency and treated with 0.1µg/ml Colcemid (Roche, USA) as mentioned previously (192). The cells were processed, spread on slides and chromosomes were stained with Giemsa staining solution as mentioned previously (192).

4.6.4. Plasmid cloning

The MyPVTag was amplified from the *Myotis polyomavirus* whole genome (National Centre for Biotechnology Information (NCBI), Accession number NC_011310.1) cloned in a TOPO vector (Invitrogen, USA) and sub-cloned into a pcDNA3 (Invitrogen, USA) backbone. SV40Tag (a generous gift from Ivan Sadowski, University of British Columbia) was also sub-cloned in pcDNA3. The pcDNA3 plasmids encoding the T-antigens were used for transfection studies.

4.6.5. Flow cytometry

To quantify DNA in T-antigen (T-ag) transfected Vero cells, the cells were seeded at a concentration of 5 x 10^5 cells/well in 6-well plates. The cells were transfected with 2.5 μg of either SV40Tag (SV40Tag in pcDNA3), MyPVTag (MyPVTag in pcDNA3) or pcDNA3 using Lipofectamine 2000 (ThermoFisher Scientific, USA). The cells were harvested and prepared for flow cytometry twenty-four hours after transfection. Briefly, cells were harvested and re-suspended in Dulbecco's Phosphate-Buffered Saline (DPBS) (ThermoFisher Scientific, USA). Cells were fixed in 70% ice-cold ethanol for 30 mins and stained for the respective T-antigens using 0.8 μg/ml mouse anti-SV40Tag (cross-reactive for SV40Tag and MyPVTag) (Molecular Probes, USA). The secondary antibody cocktail contained 0.01mg/ml propidium iodide (Molecular Probes), 0.2 mg/ml RNase A (Sigma, USA), 4.0 μg/ml goat anti-mouse immunoglobulin-Alexa488 conjugate (Molecular Probes) and 0.1% Triton X-100 (Sigma, USA) in DPBS. The cells were filtered through 64 μm nylon mesh prior to analyses. For analyzing the cell lineage of the clones, intracellular staining using the commercial BD fixative (BD Biosciences, USA) was carried out following the manufacturer's recommendation. Murine monoclonal antibodies against vimentin (1/200 dilution of Monoclonal anti-vimentin, clone VIM 13.2, mouse ascites fluid IgM) (Sigma-Aldrich, USA) and cytokeratin were used (1/200 dilution of monoclonal anti-cytokeratin 8.13, clone K8.13, mouse ascites fluid IgG2a isotype) (Sigma,

USA). Secondary antibodies used were 0.625 µg/ml goat anti-mouse IgM (µ)-FITC conjugate (Caltag/ Invitrogen, USA) and 0.25 µg/ml goat anti-mouse IgG2a-FITC conjugate (Caltag/Invitrogen, USA). Cells were analysed using FacsCalibur (BD Biosciences, USA) with forward scatter detection using a photodiode with 488/10nm bandpass filter and side scatter detection PMT with Brewster-angle beam splitter. FITC was detected with a 488 nm laser and 530/30 nm band pass filter. For each sample 50,000 events were accumulated and analyzed with CellQuest Pro (BD Biosciences, USA).

4.6.6. Nucleic acid extraction, PCR and qRT-PCR

All RNA extractions were performed using the RNeasy Plus Mini kit (QIAGEN, Germany) as per manufacturer's instructions. cDNA was prepared using the QuantiTect Reverse Transcription kit (QIAGEN) as per manufacturer's instructions. One µg of RNA was used for cDNA preparation. cDNA was used as a template for the quantification of target genes. Conventional PCR (polymerase chain reaction) to determine the cell lineage of the clones was performed using primers specific for *E. fuscus* vimentin (BBB–Vimentin–F–TCAAGAATACCCGCACCAACG and BBB-Vimentin–R– ACTGCTGACGGACGTCGCGC) and cytokeratin (BBB-Cytoker–F–GAAGACCTACAAGGTGTCCAC and BBB-Cytoker–R–CCATCTCGGGTCTCAATCTTC). Primers were designed using the annotated *E. fuscus* genome on NCBI (Accession No. vimentin - XM_008148829.1; cytokeratin - XM_008140727.1). After initial denaturation for 3 min at 94°C, the remaining 35 PCR cycles were at 94°C/30s, 60°C/30s and 72°C/1min. The final extension was at 72°C for 10 min.

Conventional PCR for the detection and identification of *E. fuscus* cytochrome B was performed using primers CytB US - CCCCHCCHCAYATYAARCCMGARTGATA and CytB DS -

TCRACDGGNTGYCCTCCDATTCATGTTA. After initial denaturation for 3 min at 94°C, the remaining 35 PCR cycles were at 94°C/30s, 55°C/30s and 72°C/1min. The final extension was at 72°C for 10 min.

Semi-nested PCR using primers specific to the 16s rRNA gene of mollicutes was performed for the detection of mycoplasma in cell lines. Primers were designed as mentioned previously (193, 194). Briefly, primers My-1-ACGGCCCADACTYCTACGGRAGGCAGCAGTA and My-2-CCRTGCACCAYTTGTCWHHHBGWWAACCTC were used for the first PCR. After initial denaturation for 3 min at 94°C, the remaining 40 PCR cycles were at 94°C/30s, 64°C/30s and 72°C/1min. The final extension was at 72°C for 10 min. Primers My-2 and My-3-GTAATACATAGCTCGCAAGCGTTATC were used for the second PCR. After initial denaturation for 3 min at 94°C, the remaining 35 PCR cycles were at 94°C/30s, 60°C/30s and 72°C/1min. The final extension was at 72°C for 10 min.

For IFN beta quantification, qPCR assays targeting the IFN beta transcripts and the normalizer (GAPDH, Glyceraldehyde-3-phosphate) were performed for the clones. Stratagene's MX3005P PCR (Stratagene, USA) cycler was used in conjunction with Quantifast SYBR Green PCR kit (QIAGEN). Primers used were Interferon beta (BBB_IFNbeta-F-GCTCCGATTCCGACAGAGAAGCA and BBB_IFNbeta-R-ATGCATGACCACCATGGCTTC) and GAPDH (BBB_GAPDH-F-GGAGCGAGATCCCGCCAACAT and BBB_GAPDH-R-GGGAGTTGTCATACTTGTCATGG). Primers were designed using the annotated *E. fuscus* genome (NCBI, Accession No. Interferon beta: XM_008145044.1 and GAPDH:

XM_008144826.1). Samples were prepared as previously mentioned (18). The products were quantified based on the amount of relative IFN beta expression. Briefly, cells were either transfected with 750ng/ml poly (I:C) (InvivoGen, USA) using Lipofectamine 2000 (ThermoFisher Scientific, USA) or mock transfected. For quantifying PED-CoV transcripts, primers were designed to amplify the PED-CoV nucleocapsid (N) gene (GenBank accession number KF272920), (PEDV-s GCAACAACAGGTCCAGATCTC) and (PEDV-r CTCCACGACCCTGGTTATTTC). For qPCR, after the initial denaturation step of 95°C for 10 minutes, the remaining 40 cycles were at 95°C/30s, 55°C/1min and 72°C/1min. The absorbance reading was taken after the 55°C step. Relative fold change in gene expression between the two groups of cells was calculated and plotted after normalizing the Ct values for IFN beta using GAPDH. Three housekeeping genes were tested (GAPDH, beta-actin and beta-2-microglobulin) and none showed variation between treated and mock treated samples. Thus GAPDH was used for normalizing the data. Difference of one Ct indicates a two-fold difference in gene expression. PCR and qRT-PCR products were confirmed on a gel and sequenced (Macrogen, South Korea). Reaction efficiencies for qRT-PCR primers were calculated to be between 95 and 105%.

4.6.7. Cell division

Total number of viable cells was determined by using a hemocytometer to count viable cells by trypan blue exclusion method. Cells were cultivated in 6-well plates, trypsinized and re-suspended in media at every time point.

4.6.8. Virus replication

Efk3 parental cell line and three subclones were inoculated with VSV-Indiana strain (Dr. Ellis' lab at the University of Saskatchewan), HSV, PED-CoV (Dr. Zhou's lab at Vaccine and Infectious Disease Organization - International Vaccine Center (VIDO-InterVac)), and MERS-

CoV (strain EMC/2012, Dr. Fouchier at Erasmus Medical Center in the Netherlands). For VSV inoculations, water soluble tetrazolium salt 50 (WST_{50}) concentration of the virus was used to infect the cells, which were seeded in 12 well plates at a concentration of 2 x 10^5 cells/well. WST_{50} was determined as that dilution of virus that produced 50% cell death as measured using the WST-1 assay. WST-1 assay is similar to the MTT assay (195). Briefly, cells were seeded in 96 well plates at a density of 1 x 10^4/well and analyzed with the WST assay at the indicated time points. 10 µl WST-1 reagent (Roche, USA) was added to each well and incubated at 37^0C for 1 hr. Colour developed was measured at 450 nm with a reference wavelength of 650 nm using Molecular Devices Vmax spectrophotometer. Intensity of the colour developed is directly proportional to the number of viable cells in the wells. Cells cultured in 12-well plates were inoculated with 100µl virus (VSV) for 1hr, rinsed with sterile PBS and medium replaced with DMEM containing 10% FBS. Cells and the supernatant were harvested at 4 and 48h post infection and frozen at -80°C. After freeze-thawing the supernatant and cells three times, virus from Efk3 parental cell line and the subclones was titrated in Vero cells using the WST_{50} assay. Virus was quantified using the WST_{50} assay and formula as described by Heldt *et al.* (195). For HSV titration, MRC5, Efk1B, Efk2F and Efk3B subclones were seeded in triplicates at a concentration of 2.5 x 10^5 cells/well in 6 well plates. The cells were inoculated with HSV at a multiplicity of infection (MOI) of 1. Viral inoculum (0.5 ml) was replaced with complete medium and plates frozen at 0 and 24 h post inoculation. Plates were freeze-thawed 3x, transferred to 15 ml tubes and centrifuged at 2000 rpm for 5 min. Supernatant was collected and serially diluted 1:10 down to 10^{-6} and titrated in Vero cells (1 x 10^5 cells/well in 24-well plates). For quantifying the virus, 100 µl of diluted virus was added to the wells and incubated for an

hour. The inoculum was replaced with 0.5 ml of DMEM+1% pooled human serum (MP Biomedical Cellect). Plaques were counted under the microscope 5 days later.

Multistep replication kinetics were determined by inoculating wells of cells in triplicate with MERS-CoV (strain EMC/2012) with a MOI of 0.01, 50% tissue culture infectious dose ($TCID_{50}$) per cell. One hour after inoculation, cells were washed once with DMEM and culture medium replaced. Culture supernatants were sampled at 0, 24, 48, 72, 96 and 120 h after inoculation. MERS-CoV was titrated by end-point titration performed in quadruplicate using Vero E6 cells cultured in DMEM supplemented with 2% fetal calf serum, 1 mM L-glutamine (Lonza, USA), 50 U/ml penicillin and 50 μg/ml streptomycin. Cells were inoculated with ten-fold serial dilutions of virus, and scored for cytopathic effect 5 days later. The $TCID_{50}$ was calculated by the method of Spearman-Karber (196).

For PED-CoV infection, cells were seeded in 24-well plate at a density of $1.2x10^5$/well and cultured overnight. Cells were inoculated with PED-CoV at an MOI of 1. Cells were harvested and total RNA extracted at 0, 24, 48, 72 and 96 h. Virus replication was quantified by qRT-PCR.

4.6.9. Statistics

Significance between T-ag data was calculated by Mann-Whitney U test for two independent samples using IBM SPSS (Version 21).

4.7. Results

4.7.1 Characterizing the MyPV-Tag (*Myotis polyomavirus* large-T antigen)

The *SV40* large T-antigen is well characterized and is known to enhance DNA replication in cells (197) and immortalize primary cells (140). Our laboratory has previously detected a novel polyomavirus in *M. lucifugus* (17). To determine if the *Myotis polyomavirus* T-ag shared the ability of its *SV40* homologue to induce DNA replication, we transfected Vero cells with plasmids encoding genes for the two T-antigens. We then confirmed that cells expressed T antigen by immunostaining and compared the DNA content of T antigen expressing cells with cells transfected with the pcDNA null plasmid. Figure 4.1 shows cells expressing *Myotis polyomavirus* and *SV40* T antigen contained more DNA than cells transfected with pcDNA plasmid. SV40Tag expressing Vero cells showed the highest increase in DNA content.

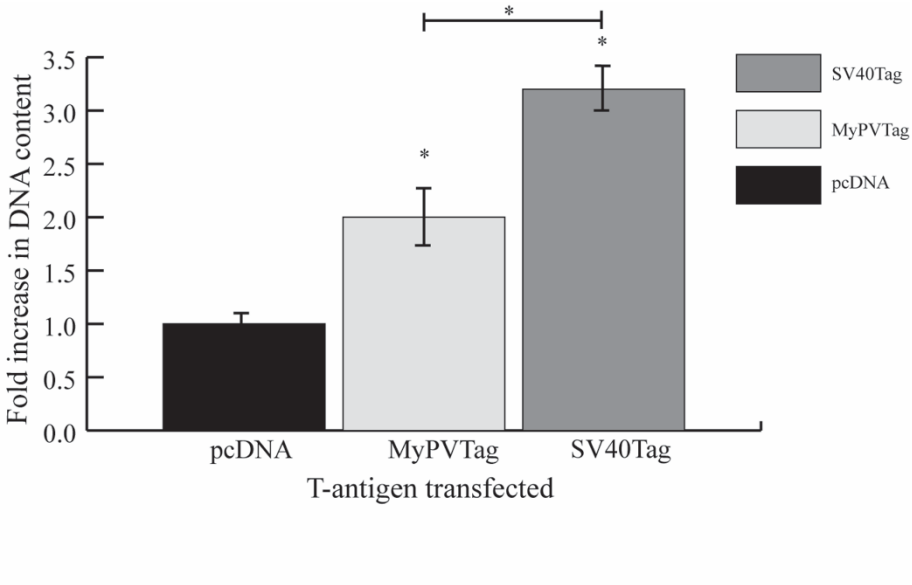

Figure 4. 1. Transfection with SV40 and MyPV T-antigens increases DNA content in cells. Vero cells were transfected with plasmids expressing either SV40Tag, MyPVTag or empty vector (pcDNA). Twenty-four hr after transfection, cells were immune-stained for cytoplasmic T-antigen and with propidium iodide to quantify DNA. The DNA content of T-antigen and pcDNA transfected cells was determined by flow cytometry and expressed as the fold increase in DNA content relative to pcDNA transfected cells. The ratio for cells transfected with pcDNA was taken as '1'. Experiments were done in triplicate and mean values plotted. Error bars represent standard deviation. Statistical difference was calculated using Mann-Whitney *U* test for two independent samples. * <0.05.

4.7.2. Establishing and immortalizing *E. fuscus* kidney cells

We attempted to immortalize primary cells derived from the kidney of *E. fuscus* by transfection with plasmids expressing SV40Tag, MyPVTag or empty vector (pcDNA). We observed clusters of cells in cultures transfected with either T-ag but after several passages, only the MyPVTag transfected primary cells (Efk) continued to replicate. The immortalized Efk cell line was cloned by limiting dilution to generate three clones (Efk1, 2 and 3). 8 clones were further isolated by end point dilution of Efk1, Efk2 and Efk3 cells. We established the clones as separate cell lines and characterized representative clones from each of the three clones i.e. Efk1, Efk2 and Efk3, along with the parental Efk3 cells for their cell type markers, interferon beta response, virus susceptibility and cell division rates. We determined the number of chromosomes in Efk-3B to rule out the possibility of chromosome number abnormality in immortalized cells (Fig. S4.1). Efk-3B had 2n=50 chromosomes, which is normal for genus *Eptesicus* (198).

4.7.3. Lineage of the Efk clones

We screened the clones for cytokeratin, a lineage-specific marker for epithelial cells (167) and vimentin, a lineage marker for fibroblasts (199). Since specific antibodies are not available for bat cytokeratin and vimentin, we used antibodies specific to the human proteins. No positive staining of the bat clones was observed with either anti-vimentin or anti-cytokeratin antibodies when cells were analyzed with flow cytometry (data not shown). We then screened the cell lines for expression of vimentin and cytokeratin transcript by conventional PCR. Five of the eight clones analyzed contained detectable transcripts for both cytokeratin and vimentin. In contrast, only vimentin transcripts were detected in primary Efk cells at passage 8 (Table 4.1).

4.7.4. Interferon beta production

We characterized the Efk clones for their capacity to respond to polyinosinic-polycytidylic acid (poly(I:C)), a synthetic analogue of double-stranded RNA (200), through analysis of interferon beta transcription. Poly(I:C)-treated primary Efk cells displayed an average of 60,000-fold increase in interferon beta transcripts when compared to mock transfected cells. All clones displayed increased interferon beta transcription following poly (I:C) treatment, with the level of increase ranging from six thousand to over sixty thousand. (Table 4.1).

Clone	Marker Type		IFN beta fold increase
	Cytokeratin	Vimentin	
1H	-	+	6,517.03
2A	-	+	33,225.42
2B	+	+	18,432.95
1A	+	+	39,786.73
1B	-	+	61,572.56
1E	+	+	6,746.85
2F	+	+	42,938.97
3B	+	+	7,912.95
Efk3 (uncloned)	-	+	10,155.68
Efk (primary cells)	-	+	60,100

Table 4. 1. Transcripts for cell lineage markers vimentin (fibroblast) and cytokeratin (epithelial) are expressed by the Efk clones. The ability of the cells to respond to poly (I:C) treatment with increased IFN beta gene expression was detected by qRT-PCR. Along with the clones, a parental cell line (Efk3) and primary kidney cells (Efk; not transfected with either T-ag) were compared. + = PCR product detected, - = no PCR product detected.

4.7.5. Cell multiplication rates

All clones displayed similar multiplication rates when assayed with the WST-1 reagent. We determined the cell division rates of three sub-clones (1B, 2F and 3B) and the parental Efk3 cell line by counting viable cells using a hemocytometer at various time points after seeding (Fig. 4.2). The three clones and parental cell line (Efk3) did not differ from one another in their multiplication rates.

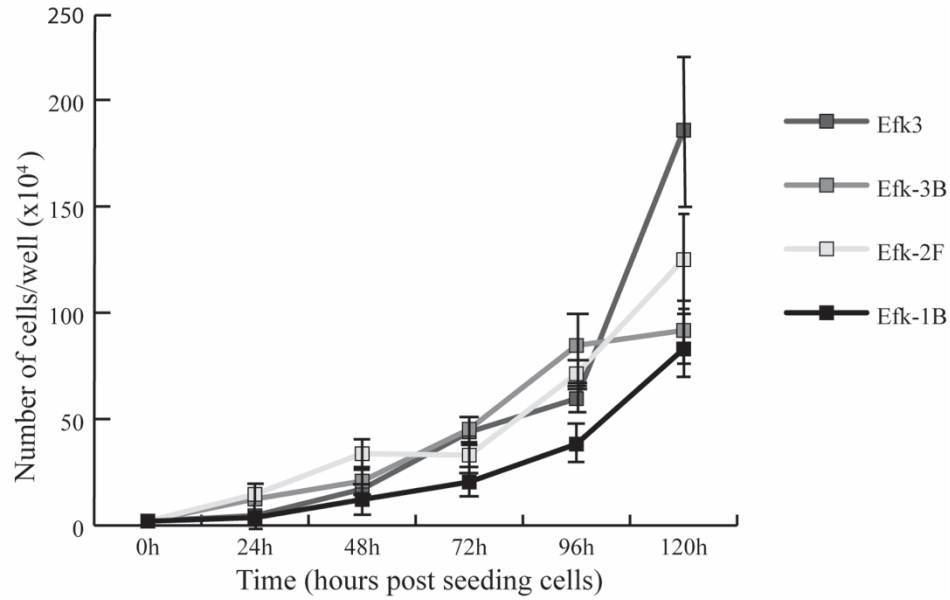

Figure 4. 2. Efk3 and Efk clones multiplication curve. The cell division curve for the three clones and Efk3 parental cell line was determined by counting viable cells at the indicated time-points.

4.7.6. Efk cells competence for virus infection

We evaluated the Efk3 parental cell line and three clones (1B, 2F and 3B) for their competence in supporting VSV, HSV, PED-CoV and MERS-CoV replication (Figs 4.3 and 4.4). As a positive control, Vero cells were infected with MERS-CoV and PED-CoV and MRC5 cells were infected with HSV and VSV. VSV caused rounding, sloughing and detachment of MRC5 cells in culture, PED-CoV caused syncytia in Vero cells and MERS-CoV caused rounding and sloughing off of Vero cells. Cytopathic effects (CPE) such as plaques or rounding and sloughing off of

cells following infection with VSV and MERS-CoV was observed with all Efk clones. Two of the three clones responded to PED-CoV infection with cytopathology. In contrast, Efk-2F did not exhibit noticeable CPE 24 h.p.i. with PED-CoV (Fig. 4.3). The positive control cell lines exhibited CPE 24 h.p.i. following infection with the respective viruses.

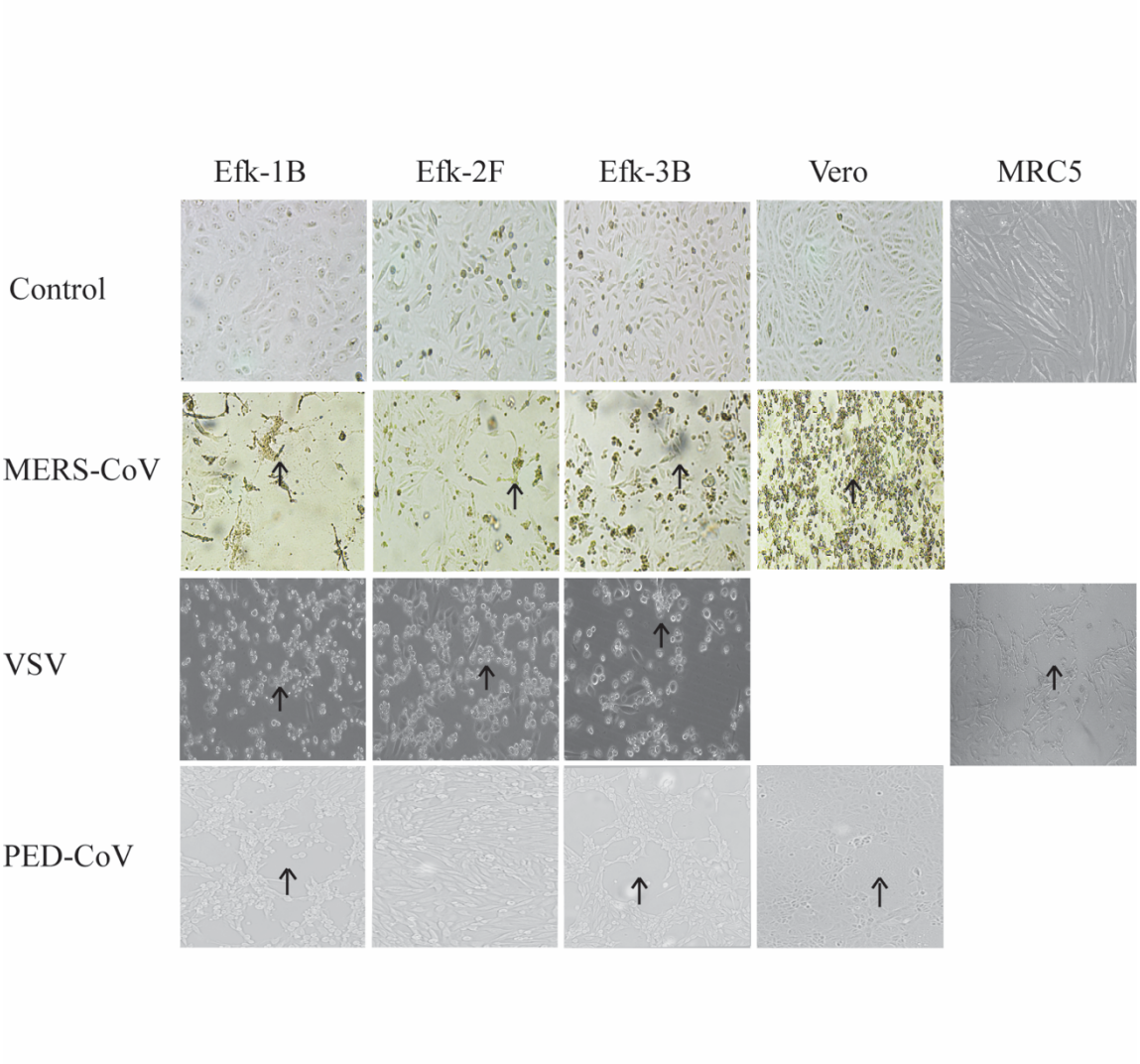

Figure 4. 3. CPE observed in Efk cells. CPE observed following infection with MERS-CoV, VSV and PED-CoV is indicated by arrows.

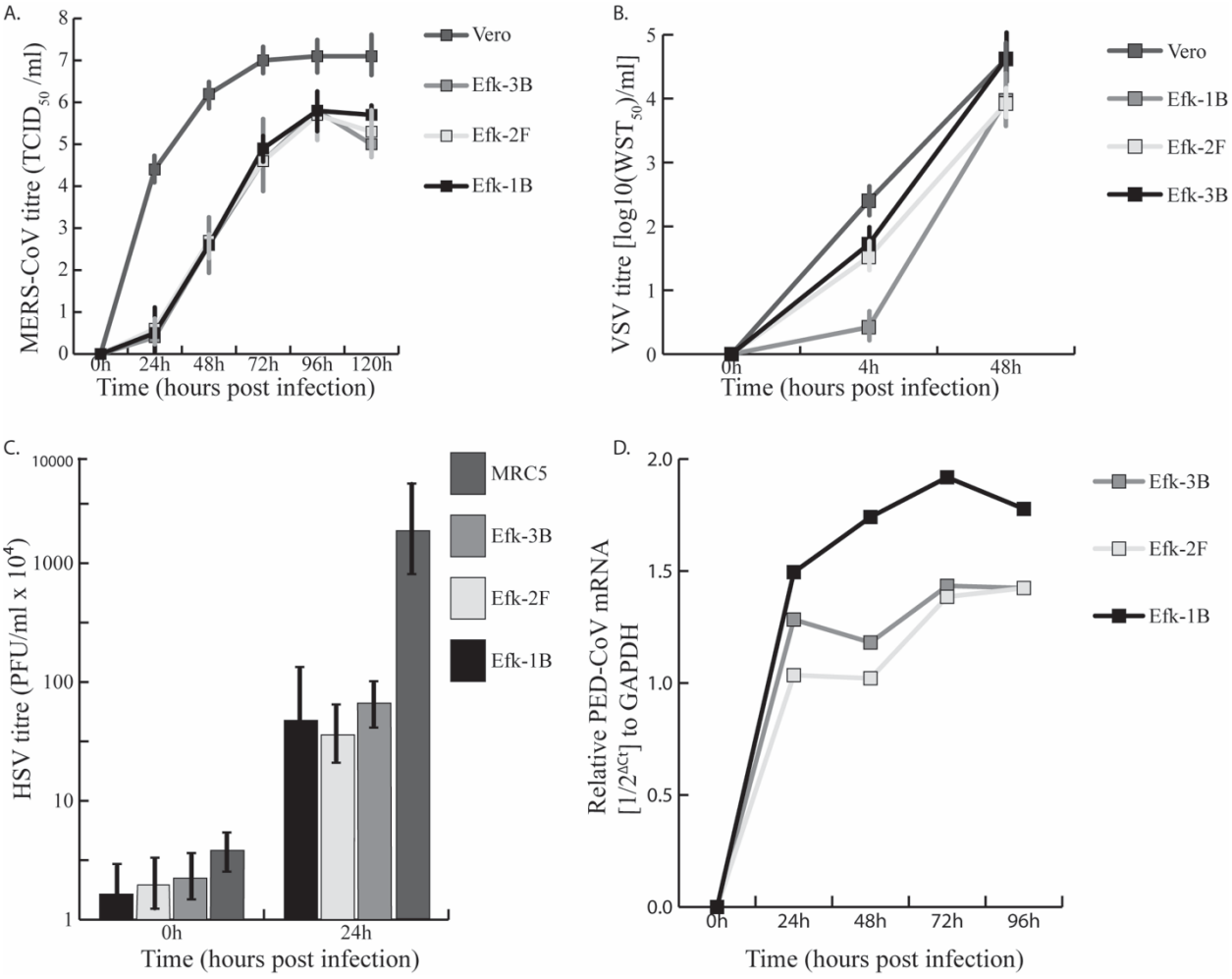

Figure 4. 4. Efk clones support replication of viruses. The amount of virus (or viral nucleic acid) produced by Efk cell clones was measured by determining $\log_{10}$ $TCID_{50}$ (MERS-CoV figure 4.A), WST_{50} (VSV, figure 4.B.), plaque assay (HSV, figure 4.C) and qRT-PCR (PED-CoV, figure 4.D). The data confirm that the viruses replicated in the Efk cell lines as well as the relevant positive control cell lines (Vero, MRC5).

4.8. Discussion

Most viruses that have been transmitted from bats to other species have been studied in animal models of human disease or in cell lines of non-bat origin. In addition, most *in vitro* studies of mammalian innate immune and anti-viral responses have been performed in human and rodent cell lines. The results from these studies may not accurately represent pathogen-host interactions

that occur in bats. Establishing bat cell lines enable researchers to study relevant virus-host interactions in a system that more closely resembles the reservoir host. Cell lines have been established from fruit bats (57, 140), *Myotis myotis* (136), *Tadarida brasiliensis* and other insectivorous bats (184). Primary embryonic cells have been developed from *E. fuscus* (190) but an immortalized *E. fuscus* cell line capable of supporting the replication of viruses from three diverse viral families is not commercially available yet.

Historically, cells have been immortalized by either using the large T-antigen from *SV40*, which is a monkey virus or by the ectopic expression of hTERT. We characterized the MyPVTag and SV40Tag for their ability to enhance DNA replication in cells. Both MyPVTag and the SV40Tag significantly increased DNA content in Vero cells. *SV40* T-ag is known to enhance DNA content in cells (201, 202) and interestingly the bat polyomavirus T-antigen shared similar properties. *Myotis polyomavirus* belongs to the same family as *SV40*. Large T-ags from both these viruses were transfected to immortalize the *E. fuscus* kidney cells, but only cells expressing MyPVTag gave rise to stable cell lines. The reason behind the inability of SV40Tag to immortalize bat cells is not known. However, it might be possible that the expression of MyPVTag, derived from a virus found in this bat, could have been favored by the cellular machinery over the SV40Tag derived from *SV40*, which has not been detected in big brown bats yet.

Clones 2B, 1A, 1E, 2F, 3B expressed transcripts for lineage-specific proteins of both epithelial and fibroblast cells and Clones 1H, 2A and 1B expressed mRNA for vimentin. Since primary bats cells at passage 8 had detectable transcript for vimentin alone, it is possible that the immortalization procedure may have altered transcription in some of the clones. There is

evidence, however, that tumor cells can co-express both epithelial and fibroblast markers (203). It is also possible that MyPVTag immortalized a mixture of both epithelial and fibroblast cells and the epithelial cells had a replication advantage during the process of limiting dilution cloning. When cells were being passaged, some cells were more strongly adhered to the plastic and were not removed by trypsinization. This process could have selected for particular cell types based on their adherent properties.

Relatively little is known about the innate immune response of insectivorous bats to viral infection. We were able to generate an insectivorous bat cell line capable of upregulating IFN beta gene expression in response to a known innate immune stimulus. An early interferon response is known to inhibit replication of some viruses (204). Interferon response in terms of interferon beta transcript upregulation by bat cell lines, mostly cell lines from fruit bats, has been demonstrated before (57, 134, 140, 205). We analyzed the capacity of the Efk clones to respond to poly (I:C) stimulation by monitoring interferon beta gene expression. This synthetic analogue of dsRNA is usually used as a pathogen-associated molecular pattern (PAMP) to stimulate interferon responses (140). The clones responded to poly(I:C), as we observed a several thousand-fold increase in interferon beta transcript when compared to mock treated cells. Immortalization of cells is sometimes known to compromise the ability of the cells to transcribe the interferon beta gene (134). The Efk clones, however, retained a remarkable but variable capacity to respond to poly(I:C).

West Nile virus, Eptesipox virus, novel group I coronavirus and American bat vesiculovirus are examples of viruses that have been detected in *Eptesicus fuscus* (1, 206). The ability of this cell

line to support the replication of viruses from three different viral families demonstrates the potential application of this cell line to isolate and study viruses in bats that have previously only been detected using PCR and sequencing. Further work can be done to identify receptors specific to these viruses. VSV, HSV, PED-CoV and MERS-CoV grew to varying levels in the Efk clones. Clone 2F did not exhibit any visual cytopathology on infection with PED-CoV although virus replication was detected by qRT-PCR. This could be due to a lower level of virus replication in 2F (Fig. 4.4.D.) as the images (Fig. 4.3) were taken 48 hrs post-infection. At this point we do not know if 2F mounts a more robust interferon response or if it lacks other factors required for PED-CoV replication during the initial 48hrs post-infection.

In conclusion, we established a stable kidney cell line from a northern latitude bat, which has the capacity to respond to a known innate immune stimulus with transcription of IFN beta. Furthermore, this cell line supported the replication of viruses from three virus families known to be harbored by bats. Not much is known about innate immune responses in bats and how they are activated during viral infections. Establishing well-characterized cell lines from relevant bat reservoir species is the first step in addressing the many questions that researchers have about innate immunity in bats (1, 12). This cell line will help us better understand the bat innate responses and how they may contribute to the absence of overt disease symptoms when bats are infected with these viruses.

4.9. Supplementary information

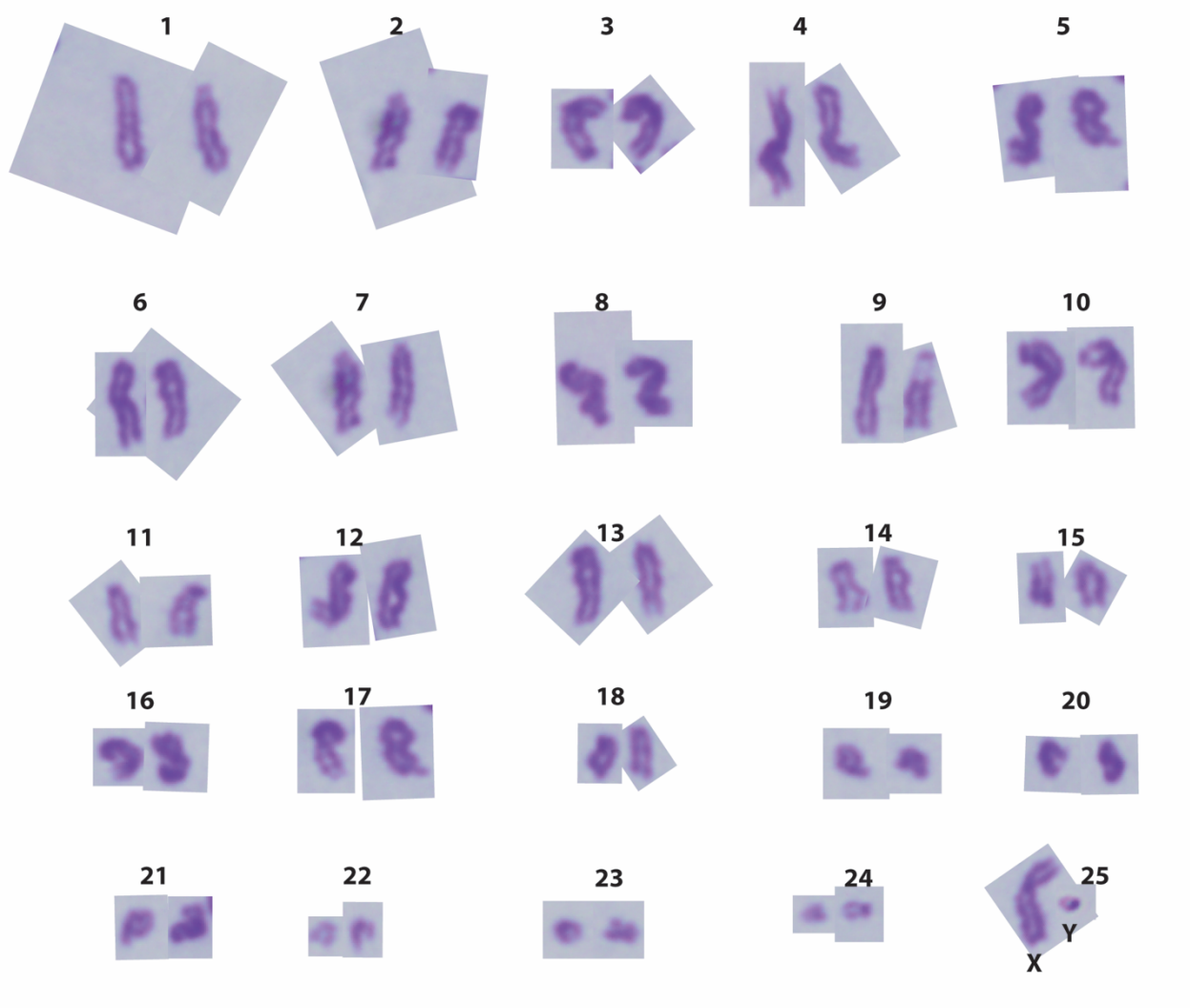

Figure S4. 1. Eptesicus fuscus cell line (Efk-3B) was confirmed to have 2n = 50 chromosomes

4.10. Transition statement

Insectivorous bats are speculated to be reservoirs of several viruses, including Middle East respiratory syndrome coronavirus (MERS-CoV). Generating the first commercially available cell line from a North American insectivorous bat species provided us with an *in vitro* model to stimulate the innate antiviral responses in these cells. Since experimentally and naturally infected bats do not demonstrate classical signs of disease, we next compared the innate immune

responses to known viral surrogates in bat and human cells. Our goal for the next study was to identify unique adaptations in bat cells that may help us explain how bats remain relatively asymptomatic when infected with emerging viruses that cause serious disease in other mammals. Since we have previously detected coronavirus and polyomavirus in Canadian brown bats, the use of this big brown bat cell line was critical in exploring the global innate antiviral immune responses.

Chapter 5: Lack of inflammatory gene expression in bats: a unique role for a transcription repressor

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5.2. Citation

Banerjee, A., Rapin, N., Bollinger, T., and Misra, V. (2017). Lack of inflammatory gene expression in bats: a unique role for a transcription repressor. Sci Rep *7*, 2232.

5.3. Contribution

My supervisor and I conceived the ideas for this chapter and we designed the experiments. I performed all the experiments for this study. Dr. Bollinger provided me with big brown bat long bones and Ms. Rapin performed the c-Rel western blot.

5.4. Abstract

In recent years, viruses similar to those that appear to cause no overt disease in bats have spilled-over to humans and other species causing serious disease. Since pathology in such diseases is often attributed to an over-active inflammatory response, we tested the hypothesis that bat cells

respond to stimulation of their receptors for viral ligands with a strong antiviral response, but unlike in human cells, the inflammatory response is not overtly activated. We compared the response of human and bat cells to poly(I:C), a viral double-stranded RNA surrogate. We measured transcripts for several inflammatory, interferon and interferon stimulated genes using quantitative real-time PCR and observed that human and bat cells both, when stimulated with poly(I:C), contained higher levels of transcripts for interferon beta than unstimulated cells. In contrast, only human cells expressed robust amount of RNA for TNFα, a cell signaling protein involved in systemic inflammation. We examined the bat TNFα promoter and found a potential repressor (c-Rel) binding motif. We demonstrated that c-Rel binds to the putative c-Rel motif in the promoter and knocking down c-Rel transcripts significantly increased basal levels of TNFα transcripts. Our results suggest bats may have a unique mechanism to suppress inflammatory pathology.

5.5. Introduction

Bats are thought to be natural reservoirs for several emerging and re-emerging viruses such as those that closely resemble severe acute respiratory syndrome (SARS), Middle East respiratory syndrome (MERS) and porcine epidemic diarrhoea (PED) – causing coronaviruses (CoV), Marburg and, possibly, Ebola filoviruses, and Hendra and Nipah paramyxoviruses, amongst others (1, 43, 47, 159, 207). These viruses are speculated to have spilled over from bats to humans and other animals, directly or through intermediate hosts, causing severe and often fatal disease. Despite evidence of bats harbouring these viruses, or viruses closely related to them, bats do not appear to show overt symptoms or clinical signs of infection (15). Infecting Pteropid, Jamaican and Egyptian fruit bats with Nipah and Hendra viruses, MERS-CoV and Ebolavirus

yielded no evidence of disease. The bats sero-converted and in some cases virus could be detected post infection (9-11, 208), but these bats did not demonstrate signs of illness. We do not completely understand why bats are less susceptible to these viral infections than other mammals that often succumb.

The immune system, based on our knowledge from humans and other mammals, can be broadly categorised into two branches – the innate immune system and the adaptive immune system (209). Both branches are distinct, although there is interaction between them. During viral infection, the innate response is the first line of defence and primes the adaptive immune response against the virus (75, 210). A virus infected cell detects several pathogen associated molecular patterns (PAMPs) associated with the virus through pattern recognition receptors (PRRs) present in endosomal compartments, cytoplasm and cell membrane [reviewed by Mogensen (65)]. Some of these PRRs, such as toll-like receptors (TLRs) 3, 7, 8, 9, Retinoic acid-inducible gene I (RIG-I) and Melanoma Differentiation-Associated protein 5 (MDA5), have specifically evolved to recognise microbial nucleic acids [reviewed by Lee and Kim (211)]. Polyinosinic:polycytidylic acid [poly(I:C)] is a known double-stranded RNA analogue which is detected by TLR3, RIG-I and MDA5. After detection, PRRs signal through mediators to activate two pathways - the antiviral cytokine (interferons) and inflammatory pathways (67).

Nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) and interferon regulatory factor 3 (IRF3) are two signal mediators that activate antiviral and inflammatory pathways in response to double-stranded RNA sensed by TLR3, RIG-I and MDA5 [reviewed by Mogensen (65)]. Five members of the NFκB family of proteins have been identified in humans, namely,

RelA (p65), RelB, c-Rel, NFκB-1 (p50) and NFκB-2 (p52). All five members form homo- or hetero-dimers and share some structural features. These dimers are bound by molecules of the inhibitor of NFκB (IκB) family and retained in the cytoplasm of the cell in an inactivated state. After PAMP recognition, downstream signals mark the inhibitors for degradation and the dimers translocate to the nucleus of the cell to cause expression of antiviral and inflammatory genes (66) (Fig. 5.1). Different combinations of the proteins have vastly different effects on gene expression (212). For instance, hetero-dimers of p50 or p52 and p65 or RelB activate transcription. In contrast, c-Rel as a homo-dimer or in association with p50 or p65, represses transcriptional activation by NFκB (213).

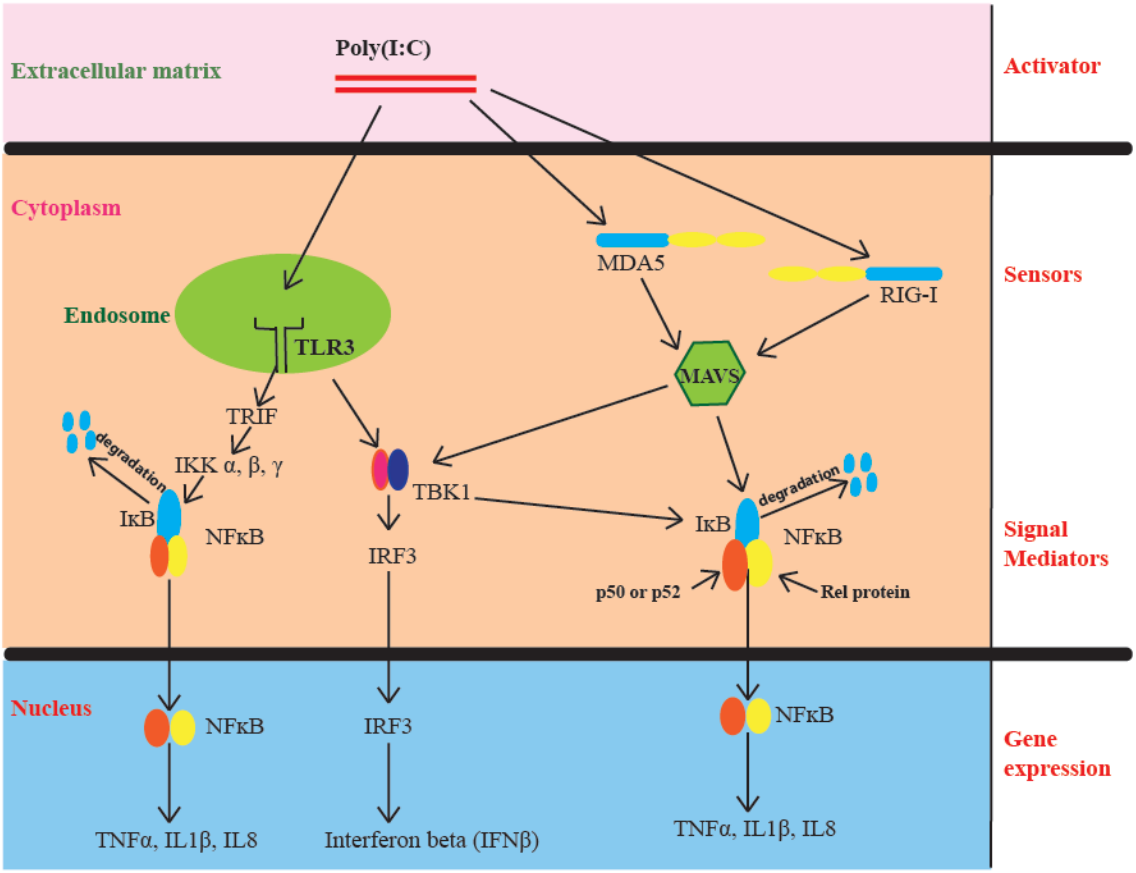


Figure 5. 1. Schematic representation of detection of double-stranded RNA in a human cell and activation of the innate immune response. RNA viruses during replication produce double-stranded RNA intermediates (PAMPs), which are detected by cellular receptors (PRRs).

Poly (I:C) is a known double-stranded RNA analogue (activator) which is detected by sensors such as TLR3 (black), RIG-I and MDA5 (blue, CARD domains in yellow) in a cell. These sensors, when stimulated by the activator, lead to the expression of interferons (IFNβ) and inflammatory genes (TNFα, IL1β, IL8) through adaptor proteins (MAVS and TRIF) and signal mediators such as NFκB (orange and yellow subunits) and IRF3. NFκB is retained in an inactive state in the cytoplasm by inhibitory molecules such as IκB (blue). Upon receiving an activation signal via a sensor, kinases (TBK1) phosphorylate IRF3, which then translocates to the nucleus to activate transcription. Kinases, such as IKK α, β or γ phosphorylate IκB inhibitors and mark them for degradation, thereby activating NFκB. Active NFκB then causes expression of downstream genes by translocating to the nucleus.

Chiroptera is a very diverse order and information about one genus or species may not apply to all bats. However, *Pteropus alecto* (black flying fox) is being extensively studied to better understand the bat immune system. Three and a half percent of *P. alecto* transcribed genes, amounting to about 500 genes, correspond to immune genes (90). *P. alecto* homologs to human TLR 1-10 have been sequenced and TLR 13 has been described. RIG-I, major histocompatibility complex I (MHC-I) and interferon regulatory factor 7 (IRF7) have been detected and characterized (91-93). The interferon pathway, immunoglobulins and the presence of microRNAs have been substantiated in this bat. Constitutive expression of interferon alpha and the ability of cells derived from *P. alecto* to mount an interferon beta (IFNβ) response to viral challenges has been demonstrated (94-100).

A robust antiviral and a controlled inflammatory response is desirable to control a viral infection. During SARS-CoV and MERS-CoV infection in humans and PED-CoV infection in pigs, the viruses inhibit an early interferon response and cause massive secretion of pro-inflammatory chemokines and cytokines, leading to excessive recruitment of immune cells (39, 214, 215). This is detrimental as an excessive inflammatory response causes tissue damage and organ dysfunction in the host (216).

In this study, we hypothesized that *Eptesicus fuscus* (big brown bat) cells would mount a strong antiviral cytokine response but a low inflammatory response to poly(I:C), synthetic single-stranded RNA (ssRNA, a viral single-stranded RNA surrogate), and CpG oligo deoxynucleotides (ODN, a viral and bacterial DNA surrogate). These are known stimulants for human TLRs 3, 7/8 and 9 respectively. We compared the response of immortalized *E. fuscus* kidney cells (Efk3) (110) as well as *E. fuscus* bone marrow derived myeloid cells stimulated with poly (I:C) with that of human fibroblast cells (MRC5). We quantified the expression of innate response genes including IFNβ, tumor necrosis factor alpha (TNFα), interleukin 8 (IL8) and others using quantitative real-time polymerase chain reaction (qRT-PCR). We observed that both bat and human cells mounted a strong IFNβ response but only human cells expressed high levels of transcripts for proinflammatory cytokines such as TNFα and IL8 after TLR agonist treatments. To further explore the low TNFα response in bat cells, we analyzed the *E. fuscus* TNFα promoter for transcription factor binding motifs and identified a potential binding site for c-Rel proto-oncoprotein, a known suppressor of gene expression (217). Ectopically expressed c-Rel bound to DNA containing this motif and the protein localized to the nucleus of bat cells in response to poly(I:C). Deletion of this motif in the promoter enhanced activation by poly (I:C) and partial knockdown of bat c-Rel RNA by specific small interfering RNA (siRNA) increased basal levels of TNFα transcripts in bat cells. We could detect c-Rel transcripts in every major big brown bat tissue, such as spleen, gut, ileum, kidney, lung, liver and the bat kidney cell line, unlike in humans, where it is found predominantly in hematopoetic cells (218). Finally, we could also demonstrate that bat c-Rel bound to the potential motif as promoters containing the motif were co-immunoprecipitated to higher levels than promoters that lacked this motif. Our results

suggest that bats might have evolved a unique mechanism to suppress an exaggerated inflammatory response to viruses.

5.6. Materials and Methods

5.6.1. Ethics statement

Long bones (femur and humerus) and organs were obtained from big brown bats submitted to Canadian Wildlife Health Cooperative (CWHC). The bats were euthanized by a protocol approved by the Committee on Animal Care and Supply of the University of Saskatchewan Animal Research Ethics Board (protocol #20090036) and were in accordance with regulations approved by the Canadian Council on Animal Care.

5.6.2. Cell culture

Eptesicus fuscus kidney cells (Efk3) were grown in Dulbecco's Minimal Essential Medium with GlutaGro (DMEM; Corning) containing 10% fetal bovine serum (FBS; Seradigm), Penicillin/Streptomycin (Gibco) and 1% GlutaMax (Gibco). MRC5 cells (ATCC CCL-171) were cultured in Minimum Essential Medium Eagle (MEM; Corning) supplemented with 10% FBS, 1/100 non-essential amino acids (NEAA; Gibco), 1/100 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; Gibco) and 1/1000 gentamycin (Gibco). HEK293T cells (Dr. Robert Brownlie, VIDO-Intervac) were cultured in DMEM with GlutaGro containing 10% FBS and Penicillin/Streptomycin. For bone marrow derived myeloid cells, bone marrow from big brown bat long bones was processed as described for mice (219). The cells were seeded in Roswell Park Memorial Institute 1640 (RPMI; Sigma-Aldrich) medium containing 10% FBS, Penicillin/Streptomycin and 20ng/ml human granulocyte-macrophage colony-stimulating factor (hGM-CSF; PeproTech).

5.6.3. TLR challenge

MRC5, Efk3 and big brown bat bone marrow derived myeloid cells were seeded at a concentration of 3 x 10^5 cells/well in 6 well plates and transfected with TLR ligands. Briefly, cell lines were transfected with 750 ng/ml long poly (I:C) (InvivoGen) or 4 μg/ml single-stranded RNA 40 (ssRNA40; InvivoGen) or 3 μM CpG ODN (InvivoGen) using Lipofectamine 2000 (Invitrogen). Cells were harvested 16 h post-transfection and RNA was extracted. For time-point experiments, cell lines were treated with the above-mentioned concentrations of TLR ligands and RNA was extracted at indicated time points.

5.6.4. Nucleic acid extraction, PCR and qRT-PCR

All RNA extractions were performed using the RNeasy Plus Mini kit (QIAGEN, Germany) as per manufacturer's instructions. cDNA was prepared using the QuantiTect Reverse Transcription kit (QIAGEN) as per manufacturer's instructions. One μg of RNA was used for cDNA preparation. cDNA was used as a template for the quantification of target genes. DNA extraction from MRC5 and Efk3 cells was performed using the DNeasy Blood and Tissue kit (QIAGEN) as per manufacturer's instructions.

Conventional PCR was performed to amplify human or big brown bat TNFα promoters, big brown bat c-Rel coding sequence (CDS) and cDNA from c-Rel transcripts in big brown bat organs using specific primers. Primers with restriction sites were used to clone the TNFα promoters and c-Rel CDS (Table 5.1). Primers without restriction sites were designed to detect c-Rel transcripts in big brown bat organs (supplementary table S5.4). Human TNFα promoter sequence was obtained from NCBI (Accession number: AB048818) and amplified by PCR from DNA extracted from MRC5 cells. The big brown bat TNFα promoter was defined as a sequence

up to 1200bp upstream of the TNFα gene (sequence submitted; GenBank accession: BK009991) and amplified by PCR from DNA extracted from Efk3 cells. Big brown bat c-Rel sequence was obtained from NCBI (Accession number: XM_008162099.1). PCR was performed using the following thermal cycle profile: initial denaturation for 3 min at 94°C, 35 PCR cycles at 94°C/30s, 55°C/30s and 72°C/1 min. The final extension was at 72°C for 10 min.

For the quantification of innate immune response genes, qRT-PCR assays targeting respective gene transcripts (supplementary table S5.4) and the normalizer (Glyceraldehyde-3-phosphate dehydrogenase; GAPDH) were performed for both MRC5 and Efk3 cells. Agilent's MX3005P PCR cycler was used in conjunction with Quantifast SYBR Green PCR kit (QIAGEN) and samples were prepared as previously mentioned (18). Primers for Efk3 cells were designed using the annotated big brown bat genome (Accession No. PRJNA72449). Primer sequences for MRC5 cells were obtained from PrimerBank (220, 221) or nucleotide database on National Centre for Biotechnology Information (NCBI). When primer sequences were not available for MRC5 or genes not annotated for big brown bat, multiple sequence alignment was performed with other mammalian homologues and primers were designed against conserved regions. One of the cytokines, IL8, is not annotated in the big brown bat genome. Primers for IL8 were designed using the annotated *Myotis lucifugus* genome. The products were quantified based on the amount of relative gene expression. All amplified products were confirmed on a gel and sequenced (Macrogen). Reaction efficiencies for qRT-PCR primers were between 95 and 105%.

For qRT-PCR, after the initial denaturation step of 95°C for 5 minutes, two step cycling for 40 cycles was performed at 95°C/10s, (51-56)°C/30s. Absorbance readings were acquired after each

cycle. The final three steps were carried out at 95°C/1 min, 55°C/30s and 95°C/30s to generate the dissociation curve. Absorbance readings for the dissociation curve were acquired at every degree from 55-95°C. The annealing temperatures were optimized for different groups of genes (supplementary table S5.4). Relative fold change in gene expression between the two groups of cells (treated and mock treated) was calculated after normalizing the Ct values using GAPDH. Three housekeeping genes were tested (GAPDH, β-actin and β-2-microglobulin) for MRC5 and two for Efk3 cells (GAPDH and β-actin). There was no variation in Ct values for the housekeeping genes between treated and mock treated samples. Thus GAPDH was chosen for normalizing the data. Difference of one Ct indicates a two-fold difference in gene expression. qRT-PCR for quantifying immunoprecipitated DNA after the ChIP assay was performed using primers (Table 5.1) designed to amplify a region spanning the putative c-Rel binding site of approximately 410 bp for the bat promoter and 480 bp for the human promoter (± the putative c-Rel binding motif). The reaction conditions were as described above.

Sequence	Primers	Features and legend
Human TNF alpha promoter	GCCGGTACCGCTGTCTGCTTGTGTGTGTG and GCCCTCGAGGGGGACACACAAGCATCAAG	KpnI and XhoI sites
E. fuscus TNF alpha promoter	GCCACGCGTAAGAATGTCTCGGGCTGTT and GCCCTCGAGGCTGTGTCTCCCAGAGGCC	MluI and XhoI sites
E. fuscus cRel CDS cloned in pCMV-HA-N	GCCGTCGACCATGCGTTTTCGATACAAATG and GCCGCGGCCGCTTACAAGTTAACCGGAAAAA	SalI and NotI sites
CD.Ri.17417.13.1 (cRel_siRNA) - sequence	rArArA rGrGrA rArGrC rUrArU rUrArU rUrUrC rArArG rArATA	r = ribose sugar
CD.Ri.17417.13.1 (cRel_siRNA) - sequence2	rUrArU rUrCrU rUrGrA rArArU rArArU rArGrC rUrUrC rCrUrU rUrArC	r = ribose sugar
CD.Ri.17417.13.2 (cRel_siRNA) - sequence	rGrGrA rArGrA rUrUrC rArUrU rArArA rArArA rGrArA rUrCA A	r = ribose sugar
CD.Ri.17417.13.2 (cRel_siRNA) - sequence2	rUrUrG rArUrU rCrUrU rUrUrU rUrArA rUrGrA rArUrC rUrUrC rCrUrU	r = ribose sugar
E. fuscus TNF alpha promoter - ChIP primers	GGCAGATGTGGCCACAGGCAGAG and CAGAGAGCTGAGTCCTTGACG	-
Human TNF alpha promoter - ChIP primers	GGGGAGAACAAAAGGATAAGG and CTCTCACTTCTCAGGGCCCCAG	-

Table 5. 1. siRNA, cloning and ChiP qRT-PCR primer sequences. For siRNA sequences, ribonucleotides are preceded by the letter 'r'. Cloning primer sequences contain restriction sites as part of the sequence.

5.6.5. Agarose Gel Electrophoresis

One percent agarose (Invitrogen, USA) gels were prepared using 0.5X TBE [Tris – 1M (VWR), Ethylenediaminetetraacetic acid disodium salt (EDTA) solution – 0.02M (Gibco) and Boric acid – 1 M; pH 8.4]. One µl SYBR Safe DNA gel stain (Invitrogen) was added for every 1 ml of gel. Ten µl of PCR or quantitative real-time PCR (qRTPCR) products were run on the gel for 1 h at 105 volts and visualized under an ultraviolet gel imaging system (AlphaImager HP).

5.6.6. Cloning TNFα promoter and c-Rel

Human TNFα promoter sequence was amplified by PCR from DNA extracted from MRC5 cells and cloned in pCAT3 vector (Promega) upstream of the chloramphenicol acetyltransferase (CAT) gene using restriction sites KpnI and XhoI. The big brown bat TNFα promoter was

amplified by PCR using DNA extracted from Efk3 cells and cloned upstream of the CAT gene in a pCAT3 vector using restriction sites MluI and XhoI. Big brown bat c-Rel coding sequence (CDS) was amplified from cDNA prepared from RNA extracted from Efk3 cells and cloned in-phase downstream of a Hemagglutinin (HA) tag in pCMV-HA-N vector (Clontech) using restriction sites SalI and NotI.

5.6.7. Generating TNFα promoter mutants

Mutant big brown bat and human TNFα promoters were generated by removing or adding the c-Rel binding site. Agilent's QuikChange II Site-Directed Mutagenesis kit was used as per a modification of the manufacturer's protocol suggested by Wang and Malcolm (222). For the bat mutant promoter, two primers (IDT) were designed to loop out the c-Rel binding site, BBB M1 – F - GCTTCATACAAAAACTGCCTTTGGATCCAAG and BBB M1 – R – CTTGGATCCAAAGGCAGTTTTTGTATGAAGC. The primers were used to amplify wild-type big brown bat TNFα promoter. For the human mutant promoter, primers were designed containing the putative bat c-Rel binding motif: Hu-M1-F-GAATGGGTTACAGGAGGGGCTTCGGATCCTCTGGGGAGATG and Hu-M1-R-CATCTCCCCAGAGGATCCGAAGCCCCTCCTGTAACCCATTC. The primers were used to amplify wild-type human TNFα promoter. Deletion and addition of the c-Rel binding site were confirmed by sequencing (Macrogen).

5.6.8. Chloramphenicol acetyl transferase (CAT) and β-galactosidase (β-gal) assay

MRC5 and Efk3 cells were seeded at a concentration of 3 x 10^5 cells/well in 6 well plates. At 60-80% confluency, 500 ng of human or big brown bat TNFα promoter (wildtype or mutant), 500 ng β-galactosidase (β-gal) expressing plasmid and 1 μg of pcDNA empty plasmid to make up a total of 2 μg DNA/well was transfected using Lipofectamine 2000. After 24 h, the medium was

replaced with fresh complete medium (DMEM). After 4 h, cell lines were transfected with 750 ng/ml poly (I:C) and incubated for 16 h. CAT and β-gal assays were performed as previously mentioned (223).

5.6.9. Partial knock-down of c-Rel, TLR3, RIG-I and MDA5 transcripts in Efk3 cells

Dicer-ready siRNA (DsiRNA) specific to big brown bat c-Rel, TLR3, RIG-I and MDA5 were designed and obtained through Integrated DNA Technologies (IDT). A 100 nM final concentration of a 1:1 mixture of two DsiRNAs (Table 5.1 and supplementary table S5.5) targeting separate regions on the respective transcript was transfected into Efk3 cells using Lipofectamine 2000. Scrambled non-specific DsiRNA (NC DsiRNA; IDT) was used as a negative control. Cy3 labelled DsiRNA (IDT) was used to confirm 100% transfection efficiency.

5.6.10. Immunofluorescence

Efk3 cells were seeded at a concentration of 3 x 10^5 cells/well in 6 well plates with glass cover-slips and transfected with 5 μg/well pCMV-HA-N plasmid expressing big brown bats c-Rel using Lipofectamine 2000. Cells were treated with 750 ng/ml poly (I:C) after 24 h and incubated for another 16 h. Media was discarded and cells were rinsed with 2ml PBS. Cover-slips were transferred to wells containing ice-cold methanol in 6-well plates and incubated for 20 mins in a freezer. Methanol was discarded and cells were washed with PBS. Cells were blocked using a blocking solution [PBS, 10% newborn calf serum (Invitrogen) and 0.1% Tween 20 (USB)]. Primary staining for c-Rel was performed using 1:2000 dilution (as used by Smith *et al.*(224)) of mouse anti-HA (Sigma). Secondary staining was performed using 4 μg/ml goat anti-mouse Alexa 488 (Molecular Probes) and 0.2 μg/ml Hoechst 33342 (Molecular Probes) in blocking solution. Cells were observed under a fluorescent microscope and images were acquired using

DP Controller (OLYMPUS, Version 3.2.1.276). Mean fluorescence was measured using Image J (Version 1.49) and calculated using a formula previously described (225).

5.6.11. Differential staining of bone marrow derived cells

Cells obtained from big brown bats bone marrow were concentrated onto a slide using Cytospin 4 (ThermoFisher). The slides were fixed in Hema 3 fixative solution (Fisher Scientific, USA) for 10 seconds, followed by 5 dips for 1 second each in Hema 3 solution I (Fisher Scientific, USA) then Hema 3 solution II (Fisher Scientific, USA). The slides were rinsed with deionized water, air dried and observed under a light microscope.

5.6.12. Chromatin immunoprecipitation assay (ChIP)

HEK293T cells were seeded in 6-well plates at a concentration of 3 x 10^5 cells/well. Cells at 60-70% confluency were co-transfected with the TNFα promoters and big brown bat c-Rel using Lipofectamine 2000 in serum free medium (OPTI-MEM, Gibco). After 4 h, serum free medium was replaced with complete medium and the cells were incubated for 16 h. After 16 h, cells were transfected with 750 ng/ml poly (I:C) and incubated for 4 h. The cells were then fixed using 1% formaldehyde (Thermo Scientific) and processed for ChIP assay as per manufacturer's instructions (Pierce Agarose ChIP kit, Thermo Scientific). For immunoprecipitating HA-tagged big brown bat c-Rel, 1/1000 dilution of mouse anti-HA antibody (Sigma) was used and 5 μg mouse IgG isotype control (Thermo Scientific) was used as the non-specific antibody control. ChIP assay positive (human anti-RNA polymerase II) and negative control (rabbit IgG) antibodies were provided with the kit. Positive control primers for human GAPDH were provided with the kit. The amount of TNFα promoter immunoprecipitated was quantified by quantitative real-time PCR (qRT-PCR) and percent input was calculated and plotted as per

manufacturer's instructions and as previously mentioned (226). The qRT-PCR products were analysed by gel electrophoresis.

5.6.13. c-Rel Western Blots

HEK293T cells were seeded at a concentration of 3x 10^5 cells/well in six well plates and simultaneously transfected with 100 nM of 1:1 cocktail of two different siRNA specific to c-Rel (table 5.1), NC siRNA or no siRNA and plasmids expressing HA-tagged c-Rel. Cells were harvested in sample buffer for western blots 24 hrs post transfections. Western blots were carried out as previously described (227). Primary antibodies used were: 1: 1,000 mouse anti-HA (Clone HA7; Sigma) and 1: 5,000 rabbit anti-GAPDH (Cedarlane). Secondary antibodies used were: 1:10,000 goat anti-mouse Alexa 488 (Molecular Probes) and 1: 10,000 goat anti-rabbit Cy5 (GE Healthcare).

5.6.14. TLR3, RIG-I and MDA5 knockdown

Dicer-ready siRNA (DsiRNA) specific to big brown bat TLR3, RIG-I and MDA5 were designed and obtained through Integrated DNA Technologies (IDT). A 100 nM final concentration of a 1:1 mixture of two DsiRNAs (supplementary Table S5.5) targeting separate regions on the respective transcripts was transfected into Efk3 cells using Lipofectamine 2000. Scrambled non-specific DsiRNA (NC DsiRNA; IDT) was used as a negative control. Transfections were carried out as previously mentioned for siRNA (228). In brief, cells were transfected with the siRNA cocktail for 24 hours, media replaced and then transfected with 750 ng/ml poly (I:C) using Lipofectamine 2000. RNA was extracted 16h after poly (I:C) stimulation for cDNA preparation and qRT-PCR.

5.6.15. Statistics

Significance of the data was determined by two-tailed Mann-Whitney *U* test for non-parametric independent samples using IBM SPSS (Version 21). In the figures, **P*<0.05 and ***P*<0.01. Actual 'P values' are mentioned in figure legends.

5.7. Results

5.7.1. TLR expression in MRC5 and Efk3 cells

To determine if the human and bat cell lines we studied expressed receptors for viral ligands, we examined these cells for TLR 2, 3, 7, 8, 9, RIG-I and MDA5 using PCR (supplementary table S5.1). Both cell lines contained transcripts for these receptors.

5.7.2. Big brown bat cells express high levels of IFNβ but low TNFα transcripts in response to poly (I:C)

To determine if the cells were capable of innate responses to viral ligands we treated the cells with poly(I:C), single-stranded RNA (ssRNA) and CpG ODNs. We used these surrogates instead of viruses capable of infecting both cells to prevent modulation of these pathways by viral proteins.

We quantified the expression of several transcription factors and downstream genes by qRT-PCR. We observed a heightened innate response with poly (I:C) in bat cells but not as much with ssRNA and CpG ODN (supplementary table S5.2). We therefore decided to further analyse the cytokine response in bat and human cells to poly(I:C). While both MRC5 and Efk3 cells responded to poly (I:C) with a robust increase in IFNβ transcripts (Fig. 5.2a), only MRC5 cells responded with increased levels of TNFα RNA (Fig. 5.2b). Efk3 cells upregulated TNFα

transcript levels by 2.4-folds only compared to 315-fold upregulation in human cells after poly (I:C) treatment (Fig. 5.2b). We therefore examined further the response of these two genes. To determine when IFNβ and TNFα transcripts are expressed following poly (I:C) treatment, we quantified IFNβ and TNFα transcripts in MRC5 and Efk3 cells at different times after poly (I:C) treatment. Both MRC5 and Efk3 cells showed highest IFNβ transcript levels at 9h post-transfection (Fig. 5.2c). TNFα transcript levels were highest at 12h post-transfection in MRC5 cells and there was relatively little expression in Efk3 cells (Fig. 5.2d). To rule out the possibility of Efk3 cells not being able to mount a TNFα response, we transfected bone marrow derived myeloid cells from big brown bat long bones with poly(I:C). The mixed population of cells (see supplemental Fig. S5.1) demonstrated an average of 1700-fold increase in IFNβ transcripts but only 11-fold increase in TNFα transcripts (Fig. 5.2e) post stimulation.

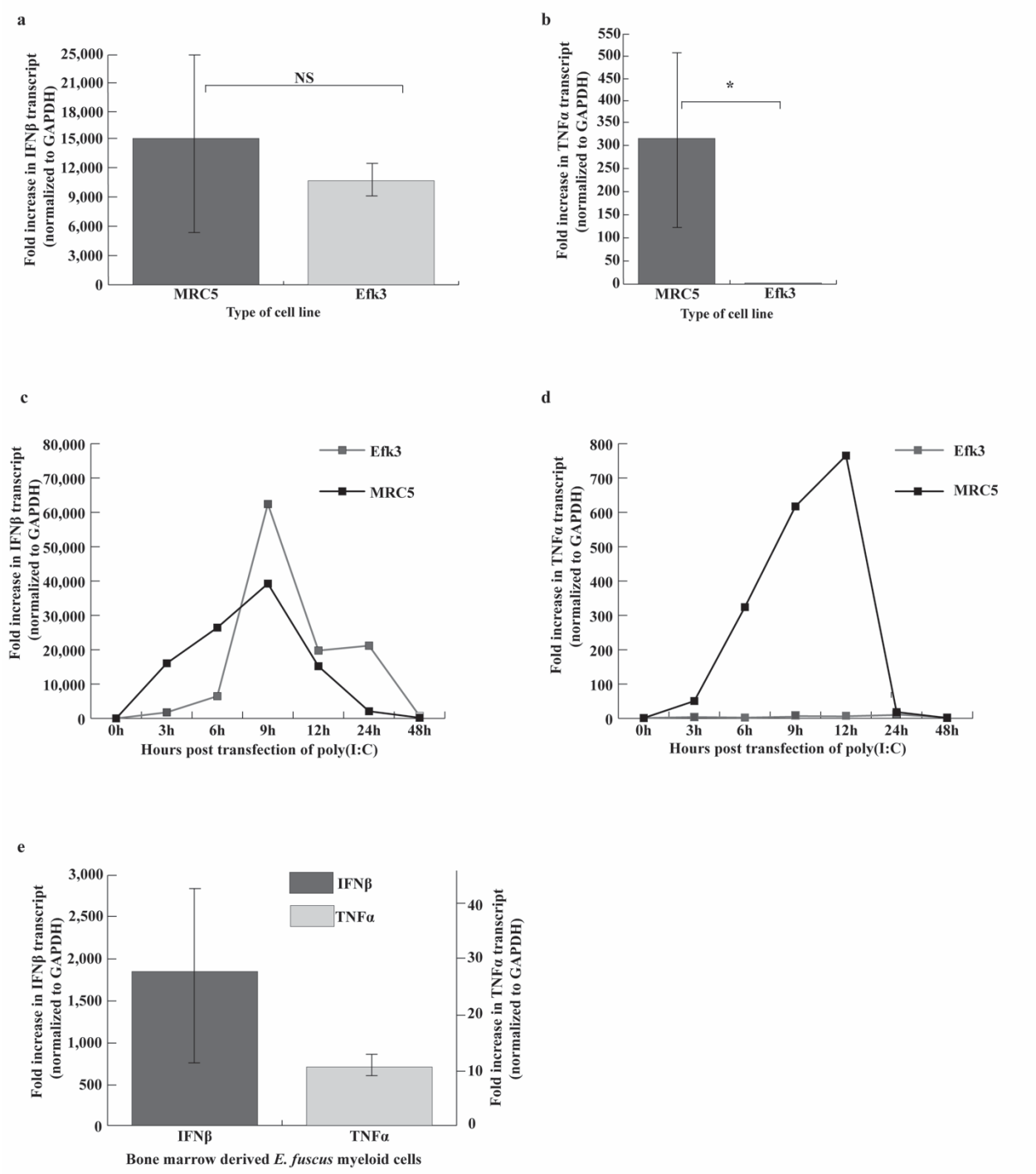


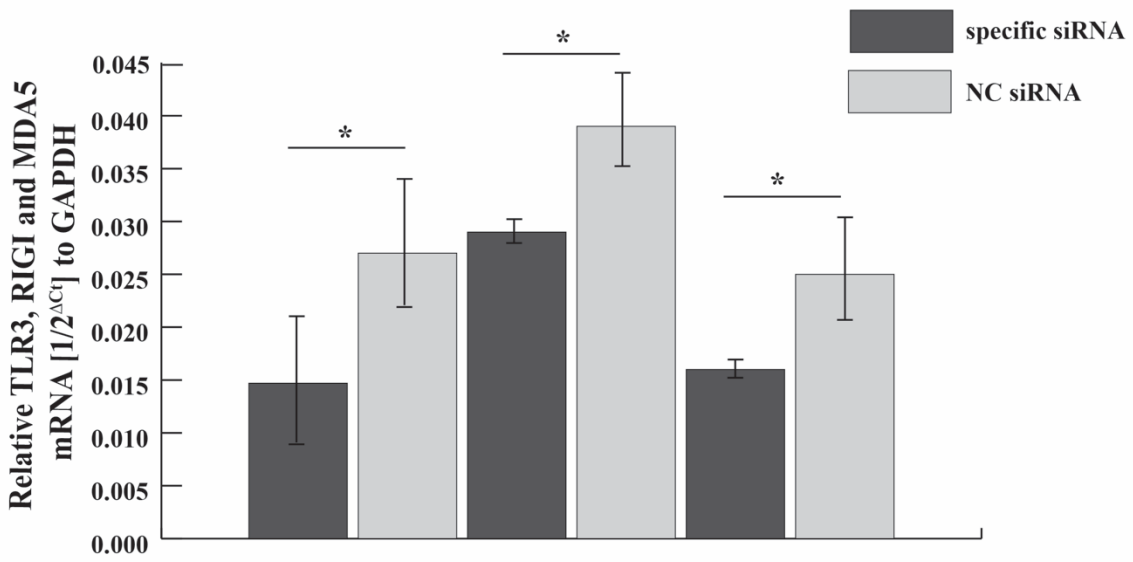
Figure 5. 2. Efk3 cells do not express high levels of TNFα transcripts in response to poly(I:C). We transfected human fibroblasts, bat kidney (Efk3) and bat myeloid cells with poly(I:C), a known TLR3 stimulant, and studied the expression of IFNβ and TNFα relative to mock transfected cells. (a) Both MRC5 and Efk3 cells responded to poly (I:C) by expressing IFNβ transcripts (mean±SD, n=3, P=0.05). (b) MRC5 cells responded to poly (I:C) by several hundred-fold expression of TNFα transcripts but Efk3 cells expressed significantly lower levels of TNFα transcripts (mean±SD, n=3, P=0.021). (c) Transcripts for IFNβ in MRC5 and Efk3

cells were quantified at several time points after poly (I:C) treatment. Both MRC5 and Efk3 cells showed highest IFNβ transcript levels 9h post poly (I:C) treatment. (d) Transcripts for TNFα in MRC5 and Efk3 cells were quantified at several time points after poly (I:C) treatment. MRC5 cells showed highest TNFα transcripts 12h post poly (I:C) treatment but Efk3 cells did not express TNFα transcripts to relatively comparable levels. (e) Big brown bat bone marrow derived myeloid cells expressed IFNβ transcripts to 1700 fold higher post poly (I:C) treatment but TNFα transcripts were expressed to an average of only 11 fold higher over mock treated cells (mean±SD, n=3). Results are expressed as fold increases over mock-treated cells normalized to GAPDH values (Materials and Methods). Statistical significance was calculated using two-tailed Mann-Whitney *U* test for two independent samples. **P*<0.05, NS = not significant. n is the number of independent experiments. SD=standard deviation.

5.7.3. Poly (I:C) signals through TLR3 to activate IFNβ in Efk3 cells

To determine if poly (I:C) upregulated IFNβ transcripts in bat cells through recognition by TLR3, we measured IFNβ transcripts in bat cells treated either with poly (I:C) added to the medium or poly (I:C) transfected in to bat cells. We compared both treatment types to mock poly (I:C) treated cells. Adding poly (I:C) to the cell culture medium did not upregulate IFNβ transcripts in bat cells (supplementary Fig. S5.2). To further identify the roles of TLR3, RIG-I and MDA5 receptors in dsRNA recognition in bat cells, we partially knocked down these receptors using siRNA. siRNA specific to these receptors significantly reduced transcripts for TLR3, RIG-I and MDA5 (Fig. 5.3a). Knocking down TLR3 transcripts significantly reduced IFNβ transcripts after poly (I:C) transfection in bat cells (Fig. 5.3b). Although knocking down RIG-I in bat cells correlated with a decrease in IFNβ transcripts post poly (I:C) transfection, it was not significant (Fig 5.3b). Knocking down MDA5 did not have any effect on IFNβ transcript levels after poly (I:C) transfection in bat cells (Fig 5.3b).

a



Type of DsiRNA	TLR3 siRNA	NC siRNA	RIGI siRNA	NC siRNA	MDA5 siRNA	NC siRNA
Receptor quantified	TLR3	TLR3	RIGI	RIGI	MDA5	MDA5

b

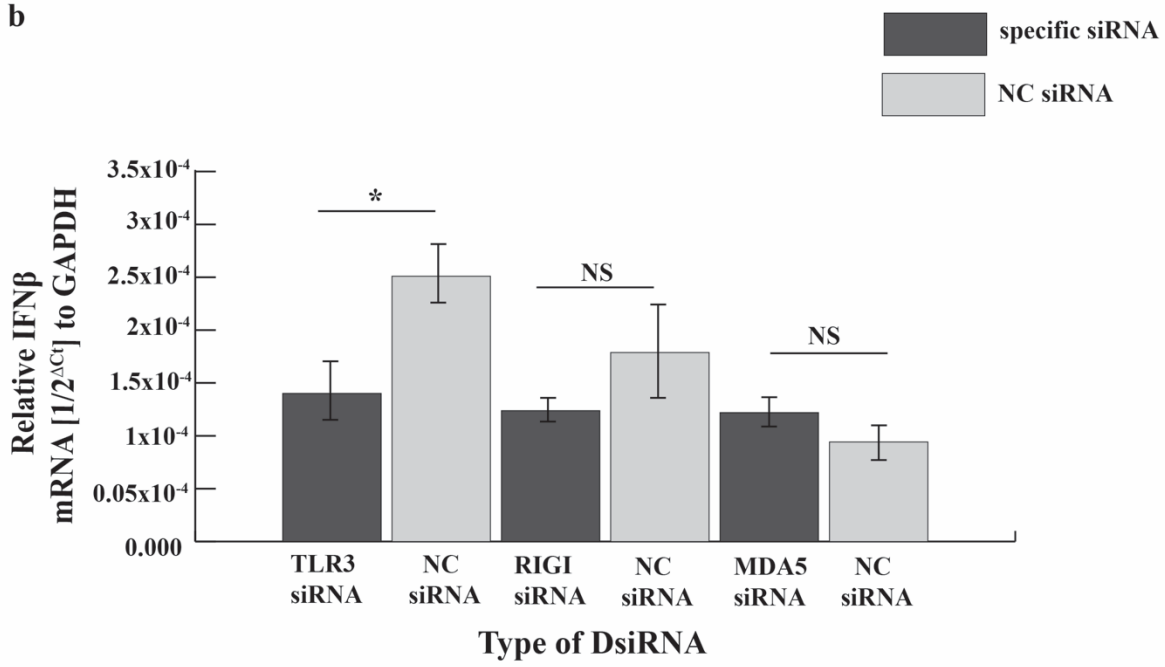


Figure 5. 3. Poly (I:C) signals through TLR3 in Efk3 cells. To identify the role of TLR3, RIG-I and MDA5 in poly (I:C) induced interferon signaling, we transfected siRNA specific to *E. fuscus* TLR3, RIG-I or MDA5 in Efk3 cells and stimulated the cells with poly(I:C). (a) siRNA specific to these receptors partially knocked down transcripts for TLR3 (P=0.043), RIG-I (P=0.021) and MDA5 (P=0.02) in poly (I:C) stimulated Efk3 cells (mean±SD, n=4). (b) Knocking down TLR3 in bat cells significantly reduced IFNβ transcripts upregulation after poly (I:C) challenge (mean±SD, n=4; P=0.02). For RIG-I knocked down bat cells, there was a decrease in IFNβ transcripts, however it was not significant (mean±SD, n=4; P=0.05). MDA5 knockdown did not correlate with decrease in IFNβ transcripts (mean±SD, n=4; P=0.083). Relative amounts of RNA are expressed as a reciprocal of Ct (the PCR cycle at which the product is measurable) normalized to Ct for GAPDH. Statistical significance was calculated using two-tailed Mann-Whitney *U* test for two independent samples. *P**<0.05, NS = not significant. NC = negative control.

5.7.4. Poly (I:C) treatment leads to the suppression of the big brown bat wildtype TNFα promoter activity

To determine if the difference in the response of EfK3 and MRC5 cells was because of inherent features in their promoters for TNFα, we cloned the human TNFα promoter (229) and the corresponding region upstream of the big brown bat TNFα coding sequences in a plasmid with the reporter gene, chloramphenicol acetyltransferase (CAT). We transfected the plasmids in MRC5 and Efk3 cells and observed that the big brown bat TNFα promoter showed decreased activity post poly (I:C) challenge in both MRC5 and Efk3 cells (Fig. 5.4). In contrast, the human TNFα promoter showed increased activity in both Efk3 and MRC5 cells after poly (I:C) challenge (Fig. 5.4).

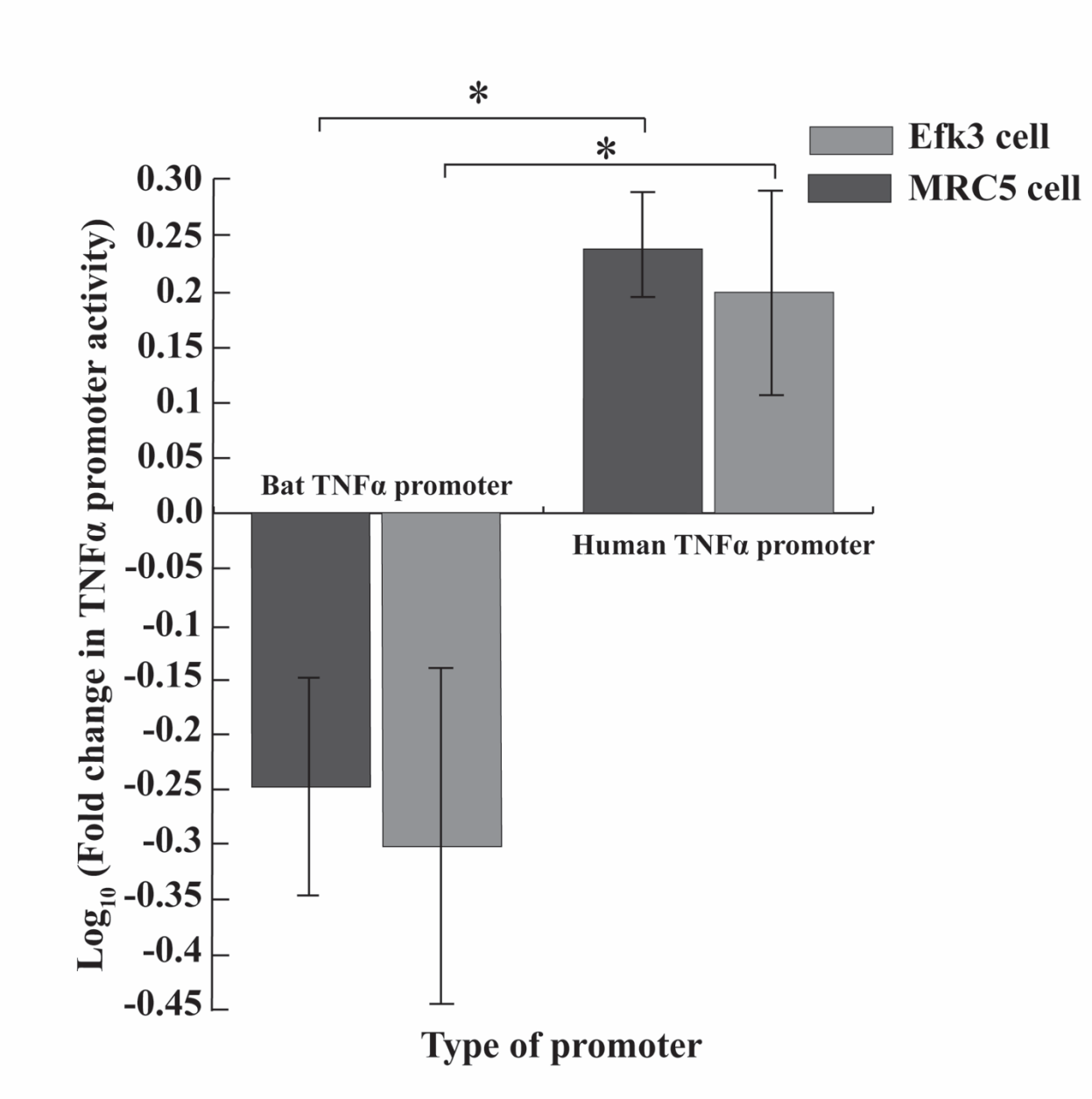


Figure 5. 4. Big brown bat TNFα promoter is functionally different than its human counterpart. We transfected both human and bat TNFα promoters individually in human and bat cells and quantified their activity after poly (I:C) treatment by measuring the expression of a downstream surrogate gene, chloramphenicol acetyltransferase (CAT). Human TNFα promoter showed increased activity after poly (I:C) treatment (as measured by CAT activity) in both Efk3 (P=0.021) and MRC5 (P=0.020) cells. In contrast, big brown bat TNFα promoter showed decreased activity after poly (I:C) treatment in both Efk3 (P=0.021) and MRC5 (P=0.020) cells (mean±SD, n=4). Results are expressed as fold increases over mock-treated cells normalized to β-galactosidase. Statistical significance was calculated using two-tailed Mann-Whitney *U* test for two independent samples. *P**<0.05.

5.7.5. Big brown bat TNFα promoter has a unique c-Rel binding site

Since the big brown bat TNFα promoter showed decreased activity post poly (I:C) stimulation, we examined the human TNFα promoter and bat nucleotide sequence 1,200 bases upstream from the TNFα coding sequence for potential transcription factor binding motifs using the bioinformatics tool PROMO (230). Both promoters contained motifs for binding NFκB although the big brown bat TNFα promoter had one less site. In addition, the bat promoter had a putative c-Rel binding motif (Fig. 5.5) not present in the human counterpart.

Figure 5. 5. Big brown bat TNFα promoter has a unique c-Rel motif. Human and big brown bat TNFα promoters were analyzed for NFκB and c-Rel transcription factor binding sites. Human TNFα promoter has three NFκB binding and no c-Rel binding site. Big brown bat TNFα promoter has two NFκB binding site and one c-Rel binding site. Other transcription factor binding sites, including sites for NFκB-1 and Rel-A, are not shown here.

5.7.6. c-Rel inhibits big brown bat wildtype TNFα promoter activity

By analyzing the nucleotide sequence of the big brown bat TNFα promoter, we identified a potential c-Rel binding site. To identify the role of this binding motif in the big brown bat TNFα

promoter, we deleted it (Fig. 5.6a) and observed the promoter's activity in response to poly (I:C) in bat cells. Deleting the c-Rel binding site in the big brown bat TNFα promoter increased the promoter activity in response to poly (I:C) (Fig. 5.6b). To further identify the role of c-Rel in repressing the bat TNFα promoter, we partially knocked down c-Rel transcripts using siRNA in bat cells (Fig. 5.6c) and quantified basal TNFα transcripts. There was a significant increase in basal TNFα transcripts in these cells (Fig. 5.6d). We further confirmed that siRNA directed against c-Rel used in this study can successfully shutdown the expression of c-Rel at a protein level (supplementary Fig. S5.3).

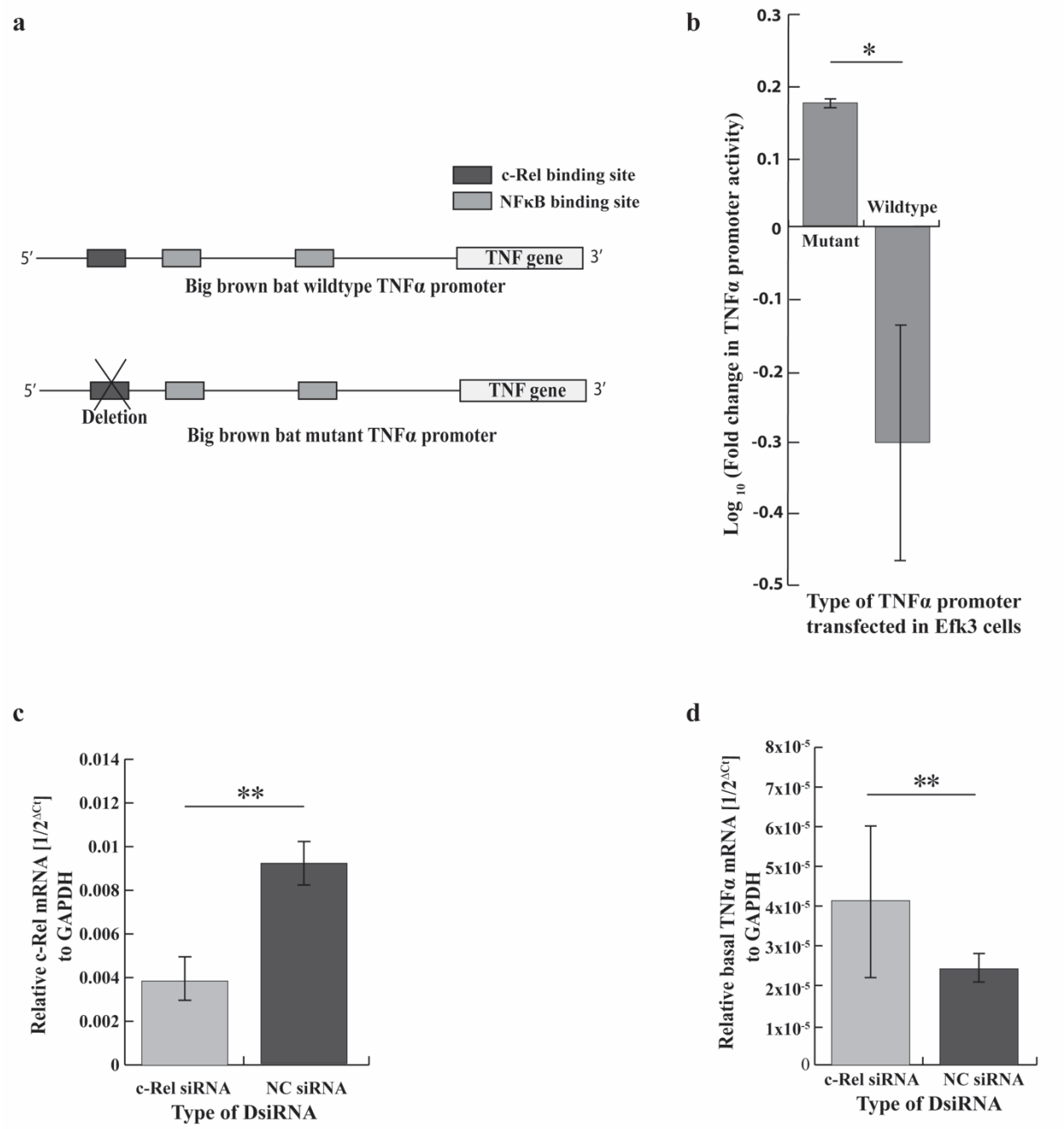


Figure 5. 6. c-Rel acts as a repressor of the big brown bat TNFα promoter. (a) Schematic representation of big brown bat TNFα wildtype and mutant promoter. In the mutant bat promoter, the putative c-Rel binding site was deleted. (b) Deleting the putative c-Rel binding site in the wildtype big brown bat TNFα promoter significantly (P=0.034) increased the promoter activity in response to poly (I:C) treatment (mean±SD, n=3). (c) DsiRNA directed against bat c-Rel significantly (P=0.009) reduced c-Rel transcripts in bat cells (mean±SD, n=5). (d) Basal TNFα transcript levels increased significantly (P=0.009) in partially c-Rel knocked down bat cells (mean±SD, n=5). For figures 5C and 5D, relative amounts of RNA are expressed as a reciprocal of Ct (the PCR cycle at which the product is measurable) normalized to Ct for

GAPDH. Statistical significance was calculated using two-tailed Mann-Whitney *U* test for two independent samples. *P**<0.05, *P***<0.01. NC = negative control.

5.7.7. Big brown bat c-Rel responds to poly (I:C) by translocating from the cytoplasm to the nucleus

Rel proteins, either as hetero-dimers or homo-dimers, translocate to the nucleus of the cell after PAMP recognition and downstream signaling by PRRs to bind to promoters and regulate gene transcription. To determine if bat c-Rel responded to poly (I:C) treatment by similar movement, we cloned big brown bat c-Rel into a vector with an influenza virus haemagglutinin (HA) tag, that could be recognized by a commercially available monoclonal antibody, and transfected the protein expressing construct into Efk3 cells. We determined the cellular location of c-Rel by immunofluorescence and observed that c-Rel localized to the nucleus of poly (I:C) treated cells (Fig. 5.7a). In mock treated cells, c-Rel was present in the nucleus and the cytoplasm (Fig. 5.7a). The mean nuclear:cytoplasm fluorescence ratio was significantly higher in poly (I:C) treated cells than mock treated cells (Fig. 5.7b).

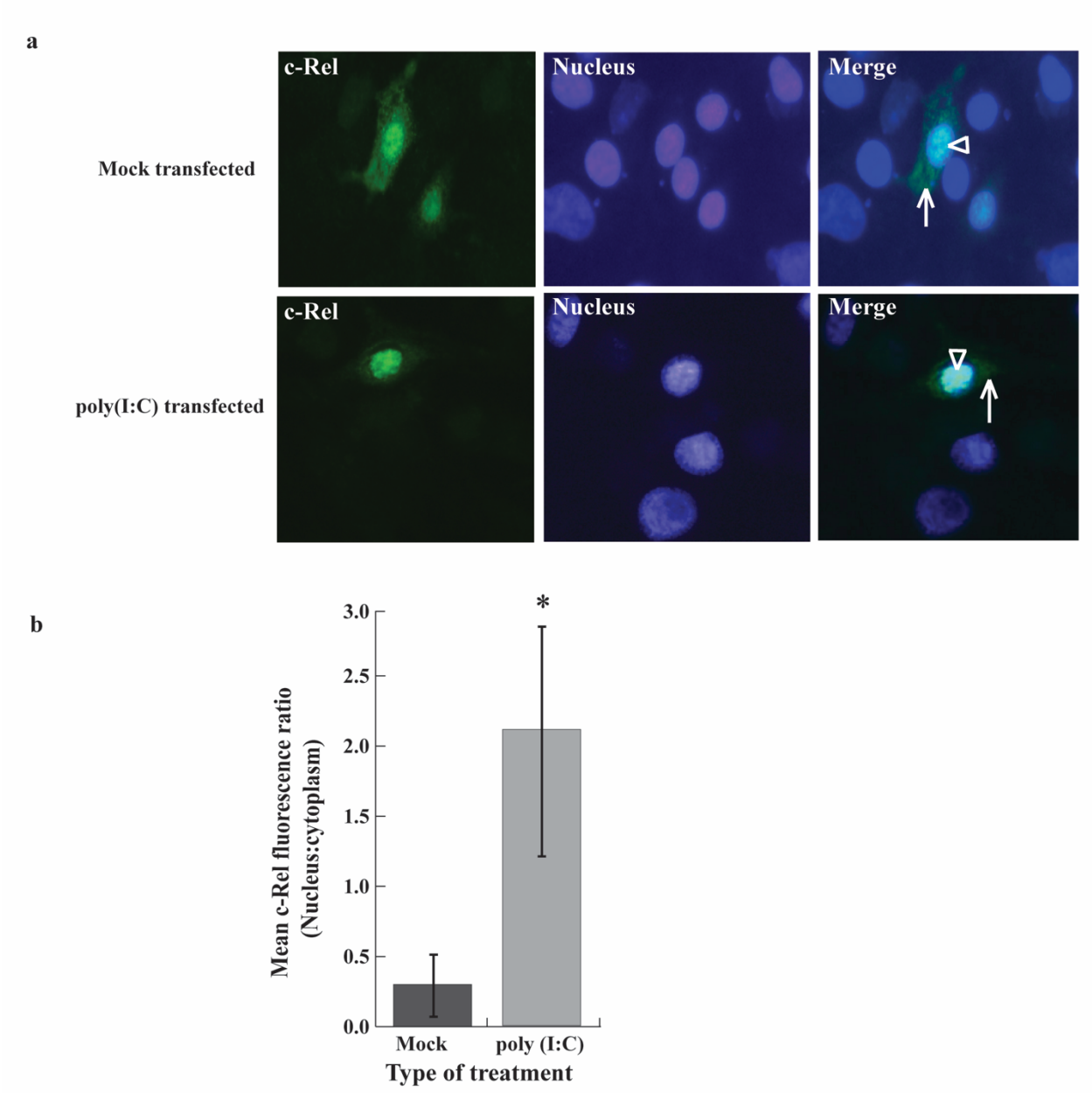


Figure 5. 7. Big brown bat c-Rel localizes in the cell nucleus after poly (I:C) treatment. To characterize the bat c-Rel, we studied the cellular location of ectopically expressed c-Rel in poly (I:C) and mock transfected cells. (a) Big brown bat c-Rel localized primarily in the nucleus after poly (I:C) challenge compared to mock challenged cells, where ectopically expressed c-Rel localized both in the cytoplasm and nucleus. (b) Mean fluorescence ratio (i.e. nucleus:cytoplasm) was significantly (P=0.021) higher in poly (I:C) treated cells than mock treated cells (mean±SD, n=4). Statistical difference was calculated using two-tailed Mann-Whitney *U* test for two independent samples. *P**<0.05.

5.7.8. Big brown bat c-Rel binds to the putative c-Rel binding site

To study if bat c-Rel bound to the putative c-Rel motif, we co-transfected into human cells plasmids expressing HA-tagged bat c-Rel and plasmids containing wildtype or mutant bat and human TNFα promoters. We then performed chromatin immunoprecipitation (ChIP) assay on these cells using antibodies against the HA-tagged c-Rel. We used qRT-PCR to detect TNFα promoters in the immunoprecipitated samples. Bat c-Rel co-precipitated significantly higher amounts of big brown bat wildtype TNFα promoter with the putative c-Rel binding motif than the promoter without the motif (Fig. 5.8a). In addition, bat c-Rel precipitated a higher amount of the mutant human TNFα promoter with the putative bat c-Rel binding site than the wildtype human promoter (Fig. 5.8b).

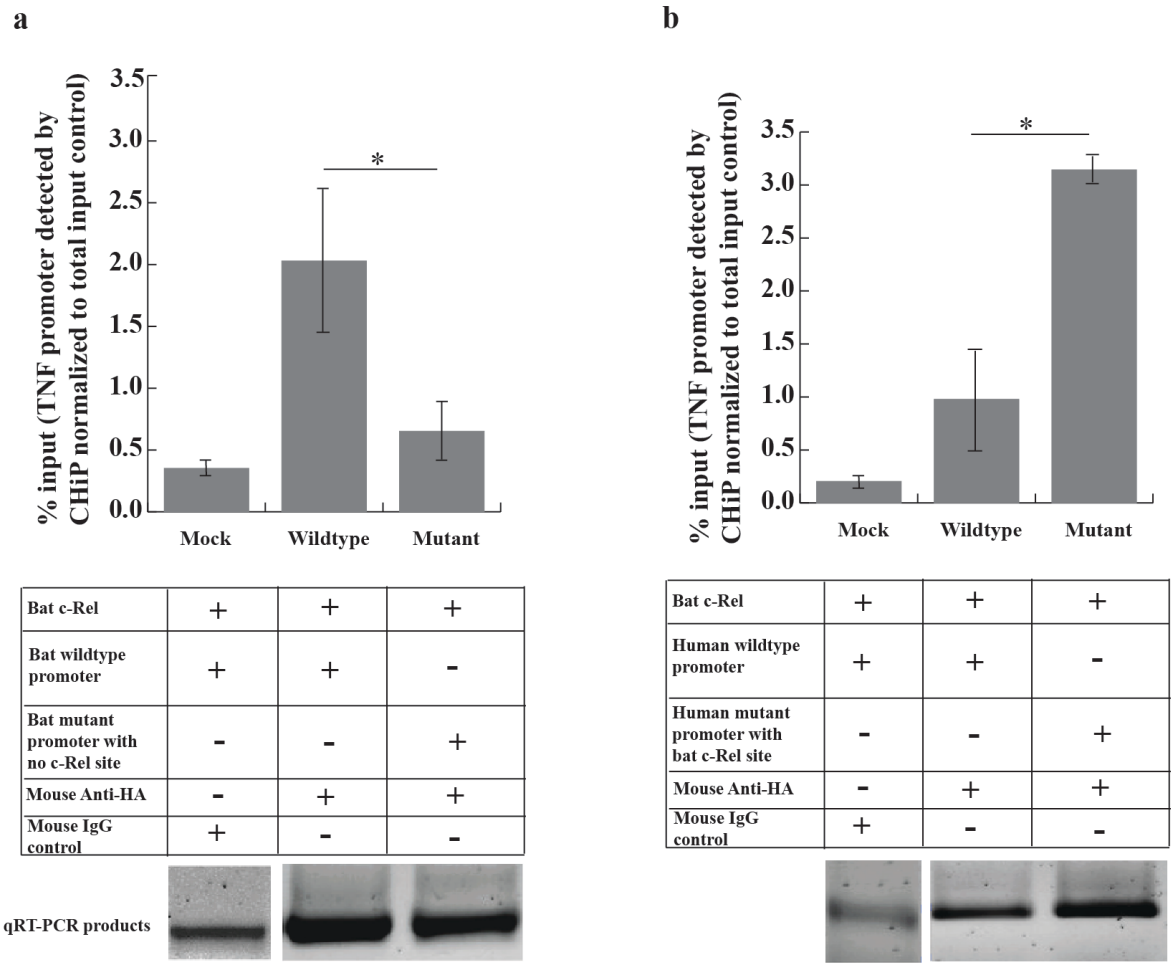


Figure 5. 8. Big brown bat c-Rel binds to the putative c-Rel binding site. We co-transfected plasmids expressing HA-tagged bat c-Rel and human/bat TNFα wildtype/mutant promoters in HEK293T cells. We immunoprecipitated c-Rel using HA-specific antibodies and quantified the amount of TNFα promoter pulled down by qRT-PCR. (a) Bat c-Rel pulled down a significantly (P=0.021) higher amount of wildtype big brown bat TNFα promoter that contained the putative c-Rel binding motif over the mutant promoter in which the motif was deleted (mean±SD, n=4). (b) Bat c-Rel pulled down a significantly (P=0.021) higher amount of mutant human TNFα promoter which contained the putative bat c-Rel binding motif over the human wildtype TNFα promoter, which lacked the c-Rel binding motif (mean±SD, n=4). qRT-PCR products obtained from quantifying the immunoprecipitated samples were electrophoresed on a gel and their representative cropped images are shown. Images have been acquired from two different gels. Statistical difference was calculated using two-tailed Mann-Whitney *U* test for two independent samples $P^*<0.05$.

5.8. Discussion

Chiroptera is a very diverse order with over 1300 species of bats. Viruses from several families have been detected in different species of bats (1) although very few of these viruses are known to cause disease in their natural hosts. West Nile virus, Eptesipox virus, a novel group I coronavirus and American bat vesiculovirus have been detected in asymptomatic big brown bats (1, 206). We do not completely understand how bats and viruses coexist. Researchers are working to identify unique adaptations that might allow bats to coexist with these viruses (12, 23, 159). Our results indicate that big brown bats may have evolved a unique mechanism to avoid an overblown inflammatory response to activation of the TLR3 pathway by viral ligands. This is in addition to other adaptations in bats currently being proposed , such as loss of the PYHIN family of genes, thereby losing the ability to sense foreign DNA in cells and activating inflammasomes (107).

To compare the innate responses of human and bat cells to viral ligands we treated the cells with surrogates of viral PAMPs. We used these surrogates instead of viruses capable of infecting both cells to prevent modulation of these pathways by viral proteins. In preliminary experiments we had determined that PED-CoV, which replicates and produces cytopathic effects in bat cells (110) does not induce an interferon response (data not shown). Coronavirus N protein is known to inhibit IFNβ production by preventing IRF3 phosphorylation (117, 231).

We observed an increase in IFNβ transcripts in Efk3 cells in response to poly(I:C), as has been previously demonstrated for other bat species (111, 136, 232), but very little increase in TNFα transcripts compared to human cells. Recently, a *P. alecto* adaptive immune cell population was characterized and a subset of cells was shown to produce TNFα on stimulation with ionomycin,

although the amount of TNFα produced was not reported (102). We challenged big brown bat bone marrow derived myeloid cells and the kidney cell line with poly (I:C) and quantified the transcripts for representative antiviral and inflammatory genes. Inflammatory cytokine transcripts for TNFα, IL8 and IL1β (supplementary table S5.2) in Efk3 cells and TNFα in big brown bat bone marrow derived myeloid cells were not expressed to levels observed in MRC5 cells. IFNβ and TNFα transcript levels observed in poly (I:C) challenged big brown bat myeloid cells were comparable to levels observed in poly (I:C) challenged *P. alecto* bone marrow derived dendritic cells by Zhou *et al*.(101).

Interferon β production in response to poly (I:C) has been studied in bat cells (110, 136) but the receptors involved in double-stranded RNA signaling have not been fully explored in these cells. Adding poly (I:C) to the culture medium did not upregulate transcripts for IFNβ in bat cells (supplementary Fig. S5.2). Transfection seems to be essential for the activation of IFNβ in Efk3 cells. Thus, dsRNA does not signal through cell surface receptors in Efk3 cells. The requirement of transfection for the activation of TLR3 in certain cell types has been further elucidated by Zhou *et al.* (70). Using siRNA, we were able to show for the first time that poly (I:C) or dsRNA is recognized primarily through TLR3 in bat cells (Fig. 5.3). However, the role of RIG-I in dsRNA recognition cannot be completely ruled out. The role of other PRRs in bats in recognizing specific ligands is yet to be explored.

TNFα plays a key role in inflammatory, infectious and malignant conditions. TNFα signaling transduction pathways are complex and are not fully understood (233). NFκB plays a central role in the regulation of TNFα gene expression. Different combinations of the subunits that make up

NFκB have vastly different effects on gene expression (212). For instance, hetero-dimers of p50 or p52 and p65 or RelB activate transcription. In contrast, c-Rel as a homo-dimer or in association with p50 and p65, repress transcriptional activation by NFκB (213) and c-Rel has been previously shown to be a repressor of certain gene promoters in human cells, such as Ephrin type-B receptor 2 (EPHB2) in colorectal cancer cells (217). We detected a putative c-Rel binding motif in the big brown bat TNFα promoter. Deleting this motif reversed the bat promoter activity after poly (I:C) treatment and partial knockdown of c-Rel RNA significantly increased basal TNFα transcript levels in bat cells demonstrating the ability of c-Rel to repress the TNFα promoter in bat cells.

The big brown bat TNFα promoter had two NFκB binding sites, which is one less than the human counterpart. We do not know if having one less NFκB binding site can be an additional reason for the low TNFα promoter activity. However, adding an additional NFκB binding site to the big brown bat promoter lacking the c-Rel binding site did not cause any further increase in promoter activity after poly (I:C) treatment (data not shown).

c-Rel might not be the only regulator of inflammatory gene expression and big brown bats may have evolved more than one mechanism to regulate inflammatory responses. We evaluated a second inflammatory gene, IL1β and found that it, like TNFα, did not respond to poly (I:C) in bat cells (supplementary table S5.2). However, while the IL1β promoter had identifiable NFκB binding sites, it did not have a specific c-Rel binding site. There is evidence that NFκB molecules comprising p50 homodimers can act as transcriptional repressors (reviewed by

Rothwarf and Karin (234)). Further work is required to identify the role of p50 homodimers in bat cells.

Zhang *et al.* have reported *c-Rel* to be under positive selection in bats based on whole-genome analysis of two distantly related species, fruit bat *P. alecto* and insectivorous bat *Myotis davidii* (14). They suggest that the selection may have been driven by the involvement of c-Rel in DNA repair pathways and the need for efficient repair of damage caused by reactive oxygen species generated during flight. In addition, Enchéry and Horvat have also speculated that the positive selection of *c-Rel* may contribute to bats' immunovirological peculiarities (56). Our results suggest that the positive selection of *c-Rel* may also have been driven by the need to dampen the destructive effects of inflammation in response to viral infections. Zhang *et al.* also identified mutations in the REL homology domain (RHD) of c-Rel that could potentially affect the binding of IκB and speculated that this may allow nuclear transport in the absence of TLR3 stimulation (14). These mutations are also present in big brown bat *c-Rel* (supplementary Fig. S5.4). However, we did find that while ectopically expressed bat c-Rel was present in both cytoplasm and nucleus, poly (I:C) stimulation increased translocation to the nucleus (Fig 5.7a). The mutations, therefore, do not completely obviate TLR3 control.

We have also shown that big brown bat c-Rel physically interacts with the putative c-Rel binding site. Promoters containing the putative c-Rel binding site were co-immunoprecipitated at higher levels by the bat c-Rel than promoters that lacked it (Fig 5.8). These results demonstrate that bat c-Rel can suppress the expression of TNFα and that its putative binding site in the promoter for the gene plays a role. However, we have not identified the mechanism by which c-Rel acts. More

work is also needed to characterize the interaction between the various NFκB subunits and their downstream effects on different promoters.

Proteins of the Rel family differ according to tissue types in humans. Rel family protein, p65 is found in virtually all cell types, whereas c-Rel complexes (eg. p50/c-Rel and c-Rel homo-dimers) are predominantly expressed in cells of hematopoietic lineage, such as lymphoid and myeloid cells (218). We detected c-Rel transcripts in a wide variety of big brown bat tissues such as spleen, gut, ileum, kidney, lung, liver and the kidney cell line (Efk3) as well (supplementary table S5.3). We further analysed the promoters of animals in different mammalian orders and could not detect a c-Rel binding motif in the sequence 1000bp upstream of their TNFα genes. We detected potential c-Rel binding sites in other bats such as *M. davidii* and *M. natalensis* (Fig 5.9). We did not detect a potential c-Rel binding site in the *P. alecto* DNA sequences that lie upstream from the TNFα coding sequences but there was one downstream of the coding sequences (data not shown). The potential role of c-Rel in the DNA repair pathway and evolution of flight in bats has been proposed by Zhang *et al.* (14). We do not yet fully understand the role of c-Rel in DNA repair pathways in different tissue types in bats and in different species of bats. Different bat species could have evolved different strategies or a combination of strategies to control an overblown inflammatory response.

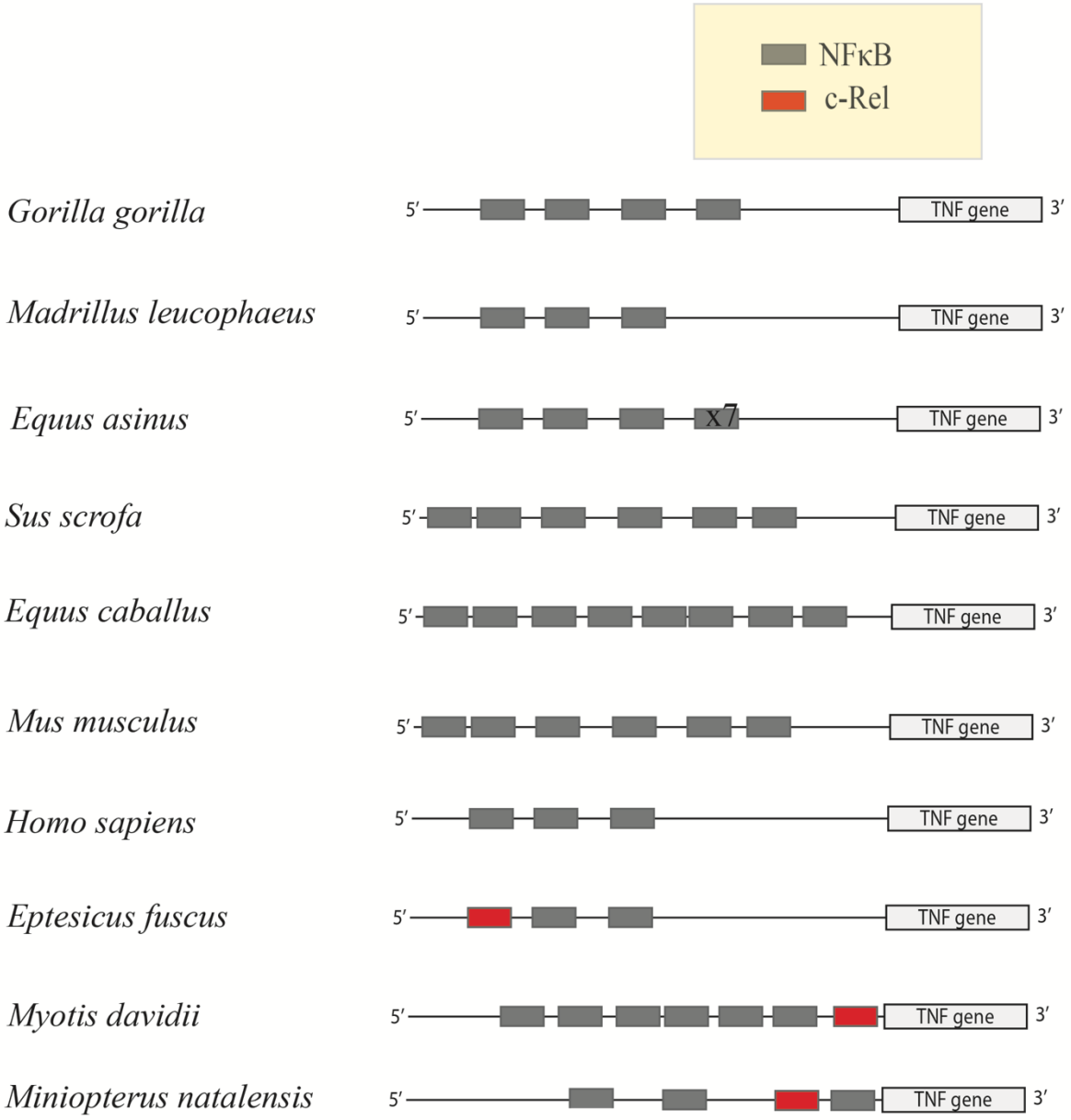


Figure 5. 9. Bats are unique in having c-Rel binding sites in their TNFα promoter. At least three species of bats, *E. fuscus*, *M. davidii* and *M. natalensis* have c-Rel binding sites upstream of the TNFα gene. c-Rel sites (red boxes) are absent in the 1000bp region upstream of the TNFα gene in other mammals represented in this figure. The number of NFκB binding motifs (gray boxes) in the TNFα promoter varied amongst mammals. All sites were predicted using PROMO. For *Equus asinus* x7 indicates 7 additional copies of the NFκB binding site.

Our study demonstrates that big brown bats have possibly evolved a mechanism to control the over expression of inflammatory genes in response to activation of their innate immune system by viral nucleic acid PAMPs. Our work raises several questions about the bat innate immune response that need to be further explored. Identifying unique defence mechanisms in bats might allow us to extend the knowledge for therapeutic purposes in spill-over hosts that often develop significant disease or succumb to infections with these viruses.

5.9. Supplementary Information

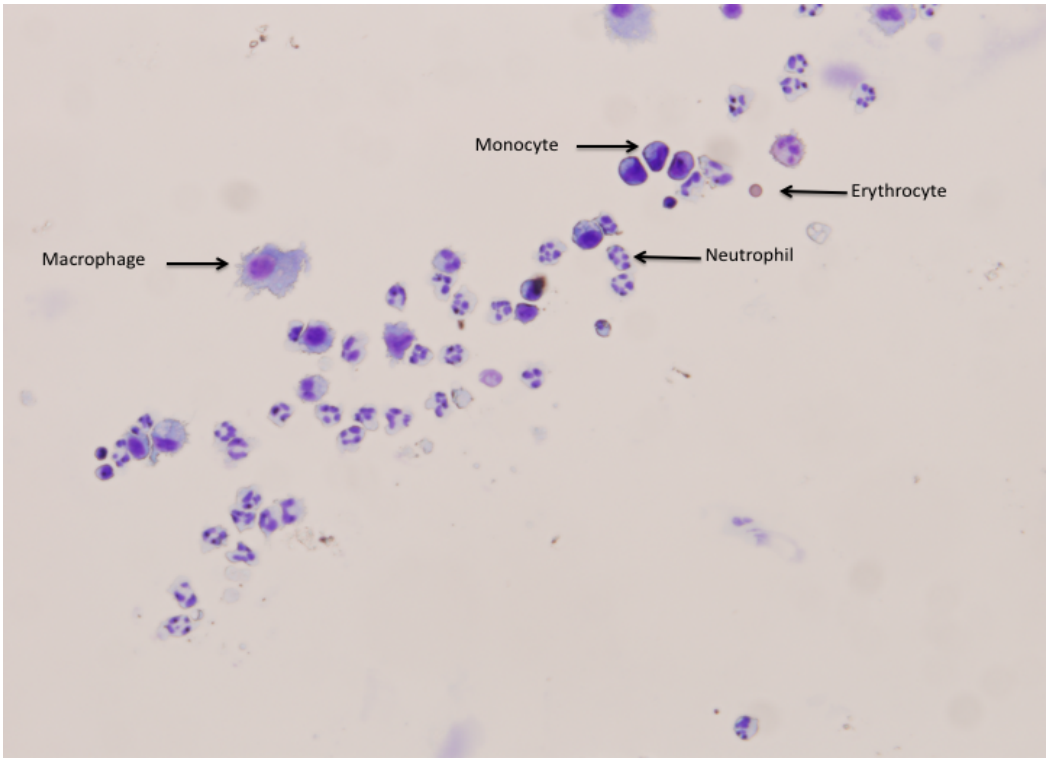


Figure S5. 1. Big brown bat bone marrow derived myeloid cells were a mixed population. Bone marrow derived cells were deposited on a slide by centrifugation and differentially stained. Various populations of myeloid cells were observed. Cell types based on morphological features are labelled in the figure.

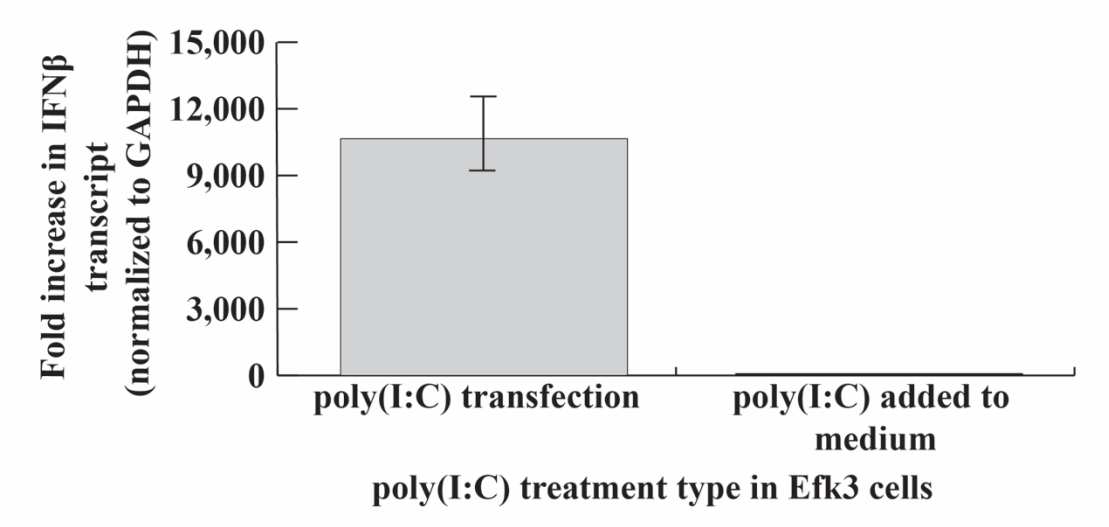
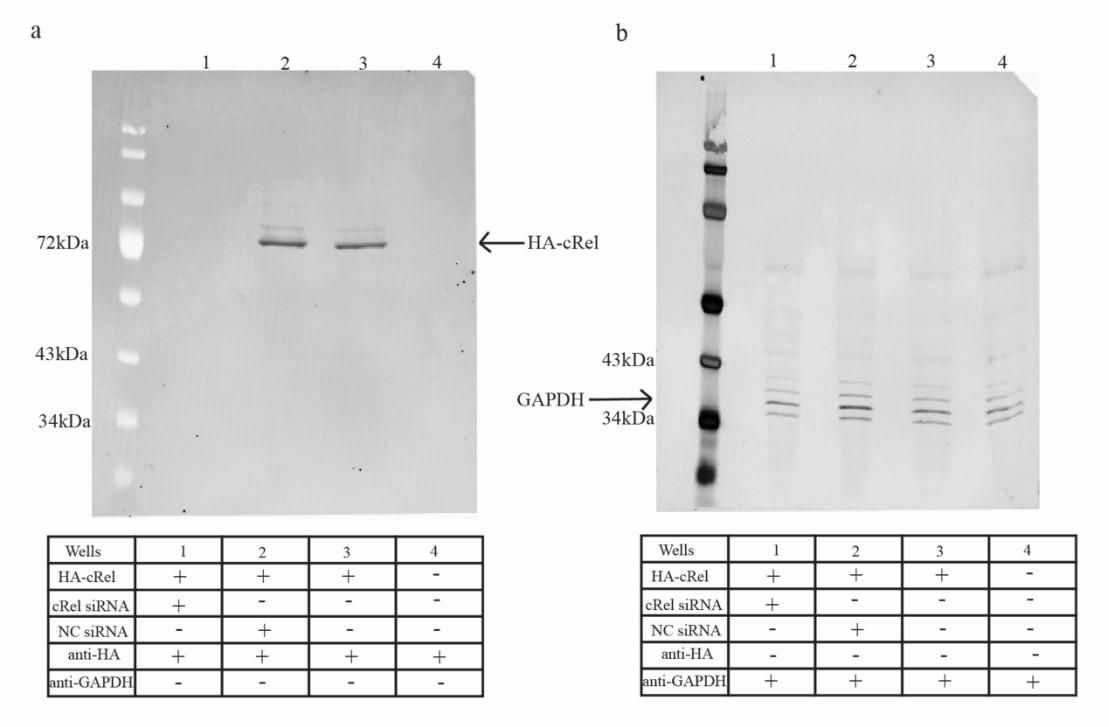


Figure S5. 2. Transfection of poly (I:C) is essential to activation of the TLR3 pathway. To determine if poly (I:C) is recognized by a cell surface receptor or an internal receptor, such as TLR3, in bat cells, we quantified IFNβ transcripts in Efk3 cells after transfecting or adding poly (I:C) to culture medium. Adding poly (I:C) to the culture medium did not upregulate IFNβ transcripts in Efk3 cells, whereas transfecting poly (I:C) induced several thousand folds of IFNβ transcripts (Mean±SD, n=4).



Wells	1	2	3	4
HA-cRel	+	+	+	-
cRel siRNA	+	-	-	-
NC siRNA	-	+	-	-
anti-HA	+	+	+	+
anti-GAPDH	-	-	-	-

Wells	1	2	3	4
HA-cRel	+	+	+	-
cRel siRNA	+	-	-	-
NC siRNA	-	+	-	-
anti-HA	-	-	-	-
anti-GAPDH	+	+	+	+

Figure S5. 3. c-Rel siRNA shuts down expression of HA-tagged c-Rel. To study the knockdown potential of c-Rel siRNA at a protein level, we simultaneously transfected HEK293T cells with siRNA specific to c-Rel, non-specific scrambled siRNA (NC siRNA) or no siRNA and plasmids expressing HA-tagged c-Rel. (a) siRNA specific to c-Rel completely shuts down the expression of HA-c-Rel from the plasmid. In contrast, cells transfected with NC siRNA or no siRNA express HA-c-Rel. (b) There was no change in GAPDH expression between different treatments. NC = Negative control.

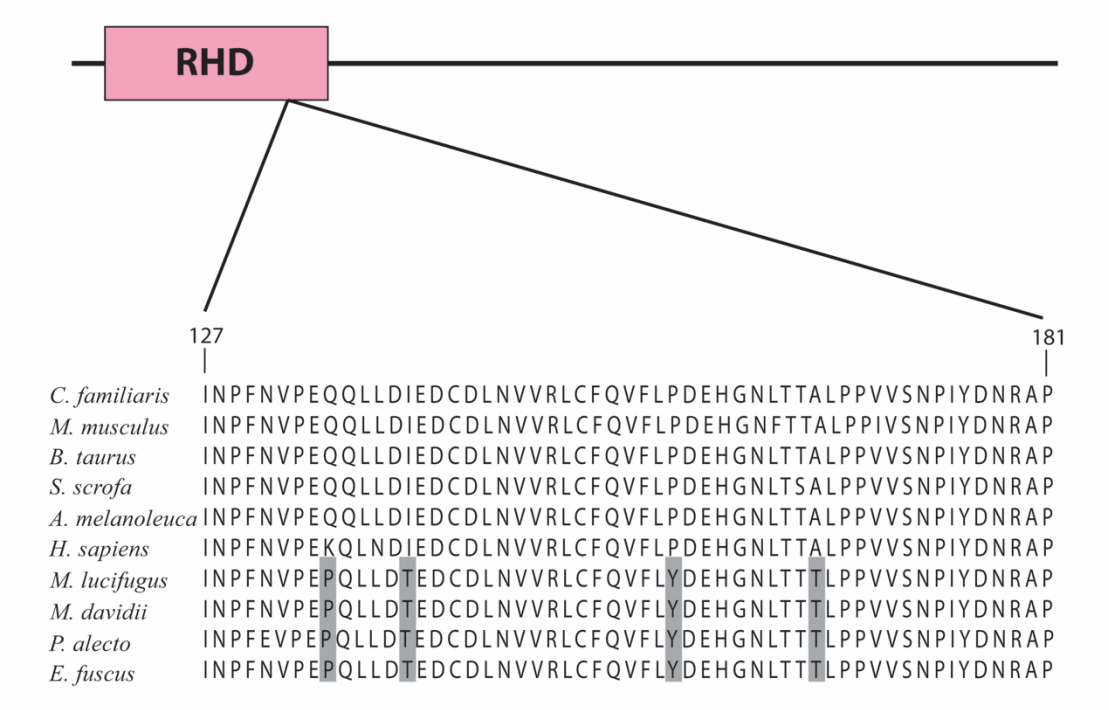


Figure S5. 4. Mutations in the c-Rel Rel homology domain (RHD). Zhang *et al.* show that 4 mutations specific to bat c-Rel exist in the downstream region of the RHD (14). IκB binding domain is located in the downstream region of the RHD and Zhang *et al.* propose mutations in this region can affect IκB binding. Differences in amino acids between bat RHD domains and those of other representative mammals are highlighted.

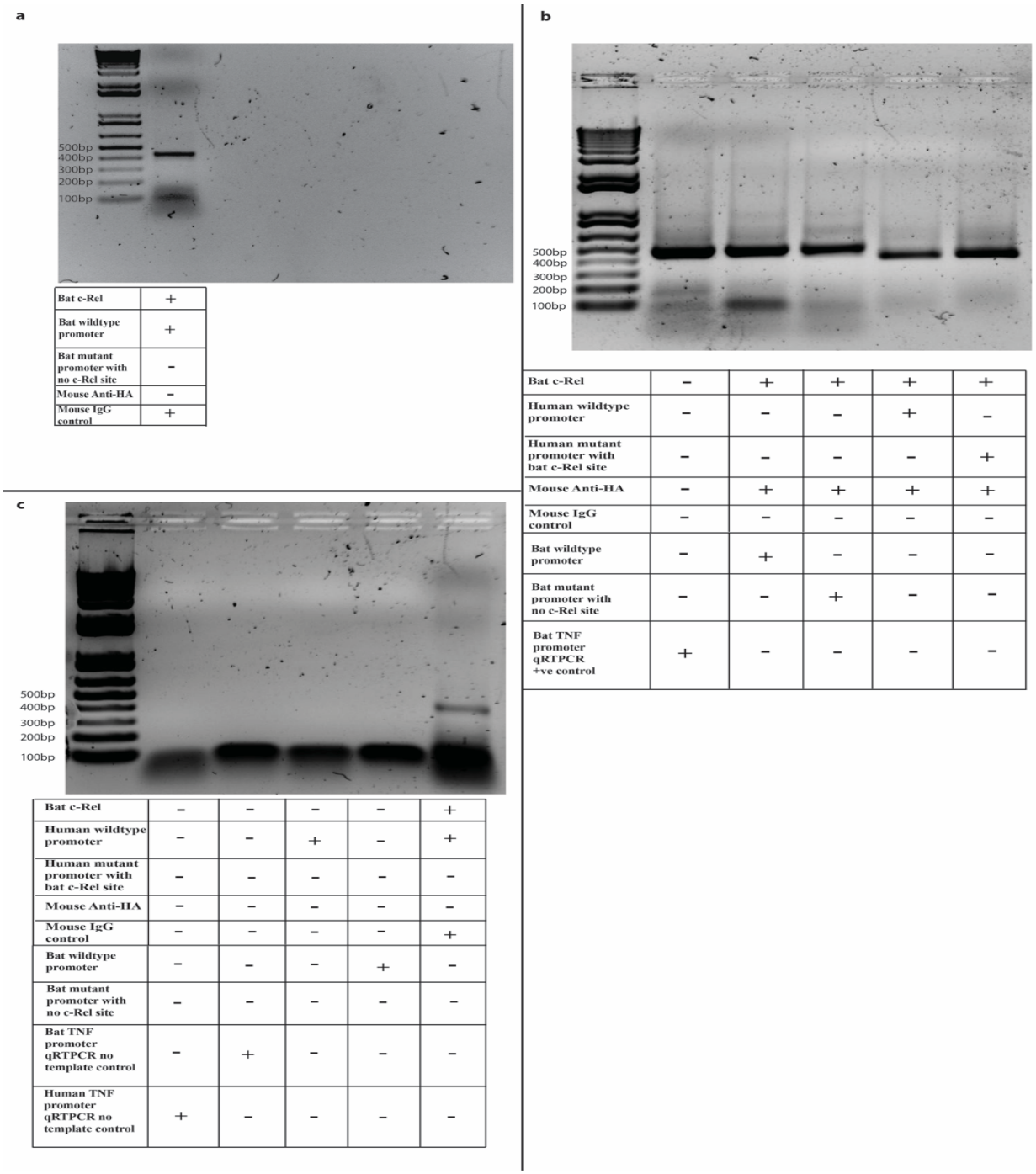


Figure S5. 5. qRT-PCR products from ChIP assay were electrophoresed on separate gels. Images for figure 8 in the article file are shown above. Cropped images were acquired from these images and scaled to fit under the qRT-PCR data in Fig. 8. ChIP and qRT-PCR controls are shown too.

Supplementary Table S5. 1. PRR transcripts detected in human (MRC5) and bat (Efk3) cells by PCR.

PRR type	MRC5	Efk3
TLR2	+	+
TLR3	+	+
TLR7	+	+
TLR8	+	+
TLR9	+	+
RIG-I	+	+
MDA5	+	+

Supplementary Table S5. 2. Fold changes observed for innate immune gene transcripts in MRC5 and Efk3 cells after different TLR ligand treatments. We transfected human fibroblasts and bat kidney (Efk3) cells with poly(I:C), a known TLR3 stimulant, and studied the expression of genes involved in the TLR3 pathway, relative to mock transfected cells. Experiments were carried out in replicates (n=3). Standard deviation values are reported along with mean fold changes.

TLR ligand	**poly (I:C)**				**ssRNA 40**				**CpG ODN**			
Gene	**Mean fold change - MRC5 cells**	**Standard deviation - MRC5 cells**	**Mean fold change - Efk3 cells**	**Standard deviation - Efk3 cells**	**Mean fold change - MRC5 cells**	**Standard deviation - MRC5 cells**	**Mean fold change - Efk3 cells**	**Standard deviation - Efk3 cells**	**Mean fold change - MRC5 cells**	**Standard deviation - MRC5 cells**	**Mean fold change - Efk3 cells**	**Standard deviation - Efk3 cells**
IRF3	1.47	0.15	1.02	2.03	1.43	0.58	-1.09	0.44	0.53	1.42	0.17	0.05

STAT 1	7.91	2.32	6.09	1.65	-1.66	0.37	-1.05	0.33	4.3	1.11	-1.35	0.09
STAT 2	3.5	0.45	4.9	1.8	1.14	0.07	1.25	0.29	1.74	0.34	1.195	1.74
MDA5	58.97	14.54	50.21	18.8	-1	0.09	1.2	0.21	6.43	0.133	-1.35	0.33
IL12A	3.2	0.58	3.11	0.7	-1.2	0.152	1.31	0.49	1.53	0.32	1.995	0.03
IL12B	3.59	1.85	2.77	3.6	5.13	0.99	-2.14	1.26	-0.95	1.99	0.36	1.97
GBP1	17.13	1.87	32	3.33	-1.86	0.65	2.46	2.1	2.14	0.21	-1.43	0.28
TLR8	0.22	1.48	-1.11	0.38	-1.2	0.095	-1.08	0.5	-0.5	1.46	1.25	0.08
TLR7	-0.28	2.72	-1.11	1.54	1.42	0.59	2.36	0.99	1.23	0.375	1.22	1.85
IRF7	50.17	17.1	80.1	21.03	2.24	1.78	-1.01	0.24	6.72	0.71	0.615	0.19
IFNβ	**15511**	**10160**	**10594.5**	**2207.8**	8.3	1.29	3.01	0.38	35.4	9.84	5.65	3.15
NFκB1	3.85	0.74	-1.23	0.28	2.66	1.08	1.41	0.38	1.35	0.34	2.035	0.268
TLR9	1.78	0.468	-1.38	0.31	-1.22	0.11	-1.14	0.75	-0.43	1.72	-1.18	0.05
TNFα	**315.16**	**208.4**	**2.4**	**0.09**	3.37	2.01	2.8	1.1	20.52	23.66	3.085	0.127
IFI6	84.58	33.08	43.26	20.95	4.7	0.45	1.07	0.22	15.99	9.58	2.885	1.56
IFI27	790.16	88.9	2.9	0.7	3.53	0.98	-1.07	0.36	34.93	5.91	1.23	1.44
IRF1	6	0.06	2.99	0.71	1.65	0.74	1.21	0.69	1.71	0.39	3.18	0.9

ISG20	310.35	30.33	0.255	2.04	-1.08	0.036	-1.04	0.55	4.38	0.82	-1.12	0.4
OAS1	2949	2062.14	229.93	187.4	9.46	1.15	1.98	1.05	81.41	7.31	1.77	0.32
RSAD2	3637.4	3121.22	49.65	35.92	3.64	0.72	1.26	1.28	66.37	7.59	1.65	0.09
RIG-I	86.34	17.71	5.92	0.1	2.51	1.55	1.51	0.41	7.36	2	-1.1	0.4
IL18	1.29	0.1	-1.9	0.7	-3.1	1.6	1.18	0.76	-0.45	1.28	-1.41	0.09
IL8	**565**	**100.26**	**-1.49**	**0.36**	2.89	1.11	1.84	0.19	27.25	8.07	1.39	0.36
IL1β	**112.3**	**14.31**	**3.16**	**2.95**	2.31	0.95	16.29	3.6	8.42	3.8	0.08	0.06
MX1	632.33	200.61	44.32	4.24	0.38	2.55	1.79	0.44	37.45	6.53	-1.65	7
TLR3	15.26	6.8	4.37	0.74	0.57	1.53	1.84	1.1	2.72	0.27	-1.065	0.04

Supplementary Table S5. 3. c-Rel transcripts were detected in several big brown bat tissues by PCR.

Big brown bat organ	**c-Rel transcript**
Spleen	+
Gut	+
Ileum	+
Kidney	+
Lung	+
Liver	+
Efk3 cell line	+

Supplementary Table S5. 4. Accession numbers, primer sequences and annealing temperatures for human and big brown bat genes and primers.

Gene	NCBI Accession - Human	NCBI accession *E. fuscus*	Primers - human (forward and reverse)	Annealing temperature	Primers– *E. fuscus* (forward and reverse)	Annealing temperature
IFNβ	NM_002176.3	XM_008145044.1	GCTTGGATTCCTACAAAGAAGCA ; ATAGATGGTCAATGCGGCGTC	54	GCTCCGATTCCGACAGAGAAGCA ; ATGCATGACCACCATGGCTTC	56
NFκB - 1	NM_003998.3	XM_008156644.1	GAAGCACGAATGACAGAGGC ; GCTTGGCGGATTAGCTCTTTT	54	GAAGCACGGATGACAGATGC ; GCCTGGCGGATGATCTCCTTT	56
IL12A	NM_000882.3	XM_008142364.1	CCTTGCACTTCTGAAGAGATTGA ; ACAGGGCCATCATAAAAGAGGT	53	TCCTGCACTTCTGAAGAGATTGA ; ACAGGGTCGTCATAAAAGAGGC	52
IL12B	NM_002187.2	XM_008147661.1	ACCCTGACCATCCAAGTCAAA ; TTGGCCTCGCATCTTAGAAAG	55	ACCTTGACCATCCAAGTCAAA ; TTTGCTTCACATTTTAGAAAG	52

IRF3	NM_001571.5	XM_008154348.1	AGAGGCTCGTGATGGTCAAG ; AGGTCCACAGTATTCTCCAGG	55	AGAAGCTAGTGATGGTCAAG ; AGGTCCACAGTGTTCTCCAGC	56
IL18	NM_001562.3	XM_008150175.1	TCTTCATTGACCAAGGAAATCGG ; TCCGGGGTGCATTATCTCTAC	51	TCTTCGTTAACCAGGGAAGCCAA ; TCTGGGGTGCATTCTCTTCAC	52
IRF7	NM_001572.3	XM_008159582.1	CCCACGCTATACCATCTACCT ; GATGTCGTCATAGAGGCTGTTG	57	CCCGCACTGCACCATCTACCT ; CAGGTCCTCGTACAGGCTGTTG	56
STAT1	NM_007315.3	XM_008146505.1	CAGCTTGACTCAAAATTCCTGGA ; TGAAGATTACGCTTGCTTTTCCT	54	CAGCTGGACGCCAAGTTCCTGGA ; TGCAGGTTGCGCTTGCTCTTCCT	56
STAT2	NM_005419.3	XM_008148645.1	GAGCCAGCAACATGAGATTGA ; GCCTGGATCTTATATCGGAAGCA	53	GAGCCAGCAGCATGAGATCGA ; GCCTGAGTCTTGTATCGGAAGAG	56
TLR3	NM_003265.2	XM_008151907.1	TCAACTCAGAAGATTACCAGCCG ;	53	TCAGCTCAGAAAATTACCGCCTG ;	52

			AGTTCAGTCAAA TTCGTGCAGAA		AGTTCAGTCAAAT TCCGGCAGAA	
TLR7	NM_01 6562.3	XM_00 815657 7.1	TCGTGGACTGCA CAGACAAG ; GGTATGTGGTTA ATGGTGAGGGT	51	TTGTGGACTGCAC GGACAAG ; GGTATGTGGTTGA TGGTGAGGGT	52
TLR8	NM_13 8636.5	XM_00 815657 6.1	GACTACAGGAAG TTCCCCAAAC ; ATACCGGGATTT CCGTTCTGG	51	GGCTGCAAGAAG TCCCCCAAAC ; TTGCAATAATTCT CACAGTAG	52
TLR9	NM_01 7442.3	XM_00 815506 6.1	CTGCCACATGAC CATCGAG ; GGACAGGGATAT GAGGGATTTGG	55	CTGCCACATGACC ATCGAG ; GGCCAGGGTCCG GAGGGCGGGGG	56
TNFα	NM_00 0594.3	XM_00 815736 0.1	CAGCCTCTTCTC CTTCCTGA ; AGATGATCTGAC TGCCTGGG	57	GCCCATGTTGTAG CAAACC ; GCCCTTGAAGAG GACCTGGG	56
IL8	NM_00 0584.3	XM_01 446059 9.1	ACTGAGAGTGAT TGAGAGTGGAC ; AACCCTCTGCAC CCAGTTTTC	51	AAACATGACTTCC AAGCTGG ; TGTGGTCCACTCT CAATCAC	52

IL1β	NM_000576.2	XM_008160259.1	ATGATGGCTTATTACAGTGGCAA ; GTCGGAGATTCGTAGCTGGA	54	ATGATGGCTTACTACAGTGACAA ; GTCGGAGATTTTCAGCTGGA	52
GBP1	NM_002053.2	XM_008157793.1	AGGAGTTCCTTCAAAGATGTGGA ; GCAACTGGACCCTGTCGTT	54	TTCAGCTGACTTTGTGAGCT ; ACTGCTGATGGCATTAACAT	52
IFI6	NM_002038.3	XM_008148008.1	GGTCTGCGATCCTGAATGGG ; TCACTATCGAGATACTTGTGGGT	57	GGTCGGCCATAGCGAACGGG ; TTGTTGTCTATCTGCCTGTGGAC	56
IFI27	NM_206949.2	XM_008159947.1	TGCTCTCACCTCATCAGCAGT ; CACAACTCCTCCAATCACAACT	56	GCCAAGATGATGTCATCAGC ; CACATCCAGGCCCCAATAG	56
IRF1	NM_002198.2	XM_008142488.1	ATGCCCATCACTCGGATGC ; CCCTGCTTTGTATCGGCCTG	56	ATGCCCATCACTCGGATGC ; CCCGACTTTGTACCGGCCTG	56
ISG20	NM_002201.5	XM_008155878.1	TCTACGACACGTCCACTGACA ;	56	CCCGAGACACCTCCCATATTC ;	56

			CTGTTCTGGATG CTCTTGTGC		CCAGCCTGGATGT CCCGGTTC	
MX1	NM_00 114492 5.2	XM_00 814569 1.1	AGGACTACGAGA TTGAGAT ; TTATGCCAGGAA GGTCTA	51	GGACATGGAGAT CAATCTT ; TGATGCCAGGAA GGTCTA	52
OAS1	NM_01 6816.3	XM_00 814291 9.1	AGTTGACTGGCG GCTATAAAC ; GTGCTTGACTAG GCGGATGAG	55	AGGTGACGGACG ACTACAGAC ; GTGCTTGACCAGG CGGATGAG	56
RSAD 2	NM_08 0657.4	XM_00 816243 0.1	TGGGTGCTTACA CCTGCTG ; GAAGTGATAGTT GACGCTGGTT	53	CGAGTACCTGGGC CGCCTG ; GAAGCGGTTGAT GACGGAGTTG	56
RIG-I	NM_01 4314.3	XM_00 814200 5.1	CTGGACCCTACC TACATCCTG ; GGCATCCAAAAA GCCACGG	55	CTGGACCCCACCT ACGTCCTC ; AGCATCCAAAAA GCCACGA	56
MDA 5	NM_02 2168.3	XM_00 813863 1.1	GCCCGCTACATG AACCCTG ; CAGCAATCCGGT TTCTGTCTT	55	GCCCGCTACATGG ACCCCG ; CAGAAACCCGAC TTCTGTCTT	56

GAPDH	NM_002046.5	XM_008144826.1	GGAGCGAGATCCCTCCAAAAT ; GGCTGTTGTCATACTTCTCATGG	57	GGAGCGAGATCCCGCCAACAT ; GGGAGTTGTCATACTTGTCATGG	56
β-actin	NM_001017992.3	XM_008156007.1	CTCGACACCAGGGCGTTATG ; CCACTCCATGCTCGATAGGAT	57	CCCGGCACCAGGGCGTGATG ; CGATGCCGTGCTCGATGGGGT	56
TLR2	NM_003264.4	XM_008144148.1	TTATCCAGCACACGAATACACAG ; AGGCATCTGGTAGAGTCATCAA	52	TTATCCAATACAAGAATACAGAG ; AGGCATCTGGTAGAGTCTTCAA	51
c-Rel	-	XM_008162099.1	-		GACGACTACTCTACCTCCTG ; ATCTTCACCGTCACTGGCTC	56
β2-microglobulin	NM_004048.2	-	GAGGCTATCCAGCGTACTCCA ; CGGCAGGCATACTCATCTTTT	55	-	

Supplementary Table S5. 5. siRNA sequences designed against *E. fuscus* (big brown bat) TLR3, RIG-I and MDA5. r = ribose sugar; BBB = big brown bat.

siRNA sequence	Duplex name	siRNA duplex sequences	Binding position on transcript
BBB TLR3 siRNA sequence	CD.Ri.26282.13.2	5' rCrUrArUrCrUrCrArArCrUrUrUrCrUrGrArCrArArArArCCT 3'	BBB TLR3 - position 337-362
BBB TLR3 siRNA sequence2		5' rArGrGrUrUrUrUrGrUrCrArGrArArArGrUrUrGrArGrArUrArGrCrU 3'	
BBB TLR3 siRNA sequence	CD.Ri.26282.13.1	5' rCrArArCrArUrGrArGrArUrArCrCrUrGrArArUrUrUrArAGA 3'	BBB TLR3 - position 972-997
BBB TLR3 siRNA sequence2		5' rUrCrUrUrArArArUrUrCrArGrGrUrArUrCrUrCrArUrGrUrUrGrArA 3'	
BBB RIG-I siRNA sequence	CD.Ri.26280.13.2	5' rArGrCrUrUrUrCrUrArArArArCrCrUrGrArGrArUrArUrUGA 3'	BBB RIG-I - position 1972-1997
BBB RIG-I siRNA sequence2		5' rUrCrArArUrArUrCrUrCrArGrGrUrUrUrUrArGrArArArGrCrUrGrA 3'	
BBB RIG-I siRNA sequence	CD.Ri.26280.13.10	5' rGrUrGrGrCrArArCrArGrUrCrArArArGrArCrArArCrUrUGG 3'	BBB RIG-I - position 1312-1337

BBB RIG-I siRNA sequence2		5' rCrCrArArGrUrUrGrUrCrUrUrUrGrArCrUrGrUrUrGrCrCrArCrCrA 3'	
BBB MDA5 siRNA sequence	CD.Ri.26279.13.2	5' rArCrUrUrGrGrArCrArArGrArArArArArArGrCrArUrCrUGA 3'	BBB MDA5 - position 1256-1281
BBB MDA5 siRNA sequence2		5' rUrCrArGrArUrGrCrUrUrUrUrUrUrCrUrUrGrUrCrCrArArGrUrGrA 3'	
BBB MDA5 siRNA sequence	CD.Ri.26279.13.1	5' rGrArArGrArUrUrCrUrUrUrUrArArArArCrArUrUrGrUrCAA 3'	BBB MDA5 - position 3301-3326
BBB MDA5 siRNA sequence2		5' rUrUrGrArCrArArUrGrUrUrUrUrArArArArGrArArUrCrUrUrCrArA 3'	

5.10. Transition statement

Pathology during coronavirus infections such as SARS and MERS is associated with acute inflammation. In the above study, we demonstrated that big brown bat and potentially other insectivorous bat cells have evolved a unique mechanism to suppress an over-inflammatory process. However, our studies were carried out using a virus surrogate, poly(I:C). Poly (I:C) is a synthetic analogue of double-stranded RNA and simulates toll-like receptor 3 and cytosolic receptors RIG-I and MDA5 to initiate an innate immune response in the cells. In theory, in an

immunology aspect, poly (I:C) mimics an RNA virus infection in a cell. After having identified the existence of a functional interferon pathway in bat cells, we wanted to further characterize the TLR3 pathway. We were also interested in identifying if the immune response patterns identified above would be reproducible using a 'whole' virus challenge model. We thus decided to study the antiviral interferon pathway in response to infection with MERS-CoV, a risk group 3 pathogen that is speculated to have evolved in insectivorous bats before spilling over to camels and eventually humans. We were interested in observing the virus-host interactions in bat and human cells to identify differences that may explain how bats remain refractory to disease when infected with coronaviruses such as MERS-CoV.

Chapter 6: Learning from an ancestral host: IRF3 mediated signaling is critical for limiting Middle-East respiratory syndrome (MERS) coronavirus propagation in bat cells

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Listed authors: *Arinjay Banerjee, Darryl Falzarano, Jocelyne Lew, Noreen Rapin and Vikram Misra*

6.1. Contribution

My supervisor (Dr. Vikram Misra) and I conceived this study along with intellectual inputs from Dr. Falzarano. I performed all the experiments. Preliminary time point qRT-PCR experiments were performed by Dr. Falzarano and Ms. Lew. Dr. Falzarano helped me with the virus titrations. Ms. Rapin helped optimize antibodies for Western blots. I was trained in the Containment level 3 laboratory at VIDO/InterVac by Dr. Falzarano and Ms. Lew.

6.2. Abstract

Insectivorous bats are speculated to be ancestral hosts of Middle-East respiratory syndrome (MERS) coronavirus (CoV). MERS-CoV causes disease in humans with a thirty-five percent case-fatality rate and has evolved proteins that can effectively counteract human antiviral responses. Since bats experimentally infected with MERS-CoV do not develop visible signs of disease, we tested the hypothesis that the virus would grow less efficiently in bat cells than in

human cells because of its inability to subvert innate antiviral responses in bat cells. We infected human and bat (*Eptesicus fuscus*) cells with MERS-CoV and observed that the virus grew to significantly higher levels in human cells. MERS-CoV also effectively suppressed antiviral interferon beta (IFNβ) response in human cells, but not in bat cells. In contrast, virus yield in interferon response factor 3 (IRF3)-depleted human and bat cells were comparable, suggesting that the IRF3-mediated antiviral innate responses were resistant to subversion by MERS-CoV. To determine if IRF3, a critical mediator of the interferon response in other mammals, also regulated the response in bats, we examined the response of IRF3 to poly(I:C), a synthetic analogue of viral double-stranded RNA. We observed that bat IRF3 responded to poly (I:C) by nuclear translocation and post-translational modifications; hallmarks of IRF3 activation. Suppression of IRF3 by small interfering RNA (siRNA) demonstrated that IRF3 was critical for poly (I:C) and MERS-CoV induced induction of IFNβ in bats cells. Our study demonstrates a unique adaptation in insectivorous bat cells that might explain the ability of bats to co-exist with several coronaviruses.

6.3. Introduction

Bats are ecologically important mammals that are speculated to be reservoirs of several emerging viruses, including coronaviruses (1, 7). Bats have been called global reservoirs of deadly coronaviruses (CoVs) (28) and over 200 different viruses have been isolated or detected in bats (1), with new viruses being detected on a regular basis. Over nine hundred coronavirus sequences from bats have been reported (24) and recently, coronaviruses have been documented to cause serious disease in humans and agricultural animals. These include severe acute respiratory syndrome (SARS)-CoV, Middle-East respiratory syndrome (MERS)-CoV, porcine

epidemic diarrhea virus (PEDV) and swine acute diarrhea syndrome (SADS) coronavirus (8, 9, 43, 45, 207, 235-237). The spill-over of these viruses to susceptible hosts, including humans and agricultural animals, often results in severe disease.

MERS-CoV causes severe respiratory illness in humans, including cough, fever and shortness of breath. Since September 2012, World Health Organization has been notified of 2,090 laboratory-confirmed cases of MERS-CoV infection, with at least 730 deaths. Twenty-seven countries have reported cases of MERS-CoV (238). SARS-CoV caused a pandemic in 2003-04 that killed ten percent of the over 8000 infected individuals (236) and PEDV was responsible for over $300 million dollars in losses to the US swine industry in 2013-14 (239). In many cases, infections with SARS- and MERS-CoVs lead to severe illness including death as only supportive care is available.

Insectivorous vespertilionid bats from the suborder Yangochiroptera have been proposed as an ancestral or primordial source of MERS-CoV as several closely related coronaviruses have been described in these bats (45, 240-242). Although virus-host interaction for MERS-CoV is being studied in human cells (113, 114, 116, 243, 244), little information exists on how MERS-CoV interacts with cells from an ancestral and/or potential reservoir host, such as bats. Moreover, we do not have an understanding of what sort of innate immune response is mounted by bat cells against MERS-CoV.

The innate immune response is the first line of defence against invading pathogens, including viruses. When the pathogen-associated molecular patterns (PAMPs) of a virus are sensed by a

cell, the cell activates various signaling pathways to counteract the ensuing infection. During coronavirus infections and subsequent virus replication, the cell recognizes viral nucleic acid through cellular receptors or pattern recognition receptors (PRRs) and mounts an antiviral response. This response is mediated through interferon regulatory factor 3 (IRF3), a key transcription factor involved in antiviral interferon signaling. IRF3 exists as a monomer in the cytoplasm of human cells. When PRRs are activated in a cell in response to viral nucleic acids, cellular kinases such as TANK-binding kinase 1 (TBK1) phosphorylate IRF3, which then dimerizes and translocates to the nucleus of the cell. Once in the nucleus, phosphorylated IRF3 binds to the promoter of antiviral interferon genes and enhances expression of interferons, such as interferon beta (IFNβ) [reviewed by Kawai *et al.* (66)] (Fig. 6.1).

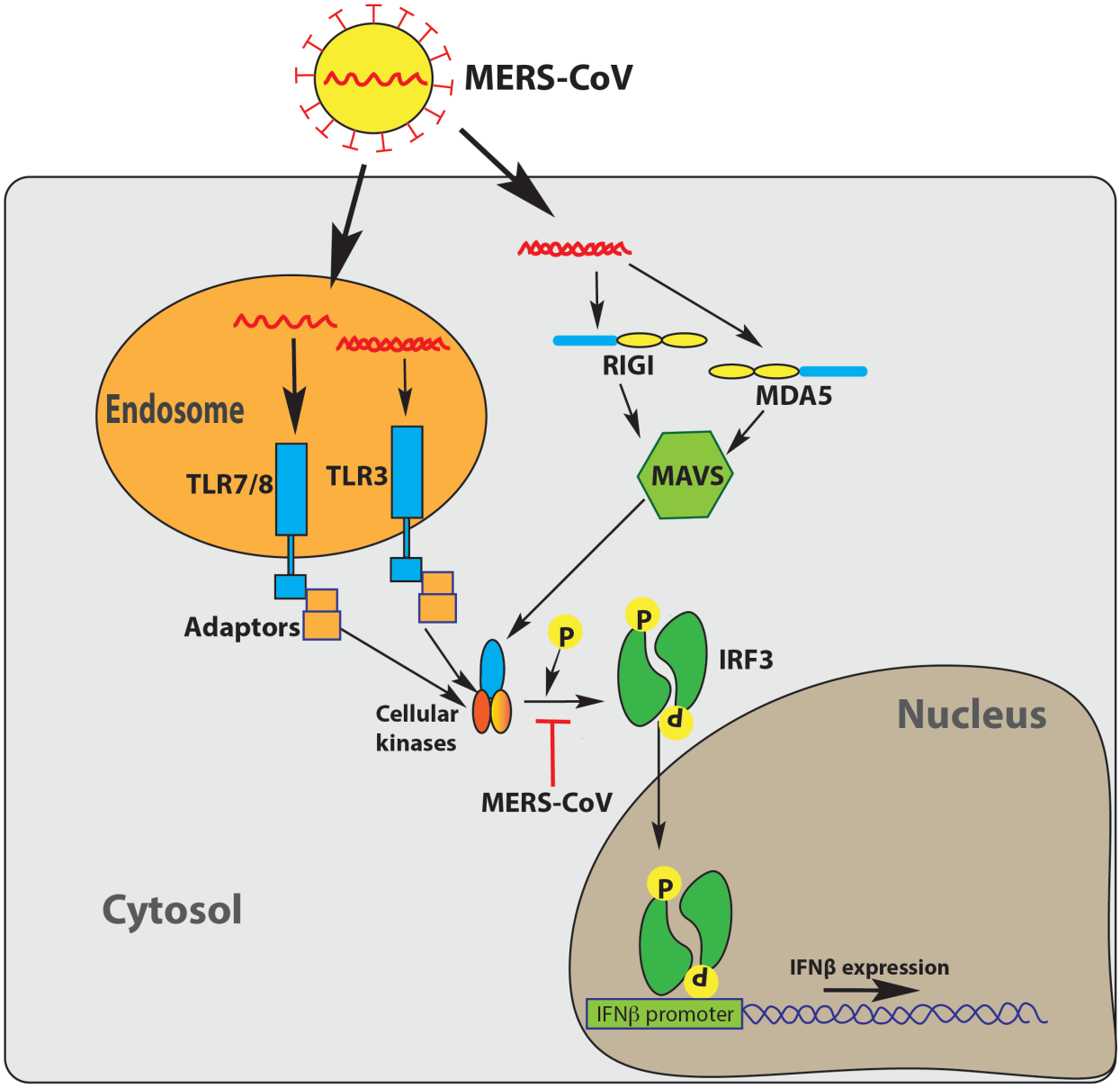

Figure 6. 1. Schematic representation of IRF3 mediated antiviral IFNβ expression and inhibition of IRF3 activation by MERS-CoV. Human cells identify viral single-stranded (ss) and double-stranded (ds) RNA through evolutionary conserved cellular receptors. Endoplasmic Toll-like receptor 3 (TLR3) and cytosolic retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated protein 5 (MDA5) recognize double-stranded (ds) RNA. Endoplasmic TLRs 7 and 8 recognize single-stranded (ss) RNA. These cellular receptors then signal through various adaptor proteins such as mitochondrial antiviral-signaling protein (MAVS), myeloid differentiation primary response 88 (MyD88) and TIR-domain-containing adapter-inducing interferon-β (TRIF). These signals converge to activate cellular kinases such as TANK binding kinase 1 (TBK1) and inhibitor of nuclear factor kappa B kinase subunit epsilon (IKKε). These kinases phosphorylate interferon response factor 3 (IRF3), leading to its dimerization and subsequent localization to the nucleus of the cell to initiate antiviral interferon gene expression.

MERS-CoV proteins such as M, ORF 4a, ORF 4b and ORF 5 inhibit IRF3-mediated activation of antiviral interferon responses in human cells (116).

Interferons are key molecules that activate interferon stimulated genes (ISGs) such as 2'-5'-oligoadenylate synthetase 1(OAS1) and interferon-induced GTP-binding protein Mx1 in infected and neighboring cells [reviewed by Ivashkiv *et al.* (245)]. ISGs inhibit virus replication in these cells through various mechanisms that have been reviewed elsewhere (89).

Viruses have evolved different mechanisms to counteract the antiviral interferon responses [reviewed by de Wit *et al.* and Schulz *et al.* (38, 112)]. Like other viruses, coronaviruses such as SARS-CoV, MERS-CoV and PEDV have evolved different strategies to inhibit interferon signaling in host cells (116, 117, 246, 247). MERS-CoV structural and accessory proteins inhibit interferon production in human cells, predominantly through the inhibition of IRF3 mediated signaling (116) (Fig. 6.1). However, we do not know if MERS-CoV can subvert interferon signaling in bat cells in a similar manner.

Most studies on MERS-CoV-host interactions have been performed in human cells by ectopically expressing MERS-CoV proteins. Little research has been done to study the virus in human cells, largely due to containment requirements. To our knowledge, there are no studies that have looked at the effect of MERS-CoV infection on IRF3-mediated innate immune responses in bat cells. Since vespertilionid bats have been speculated as ancestral hosts of MERS-CoV (45, 48, 132, 240), we tested the hypothesis that MERS-CoV cannot effectively shut-down interferon responses in cells from these bats. To test our hypothesis, we infected big brown bat (vespertilionid bat; *Eptesicus fuscus*) kidney cells (110) and human lung cells with

MERS-CoV. We observed that MERS-CoV propagated to higher levels in human cells than bat cells. Consistent with other studies, human cells infected with MERS-CoV did not express transcripts for IFNβ. However, big brown bat cells infected with MERS-CoV expressed robust amounts of IFNβ transcripts. By performing small interfering RNA (siRNA) mediated knock-down of IRF3, immunofluorescent microscopy and immune blot analysis, we have shown that IRF3 is critical for the poly (I:C) and MERS-CoV-mediated antiviral IFNβ response in bat cells.

Our data suggest that a functional IRF3 signaling pathway in big brown bat cells is critical to limit MERS-CoV replication. This and other antiviral signaling pathways warrant further investigation to identify adaptations that allow bats to mount an antiviral response to a viral infection. Understanding cellular and molecular virus-host interactions in reservoir hosts may help in predicting factors that contribute to the emergence of these viruses from their natural host.

6.4. Materials and Methods

6.4.1. Cell culture and virus infection

Eptesicus fuscus kidney cells (110) (Efk3 or bat cells) were grown in Dulbecco's Minimal Essential Medium with GlutaGro (DMEM; Corning) containing 10% fetal bovine serum (FBS; Sigma), penicillin/streptomycin (Gibco) and 1% GlutaMax (Gibco). Human lung (MRC5) cells (ATCC CCL-171) were cultured in Minimum Essential Medium Eagle (MEM; Corning) supplemented with 10% FBS, 1/100 non-essential amino acids (NEAA; Gibco), 1/100 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; Gibco) and 1/1000 gentamycin (Gibco).

Vero (green monkey kidney) cells were grown in DMEM supplemented with 10% FBS and penicillin/streptomycin. Cells were incubated in a humidified incubator at 37°C with 5% CO_2. For virus infection studies, MRC5 and Efk3 cells were seeded at a concentration of 3 x 10^5 cells/well in a six well plate. Based on the experiment (refer to results), the cells were infected with varying multiplicity of infection (MOI) of MERS-CoV (strain EMC/2012) in a containment level 3 laboratory. After 1 h, the inoculum was removed, cells were rinsed three times with media to remove residual inoculum and fresh complete medium was added on the cells.

6.4.2. Virus titration

MERS-CoV virus infections and titrations were done in a containment level 3 laboratory. For titrating the amount of virus in supernatants from infected MRC5 and Efk3 cells, Vero cells were seeded in 96-well plates at a concentration of 10^5 cells/well in 100 µl of complete media. The plates were incubated at 37°C overnight. Next day, media was taken off the cells and 50 µl of 1:10 serially diluted virus containing supernatant was added to the plates. The plates were incubated at 37°C for 1 h. After incubation, the virus containing supernatant was discarded and 100 µl of complete media was added to the plates. The plates were incubated at 37°C for three days. Cytopathic effect was observed under a microscope. Tissue culture infectious dose 50/ml ($TCID_{50}$/ml) was calculated using the Spearman and Karber algorithm (248, 249).

6.4.3. TLR challenge

MRC5 and Efk3 cells were seeded at a concentration of 3 x 10^5 cells/well in 6 well plates and transfected with 750 ng/ml poly (I:C) (InvivoGen) using Lipofectamine 2000 (Invitrogen) as previously described (106). Briefly, 750 ng/ml poly (I:C) was mixed in a total volume of 250 µl of TransfectaGro (Corning) and 12 µl of lipofectamine 2000. This mixture was incubated at

room temperature for 15 minutes and added to cells in complete medium. Cells were harvested 16h post-transfection and RNA was extracted.

6.4.4. Nucleic acid extraction and qRT-PCR

All RNA extractions were performed using the RNeasy Plus Mini kit (QIAGEN, Germany) as per manufacturer's instructions. cDNA was prepared using iScript gDNA clear kit (Bio-Rad) as per manufacturer's instructions. 500 ng of RNA was used for cDNA preparation. cDNA was used as a template for the quantification of target genes.

qRT-PCR assays targeting respective cellular genes and the normalizer (Glyceraldehyde-3-phosphate; GAPDH) were performed for both MRC5 and Efk3 cells. Primer sequences for human and bat genes have been published before (see supplementary data here (106)). Bio-Rad's CFX96 Touch PCR thermocycler was used in conjunction with Bio-Rad's Ssofast Evagreen supermix (Bio-Rad) and samples were prepared as previously described (18). For qRT-PCR, after the initial denaturation step of 95°C for 5 minutes, two step cycling for 40 cycles was performed at 95°C/10s, 56°C/30s. Absorbance readings were acquired after each cycle. The final three steps were carried out at 95°C/1 min, 55°C/30s and 95°C/30s to generate the dissociation curve. Absorbance readings for the dissociation curve were acquired at every degree from 55-95°C. Relative fold change in gene expression between the two groups of cells (treated/infected and mock treated/infected) was calculated after normalizing the Ct values using GAPDH. Difference of one Ct indicates a two-fold difference in gene expression. Primer sequences can be found in table 6.1 and here (106).

6.4.5. Agarose Gel Electrophoresis

One percent agarose (Invitrogen, USA) gels were prepared using 0.5X TBE [Tris – 1M (VWR), Ethylenediaminetetraacetic acid disodium salt (EDTA) solution – 0.02 M (Gibco) and Boric acid – 1M; pH 8.4]. 1 μl SYBR Safe DNA gel stain (Invitrogen) was added for every 1 ml of gel. 10 μl of PCR or qRT-PCR products were run on the gel for 1h at 105 volts and visualized under an ultraviolet gel imaging system (AlphaImager HP).

6.4.6. Knock-down of IRF3 transcripts in Efk3 and MRC5 cells.

Dicer-ready siRNA (DsiRNA) specific to big brown bat and human IRF3 was designed and obtained through Integrated DNA Technologies (IDT). A 100 nM final concentration of a 1:1 mixture of two DsiRNAs per cell line (Table 6.1) targeting separate regions on the big brown bat and human IRF3 transcript was transfected into Efk3 and MRC5 cells using Lipofectamine 2000. Scrambled non-specific DsiRNA (NC DsiRNA; IDT) was used as a negative control.

Name	Sequence - human (5' - 3')	Sequence - bat (5' - 3')	Feature
IFNβ	GCTTGGATTCCTACAAAGAAGCA	GCTCCGATTCCGACAGAGAAGCA	Forward primer
	ATAGATGGTCAATGCGGCGTC	ATGCATGACCACCATGGCTTC	Reverse primer
GAPDH	GGAGCGAGATCCCTCCAAAAT	GGAGCGAGATCCCGCCAACAT	Forward primer
	GGCTGTTGTCATACTTCTCATGG	GGGAGTTGTCATACTTGTCATGG	Reverse primer
TNF	CAGCCTCTTCTCCTTCCTGA	GCCCATGTTGTAGCAAACC	Forward primer
	AGATGATCTGACTGCCTGGG	GCCCTTGAAGAGGACCTGGG	Reverse primer
OAS1	AGTTGACTGGCGGCTATAAAC	AGGTGACGGACGACTACAGAC	Forward primer
	GTGCTTGACTAGGCGGATGAG	GTGCTTGACCAGGCGGATGAG	Reverse primer
siIRF3 - 1 (Duplex)	5' rGrUrGrGrArGrGrCrArGrUrArCrU rUrCrUrGrArUrArCrCCA 3'	5' rCrArArGrArArGrCrUrArGrUrGrA rUrGrGrUrCrArArGrGTT 3'	r-ribose sugar
	5' rUrGrGrGrUrArUrCrArGrArArGrUrArC rUrGrCrCrUrCrCrArCrCrA 3'	5' rArArCrCrUrUrGrArCrCrArUrCrArC rUrArGrCrUrUrCrUrUrGrGrU 3'	r-ribose sugar
siIRF3 - 2 (Duplex)	5' rArCrUrGrUrGrGrArCrCrUrGrCrArC rArUrUrUrCrCrArACA 3'	5' rCrUrGrCrCrArArCrCrUrGrGrArA rGrArGrGrArArUrUrUCA 3'	r-ribose sugar
	5' rUrGrUrUrGrGrArArArUrGrUrGrCrArG rGrUrCrCrArCrArGrUrArU 3'	5' rUrGrArArArUrUrCrCrUrCrUrUrCrC rArGrGrUrUrGrGrCrArGrGrU 3'	r-ribose sugar

Table 6. 1. Primer and siRNA sequences.

6.4.7. Immunofluorescence

Efk3 and MRC5 cells were seeded at a concentration of 3 x 10^5 cells/well in 6-well plates (Thermo Scientific) with glass cover-slips. Cells were treated with 750 ng/ml poly (I:C) after 24 h and incubated for another 16 h. Media was discarded and cells were rinsed with 2 ml PBS. Cover-slips were transferred to wells containing ice-cold methanol in 6-well plates and incubated for 20 mins in a freezer. Methanol was discarded and cells were washed with PBS. Cells were blocked using a blocking solution [PBS, 10% donor calf serum (Sigma) and 0.1% Tween 20 (USB)]. Primary staining for IRF3 and GAPDH was performed using 1:100 dilution of rabbit anti-IRF3 (Abcam; Catalogue number: ab68481; RRID: AB_11155653) and mouse anti-GAPDH

(EMD Milipore; Catalogue number: AB2302; RRID: AB_10615768). Secondary staining was performed using 4 μg/ml goat anti-mouse Alexa 488 (Molecular Probes; Catalogue number: A-11001; RRID: AB_2534069), 0.1 μg/ml goat anti-rabbit Cy5 (GE Healthcare; Catalogue number: PA45012; RRID: AB_772204) and 0.2 μg/ml Hoechst 33342 (Molecular Probes; Catalogue number: H3570) in blocking solution. Cells were observed under TCS SP5 confocal microscope (Leica).

6.4.8. Cell fractionation

MRC5 and Efk3 cells were seeded at a concentration of 3x 10^5 cells /100 mm plate in 10 ml of media. 24 h after the cells were seeded, cells were mock transfected or transfected with 750 ng/ml poly (I:C) using Lipofectamine 2000. 12 h after transfection, cells from two 100 mm plate/per treatment type were trypsinized and pooled. Cell fractionation was carried out as per manufacturer's recommendation using the NE-PER nuclear and cytoplasmic extraction kit (ThermoScientific).

6.4.9. Western Blots

Efk3 and MRC5 cells were seeded at a concentration of 3x 10^5 cells/well in six well plates and simultaneously transfected with 100 nM of 1:1 cocktail of two different siRNA specific to IRF3 (see table 6.1) and NC siRNA. Cells were transfected with 750 ng/ml poly (I:C) using lipofectamine 2000 (Invitrogen) or mock transfected with lipofectamine 2000. Cells were harvested in sample buffer for western blots 48 hrs post transfections. Western blots were carried out as previously mentioned (227). Briefly, samples were denatured in a reducing sample buffer and run on a reducing gel. Proteins were blotted from the gel onto polyvinylidene difluoride (PVDF) membranes and detected using primary and secondary antibodies. Primary antibodies used were: 1: 1,000 mouse anti-GAPDH (EMD Milipore; Catalogue number: AB2302; RRID:

AB_10615768), 1: 1,000 rabbit anti-IRF3 (Abcam; Catalogue number: ab68481; RRID: AB_11155653), 1:1,000 rabbit anti-calnexin (Santa Cruz Biotechnology; Catalogue number: sc-11397; RRID: AB_2243890) and 1:1000 rabbit anti-Lamin B1 (Abcam; Catalogue number: ab16048; RRID: AB_10107828). Secondary antibodies used were: 1:10,000 goat anti-mouse Alexa 488 (Molecular Probes; Catalogue number: A-11001; RRID: AB_2534069) and 1: 10,000 goat anti-rabbit Cy5 (GE Healthcare; Catalogue number: PA45012; RRID: AB_772204). Blots were observed and imaged using a Typhoon Scanner (Amersham Biosciences).

6.4.10. Phylogenetic analysis

Mammalian IRF3 nucleotide sequences were obtained from National Centre for Biotechnology Information's (NCBI) database (Table S6.1). The evolutionary history was inferred by using the Maximum Likelihood method (1000 Bootstrap) based on the Tamura-Nei model (250). The tree with the highest log likelihood is shown. The percentage of trees in which the associated taxa cluster together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale with branch lengths measured in the number of substitutions per site. The analysis involved 30 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1213 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (251).

6.4.11. Statistics

Significance of the data was determined by two-tailed Mann-Whitney *U* test for non-parametric independent samples using IBM SPSS (Version 21).

6.5. Results

6.5.1. MERS-CoV propagates to lower levels in big brown bat cells.

Since there are few studies on the interactions of MERS-CoV with the innate immune responses in insectivorous bats, the likely ancestral host for the virus, we compared the dynamics and consequence of MERS-CoV infection in big brown bat (Efk3) and human (MRC5) cells. We observed that MERS-CoV propagated to significantly higher levels in human cells than big brown bat cells that were infected with either a low (Fig. 6.2A) or high (Fig. 6.2B) multiplicity of infection (MOI). Cytopathic effect (CPE; cell death) observed as a result of viral infection with a MOI of 10 infectious units/cell was also more pronounced in human cells 24 hours post-infection (hpi) (Fig. 6.2E).

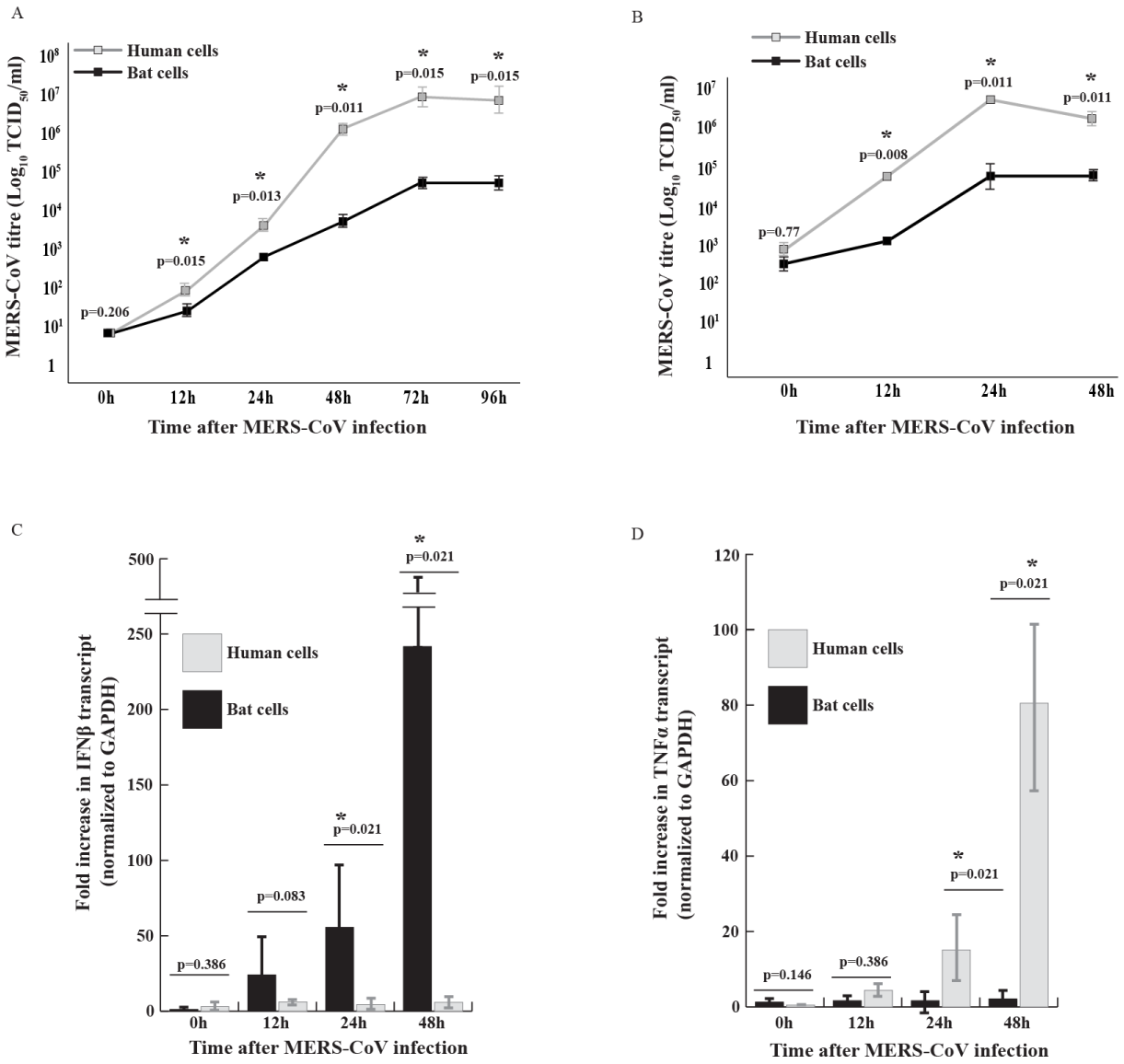
A
Human cells
Bat cells
MERS-CoV titre (Log10 TCID50/ml)
p=0.206
p=0.015
p=0.013
p=0.011
p=0.015
p=0.015
0h
12h
24h
48h
72h
96h
Time after MERS-CoV infection
B
Human cells
Bat cells
MERS-CoV titre (Log10 TCID50/ml)
p=0.77
p=0.008
p=0.011
p=0.011
0h
12h
24h
48h
Time after MERS-CoV infection
C
Fold increase in IFNβ transcript (normalized to GAPDH)
Human cells
Bat cells
p=0.386
p=0.083
p=0.021
p=0.021
0h
12h
24h
48h
Time after MERS-CoV infection
D
Fold increase in TNFα transcript (normalized to GAPDH)
Human cells
Bat cells
p=0.146
p=0.386
p=0.021
p=0.021
0h
12h
24h
48h
Time after MERS-CoV infection
E
Mock infected
MERS-CoV infected (24 hpi)
Human cells (MRC5)
Bat cells (Efk3)

Figure 6. 2. MERS-CoV replication is attenuated in bat cells and does not inhibit an IFNβ response in these cells. To assess if MERS-CoV would replicate at the same rate in human (MRC5) and bat (Efk3) cells, we infected both cell types with MERS-CoV and assessed viral replication at several time-points (by $TCID_{50}$/ml). Transcript levels for IFNβ and TNFα were quantified by qRT-PCR at the indicated time-points. (A) MERS-CoV replication in human and bat cells that were infected with a low multiplicity of infection (MOI) of 0.01 (mean±SD, n=3). (B) MERS-CoV replication in human and bat cells that were infected with a high MOI of 10 (mean±SD, n=3). (C) IFNβ transcript levels at different times after MERS-CoV infection in bat and human cells (mean±SD, n=4). (D) TNFα transcript levels at several time points in MERS-CoV infected human and bat cells (mean±SD, n=4). (E) Cytopathic effects (CPE) observed in human and bat cells twenty-four hours after MERS-CoV infection (MOI = 10). qRT-PCR results are represented as fold increases over mock-infected cells, normalized to GAPDH values (see Methods). Statistical significance was calculated using Mann-Whitney *U* test for two independent samples. SD = standard deviation. n = number of biological replicates.

6.5.2. In contrast to human cells, MERS-CoV induces IFNβ transcripts in bat cells

MERS-CoV, like other viruses, has evolved strategies to counteract the cellular defensive responses (Fig. 6.1) (38, 116). To determine if MERS-CoV could efficiently shut-down antiviral interferon responses in bat cells, we infected big brown bat kidney cells and human lung cells with MERS-CoV at a MOI of 10 tissue culture infectious dose 50 ($TCID_{50}$)/cell. We extracted RNA from these cells at 0, 12, 24 and 48 hpi and determined fold increase in IFNβ transcripts in infected cells relative to mock infected cells after normalization with transcripts for glyceraldehyde-3-phosphate (GAPDH). We observed that MERS-CoV infection did not lead to an increase in IFNβ transcripts in human cells (Fig. 6.2C). However, MERS-CoV infection produced a significant increase in IFNβ transcripts at later time points of 24 and 48hpi in bat cells (Fig. 6.2C).

Infection with MERS-CoV is associated with a delayed but an exaggerated inflammatory response (39). We have previously shown that big brown bat cells actively suppress a strong inflammatory response when treated with poly(I:C). In this study, we also tested the ability of

big brown bat cells to suppress MERS-CoV mediated expression of TNFα, a key systemic inflammatory cytokine. We observed that human cells infected with MERS-CoV expressed significantly higher levels of TNFα transcripts compared to big brown bat cells (Fig. 6.2D).

6.5.3. IRF3 localizes in the nucleus of big brown bat cells in response to poly (I:C)

Since bat cells showed an increase in IFNβ transcripts after MERS-CoV infection, we hypothesized that IRF3-mediated signaling in big brown bat cells, unlike in humans cells, is resistant to MERS-CoV mediated subversion. However, there is currently no information about the role of IRF3 in interferon signaling in bats. To test if IRF3 is critical for an interferon response in big brown bat cells, we treated bat cells with poly(I:C), a synthetic double-stranded analogue of viral RNA and observed the cellular location of IRF3 by immunofluorescent microscopy. In both human and bat cells, IRF3 localized to the nucleus of the cells after poly (I:C) treatment; a hallmark of IRF3 activation (Fig. 6.3A). We separated bat and human cells treated with poly (I:C) into cytoplasmic and nuclear fractions. We performed immune blots for phosphorylated IRF3 (pIRF3) but none of the commercial phospho-IRF3 antibodies that we purchased cross-reacted with the big brown bat pIRF3. However, numerous studies have shown that pIRF3, when detected with an anti-IRF3 antibody, displays several apparent higher molecular weight bands with a lower electrophoretic mobility than the unmodified IRF3 (laddering effect) (252-255). We observed a similar laddering effect with IRF3 in the nuclear fractions of poly (I:C) treated human and bat cells (Fig. 6.3B; arrow). In the figure, cytoplasmic fraction is stained for calnexin, an integral protein of endoplasmic reticulum and nuclear fractions are stained for lamin, a nuclear membrane protein.

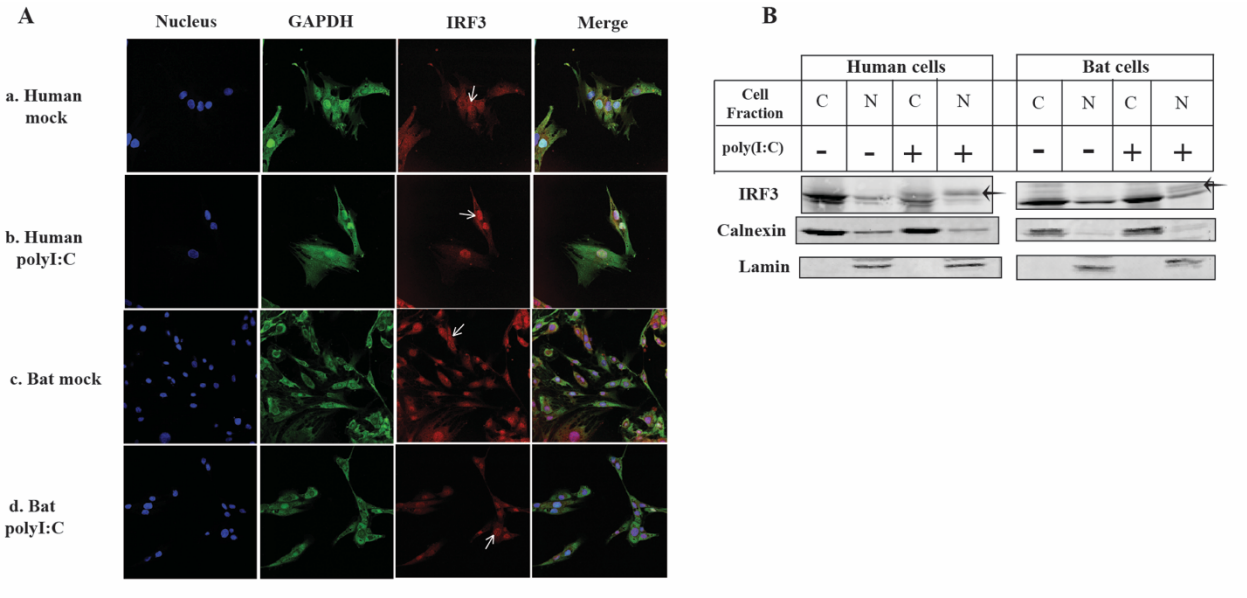

Figure 6. 3. Human and bat IRF3 localizes to the nucleus of the cell following poly (I:C) treatment. To determine if bat IRF3 responded to poly (I:C)-mediated activation by post-translational modification and nuclear translocation like human IRF3, we performed immunofluorescent microscopy and immunoblots on poly (I:C)-treated and mock-treated cells. (A) The cellular location of endogenous IRF3 in mock and poly (I:C) treated human and bat cells. IRF3 is stained red. GAPDH is stained green to highlight the cellular cytoplasm and the nucleus is stained blue. (B) Immune blots of the nuclear and cytoplasmic fractions of mock and poly (I:C) treated human and bat cells. C = cytoplasmic fraction, N = nuclear fraction, black arrow = higher molecular weight IRF3, calnexin = cytoplasmic marker and lamin = nuclear marker.

6.5.4. IRF3 is critical for antiviral interferon beta (IFNβ) production in big brown bat cells

To determine the role of IRF3 in interferon signaling in big brown bat cells, we knocked-down IRF3 in human and bat cells using siRNA and quantified the increase in IFNβ transcripts after poly (I:C) treatment by quantitative real-time polymerase chain reaction (qRT-PCR). We confirmed the reduction in expression of IRF3 in IRF3-specific siRNA-treated bat and human cells cells by performing immune blots (protein panel; Figs 6.4A and 6.4B). We observed that knocking-down IRF3 in both human and bat cells significantly reduced the expression of IFNβ transcripts in response to poly (I:C) in these cells relative to cells treated with non-specific

negative control siRNA (ncsiRNA; Figs 6.4A and 6.4B). We analysed qRT-PCR products on an agarose gel to support the qRT-PCR results (qPCR panel; Fig. 6.4). We also noticed in the immune blots that poly (I:C) treatment alone reduced the amount of IRF3 in mock siRNA (ncsiRNA) treated human cells (protein panel; Fig. 6.4B). We did not observe this phenomenon in bat cells (protein panel; Fig. 6.4A).

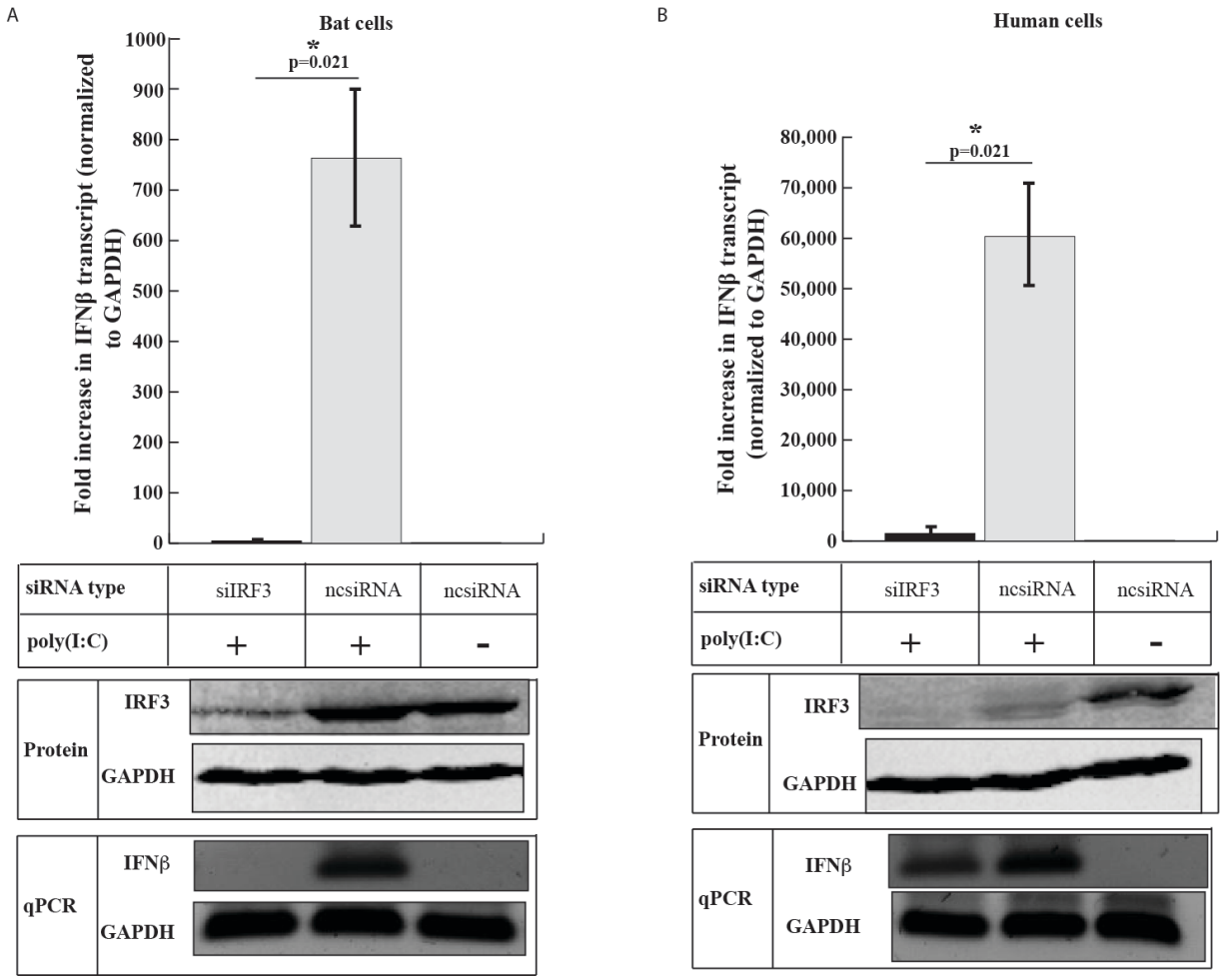

Figure 6. 4. IRF3 is required for IFNβ signaling in response to poly (I:C) in human and bat cells. To determine the role of IRF3 in antiviral interferon signaling, we partially knocked-down IRF3 in both bat (Efk3) and human (MRC5) cells and quantified the increase in IFNβ transcripts in these cells in response to poly (I:C) by quantitative real-time PCR (qRT-PCR). (A) Fold increase in IFNβ transcript levels in IRF3 knocked-down bat cells (siIRF3) and negative control siRNA (ncsiRNA) treated cells on stimulation with poly (I:C) (mean±SD, n=4). (B) Fold increase in IFNβ transcript levels in IRF3 knocked-down human cells (siIRF3) and negative control siRNA (ncsiRNA) treated cells on stimulation with poly (I:C) (mean±SD, n=4). IRF3

knockdown was confirmed by immune blots (protein panel) and qRT-PCR products were analysed on a gel to support the mean fluorescence (Ct) data (qPCR panel). Results are represented as fold increases over mock poly (I:C) transfected cells, normalized to GAPDH values (see Methods). Statistical significance was calculated using Mann-Whitney *U* test for two independent samples. *P<0.05. SD = standard deviation. n = number of biological replicates.

6.5.5. IRF3 mediated signaling plays a critical role in inhibiting MERS-CoV propagation in big brown bat cells

To determine if IRF3-mediated antiviral signaling suppressed MERS-CoV replication in big brown bat cells, we knocked-down IRF3 in both bat and human cells using siRNA and infected them with MERS-CoV. The knockdown of IRF3 in human and bat cells was confirmed by immune blots (Fig. 6.5A; protein panel). Knocking-down IRF3 significantly increased the virus titre in bat cells 48hpi. The increase in virus titre in human cells was not significant (Fig. 6.5A). Knocking-down IRF3 significantly reduced the expression of IFNβ transcripts in virus infected bat cells (Fig. 6.5B) and increased virus produced at 48 hpi to levels similar to virus-infected human cells (Fig. 6.5A). IRF3-reduced bat cells also displayed dramatic cytopathic effect (Fig. 6.5D) compared to scrambled or ncsiRNA treated control cells. Consistent with the IFNβ transcript expression pattern, there was a decrease in 2', 5'-oligoadenylate synthase 1 (OAS1) transcripts in IRF3 knocked-down bat cells infected with MERS-CoV (Fig. 6.5C). The effects of reducing IRF3 in human cells was not as dramatic as in bats cells (Figs. 6.5A, 6.5B, 6.5C, and 6.5D), most likely due to the fact that MERS-CoV had already effectively suppressed IRF3 signaling in these cells.

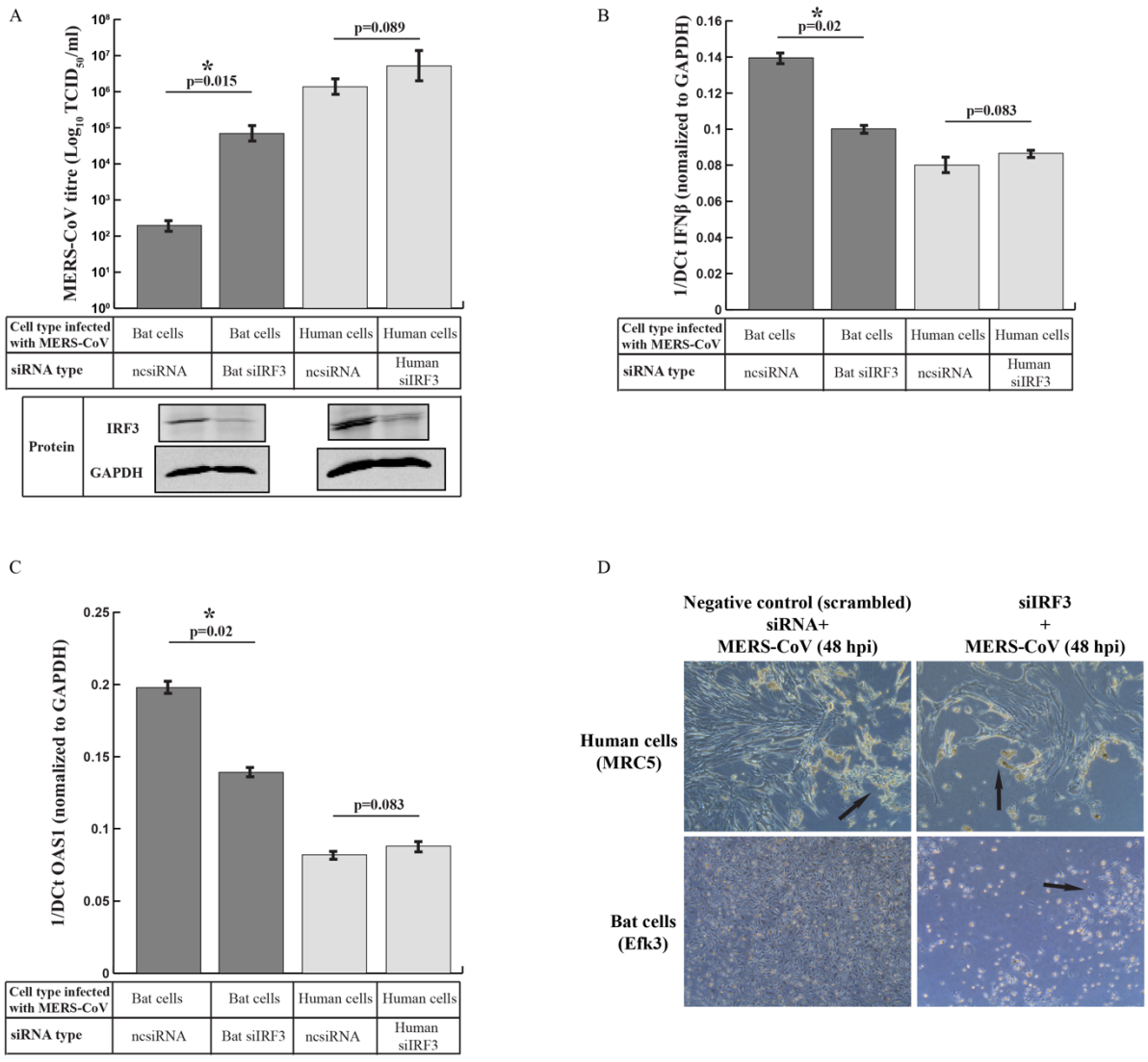

Figure 6. 5. Knock-down of IRF3 increases MERS-CoV titre and decreases IFNβ transcripts in infected big brown bat cells. To determine if IRF3 mediated signaling is crucial for limiting MERS-CoV replication in big brown bat (Efk3) cells, we knocked-down IRF3 in bat and human (MRC5) cells and infected them with MERS-CoV. A multiplicity of infection (MOI) of 0.01 infectious unit/cell was used for virus propagation studies and an MOI of 10 infectious units/cell were used for cytokine studies. We quantified virus replication in these cells by $TCID_{50}$ assay 48 hours post infection (hpi) and transcripts for IFNβ and OAS1 by qRT-PCR 24hpi. (A) MERS-CoV titres in human and bat cells that were either treated with siIRF3 (IRF3 knockdown) or ncsiRNA (mock/no IRF3 knockdown) 48hpi (mean±SD, n=4). IRF3 knockdown in human and bat cells by siRNA was confirmed by immunoblotting (protein panel). (B) IFNβ transcript levels in siIRF3 (IRF3 knockdown) or ncsiRNA (mock/no IRF3 knockdown) treated bat and human cells infected with MERS-CoV (mean±SD, n=4). (C) Interferon stimulated gene, OAS1, transcript levels in siIRF3 (IRF3 knockdown) or ncsiRNA (mock/no IRF3 knockdown) treated bat and human cells infected with MERS-CoV (mean±SD, n=4). (mean±SD, n=4). (D) Cytopathic effect (CPE) observed in siIRF3 (IRF3 knockdown) or ncsiRNA (mock/no IRF3

knockdown) treated bat and human cells 48hpi with MERS-CoV. qRT-PCR results are represented as fold increases over mock siRNA transfected cells, normalized to GAPDH values (see Methods). Statistical significance was calculated using Mann-Whitney *U* test for two independent samples. *P<0.05. SD = standard deviation. n = number of biological replicates.

6.6. Discussion

Bats are speculated to be evolutionary hosts for MERS-CoV (45, 240), an emerging coronavirus that continues to cause outbreaks in the Kingdom of Saudi Arabia. Several coronaviruses have been detected in bats in the absence of apparent disease and Munster *et al.* have shown that Jamaican fruit bats experimentally inoculated with MERS-CoV do not show clinical signs of disease (9). This suggests that, at least in some bat species the effects of infection are not as dramatic as in humans. In their study, Munster *et al.* reported an interferon response in MERS-CoV infected fruit bats, but did not explore the mechanisms that might be involved. We have studied the molecular interactions of MERS-CoV with innate and intrinsic responses in bat cells to determine if and why bat cells are resilient to MERS-CoV mediated shutdown of antiviral signaling.

A recent study identified dipeptidyl peptidase 4 (DPP4), the receptor for MERS-CoV infection in the bat *Eptesicus serotinus*. Although DPP4 is present in the respiratory tract tissue of humans, in *E. serotinus,* an insectivorous bat from the same genus as *E. fuscus* (big brown bat), DPP4 is present in the kidney and the intestinal tract. The authors suggest that insectivorous bats likely transmit MERS-like-CoVs via the fecal-oral route (148). We have previously shown that *E. fuscus* kidney cells (Efk3) are susceptible to MERS-CoV infection (110). We infected bat kidney cells (Efk3) and human lung (MRC5) cells with MERS-CoV and observed that the virus grew to lower titres in bat cells than human cells (Figs 6.2A and 6.2B). We also observed an increase in

IFNβ transcripts in MERS-CoV infected bat cells in contrast to human cells (Fig. 6.2C). Our observation describes the inability of MERS-CoV to shut-down interferon responses in an insectivorous bat cell line. Our data support the observation by Munster *et al.* (9) where MERS-CoV infected Jamaican fruit bats did not display clinical signs of disease and the bats demonstrated an increase in IFNβ transcripts.

Infection with MERS- and SARS-CoVs leads to an over induction of pro-inflammatory cytokines. This 'cytokine storm' causes massive infiltration of white blood cells and an over-amplification of the inflammatory response, which in turn damages host tissue (39, 40, 214). Tumor necrosis factor alpha (TNFα) is a key pro-inflammatory cytokine. We have previously shown that the synthetic double-stranded RNA surrogate, poly (I:C) does not induce a strong TNFα response in big brown bat cells, unlike in human cells (106). We observed a similar response on infecting human and big brown bat cells with MERS-CoV. Human cells had significantly higher levels of TNFα than bat cells (Fig. 6.2D). Big brown bats can probably limit an excessive TNFα response when infected with MERS-like-CoVs and potentially other bat CoVs. In future, *ex vivo* studies with bat immune cells and *in vivo* studies in bats will shed more light on evolutionary adaptations that allow them to co-exist with their viruses.

IRF3 is a key transcription factor that is involved in innate antiviral signaling and mediates antiviral interferon production in cells infected with viruses (86). As such, several viruses have evolved mechanisms to shut down signaling through IRF3 (38, 112). We and others have shown that bat cells can produce IFNβ in response to viral infection and poly (I:C) treatment (57, 97, 110, 111, 134). However, the role of IRF3 in the interferon signaling pathway in bats is largely

unknown. In this study, we have shown that IRF3 is critical for antiviral IFNβ expression in big brown bat cells in response to poly (I:C) and MERS-CoV (Figs 6.4A and 6.5B). We have also shown that big brown bat IRF3 demonstrates classical signs of activation in the presence of an activation signal i.e. poly (I:C) by translocating to the nucleus after undergoing post-translational modifications (Figs 6.3A and 6.3B). In Figure 6.3B, although cytoplasmic fractions (C) are free of nuclear contamination (lamin), the nuclear fractions (N) exhibit some cytoplasmic (calnexin) carry-over. This however does not affect the observation that increased amounts of higher molecular weight band is present in the nuclear fractions of poly (I:C) treated human and bat cells.

Several studies have shown that MERS-CoV has evolved multiple proteins that can effectively inhibit IRF3 mediated antiviral signaling in human cells (38, 113, 114, 116). Since MERS-CoV infection led to an increase in IFNβ transcripts in bat cells unlike in human cells, we compared different mammalian IRF3 nucleotide sequences to determine their similarity. We observed that bat IRF3 nucleotide sequences clustered separately from human and non-human primate IRF3 sequences (Fig. 6.6). In fact, bat IRF3 sequences seem to have evolved from a common ancestor for bats and felines. Bat and feline IRF3 sequences possibly evolved from a common ancestor shared by camels (Fig. 6.6), the reservoir of MERS-CoV (49, 237, 241). Bats have been hypothesized to be persistently infected with coronaviruses (50) and besides bats, cats have been shown to be persistently infected with enteric coronaviruses (256, 257). There is indeed a need to explore the role of IRF3 in persistent infections.

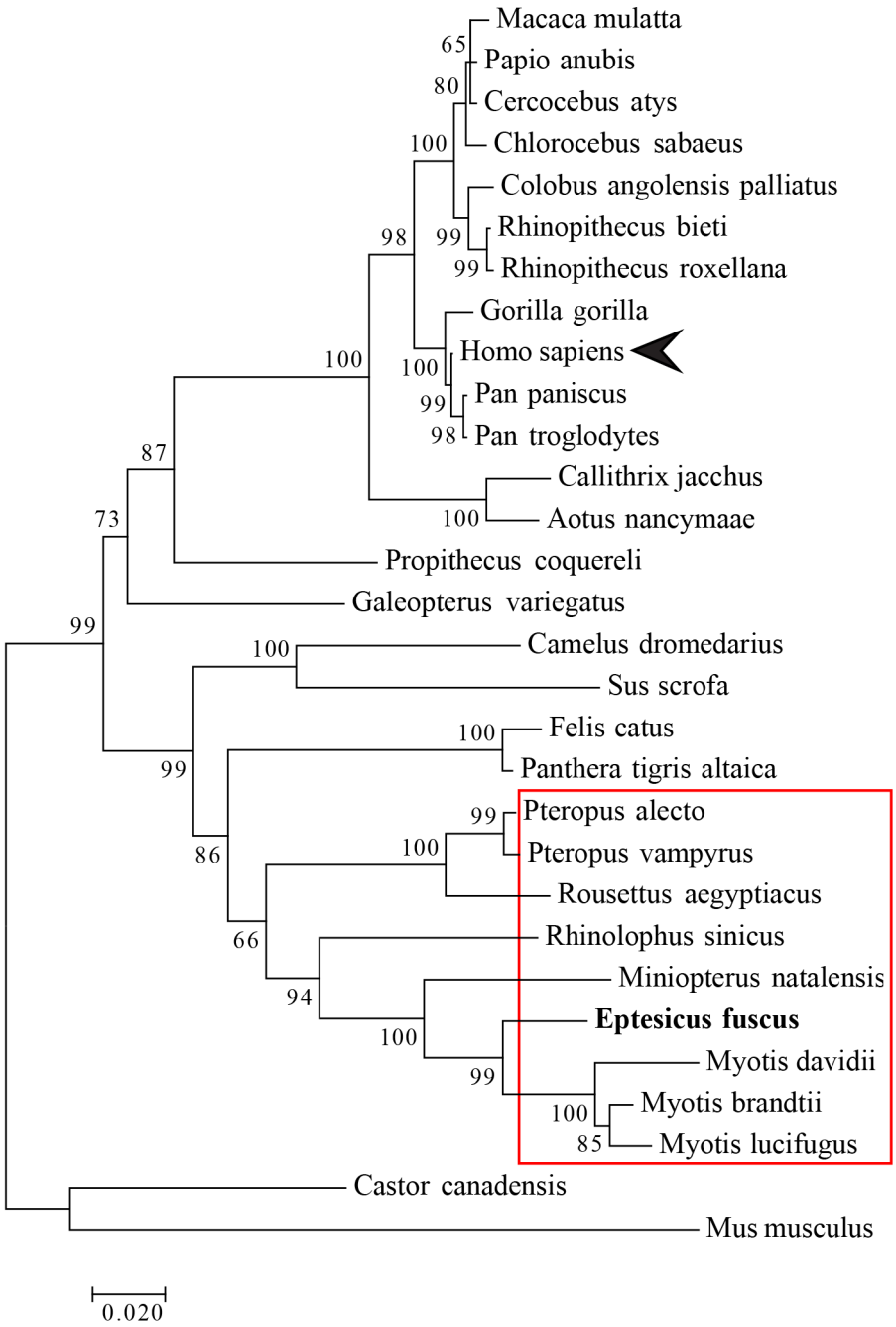

Figure 6. 6. Bat IRF3 nucleotide sequences are divergent from their human counterpart. IRF3 nucleotide sequences for several other mammals and big brown bat IRF3 sequence (bold) were aligned to identify similarities. The maximum likelihood tree for IRF3 nucleotide sequences (1000 Bootstrap) is represented here. Bar represents nucleotide substitution per site. For IRF3 nucleotide sequence information, see supplementary table S6.1.

We noted that after poly (I:C) treatment, there was a degradation of IRF3 in human cells unlike in big brown bat cells [Fig. 6.4; ncsiRNA and poly (I:C) treatment groups]. Saitoh *et al.* have shown that peptidyl-prolyl cis-trans isomerase NIMA-interacting 1 (Pin1) negatively regulates IRF3 in human cells. Pin1 selectively ubiquitinates and marks phosphorylated human IRF3 for degradation, thus limiting IRF3 mediated signaling (258). We did not observe a similar degradation of IRF3 in big brown bat cells. Preliminary data indicate that IRF3 in poly (I:C) treated bat cells degrade slower than poly (I:C) treated human cells (Fig S6.1). A delayed or absent negative feedback mechanism in bat cells might explain why bat cells have an unusual constitutive expression of IFNα (97), which is speculated to prime the bat antiviral responses. More work is needed to confirm the role of IRF3 in constitutive IFN expression in bats.

Knock-down of IRF3 in MERS-CoV infected big brown bat cells decreased IFNβ transcripts with a concomitant increase in levels of MERS-CoV (Figs 6.5B and 6.5A) suggesting that IRF3 signaling was responsible for suppressing replication of the virus in bat cells. However, there could be other signaling molecules or transcription factors in bat cells that activate IFNβ expression and more studies are required to completely dissect the IFNβ expression pathway in bats. Knocking down IRF3 in human cells did not have an effect on the levels of IFNβ transcript or MERS-CoV replication (Figs. 6.5B and 6.5A). This is probably because MERS-CoV can effectively shutdown IRF3-mediated expression of IFNs in human cells and the inclusion of siRNA did not significantly further suppress it. We observed more OAS1 transcripts in cells that expressed higher levels of IFNβ transcripts. OAS1 is an ISG and its expression is largely driven by IFN expression. This data further supports our observation that IRF3 is critical for IFN and subsequent downstream gene expression in big brown bat cells.

Our observation that IRF3 plays a critical role in controlling MERS-CoV propagation in big brown bat cells opens up a new line of investigation. Our data suggest that although MERS-CoV can effectively suppress an antiviral IFNβ response in human cells, it is unable to subvert antiviral interferon responses in big brown bat cells (Fig. 6.7). This study represents yet another unique observation in bat cells that may explain the ability of bats and their viruses to co-exist. This is in addition to other recent developments in the field of virus-bat interactions, such as a dampened stimulator of interferon gene (STING) mediated response in *Pteropus alecto* cells (108).

IRF3 is activated as a result of phosphorylation by cellular kinases and in the future, as more bat-specific reagents become available, it will be interesting to identify kinases in bat cells that phosphorylate bat IRF3. Knock-in studies to explore the resilience of bat kinases to MERS-CoV mediated inactivation in human cells, and the ability of bat kinases to activate human IRF3 will lead to the identification of novel antiviral signaling molecules.

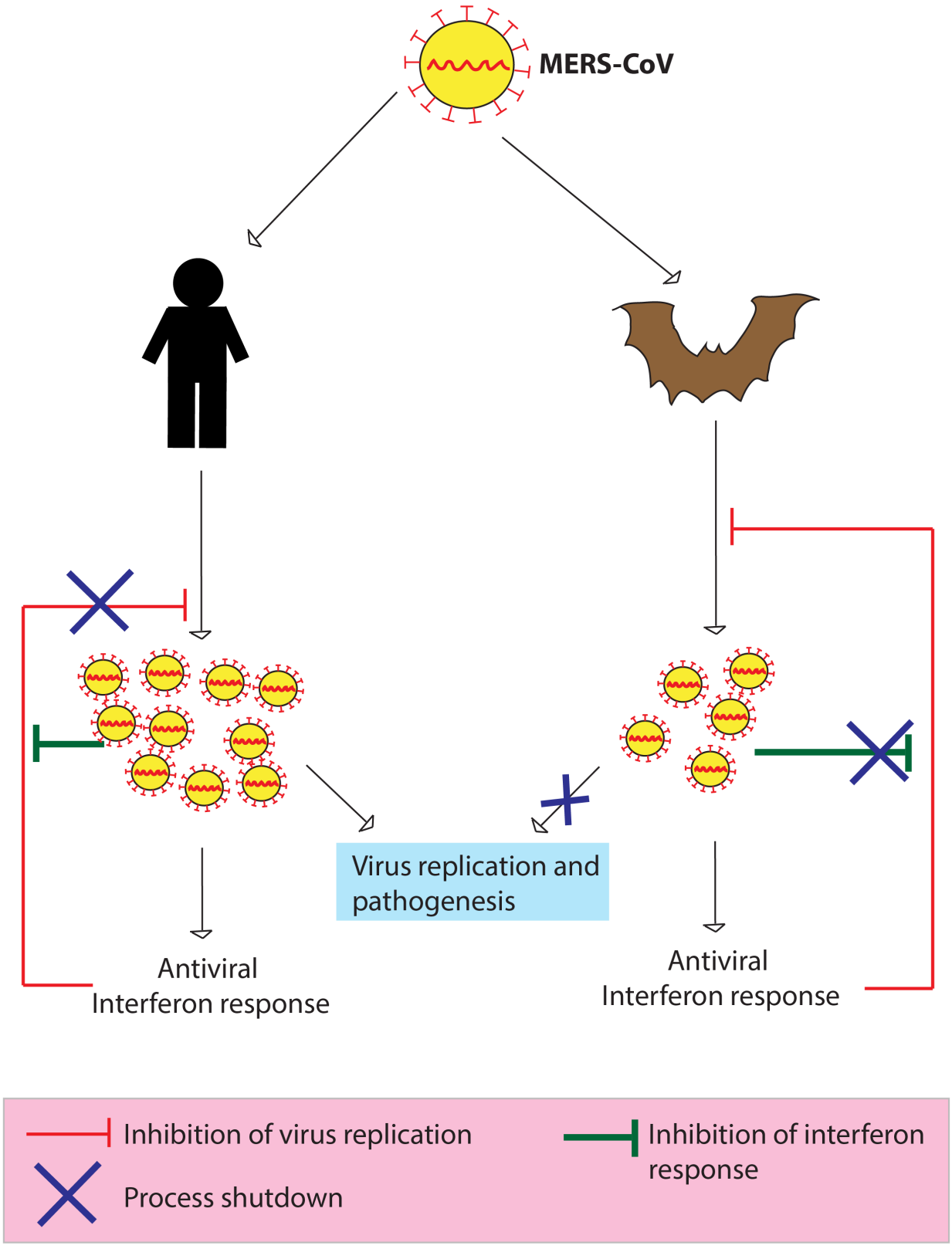
MERS-CoV
Virus replication and pathogenesis
Antiviral Interferon response
Antiviral Interferon response
Inhibition of virus replication
Process shutdown
Inhibition of interferon response

Figure 6. 7. Big brown bat cells resist MERS-CoV mediated shut-down of antiviral interferon responses. MERS-CoV can infect both human and bat cells. This virus has evolved proteins that can effectively suppress an antiviral interferon beta response in human cells allowing it to propagate to higher levels in human cells, which in-turn may cause increased pathogenesis and severe disease. In bat cells, MERS-CoV propagates to lower levels. Bat cells can mount an effective antiviral interferon beta response when infected with MERS-CoV, which in-turn suppresses virus replication in these cells. The ability of bat cells to control MERS-CoV propagation is a first step in understanding how bats might control infection with these viruses and their ability to co-exist with coronaviruses worldwide.

Bats make up over 1200 different species and in the past we have noticed differences in the genetic makeup between bat species (106). It is critical to carry out these studies in cells from different bat species to identify species-specific adaptations in virus-host interactions (259). As more knowledge about virus-host interactions in this intriguing mammalian Order become available, we may be able to adapt some of these strategies to identify new therapeutic targets or molecules to treat spill-over species such as humans and agricultural animals. Emerging viruses from wildlife is a continuing problem and learning about how ancestral hosts and reservoir species co-exist with some of these viruses will help us develop prevention and treatment strategies for other species.

6.7. Supplementary Information

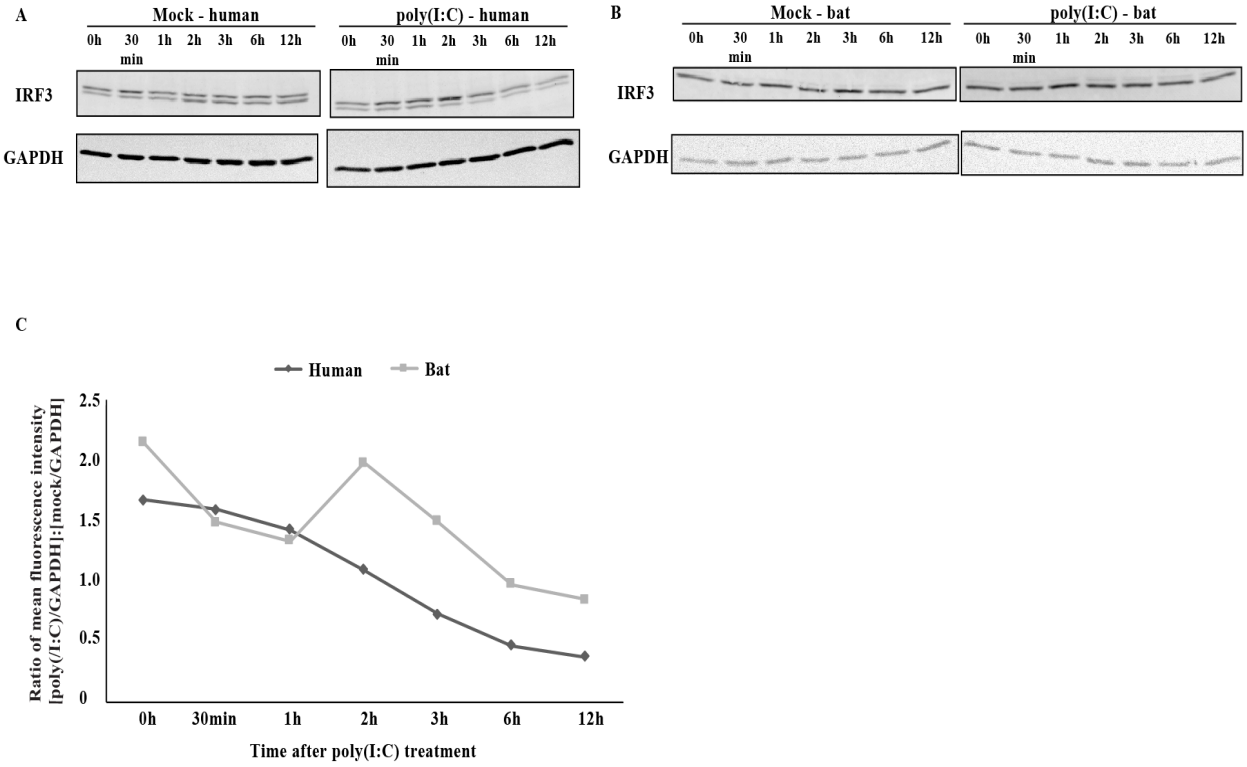

Figure S6. 1. IRF3 persists longer in poly (I:C) treated big brown bat cells than human cells. To determine if the degradation kinetics (i.e. negative feedback) of IRF3 differed in human (MRC5) and big brown bat (Efk3) cells, we seeded bat and human cells in 6-well plates at a concentration of $3x10^5$ cells/well. Next day, when the cells were 70% confluent, 750 ng/ml poly (I:C) was transfected in each well using Lipofectamine 2000. Cells were harvested at 0, 30 min, 1h, 2h, 3h, 6h and 12h post poly (I:C) transfection in sample buffer and separated on a reducing gel. IRF3 was detected by immune blotting. The mean fluorescence intensity of IRF3 and GAPDH in each sample was quantified using SoftMax Pro 7 (Molecular Devices) and adjusted for background. The ratio of IRF3 and respective GAPDH intensity in poly (I:C) treated samples were normalized by the ratio of IRF3 and the respective GAPDH intensity in mock treated samples. (A) There was a visible decrease in the amount of IRF3 in poly (I:C) treated human cells at later time points, compared to bat cells that were treated with poly (I:C). There was no decrease in IRF3 in mock transfected human and bat cells. (B) The amount of IRF3 ((poly (I:C) IRF3)/(poly (I:C) GAPDH) : (mock IRF3)/(mock GAPDH)) in poly (I:C) treated human cells decreased with time. The ratio of mean fluorescent intensity of IRF3 in poly (I:C) treated bat cells increased between 1 and 2hrs post treatment and remained higher than human cells at later time points.

Supplementary Table S6. 1. Accession numbers of IRF3 nucleotide sequences.

Species name	Accession
Aotus nancymaae	XM_012460874
Callithrix jacchus	XM_002762377
Camelus dromedarius	XM_010993178
Castor Canadensis	XM_020159968
	NM_001305969
Cercocebus atys	XM_012080898
Chlorocebus sabeaus	XM_007997589
Colobus angolensis palliatus	XM_011946361
Eptesicus fuscus	XM_008154348
Felis catus	XM_003997503
Galeopterus variegatus	XM_008575391
Gorilla gorilla	XM_019015447
Homo sapiens	NM_001571
	NM_001135797
Macaca mulatta	XM_001115379
Miniopterus natalensis	XM_016206049
Mus musculus	NM_016849
Myotis brandtii	XM_014550465
Myotis davidii	KU161111
Myotis lucifugus	XM_014449832
Pan paniscus	XM_003814337
Pan troglodytes	XM_016936566
Panthera tigris altaica	XM_007074177
Papio anubis	XM_009194993
Propithecus coquereli	XM_012643169
Pteropus alecto	XM_006905022
Pteropus vampyrus	XM_011374528
Rhinolophus sinicus	XM_019741528
Rhinopithecus bieti	XM_017882166
Rhinopithecus roxellana	XM_010369491
Rousettus aegyptiacus	XM_016122379
Sus scrofa	NM_213770

Chapter 7: General Discussion

7.1. Summary

Multiple studies have now shown that bats are potential reservoirs of several viruses, including coronaviruses that cause significant disease and mortality in humans and agricultural animals (1, 7, 20, 23, 24, 28, 43, 122, 159, 241). Although some viruses such as Marburg virus, Nipah and Hendra viruses have been successfully isolated from bats, coronavirus strains that caused SARS, MERS and PEDV are yet to be isolated from bats. Viruses very similar to these coronaviruses have been detected in bats, but there is always a chance that bats are not reservoirs but rather ancestral hosts for these viruses. However, it is still intriguing how bats as mammals can successfully co-exist with some of these viruses that are often lethal in other mammalian species such as humans. These survival strategies also hint at co-evolutionary adaptations between coronaviruses and their hosts i.e. bats.

MERS-CoV is speculated to have evolved and spilled over from insectivorous bats. MERS-CoV induces a strong self-damaging inflammatory response in infected humans (39) and counteracts antiviral immune responses in human cells (113, 114, 116, 243) to cause serious disease. My doctoral thesis elucidates some unique mechanisms that big brown bat cells have evolved to abrogate virus propagation and possibly associated disease. My thesis provides definitive clues about the unique mechanism that big brown bat cells and possibly other bat species have evolved to suppress coronavirus mediated inflammation, the primary cause of poor prognosis and severe disease in SARS and MERS human patients (38). My thesis concludes with the observation that a key antiviral signaling pathway that is known to be suppressed by MERS-CoV in human cells,

remains active in big brown bat cells. Thus, to summarize, my thesis identifies two unique adaptations to virus infection in big brown bat cells: (i) big brown bat cells very effectively control an exaggerated virus-mediated inflammatory response and (ii) big brown bat cells are resistant to MERS-CoV mediated modulation of the antiviral signaling pathway (Fig. 7.1).

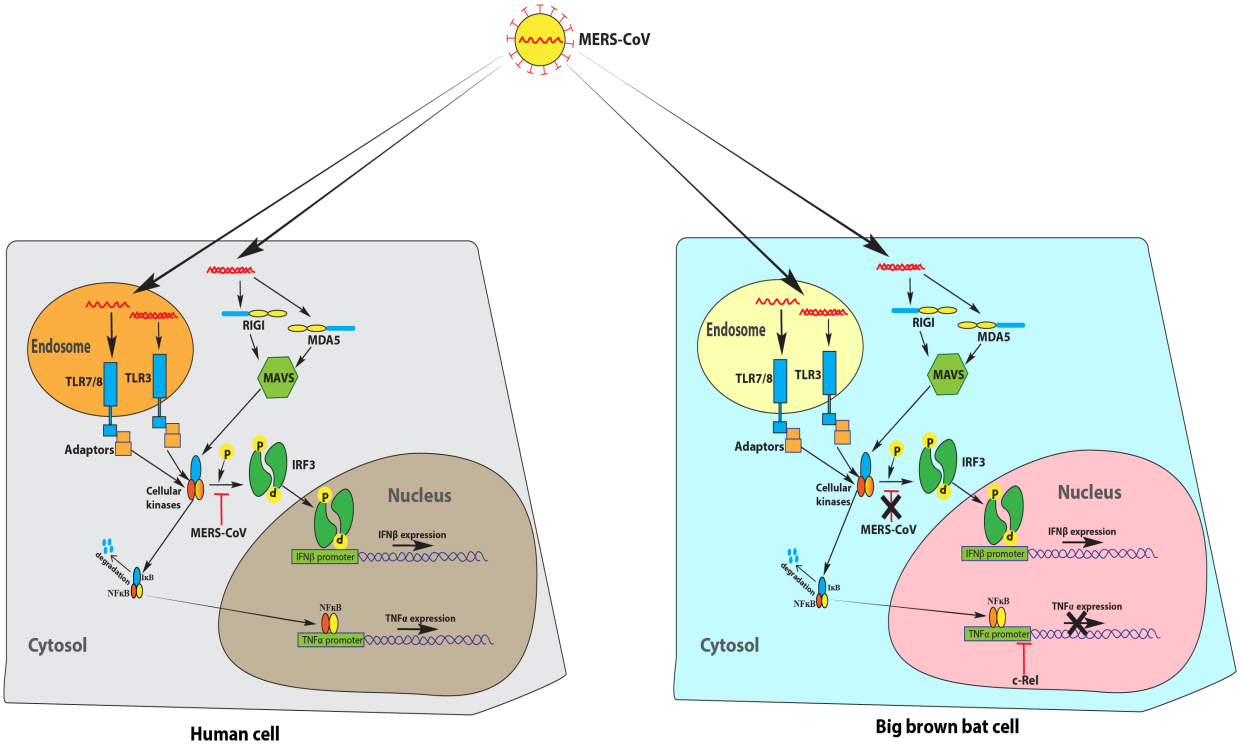

Figure 7. 1. Big brown bat cells mount an antiviral interferon response and limit TNFα response in response to poly (I:C) treatment and MERS-CoV infection. Both human (MRC5) and big brown bat (Efk3) cells can respond to double-stranded RNA [poly(I:C)] by producing interferon beta (IFNβ) transcripts. In this thesis, I have identified two unique responses in big brown bat cells that differ from their human counterpart. Big brown bat cells can very actively suppress MERS-CoV or poly (I:C) induced expression of TNFα, a key systemic inflammatory cytokine. In big brown bat cells stimulated with dsRNA, c-Rel actively suppresses the activity of the TNFα promoter, thus limiting TNFα gene expression. Furthermore, big brown bat cells are resistant to MERS-CoV-mediated subversion of IFNβ responses. Big brown bat cells infected with MERS-CoV actively signal through interferon response factor 3 (IRF3) to produce IFNβ and limit virus propagation, unlike human cells where MERS-CoV can very effectively suppress IFNβ production, leading to higher levels of virus propagation.

7.1.1. Big brown bat cells can control a virus-mediated inflammatory response

Infection with MERS- and SARS-CoVs produce an exaggerated inflammatory response or a 'cytokine storm' in humans (38, 39, 115). This cytokine storm causes massive infiltration of leucocytes in the lungs which eventually contributes to the pathology observed in clinical patients. My thesis (Chapter 5) explored the ability of bat cells to resist an exaggerated inflammatory response and tested the hypothesis that *big brown bat kidney cells have evolved a unique mechanism to control surrogate virus-mediated inflammatory responses.* Indeed, we were able to identify a unique role for the molecule c-Rel, which actively suppressed the expression of TNFα, a key systemic inflammatory cytokine in bat cells (Fig. 7.1). It is of particular interest to test the ability of big brown bat c-Rel to suppress an exaggerated inflammatory response in human cells.

7.1.2. Big brown bat cells actively control MERS-CoV propagation via a functional antiviral signaling pathway

Coronaviruses such as MERS-CoV, like other viruses have evolved proteins that can effectively suppress innate antiviral responses mediated via IRF3 in human cells (112, 114, 116, 243). In Chapter 6 of my thesis, I tested the hypothesis that *big brown bat kidney cells are resistant to MERS-CoV-mediated subversion of IFNβ signaling.* Indeed, we have identified that big brown bat cells are resistant to MERS-CoV protein mediated shut down of IRF3-mediated innate antiviral signaling (Fig. 7.1). Further work (discussed in section 7.2) is required to identify key bat cellular molecules, such as kinases that are resistant to MERS-CoV mediated subversion.

7.2. Challenges, limitations and future prospects

My thesis has identified two unique adaptations in big brown bat cells that are effective against coronaviruses. By performing phylogenetic analysis with sequences from other bats, our studies suggest that these interactions are likely present in other bat species as well. However, this remains to be tested in cell lines from other bat species.

Although epithelial and fibroblast cells do mount an innate immune response to invading viruses, the ideal cell types to fully explore innate antiviral signaling are immune cells, such as dendritic cells, macrophages and leucocytes. Although we have explored big brown bat bone marrow-derived myeloid cells for innate antiviral responses, we were not able to study these cell types in detail due to lack of reagents to cultivate and sort different immune cell types from this bat. It will be interesting to test my hypotheses in immune relevant cells in the future.

Insectivorous bats are speculated to be the ancestral or primordial hosts of MERS-CoV. My thesis is the first to report that insectivorous bat cells are resistant to MERS-CoV mediated subversion of innate antiviral responses. Our study also shows that big brown bat cells signal through IRF3 to inhibit MERS-CoV propagation in these cells. There are still several observations and unanswered questions that need to be explored in the future. MERS-CoV proteins have been shown to inhibit the IFN-mediated (JAK/STAT) signaling cascades to produce ISGs in human cells (38). However, in big brown bat cells infected with MERS-CoV, we see an increase in OAS1 (ISG) expression. Future studies will further explore the resilience of the bat JAK/STAT pathway to MERS-CoV-mediated shut-down. Understanding how bat cells regulate their innate antiviral responses against emerging viruses will allow us to design therapeutic strategies for other mammals such as humans.

MERS-CoV ORF4a has been shown to bind double-stranded RNA to inhibit the activation of PRRs such as RIG-I and MDA5 (243). We have observed that dsRNA [poly(I:C)]-mediated signaling in big brown bat cells is primarily driven through TLR3 and RIG-I (106) and since our bat cells produce IFNβ on infection with MERS-CoV (refer to Chapter 6), it is highly unlikely that ORF4a inhibits dsRNA-mediated PRR activation in bat cells. This phenomenon needs to be tested in bat cells for confirmation.

My thesis provides definitive evidence that IRF3 plays a critical role in controlling the propagation of MERS-CoV in big brown bat cells via an active IRF3 signaling pathway. Thus it is of interest to further dissect this pathway to identify unique molecules, such as bat-specific cellular kinases that enable the activation of IRF3 in bat cells infected with MERS-CoV, unlike human cells where the virus inactivates or inhibits the ability of cellular molecules to activate IRF3 (113, 114, 116). From what is known in human cells, the cellular kinase TBK1 plays a major role in activating (or phosphorylating) IRF3 in response to a virus infection. In fact, studies have shown that SARS- and MERS-CoVs block IRF3 activation or phosphorylation in human cells by inhibiting TBK1 activity (114, 260). We do not currently know if bat TBK1 is resilient to inactivation by MERS-CoV or if bat cells have a separate kinase that can also activate IRF3 independently of TBK1. A second possibility would be that MERS-CoV proteins do not interact with bat TBK1 because of structural and sequence differences between human and big brown bat TBK1. Human and big brown bat TBK1 amino acid sequences share a 94% identity, which means that both TBK1 molecules potentially share similar functional properties. However, the few differences that we do observe between the two sequences (eg. amino acid at positions

467-480) might be enough to prevent MERS-CoV proteins from interacting with bat TBK1 (Fig. 7.2). We currently do not know how MERS-CoV proteins interact with human TBK1 and which regions of TBK1 are important for this interaction. This theory needs to be tested in the laboratory for confirmation.

It is now obvious that bat cells differ from their human counterparts when it comes to virus sensing and innate/intrinsic antiviral responses. Several studies have shown that bat cells from different species have unique adaptations when it comes to responding to virus infections (reviewed in Chapter 2). However, it is difficult to predict with confidence that some of these viruses have directly come from bats. For example, MERS-CoV-like viruses have only been detected in bats and not isolated. We do not know if these viruses can suppress the antiviral responses in their wildlife reservoir species. It is important to reconstruct the evolutionary history of viruses that are speculated to have spilled over from bats to fully understand the adaptations that these viruses have gone through to infect humans and cause disease. The MERS-CoV isolate that we are currently working with is a human isolate (EMC/2012; NCBI accession/version: JX869059.2). We would ideally want to study the original ancestral virus that is speculated to have evolved in bats before spilling over to humans. One way of studying the adaptations that the ancestral MERS-CoV virus would have had in its bat host is to serially passage the human MERS-CoV strain in bat cells to generate a bat-adapted MERS-CoV strain.

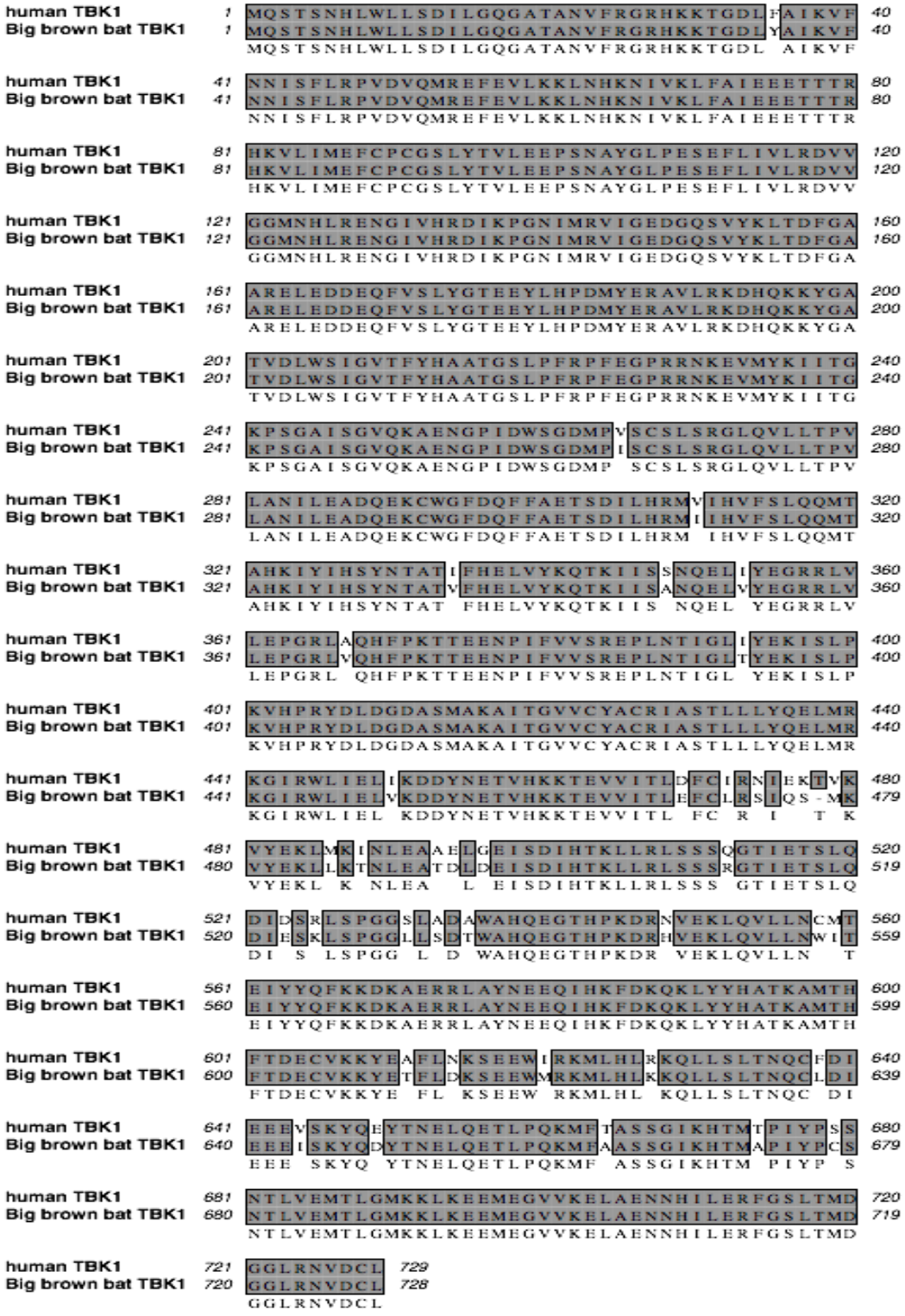

Figure 7. 2. Amino acid alignment of human and big brown bat TBK1. Human and big brown bat TBK1 amino acid sequences are 94% identical (gray box). Some differences (unboxed) can also be observed between the two sequences. Alignment was performed using the ClustalW algorithm on MacVector [version 16.0.5 (29)].

We have generated big brown bat cells (Efk3) that are persistently infected with MERS-CoV (Fig. 7.3.). Preliminary studies with the persistently infected bat cells indicate that MERS-CoV ORF5 is downregulated in these cells. Coronavirus accessory proteins regulate host responses and co-evolve rapidly with a change in host to adapt to their new host (33-35). Further studies are required to fully understand the role of accessory proteins (or the lack of in bat cells) in modulating innate and intrinsic antiviral responses. We also do not know if these accessory proteins play a host-specific role in regulating cellular homeostasis, which can also affect antiviral responses and virus propagation in infected cells.

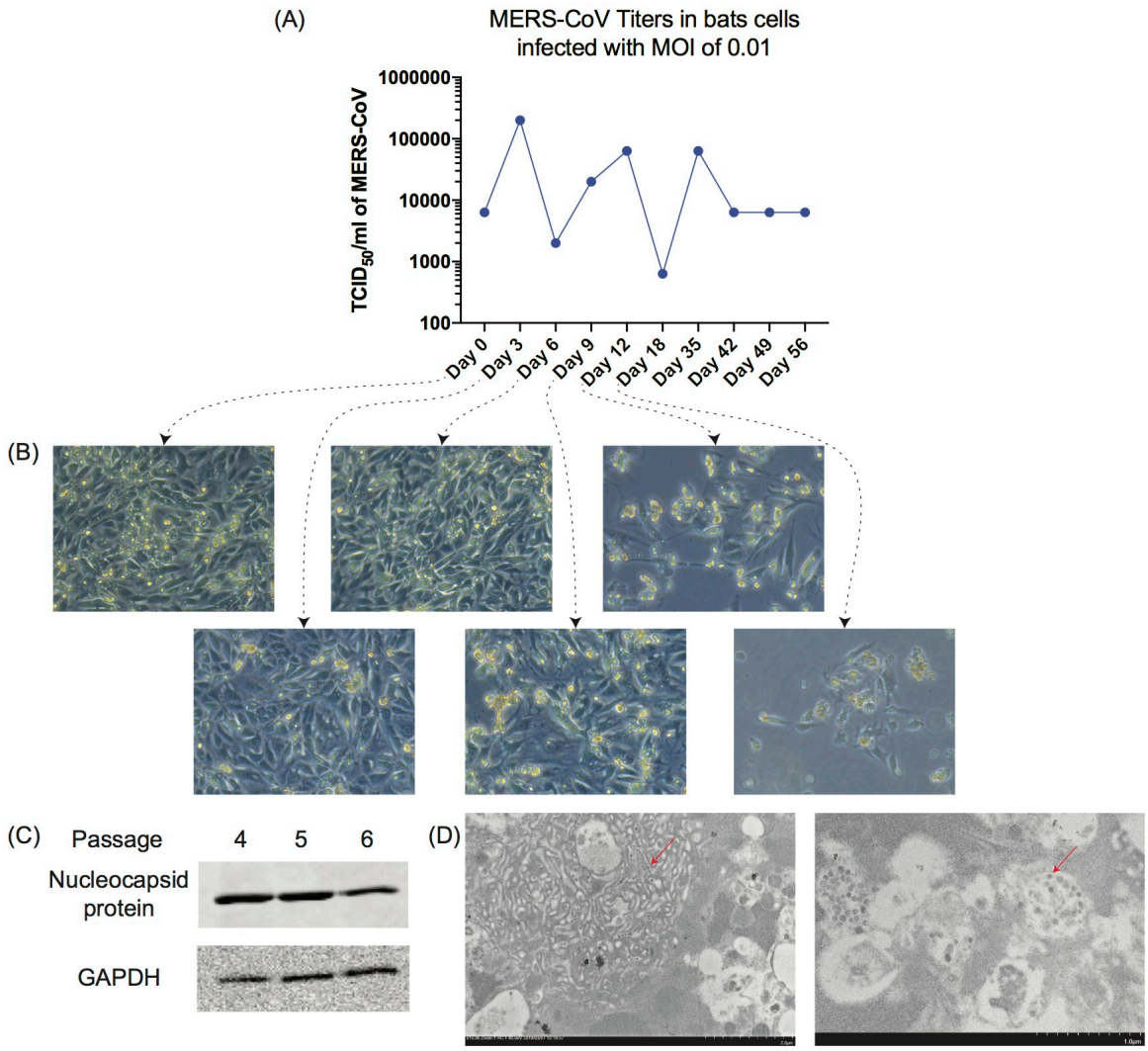

Figure 7. 3. **Big brown bat kidney epithelial cells (Efk3) persistently infected with MERS-CoV.** Efk3 cells were infected with MERS-CoV (MOI of 0.01) and maintained for 9 days. The cells were passaged on Day 10 for the first time and were subsequently passaged every 7 days. We harvested the supernatant from these cells at the indicated days and titrated them on Vero cells. To confirm the presence of virus in these cells, we performed immune-blots for MERS-CoV nucleocapsid protein and detected virus particles by electron microscopy. (A) Virus titres in the supernatant of persistently infected Efk3 cells. (B) Persistently infected Efk3 cells. (C) Immune blots for MERS-CoV nucleoprotein in persistently infected Efk3 cells at passage 4,5 and 6. GAPDH was used as a loading control. (D) Electron micrograph of formalin-fixed persistently infected Efk3 cells. Coronavirus-like particles are indicated by red arrows.

We can detect virus in the supernatant of these persistently infected bat cells (Fig. 7.3.), but we do not know if the virus that propagates in these cells is different in terms of its genomic

sequence from the original EMC/2012 strain, although the bat-adapted virus is likely to have certain mutations because of the selective pressure of the bat innate antiviral responses. This is work in progress and it will be exciting to sequence the virus from these cells. We might just have a bat-adapted MERS-CoV strain in our hands. Although we may never know if it represents the original virus that might have spilled over from bats, it will be our best model of a bat-adapted MERS-CoV strain. Future studies will allow us to monitor the molecular evolution of the bat-adapted MERS-CoV strain as we infect bat, camel and human cells, and humanized mouse models with this virus.

Identifying the specific molecules in bat cells that activate IRF3 in MERS-CoV infected cells will allow us to test these molecules for functionality in human cells and humanized mouse models by using CRISPR/Cas9 technology to knock-in bat molecules. By further studying bat molecules such as MERS-CoV resistant kinases, we might be able to develop novel therapeutic molecules in addition to identifying novel therapeutic targets in spillover species such as humans.

The biological significance of the data generated in my thesis remains to be tested in an animal model. In the future, we will test the unique adaptations in big brown bat cells that have been identified in this thesis in a relevant bat model. Furthermore, dissecting the molecular mechanisms behind regulation of virus infection in bat cells may also allow us to predict the factors that disturb the virus-bat interactions, leading to increased virus replication and catastrophic spillovers and outbreaks.

Zoonotic coronavirus outbreaks in humans (38, 237) and agricultural animals (8) continue to occur and it is important to identify unique treatment opportunities. Overall, my thesis provides important clues about how bats may survive infections with emerging coronaviruses and possibly other viruses, while providing us with an opportunity to adapt these survival strategies to design treatment strategies for humans and other spill over mammals during coronavirus outbreaks.

Chapter 8: References

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