Novel Approaches in the Treatment of Virus- Induced Inflammatory Disease

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Novel approaches in the treatment of virus-induced inflammatory disease

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Abstract

This PhD thesis combines four chapters on different fields of basic research and sets the focus on two circulating viruses of global concern, the orthomyxovirus influenza A virus (IAV) and the alphavirus Ross River virus (RRV). The first three chapters include swine influenza A virus (sIAV) surveillance for the detection and characterisation of IAV subtypes, an *in vitro* high throughput screening (HTS) on host micro RNAs (miRNAs) for the discovery of novel anti-IAV (H7N9) targets and their underlying mechanisms, and an approach to reduce disease pathogenesis in mice infected with H7N9 by targeting the pro-inflammatory factor CCL2. In a fourth chapter, drug repurposing with the interleukin-1 (IL-1) inhibitor anakinra was investigated to treat RRV-induced bone loss in mice. By combining these four chapters, a broad range of drug discovery is covered in this PhD thesis; Surveillance, HTS target discovery and the application of drug repurposing in animal models of viral diseases.

IAVs are important human and animal pathogens. Seasonal IAVs infect humans every year, and occasionally zoonotic viruses emerge to cause pandemics/epidemics with significantly higher morbidity and mortality rates. For example, swine are considered as “mixing vessels” linked to the generation of pandemic IAVs due to their susceptibility to both avian and human IAVs. IAVs are a serious global health threat not only due to reassortment in swine, but also in birds. In March 2013, the public health authorities of China reported three cases of laboratory confirmed human infection with avian IAV (H7N9), and there have been several cases reported across South East Asia and recently in North America. Most patients experience severe respiratory illness, and morbidity with mortality rates near 40%. Following H7N9 infection, there is excessive expression of pro-inflammatory factors contributing to fatal disease outcome. No vaccine is currently available for this strain and the use of antivirals is complicated due the frequent emergence of drug resistant strains. Thus, there is an imminent need to identify new drug targets for therapeutic intervention and disease control. Despite obvious differences, such as the routes of infection and disease

signs caused by IAV and RRV, their RNA nature, high mutation rate and the global emergence of both viruses demand similar attention and efforts in disease control and drug development.

Arthritogenic alphaviruses cause sporadic, sometimes large, outbreaks worldwide. These viruses particularly affect joints of the extremities and can lead to debilitating and potentially chronic arthritis/arthralgia. Similar to IAV infections, the host's innate immune response plays a crucial role in inducing elevated levels of various pro-inflammatory host factors, leading to typical arthritogenic alphavirus disease signs such as tissue destruction and bone loss in the joints. Thus, using drugs targeting the host's immune system may be a promising approach to treat inflammatory diseases in both IAV and RRV, where there is imminent need for the development of novel therapeutics.

The objectives of the first chapter of this PhD thesis were to investigate the prevalence of sIAV in commercial swine herds between June and August of 2014, identify and characterize viruses that circulate in the population. A total of 1,878 oral fluid samples were collected from pigs of all ages from 201 commercial farms located in North and South Carolina. Sixty-eight oral fluid samples from 35 farms were found positive in the M-gene PCR. The overall rate of IAV positive samples was 3.6%, comparable to other active surveillance studies conducted in the past. However, on a herd level, the percentage of IAV-positivity was significantly higher reaching 17.4%. The majority of the IAVs subtyped had an H1 hemmaglutinin, indicating a significant higher prevalence over H3 viruses, which may have important implications for the vaccination strategies followed by swine producers.

In the second chapter of this PhD, a HTS assay was performed using microRNA inhibitors to identify new host miRNA targets that reduce IAV (H7N9) replication *in vitro*. Validation studies lead to a top hit, hsa-miR-664a-3p, that had potent antiviral effects in reducing H7N9 replication by two logs. In silico pathway analysis revealed that this microRNA targeted the LIF and NEK7 genes with effects on pro-inflammatory factors. In follow up studies using siRNAs, anti-viral properties were shown for LIF. Furthermore,

inhibition of hsa-miR-664a-3p also reduced virus replication of pandemic IAV strains H1N1 and H3N2.

In the third chapter on IAVs, the potent inhibitor of CCL2-synthesis bindarit was examined as a countermeasure for H7N9-induced inflammation in a mouse model. Bindarit treatment of mice was not shown to have efficacy for H7N9 infection as indicated by in increased weight loss, increasing virus titre in the lungs, and by increased pulmonary cellular infiltration and pro-inflammatory cytokines. Consequently, the results suggest that bindarit may be ill-advised in the treatment of IAV (H7N9) infection.

In the fourth chapter on the arthritogenic alphavirus RRV, the study was designed to test a novel approach for the treatment of RRV-induced arthritis using the IL-1 receptor antagonist anakinra in mice. Anakinra demonstrated a significant effect on arthritogenic alphavirus disease by reducing RRV-induced bone loss in the mouse model. In histological analysis of the knee joint, treatment with anakinra reduced epiphyseal growth plate thinning, the loss of epiphyseal bone volume and osteoclastogenesis in the tibia. In conclusion, the safety profile of anakinra as a widely used therapeutic in patients diagnosed with rheumatoid arthritis (RA), together with our experimental data, suggests the potential use of anakinra for patients affected by arthritogenic alphavirus disease, in particular those who already suffer from pre-existing medical conditions such as RA.

Overall this PhD thesis implements modern strategies to fight viral disease of global impact. The surveillance of sIAV helps to early detect potentially pandemic strains and to develop more specific vaccines. Our HTS approach successfully unravelled novel targets against the potentially pandemic IAV (H7N9) strain and showed that inhibition of miRNAs can lead to the rapid discovery of novel countermeasure and help to unravel underlying mechanisms important for virus replication. Finally, targeting inflammatory factors in two mouse models of inflammatory virus disease showed the potential of drug repurposing in order to find a quicker way to treat disease signs, while novel classes of drugs are being developed.

Statement of originality

This work has not previously been submitted for a degree or diploma in any university. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made in the thesis itself.

_____

Stefan Wolf

Gold Coast, 18.08.16

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Abbreviations

AIDS	Acquired immune deficiency syndrome
ALRI	Acute lower respiratory infections
ARDS	Acute respiratory distress syndrome
AZT	Azidothymidine
BAL	Bronchoalveolar lavage
BFV	Barmah Forest virus
C3	Complement component 3
CAPS	Cryopyrin-associated periodic syndrome
CDC	Centres for Disease Control and Prevention
CR3	Complement receptor 3
CHIKV	Chikungunya virus
DC	Dendritic cells
DNA	Deoxyribnonucleic acid
HA	Hemagglutinin
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HTS	High throughput screening
IAV	Influenza A virus
IFN	Interferon
IL	Interleukin
IL-1RA	Interleukin-1 receptor antagonist
PCR	Polymerase chain reaction
miRNA	Micro RNA
LIF	Leukemia inhibitory factor
IP-10	Interferon gamma-induced protein 10
IPA	Ingenuity Pathway Analysis

M1	Matrix capsid protein 1
MAYV	Mayaro virus
MBL	Mannose binding lectin
MDCK	Madin-Darby Canine Kidney Cells
MIF	Macrophage migration inhibitory factor
MIP	Macrophage inflammatory protein
MCP-1/ CCL-2	Monocyte chemotactic protein 1
mRNA	Messenger RNA
NA	Neuraminidase
NEK7	NIMA-related kinase 7
NEP	Nuclear export protein
NF-κB	Nuclear factor kappa-B
NK	Natural killer cells
NLS	Nucleolar localization sequence
NP	Nucleoprotein
NS1	Non-structural protein 1
NSAID	Non-steroidal anti-inflammatory drugs
OAT	Organic anion transporter
OB	Osteoblast
ONNV	O'nyong-nyong virus
OPG	Osteoprotegerin
PA	Acidic polymerase
PB2	Basic polymerase 2
p.i.	Post infection
RA	Rheumatoid arthritis
RANK	Receptor activator of NF-κB
RANKL	RANK ligand
RISC	RNA-induced silencing complex

RNA	Ribonucleic acid
RNP	Ribnucleoprotein
RRV	Ross River virus
RSV	Respiratory syncytial virus
RT-qPCR	Quantitative reverse transcription PCR
s.c.	Sub-cutaneous
siRNA	Small inhibiting RNA
TCID50	50% tissue culture infective dose
TRAP	Tartrate-resistant acid phosphotase
TNF	Tumor necrosis factor
WHO	World Health Organization

Acknowledgment of papers included in this thesis

Included in this thesis are papers in chapters 2, 3, 4 and 5 which are co-authored with other researchers. My contributions to each co-authored paper are outlined at the front of the relevant chapter. The bibliographic details for these papers including all authors are:

Chapter 2: Stefan Wolf, Constantinos S. Kyriakis, Les P. Jones, Byoung-Shik Shim, Jarod M. Hanson, Ming Zhang and Ralph A. Tripp. Molecular Surveillance and Preliminary Subtyping of Influenza A Viruses in Swine, Southeastern United States, Summer 2014. **2016, manuscript in preparation.**

Chapter 3: Stefan Wolf, Weilin Wu, Cheryl Jones, Olivia Perwitasari, Suresh Mahalingam, Ralph A. Tripp. MicroRNA Regulation of Human Genes Essential for Influenza A (H7N9) Replication. *PLoS One,* ***2016 May 11;11(5):e0155104. Copyright: © 2016 Wolf et al.***

Chapter 4: Stefan Wolf, Scott Johnson, Olivia Perwitasari, Suresh Mahalingam and Ralph A. Tripp. Targeting the pro-inflammatory factor CCL2 (MCP-1) with bindarit for influenza A (H7N9) treatment. *Clinical and Translational Immunology,* **2016 Oct, manuscript submitted.**

Chapter 5: Stefan Wolf, Lara Herrero, Andreas Suhrbier and Suresh Mahalingam. Inhibition of IL-1 signaling reduces bone loss in arthritogenic alphavirus disease. *Arthritis and Rheumatology: Brief Reports,* **2016, manuscript in preparation.**

Publications and conference proceedings in the course of PhD

Journal articles

1. **Stefan Wolf**, Constantinos S. Kyriakis, Les P. Jones, Byoung-Shik Shim, Jarod M. Hanson, Ming Zhang and Ralph A. Tripp. Molecular Surveillance and Preliminary Subtyping of Influenza A Viruses in Swine, Southeastern United States, Summer 2014. **2016, manuscript in preparation.**
2. **Stefan Wolf**, Weilin Wu, Cheryl Jones, Olivia Perwitasari, Suresh Mahalingam and Ralph A. Tripp. MicroRNA Regulation of Human Genes Essential for Influenza A (H7N9) Replication. *PLoS One,* ***2016 May 11;11(5):e0155104. Copyright: © 2016 Wolf et al.***
3. **Stefan Wolf**, Scott Johnson, Olivia Perwitasari, Suresh Mahalingam and Ralph A. Tripp. Targeting the pro-inflammatory factor CCL2 (MCP-1) with bindarit for influenza A (H7N9) treatment. *Clinical and Translational Immunology,* **2016 Oct, manuscript submitted.**
4. **Stefan Wolf**, Lara Herrero, Andreas Suhrbier and Suresh Mahalingam. Inhibition of IL-1 signaling reduces bone loss in arthritogenic alphavirus disease. *Arthritis and Rheumatology: Brief reports,* **2016, manuscript in preparation.**
5. Lara Herrero, Penny Rudd, Xiang Liu, **Stefan Wolf** and Suresh Mahalingam. Mouse Models of Chikungunya Virus. *Methods Mol Biol*. **2016;1426:211-24.**
6. Lara Herrero, Adam Taylor, **Stefan Wolf** and Suresh Mahalingam. Arthropod-borne arthritides. *Best Pract Res Clin Rheumatol.* **2015 Apr;29(2):259-74.**
7. Lara Herrero, Andrew Zakhary, Michelle Gahan, Michelle Nelson, Belinda Herring, Andrew Hapel, Paul Keller, Maheshi Obeysekera, Weiqiang Chen, Kuo-Ching Sheng, Adam Taylor, **Stefan Wolf**, Jayaram Bettadapura, Shobha Broor, Lalit Dar and Suresh Mahalingam. Dengue virus therapeutic intervention strategies based on

viral, vector and host factors involved in disease pathogenesis. *Pharmacol Ther.* **2013 Feb;137(2):266-82. doi.**

8. **Stefan Wolf**, Olivia Perwitasari, Suresh Mahalingam and Ralph A. Tripp. miRNA therapeutics against influenza (review) *Frontiers.* **2016 manuscript in preparation.**
9. Weiqiang Chen, Suan Sin Foo, Ali Zaid, Terk-Shin Teng, Lara J. Herrero, **Stefan Wolf**, Kothila Tharmarajah, Luan V. Dinh, Caryn van Vreden, Rachel Li, David M. Ojcius, Helder I. Nakaya, Luke A.J. O'Neill, Avril A.B. Robertson, Nicholas J. King, Andreas Suhrbier, Mathew A. Cooper, Lisa F.P. Ng and Suresh Mahalingam. Specific Inhibition of NLRP3 in Chikungunya Disease Reveals a Novel Role for Inflammasome in Alphavirus-Induced Inflammation. *Nature Microbiology*. **2016 manuscript submitted 17.08.16.**
10. **Stefan Wolf**, Adam Taylor, Andreas Suhrbier and Suresh Mahalingam. The effect of ambient temperature on viral disease. **2016 manuscript in preparation.**

Conference presentations

1. *3rd International Symposium on Neglected Influenza Viruses.* **The Georgia Center, Athens Georgia USA. 15 – 17 April 2015**
2. *9th International Symposium on Avian Influenza.* **The Georgia Center, Athens Georgia USA. 12 – 15 April 2015**
3. **Stefan Wolf**, Weilin Wu, Olivia Perwitasari, Abhijeet Bakre, Suresh Mahalingam & Ralph Tripp. microRNA regulation of human genes essential for influenza H7N9 replication. Poster presentation at *The 4th isirv Antiviral Group Conference*. **The University of Texas at Austin, Texas, USA. 2 – 4 June 2015**
4. **Stefan Wolf**, Weilin Wu, Olivia Perwitasari, Abhijeet Bakre, Suresh Mahalingam & Ralph Tripp. microRNA regulation of human genes essential for influenza H7N9 replication. Poster presentation at *The 4th Southeastern*

Immunology Symposium. **Emory University School of Medicine, Emory University, Atlanta, USA. 13 – 14 June 2015**

5. **Stefan Wolf**, Weilin Wu, Cheryl Jones, Olivia Perwitasari, Suresh Mahalingam and Ralph A. Tripp. MicroRNA Regulation of Influenza A (H7N9) Replication. Oral presentation at *The 2016 International Student Research Forum*. **University of Chinese Academy of Sciences Yanqihu campus, Beijing, China. 12 – 16 June 2016**

Patents

1. **Stefan Wolf** and Ralph A. Tripp. miR-664 (has-miRNA-664a-3p) inhibitor of Influenza virus. **The University of Georgia Research Foundation. Nov 2014.**

Chapter 1 – Literature review

1.1. General introduction

Ribonucleic acid (RNA) viruses including influenza A virus (IAV) and alphaviruses such as Ross River virus (RRV) and chikungunya virus (CHIKV) are on the rise and can cause debilitating reactions by the immune system with sometimes fatal outcomes. Even though IAV is a negative single stranded RNA virus of the orthomyxoviruses and alphaviruses are positive single stranded RNA viruses of the togaviridae, they share many common features. Both types of viruses are widespread across the world, infect high numbers of people every year and are considered emerging. Where the emergence of the mosquito-borne viruses is mainly linked to the spread of their vectors, in particular the mosquitos of the *Aedes* genus, IAVs are emerging from animal sources, causing worldwide pandemics as seen in 2009 for the H1N1 swine influenza virus (sIAV) strain. Furthermore, there is clear evidence for an overreacting pathogenic immune response towards both viruses, which can result in debilitating and chronic disease in the case of arthritogenic alphavirus disease and pulmonary disease during IAV infection. Consequently, this PhD thesis covers a wide spectrum of fields with a focus on the research of virus-induced inflammatory disease. IAV pandemics with avian or swine origin during the last decade have helped to unravel the importance of surveillance programs on the evolution of IAV strains. Novel strains of IAV are being created in real time and come with the potential of pandemic outbreaks, so that there is the imminent need for the development of novel therapeutics. The occurrence of resistance towards common antivirals such as oseltamivir or rimantadine require novel approaches in the fight against IAV. Targeting the host cell mechanisms required for virus replication has been shown to be of great potential to develop novel antivirals without the risk of resistance. In the meantime, as novel antivirals are being developed, there is great potential for currently approved therapeutics to counter inflammatory processes that are associated with infectious disease such as IAV and alphaviral arthritogenic disease. Thus, drug repurposing and high throughput screening

methods are valuable tools for the fast and safe discovery of novel targets and compounds to treat such conditions. Therefore, this thesis combines approaches for IAV surveillance to understand the evolution of novel strains, high-throughput attempts to discover novel targets for the treatment of virus-induced inflammation, and the use of existing therapeutics to treat symptoms that are related to an overly aggressive attempt by the immune system to fight virus replication.

Overview

The first chapter of this PhD thesis focuses on an IAV surveillance study in swine in the Southeastern USA. The study was carried out in collaboration with Smithfield foods over the course of 1 year. From the collected field samples, we were able to show evolutionary patterns of sIAV, which is important because zoonotic IAV have jumped species and caused pandemics in humans in the past. The second chapter elaborates on the use of high throughput technologies to discover underlying host mechanisms required for the replication of IAV (H7N9) with a focus on micro RNAs (miRNAs). Ten miRNA top hits were validated and led to one candidate with potent antiviral activity. Follow-up studies discovered downstream mechanisms and target molecules important for virus replication. In chapter three, an immunomodulatory pre-clinical compound was investigated for its potential use in the treatment of IAV (H7N9) disease *in vivo*. In humans and animals, MCP-1 (CCL-2, monocyte chemotactic protein 1) and many other pro-inflammatory cytokines are highly upregulated during disease, however, whether they are protective or harmful for the host remains unclear. The potent CCL-2 inhibitor bindarit increased disease signs in a mouse model of IAV (H7N9) disease, which indicates an important role of CCL-2 in antiviral host defence against this particular virus. Chapter four explored the role of the pro-inflammatory cytokine interleukin-1 beta (IL-1β) in an animal model of RRV-induced arthritogenic disease. IL-1β and other pro-inflammatory factors are upregulated in humans and animals suffering from the disease and have been linked to bone loss in rheumatoid arthritis (RA). Inhibition of

IL-1β with anakinra, an FDA-approved and strong antagonist of the IL-1 receptor, reduced bone loss in mice without altering virus replication. Thus, anakinra may be of value for the treatment of arthritogenic alphavirus disease caused by RRV or CHIKV.

1.2. Influenza A viruses with special focus on H7N9

1.2.1. Classification

IAV causes moderate to severe disease in humans of all ages throughout the entire world (1). There are three types of influenza that can infect humans; A, B and C. IAV usually perpetuates in their natural hosts, such as wild birds or bats, where they rarely evolve and usually are not pathogenic. However, spillovers can potentially lead to worldwide pandemics with high mortality. On the other hand, Influenza B and C only infect humans, cause milder disease and are more stable than type A. Influenza is primarily spread via large virus-laden droplets from human-to-human, which are produced when infected people cough or sneeze. Transmission may occur through direct contact or indirect contact when touching surfaces contaminated with influenza and then unknowingly spreading the virus to eyes, nose or mouth (2). Influenza infection is caused by a segmented RNA virus belonging to the orthomyxoviridae family. IAVs are designated by subtype according to their surface hemagglutinin (HA) and neuraminidase (NA) proteins. To date, 18 HA and 11 NA subtypes have been identified (2). HA and NA are the surface antigens and the most important proteins for IAV fitness. These two proteins periodically change due to evolution within an immune or partially immune population, a process which is commonly referred to as antigenic drift of shift (3). In antigenic drift, mutants emerge as the predominant virus and differ to the antecedent virus, which is suppressed by specific antibodies among the population as a result of previous infections. Since the protection from past exposures to the antecedent virus is incomplete, antigenic drift may result in an epidemic. Antigenic shift appears at irregular intervals of 10 to 40+ years and involves both surface antigens

hemagglutinin and neuraminidase (4). Viruses undergo major antigenic changes from previous subtypes, to which humans fail to have protective antibodies against. Antigenic shifts may happen due to genetic recombination between IAVs infecting humans and/or animals. Antigenic shift can lead to pandemics, if the newly reassorted strain is efficiently transmitted from person-to-person. Typically, high attack rates during a pandemic affect all age groups and results in a markedly increased mortality (2).

1.2.2. Genome and virion replication

IAVs are negative-sense RNA viruses with 8 gene segments encoding at least 10 proteins. Segments 1-6 encode basic polymerase 2 (PB2), PB1, acidic polymerase (PA), hemagglutinin (HA), nucleoprotein (NP) and neuraminidase (NA), respectively. Segment 7 encodes the matrix capsid protein (M1) and through alternate splicing, the M2 protein. In a similar manner, segment 8 encodes the non-structural protein (NS1) and nuclear export protein (NEP) (5) (Figure 1). Most IAVs also encode two extra proteins in alternate open reading frames within the PB1 gene, the PB1-F2 and PB1-N40 proteins (6). IAVs are coated in an outer lipid layer which originates from the plasma membranes of the host in which the virus was propagated (7). Outside the lipid envelope, there are spikes, which consist of HA, NA and M2 protein. The M1 protein underlies the lipid layer and is important for attachment of the ribonucleoprotein (RNP), which is a complex structure mostly composed of the NP protein. The NP protein wraps eight different RNA segments of the IAV genome. RNPs contain about 50 copies of RNA-dependent RNA polymerase per virion, which consists of PB1, PB2 and PA (8). NEP is found within the virion, where PB1-F2, PB1-N40 and NS1 are nonstructural proteins (6).

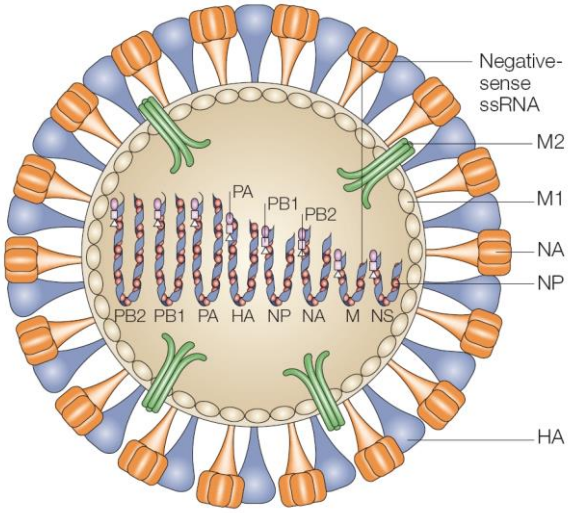


Figure 1: Influenza A virion with two surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA), and the M2 ion-channel protein are embedded in the viral envelope, which is derived from the host's plasma membrane. The ribonucleoprotein complex comprises a viral RNA segment associated with the nucleoprotein (NP) and three polymerase proteins (PA, PB1, and PB2). The matrix (M1) protein is associated with both ribonucleoprotein and the viral envelope. Source Horimoto et al. 2005 (9).

IAVs modify cellular processes for propagation and alter host responses to infection. IAV binds to sialic acid on the surface of cellular glycoproteins via HA. Clathrin-dependent endocytosis and macropinocytosis lead to internalisation of the virion. After internalisation, the pH drops which results in structural changes of the HA and fusion of the virion membrane with the endosomal membrane occurs. At the same time, the M2 protein initiates the influx of H+ protons from the endosome into the virus particle which starts the dissociation of the RNPs form the M1 protein and releases the RNPs into the cell cytoplasm. The nucleolar localization sequence (NLS) on the NP aids to actively transport the viral RNPs into the nucleus, where viral messenger RNA (mRNA) synthesis is initiated by the polymerase complex (PA, PB1 and PB2). Virus mRNAs are translocated to the cytoplasm for translation. During early infection, NP and NS1 are predominantly expressed for NP to encapsulate newly synthesized genomic and complementary RNAs (virion RNA and complementary RNA) and for NS1 to regulate several cellular processes, particularly interferon (IFN) antiviral responses. HA, NA and M2 are translated, folded and glycosylated in the ER and Golgi apparatus before they are transported to the cell surface. The PA, NEP and M1 proteins localize in the nucleus, where they potentially direct the shift from mRNA

expression to virion RNA expression e.g. RNPs. NP and M1 can both interact with the cytoskeletal proteins, but precise mechanisms of RNP packaging into budding virions is unclear. Virus budding is cooperatively mediated by the M1, M2, HA and NA proteins, with a specific role for the M2 protein in membrane scission. Finally, the NA cleaves the sialic acids from the cell surface and virus glycoproteins, preventing HA binding and aggregation of newly formed virus particles.

1.2.3. Epidemiology

Seasonal influenza

IAV is one of the most contagious infectious diseases in the world and is still a serious public health issue. Despite readily available vaccines and therapeutics for circulating strains, IAV is a global health threat affecting humans, wildlife, and agricultural species. Acute lower respiratory infections (ALRI) are the second most common cause of illness in all age groups worldwide with 429.2 million cases in 2004 and are the third most common cause of death with 4.2 million or 7% of total deaths in 2004 (10). The contribution of ALRI to illness and death varies strongly among different age and socioeconomic groups. A study by the WHO in 1990 revealed that the number of deaths attributed to ALRI was 12 times higher in developing countries than in developed countries. Viral infections with viruses such as respiratory syncytial virus (RSV) and IAVs are responsible for the majority of episodes of ALRI in children and the elderly. A recent WHO report estimated that in 2008 18% of all mortality in children under 5 years was attributed to acute respiratory infections. (11). Even though the flu reoccurs every year, IAV is an underappreciated contributor to global morbidity and mortality with significant economic consequences. According to current estimations, seasonal influenza affects 5-10% of the world's population each year, resulting in between 250,000 and 500,000 deaths (12). In the USA, influenza caused 24.7 million cases in 2003 alone, resulting in 31.4 million outpatient visits, over 334,000 hospitalisations

and approximately 41,000 deaths. The economic burden was estimated to be around 87.1 billion USD (13).

Influenza pandemics

During an influenza pandemic, a non-human IAV strain gains the ability for efficient and sustained human-to-human transmission leading to a global spread. Historic records indicate that influenza pandemics have been around for many centuries. Hippocrates described an outbreak for the year 412 BC that could have been caused by influenza (14). The first convincing report on an influenza pandemic described an outbreak in 1580, which commenced in Asia, spread over Europe and Africa to America (15). Four major pandemics have been recorded for the last century, most prominently the Spanish flu (H1N1) in 1918 with an estimated 50 million deaths, which led to the name “the last great plague of mankind” (16). The pandemic started early in 1918 with reports of outbreaks of unusual respiratory disease associated with an increased number of deaths among young people. The disease spread rapidly killing many American soldiers on their voyage to Europe, where initial infections occurred in Madrid, Spain, hence the name “Spanish flu”. After the initial wave there was a sharp decline in infections during the summer months, which was followed by a second, more severe wave in autumn of 1918 and a third wave in early 1919, both linked to global spread of the infection. In under 10 months, more than 50 million people were killed worldwide, more than the total number of victims during the war from 1914-18 (16). In March 1957 the so called “Asian flu” (H2N2) originated in the Yunan province in China, spreading rapidly to South-East Asia and Japan and subsequently to Australia, Indonesia and India. By summer the pandemic had reached Europe, Africa and the Americas, reaching the whole world within just 6 months. A second wave occurred in the autumn of 1957, altogether affecting 40-50% of the world population with 25-30% showing symptoms. The mortality was approximately 1/4000, predominantly occurring in the very young and very old. The total death toll probably exceeded 1 million (14, 17, 18). The 1968 Hong Kong flu (H3N2) also originated in China, spreading to Hong Kong in the summer of

1968 causing a major outbreak of 500,000 within only two weeks. Even though the pandemic receded after only 6 weeks, by August it had spread to Taiwan, the Philippines, Singapore and Vietnam, and by September to India, Iran and Australia. The pandemic was later introduced to North America by troops from the Vietnam war, where it peaked in December, infecting 30-40% of the population and leading to 56,000 excessive deaths compared to regular flu seasons. The Hong Kong flu was relatively mild, possibly due to partial immunity in the population to the N2 subtype which the pandemic shared with the Asian flu. Worldwide the total death toll of the 1968 Hong Kong pandemic was estimated to be between 500,000 and 1 million. The most recent global IAV pandemic occurred in North America in February/March of 2009 and was referred to as “swine flu” by the media. By June 73 countries had reported more than 26,000 laboratory-confirmed cases and the WHO declared a full-fledged pandemic. By August literally all countries had laboratory-confirmed cases (19). In Mexico, where the first cases were noted, an observational study showed that 6.5% became critically ill and of those 41% died (20). Mortality among children, young adults and pregnant women was much higher than in a typical flu season, however, older adults fared relatively well, and the total number of influenza-related deaths worldwide (105,700 – 395,600) were similar to a relatively mild influenza season (21). However, the proportionally higher mortality among children and young adults increased the severity in terms of years of life lost. Over the last decades, sporadic cases of severe IAV disease and deaths in humans have been caused by several avian IAVs, in particular the H5N1 virus in 1997 and H7N9 in 2013. These sporadic outbreaks of avian influenza may be potential forerunners of a pandemic, but the likelihood is difficult to estimate because there is no data available on how frequently zoonotic episodes occurred silently in the past.

Influenza A (H7N9)

Human infections with a new avian IAV (H7N9) were first reported in China in March 2013 (22). Most of the infections are believed to result from exposure to infected poultry or contaminated environments, as H7N9 viruses have been found in poultry in China. While

some mild illnesses in humans infected with H7N9 have been seen, most patients experienced severe respiratory illness, such as pneumonia (97.3%) and acute respiratory distress syndrome (71.2%), leading to high rates of intensive care unit admissions and mechanical ventilation (23). Human mortality attributed to IAV (H7N9) has been over 38% with 650 confirmed cases since 2013 (24, 25). No evidence of sustained human-to-human spread of H7N9 has been recorded, however, population immunity to the virus is considered very low, and the virus has significant ability to cause human disease with evidence for limited person-to-person spread under rare circumstances (25). After H7N9 broke out in China, the virus has rapidly spread to other countries and was reported in Malaysia on February 12, 2014 (26). Recently, the first documented case of H7N9 in humans has been reported for North America in Canada (27). While H7N9 has rarely been a topic in the news media, H5N1 attracted wide attention during its emergence in 2003 and 2004 as the so called “bird flu”. When the virus spread from Asia to Europe and Africa, it was entrenched in poultry in some countries, leading to millions of infected poultry, hundreds of human infections and many human deaths (28).

1.2.4. Clinical disease

IAV attaches to and penetrates respiratory epithelial cells in the trachea and bronchi after respiratory transmission. Viral replication occurs and results in the destruction of host cells (3). The incubation period for IAV is usually 2 days. Influenza illness can be from asymptomatic to severe with an abrupt onset of fever, muscle and body pains, sore throat, runny or stuffy nose, dry cough, fatigue, headache and vomiting and diarrhea, though this is more common in children than adults (Figure 2). Non-steroidal anti-inflammatory drugs (NSAIDs) and paracetamol may offer partial relief of symptoms. Systemic symptoms and fever usually last 2 to 3 days and rarely more than 5 days. Adults can transmit influenza from the day before symptoms to approximately 5 days after symptoms onset. Children can transmit influenza to others for 10 or more days (2). The most frequent complications during

an infection with IAV is pneumonia, generally with a secondary bacterial infection (29). An elevated risk exists for patients suffering from myocarditis and the virus can worsen chronic bronchitis and other chronic pulmonary diseases (1). Death is reported in less than 1 per 1,000 cases (2). The risk for complications and hospitalisations from influenza are higher among persons 65 years of age and older, young children and persons of any age with certain underlying medical conditions.

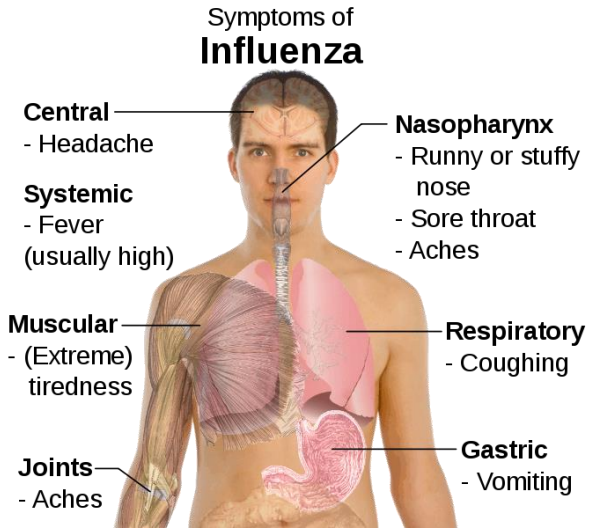


Figure 2: Common symptoms during influenza A infection. Häggström 2014 (30).

The disease caused by IAV (H7N9) is characterised by a rapidly progressing severe pneumonia. There are no disease specific symptoms and patients show those typical for acute respiratory infection, such as fever, cough and shortness of breath. The main complications include the acute respiratory distress syndrome (ARDS), septic shock and multi-organ failure, which all require intensive care unit admission and mechanical ventilation. Most patients infected with H7N9 experienced severe respiratory illness, such as pneumonia (97.3%) and acute respiratory distress syndrome (71.2%), leading to high rates of intensive care unit admissions (23). The mortality rate attributed to IAV (H7N9) infection in human is >38%, with 175 deaths from 450 confirmed cases reported within a 20-month period (24). Severe illness is more common among the elderly with underlying chronic conditions. However, on-going active influenza-like illness surveillance also showed patients with mild symptoms and healthy children and young adults with contact tracing. (31).

Similar to H7N9, the case fatality rate for IAV (H5N1) infections is much higher compared to that of seasonal influenza. In many patients, the disease caused by H5N1 shows an unusually aggressive clinical picture with rapid deterioration of disease and high fatality (28). The incubation period for IAV (H5N1) infection is longer than that for normal seasonal influenza with 5 days on average (22). Initial presentation includes high fever, usually with a temperature above 38°C and other influenza-like symptoms such as cough and sore throat. Further disease signs that have been reported include diarrhea, vomiting, abdominal pain, chest pain and bleeding from the nose and gums (28).

1.2.5. Animal models

IAV was first isolated from pigs in 1930 and later from humans in 1933 (32, 33). Shortly after, ferrets were established as an excellent laboratory model for human influenza. The histopathological changes in the respiratory tract of pigs, ferrets and mice describes changes comparable with those seen in human IAV infection. In 1962, viral replication was shown to happen in alveolar cells in experimental IAV infection in ferrets and mice (34). These animal models share many histological features with humans infected with IAV including degeneration of alveolar lining, hyperaemia and congestion, septal inflammatory infiltrates, the appearance of macrophages with necrotic cellular debris in air spaces and intraalveolar oedema and haemorrhage. Furthermore, animal models using non-human primates have been developed since the 1941 (35) including histopathological findings comparable to human IAV pneumonia with intraalveolar oedema and interstitial inflammatory cell infiltrates and changes including alveolar epithelial hyperplasia at day seven (36). Shortly after the outbreak of the novel avian-origin IAV (H7N9) occurred in March 2013, animal models were being developed. A/Anhui/1/2013 (H7N9) was intranasally administered to mice and ferrets. Animals developed typical clinical signs including body weight loss (mice and ferrets), ruffled fur (mice), sneezing (ferrets) and death (mice). Peak virus shedding was observed from the respiratory tract on day 2 post infection (p.i.) for mice and 3-5 days p.i. for

ferrets. Furthermore, virus could be detected in the brain, the liver, the spleen, the kidney and the intestine from mice and in the heart, the liver and the olfactory bulb from ferrets. These animal models enabled detailed studies of the pathogenesis of the disease and built the foundation for drug and vaccine evaluation (37). As more animal models were being developed in macaques, pigs and guinea pigs, the attachment patterns were being investigated for each animal species. The attachment patterns of IAV (H7N9) in macaques and mice, and to a lesser extent in pigs and guinea pigs, resembled the pattern in humans more closely than the one in ferrets (38).

1.2.6. Pathogenesis

Host factors - cellular and soluble

The pathogenicity and virulence of seasonal IAV is determined by several interacting factors. Host factors such as the presence of target receptors and the availability of enzymes in the host cells are essential for viral entry and replication. The state of immunocompetence and the specific immunity against certain viral epitopes of the individual host are of great importance. Furthermore, the ability of the immune system to control the viral replication effectively without causing serious collateral damage for the host by its inflammatory response is vital. On the other side, the virus' ability to bind to the host cell and to shed virus are important viral factors. Restriction of cytopathic effects allow for an appropriate balance between viral replication and control by the host. The evolution of antigenic variation driven by selective pressure of the immune response and by recombination with different virus strains from zoonotic disease allow to escape from the immunosurveillance. In addition, the modulation of the immune response to attenuate effective host defence mechanisms is an important feature of viral fitness (3).

In studies on fatal H7N9 infections in humans, evidence for a strong impact of the innate immune system leading to immunopathological changes was observed (39). Infected children usually suffer only from mild disease (40), where elderly subjects are generally

more severely affected (41). This suggests that there is little or no cross-protection from earlier influenza episodes and that the decline in primary adaptive immunity with age may be a determining factor. Greatly elevated cytokine and chemokine levels are associated with severe lung damage and airway compromise in patients suffering from H7N9 disease (23). The robust proliferation of H7N9 in the lower respiratory tract causes an excessive activation of the innate immune response, which leads to the production of inflammatory mediators in intrapulmonary macrophage and alveolar cells (42). The major histological characteristics include diffuse alveolar damage, hyaline membrane and fibroproliferation in the lung and spotty necrosis in the liver (Figure 3). Histological observations showed depletion of T lymphocytes, a varying number of neutrophils and highly abundant and activated macrophages in the alveoli (39).

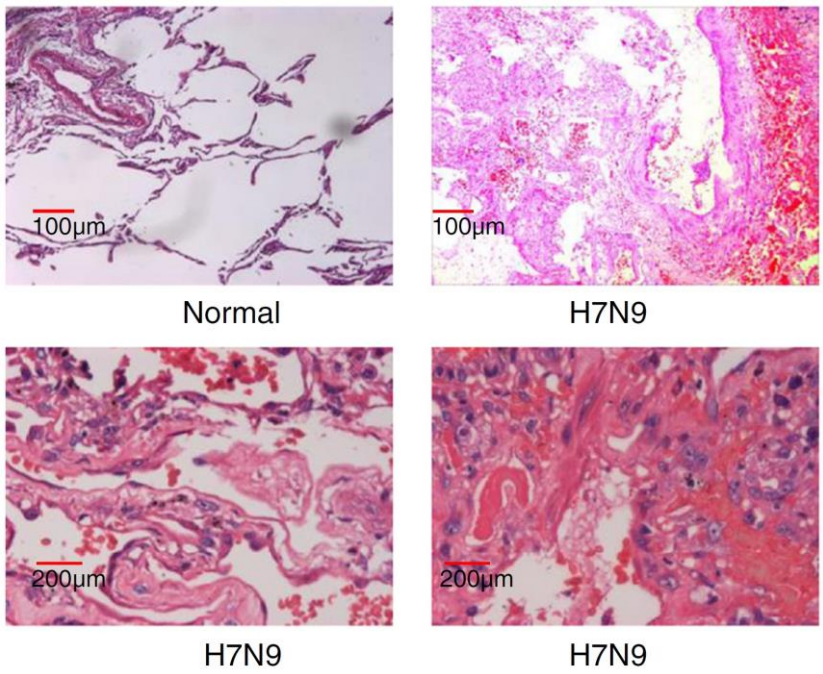


Figure 3: Micrographs of lung tissue, pathophysiology, and treatment from a patient with acute respiratory distress syndrome and H7N9 influenza. Hematoxylin and eosin staining from a normal sample and the H7N9 patient's lungs. Diffuse alveolar damage, including edema, hyaline membranes, and inflammation, were seen in these images. Guo et al. 2014 (43).

In addition, high levels of intrapulmonary inflammatory mediators, for example interferon gamma-induced protein 10 (IP-10) and IL-6 can be detected. In plasma studies on cytokine levels, IL-6 and IL-8 were strongly upregulated, whereas IL-10, macrophage

inflammatory protein-1β (MIP-1β) and IFN-γ were increased at intermediate levels. IL-1β, tumor necrosis factor-α (TNF) and MIP-1α on the other hand were only found at minimal concentrations. IL-8 is known to promote the infiltration of neutrophils, whereas MIP-1α and MIP-1β attract monocytes and macrophages. Whether the greatly elevated cytokine and chemokine levels explain why H7N9 causes such severe disease or if they simply correlate with inflammation and pathology has yet to be determined (23). Taken together, a cytokine storm, or the upregulation of pro-inflammatory cytokines and chemokines, such as IL-6, IL-8, IFN-α, IP-10, MIG, MIP-1β, and CCL-2, is described in H7N9-infected patients with lung injury and severe pneumonia (40). In mice infected with the H7N9 A/Anhui/A/2013 strain, the cytokine levels of C57BL/6 and BALB/c were compared (44). Remarkably, C57BL/6 exhibited more severe lung injury, slower recovery from lung damage, less effective viral clearance, higher levels of IL-6, CCL-2 and IL-1β and lower levels of TNF and IFN-γ than infected BALB/c mice. This suggests that TNF and IFN-γ may help to suppress viral gene expression and increase viral clearance while IL-6 and CCL-2 may contribute to lung injury during H7N9 disease.

Attachment patterns throughout the respiratory tract are important for both pathogenicity and transmissibility and there is a distinction between IAV strains from human and avian origin (45-47). Human IAVs generally attach better to upper respiratory tract epithelium, where avian IAV such as H5N1 preferably attaches to lower respiratory tract epithelium. Intriguingly, IAVs (H7N9) attaches to both the upper and lower respiratory tract in humans, which further strengthens the pandemic potential of H7N9 (38). This preference of attachment can be linked to the pattern of sialic acids on the surface of the epithelium in the respiratory tract. While the ratio of α2,6 sialic acids to α2,3 sialic acids is greater in the upper respiratory tract, the ratio is smaller in the lower respiratory tract (48). Seasonal IAVs preferably bind α2,6 sialic acids, which are more abundant in the upper respiratory tract. Anatomically the upper respiratory tract is easier accessible by the virus. Preference for binding of α2,6 sialic acids, due to the greater exposure of the upper respiratory tract to the environment compared to the lower respiratory tract, thus facilitates virus spread from

human-to-human. Avian IAV such as H5N1 on the other hand show stronger binding to α2,3 sialic acids, which hinders human-to-human spread. Mutations leading to changes in the binding affinity of avian IAV towards α2,6 sialic acids, can increase the pandemic potential of avian IAV. Avian IAV (H7N9) has been shown to be able to bind to both α2,6 sialic acids and α2,3 sialic acids, (49) which may increase the pandemic potential of this particular avian IAV strain.

1.2.7. Current treatments

There are a number of efficacious drugs available in the treatment of IAV infections; the M2 ion channel inhibitors amantadine and rimantadine and the neuraminidase inhibitors zamamivir and oseltamivir (50, 51). Early treatment with these antiviral drugs reduces the duration of symptoms and time to recover from IAV infection, however, the use of antiviral drugs is complicated by the emergence of drug resistant viruses (52, 53). Genetic diversity of IAV is mainly acquired through the mistake-prone RNA-dependent RNA polymerase, which leads to the development of drug resistant strains against the common influenza antivirals such as M2 ion channel inhibitors and the neuraminidase inhibitors (54). Thus, novel approaches target either the virus polymerase directly or target the host cell in order to limit the evolutionary pressure on the virus and to avoid the development of drug resistant strains. In addition, the use of antiviral drugs may have an effect on population vulnerability due to lack of seroconversion, as well as driving drug resistance among circulating strains (55). To prevent further spread of these infectious diseases, new drug and vaccine development is needed. Unfortunately, difficulties remain in the development of new drugs as viral and host factors associated with the infection of the ever changing IAVs are poorly understood.

No vaccine is currently available for the prevention of H7N9 infections (56). Furthermore, unusual characteristics from hallmark mutations in the virus, differing from other avian IAVs, increase the difficulty to fight against H7N9 (57). Consequently,

oseltamivir-resistant H7N9 strains were recently described in Taiwan (58). The development of new drugs and vaccines against H7N9 may take many years and treatment is needed now. Therefore, drug repurposing or the use of clinical drug candidates may help to reduce the impact of disease in the meantime. In H7N9 disease, host's immune response against the virus is believed to contribute to tissue destruction and the resulting pathology (39). Thus, treatment with corticosteroids was evaluated; however, it was shown to increase the mortality of human patients suffering from acute H7N9 infection (59). Lack of understanding of the host's immune response towards H7N9 infection is an issue for the discovery of novel therapeutics and target identification for drug repurposing.

1.2.8. Vaccines

The WHO keeps track of the world epidemiological situation twice a year and gives out recommendations for new vaccine strains in accordance with the available evidence. In general, influenza vaccines are trivalent, containing a mixture of influenza A and B strains for the predictably most circulating strains of the coming season. Reassortant strains are commonly used for vaccine production, with the surface glycoproteins HA and NA of the circulating epidemic virus and the internal proteins of a standardised production strain. The virus is grown in chick embryos or cell cultures for the production of vaccines. The two available influenza vaccines are either inactivated (killed) for injection or attenuated for nasal delivery (60). The Centres for Disease Control and Prevention (CDC) conducts studies each year to determine the effectiveness of influenza vaccine to protect against flu illness. Recent studies showed a reduction of the risk to acquire flu illness by about 50%-60% among the overall population during season when most circulating influenza viruses are like the vaccine viruses (61). A recent study on the vaccination rate among risk groups reported that an average of 75% of adults over 60 years, 45% of children aged 6-23 month, 32% of children aged 5-2 years, 59% of pregnant women, 78% of healthcare workers and 90% of individuals

with chronic conditions were vaccinated during the 2013-14 Northern Hemisphere or 2014 Southern Hemisphere influenza vaccination activities (62).

1.2.9. CCL-2 and bindarit

Bindarit (2-methyl-2-[[1-(phenylmethyl)-1H-indazol-3-yl]-methoxy]-propanoic acid) represents a novel class of inhibitors effective in selectively reducing monocyte chemotactic proteins synthesis (63). It has been shown to be a potent inhibitor of CCL-2 synthesis, and has been used to inhibit chemokine production by human intestinal epithelial cells to treat colitis (64). CCL-2 is a critical mediator of neuroinflammation in a myriad of disease states, including multiple sclerosis (65), human immunodeficiency virus 1 (HIV-1) induced encephalitis (66), Guillain-Barré Syndrome (67), Alzheimer's disease (68), ischemia (69), neurotrauma (70), epilepsy (71), neurogenic hypertension (72) and alcoholism (73). It has been studied as an ideal target for therapeutic intervention in these disease states. More importantly, bindarit has also been successfully used to alleviate virus-induced inflammation in animal models of disease. It is strongly therapeutic in a mouse model of CHIKV infection (74). Bindarit was also highly effective in treating arthritis resulting from infection with the related alphavirus RRV (75). In both these models of alphavirus infection, bindarit was able to reduce arthritic inflammation without having any detrimental effect on virus clearance. Bindarit has an innovative mechanism of action, a substantial and encouraging set of clinical tolerability data (more than 600 subjects, healthy volunteers and patients, treated up to a maximum dose of 2400 mg/day for as long as 6 months), and has the potential to be beneficial in a range of diseases (76).

1.2.10. RNA interference and drug repurposing

Recent advances in the understanding of RNA interference (RNAi) have led to genome-wide screens to determine and validate host cell genes that may be required for IAV replication (77). Small interfering RNA (siRNA) can be readily developed to target viral

or host genes and have been successfully applied in disease intervention approaches. For example, siRNA targeting RSV has been efficacious for silencing virus replication (78). In the case of IAV, inhibiting the host gene CAMK2B prevented virus replication *in vitro* (79) and knocking down trypsin also inhibited virus replication and apoptosis (80).

In addition to host gene involvement during viral infection, the magnitude and tempo of host gene expression may be altered by factors such as miRNA. Paired analysis of miRNA inhibitors and mimics enables gain and loss of function studies for a given miRNA. miRNAs have been used to validate the impact of host genes on virus replication and have been used as therapeutics (81) with the ability to negatively affect IAV replication (82). Therefore, host miRNA may play a role in host gene modulation during IAV infection. miRNAs are involved in the degradation of cytokine transcripts and modulate the expression of negative regulators of cytokine expression and signalling pathways (83). Furthermore, miRNAs have been shown to play a critical role in T and B cell development (84). miRNA can not only be used as a tool for screening, but also as a therapeutic itself. The drug miravirsen is an anti-miRNA drug candidate currently in clinical testing for treatment of hepatitis C virus (HCV) infections. Miravirsen is thought to work mainly by hybridising to mature miR-122 and blocking its interaction with HCV RNA. miR-122 is a liver specific miRNA with an important role in the life cycle of HCV (85). Miravirsen has entered phase II clinical trials where antiviral activity against HCV and no cytotoxicity was demonstrated (86).

Development of new therapeutics is a time consuming and expensive process associated with frequent failure. Thus, repurposing older and existing drugs is an option that allows for more rapid drug availability. The idea behind drug repurposing comes from the promiscuous nature of drugs, in which they can target multiple molecules or pathways. An example of successful drug repurposing is the development of the first anti-retroviral drug azidothymidine (AZT). AZT was originally developed for cancer treatment in the 1960, but was withdrawn due to its lack of efficacy. In 1987 AZT was found to be effective against HIV and became the first FDA-approved drug for the treatment of HIV and acquired immune deficiency syndrome (AIDS) (87). By using a siRNA screening, the pro-viral factor organic

anion transporter-3 (OAT-3) was discovered to be important for IAV replication. The prototypical OAT-inhibitor probenecid is currently widely used against gout and other hyperuricemic disorders. Probenecid was shown to effectively reduce IAV replication *in vitro* and *in vivo* and reduced IAV-associated mortality and morbidity *in vivo* (88). Probenecid may be used as an adjunct to antiviral therapy, but has yet to be tested in humans in the context of IAV.

1.2.11. miRNA

miRNAs are small noncoding single-stranded RNA molecules composed between 18-23 nt (nt) which function in RNA silencing and post-transcriptional regulation of gene expression in eukaryotes. The miRNA family is a global regulatory network controlling homeostasis, inflammatory responses affecting immunity and disease pathogenesis (89).. They are found in plants, animals and humans and are known to comprise more than 2,000 species in the human genome. miRNAs are transcribed in the nucleus as primary miRNAs of 2 kb in length. Primary miRNAs are processed by Drosha and Pasha into 70-100 nt stem-loop structures named pre-miRNAs. Pre-miRNAs are then exported to the cytoplasm where they are further processed by Dicer into mature double-stranded miRNAs (90). miRNAs function in a similar matter like siRNAs as one of the miRNA strands associates with the RNA-induced silencing complex (RISC), providing specificity for RISC to bind to complimentary mRNA which in turn mediates its degradation (Figure 4). Importantly, the miRNA's specificity is primarily mediated through the "seed site", which is a 6-8 nt region of the miRNA. The small complementary region enables the miRNA to potentially target numerous mRNAs (90, 91). Due to the ability to widely regulate gene functions, miRNAs potentially regulate almost every cellular process including the ones required for disease.

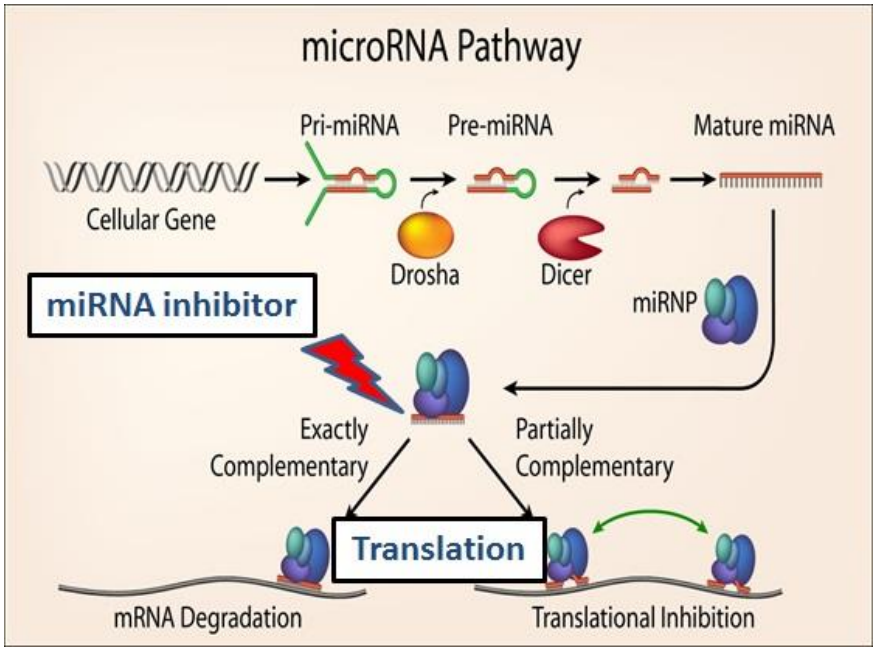


Figure 4: Micro RNAs are processed from Pri-miRNA to Mature miRNA by Drosha and Dicer. miRNA inhibitors inhibit the function of micro RNAs while allowing translation of target genes. Adapted from Sharp Lab (92).

Commercial tools for miRNA screenings are readily available and arrays of plates targeting all known miRNAs are available similar to genome wide siRNA libraries. These miRNA libraries can be equipped with both miRNA mimics and inhibitors, which allows gain and loss of function studies for any given miRNA. miRNA screening is a valuable tool for genome-wide gene screens. However, miRNAs show broad activities, thus the identification of a given phenotype may require a more specific validation process of the targeted host genes. In addition, the resulting phenotype may be the outcome of the regulation of translation of more than a single gene product and therefore an effect linked to partial inhibition of multiple genes (90).

miRNA screenings have been carried out for IAVs as both full and focused screenings in order to better understand miRNA regulation of host genes. Identified host genes during IAV infection have further been validated in siRNA screenings (93-95). miRNA mimics and inhibitors were both shown to either increase or decrease IAV replication, however, the effects were not as distinct as what was seen by gene-silencing using siRNA, which is consistent with the miRNAs role in regulating gene expression (93-95).

miRNA screenings are not as advanced as siRNA screening in practice or interpretation, but have drawn increasing attention in recent years for initial target identification, as a validation tool to correlate with siRNA screen data and to help identify high-confidence hits (91). Furthermore, miRNAs also represent potential drug targets. For example, a modified oligonucleotide inhibitor of miR-122 is currently undergoing clinical trials and showed efficacy in reducing miR-122, which is critical for hepatitis C infection, replication and hepatitis C virus RNA levels (96, 97).

1.3. Active surveillance and isolation of influenza A virus among swine

1.3.1. Surveillance

Despite the substantial contribution of sIAV to the reassortment and the emergence of IAV, surveillance for sIAV is lacking. Current epizootic events linked to sIAV and pandemic A/Ca/04/09 H1N1 influenza virus (pH1N1) highlight the necessity for surveillance systems capable of detecting IAVs with pandemic potential in swine. The 2009 pandemic revealed significant gaps in IAV surveillance capacity which compromised the assessment and monitoring of the pandemic. The overarching objectives of sIAV surveillance are to minimise the impact of emerging IAV disease by providing useful information, so appropriate control and intervention measures can be addressed. To achieve these objectives, various capacities need to be addressed that include but are not limited to a) determine the prevalence of sIAV in swine farms b) identify circulating sIAV subtypes and their relationship to global and regional sIAV subtypes, c) assist in developing an understanding of the relationship of sIAV strains to disease severity, d) monitor antiviral sensitivity in isolated viruses, e) assist in defining the antigenic character and genetic makeup of isolated viruses.

1.3.2. Swine influenza viruses

IAV infects multiple species, including humans and domestic birds, and infection in swine can cause acute respiratory disease (98) which is usually self-limiting (99). Infection of swine with IAV virus is common and generally occurs throughout the year (100); however, seasonal peaks occur similar to the pattern of disease seen in humans (101). sIAV is not classified as a reportable disease (98), which is one factor that has complicated coordinated surveillance efforts. Another factor is that sIAV was largely stable in U.S. swine populations as classical H1N1 for decades, needing little investigation until emergence of novel reassortants in the late 1990's sparked extensive virus reassortment and evolution (100); thus extensive sIAV surveillance networks and technologies are absent (Figure 5).

Commercial concerns regarding stigmatisation by the public or government due to swine influenza reporting and loss of revenues from decreased pork demand and import bans by other countries have greatly limited surveillance. The labelling of the 2009 pH1N1 as “swine flu” directly affected the U.S. pork industry, resulting in nearly $1.3 billion in economic losses (102).

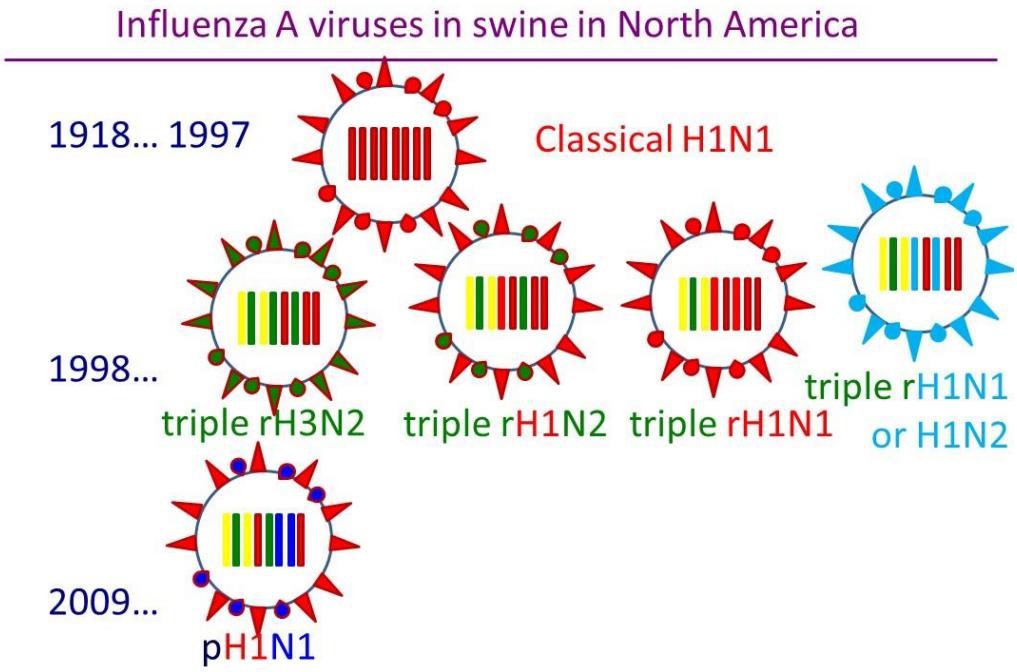


Figure 5: Evolution of swine influenza before and after 2009. In the late 90’s the classical H1N1 influenza A viruses reassorted and formed triple reassortant strains. In 2009, the swine influenza pandemic strain pH1N1 evolved. Image kindly provided by CS Kyrakis.

1.3.3. Diagnosis

Despite sIAV being an important disease, diagnosis is hindered because sIAV infection may occur throughout the year and produce a disease phenotype similar to other swine respiratory pathogens; i.e. there are no obvious hallmarks that would provide early warning. For example the clinical signs associated with sIAV are often attributed to Porcine Respiratory Disease Complex (PRCD), a polymicrobial pneumonia caused by several common swine respiratory viruses and bacteria (103). Similarly, sIAV can cause uncomplicated infections in pigs producing mild or limited clinical signs (102, 104). Thus, husbandry and surveillance methods that bring swine workers into close contact with swine

infected with a potentially zoonotic IAV strain pose risks to workers, their families, and subsequently the community. Additionally, the existing surveillance approaches for developing early warning systems in swine are lacking as seen by the swine-originated pH1N1 outbreak in 2009 (105).

1.3.4. Clinical signs

Swine infected with IAV develop a fever and begin shedding virus by 24 hours p.i. with peak virus excretion generally at 3-5 days p.i. Circulating antibodies can be detected within 10–14 days of infection (104). It has been noted that for individual pigs there is a window during infection in which the virus has been cleared but antibodies have not developed; thus, the pig appears naive (106). So, diagnostic tests to detect sIAV should be performed within the first week of infection.

sIAV surveillance studies have been reported in the U.S. (35), but studies are limited, particularly for surveillance performed by commercial producers. However, it has been demonstrated that sIAV is common among swine, and that the percentage of swine seropositive against classical swine H1N1 viruses ranged from 20 to 51 % from 1976 through 1989 (106). In contrast, over the same period, serologic evidence of H3 virus exposure was only 1%. Frequency of H3 infection is now much higher, suggesting that swine are exposed to and reassort with human H3 and avian viruses to a greater extent than in the past (107). Diagnostics for swine is challenging, as swine can be infected with multiple types of IAVs (99). However, nucleic acid-based detection is available using RT-qPCR protocols, primers and reagents developed for the detection and quantitation of sIAV. To detect a broad range of IAV subtypes, primers for RT-qPCR are designed to target the conserved M-or NP-genes (108). The limit of detection using this procedure ranges from 10^{-1} - 10^{1} $TCID_{50}$/mL (50% tissue culture infective dose/mL) depending on the strain, and additional tests are required to subtype or identify pH1N1 viruses (109). Virus isolation is still the gold standard test. Currently, sIAV virus isolation is typically done with Madin-Darby

Canine Kidney (MDCK) cells; however, RT-qPCR provides an accurate and sensitive technique and is routinely used to screen a large number of samples in a short period of time. The disadvantage of RT-qPCR is that it detects only the viral RNA and not viable virus, thus it is often paired with virus isolation when conducting surveillance.

1.3.5. Conclusion

In conclusion, it is known that swine are naturally infected with H1N1, H3N2, and H1N2 viruses. sIAV antigenically similar to human H3N2 can infect swine and cause disease. Variants of human H3N2 viruses have been transmitted to pigs, and these variants can persist in pigs after they have disappeared from the human population, e.g. A/Port Chalmers/1/73 variant of H3N2 has continued to circulate and cause disease in pigs in Europe (110). Swine are susceptible to experimental infection with all subtypes of avian IAVs. These and other previously discussed features are some of the reasons why it is critical to support swine surveillance, as our strength is currently in monitoring and reacting to newly emerging sIAV and it is necessary that there is greater collaboration between academia, public health entities and industry.

1.4. Alphaviruses – Ross River virus

1.4.1. Classification

The *Togaviridae* family consists of two genera, namely *Alphavirus* and *Rubivirus,* with the latter including Rubella virus as its only member (111). The genus *Alphavirus* consists of 30 different species, such as the Australasian RRV and Barmah Forest virus (BFV), the African o'nyong-nyong virus (ONNV), the South American Mayaro virus (MAYV), the African/Asian CHIKV and the wide spread Sindbis group viruses (SINV) (112-114). Alphaviruses are distributed globally and are commonly segmented into "Old World" and "New World" viruses. New world viruses (Venezuelan, Eastern and Western equine encephalitis viruses) are generally linked to encephalitic disease and old world viruses (e.g. CHIKV, RRV, BFV, ONNV, SINV and the MAYV) to arthritis. Old world viruses are also referred to as arthritogenic alphaviruses, with RRV and CHIKV causing epidemic polyarthritis in humans (115, 116). While RRV is mainly found within Australia and Papua New Guinea, CHIKV has raised worldwide attention after an outbreak with up to 6.5 million cases occurred in India and surrounding islands between 2004-2011. This epidemic began in Kenya, spread across the Indian Ocean to India and later to South East Asia and the Pacific Ocean, affecting nearly 40 countries including imported cases to several European countries, the USA and Japan possibly introduced through travel of infected individuals after local outbreaks of disease (116-118). Two hotspots of this epidemic were Grande Comore Island and La Reunion in 2005-2006 with an attack rate of 50% and 38% of the population, respectively. The emergence of CHIKV has recently reached new levels, as an epidemic is currently affecting the Caribbean, reaching areas in Brazil, Mexico and even Florida, USA, with an estimated 1.6 million cases (118, 119). Although rarely fatal, Alphaviruses are responsible for debilitating febrile illness, rashes, headaches and acute and chronic symmetrical peripheral polyarthralgia-polyarthritis in infected individuals (Table 1) (112, 116). Alphaviruses are sustained by arthropod-mammal transmission cycles and are therefore considered arthropod-borne viruses (arboviruses), with mosquitoes as principle vectors of

transmission. The main focus of this thesis is on the alphavirus RRV, as the virus has been used extensively studied in our arthritogenic animal model of alphavirus-induced inflammation. With over 30 mosquito species, RRV has a wide vector range with *Aedes* and *Culex* as specifically important genera. The main mammal enzootic hosts are the macropods kangaroos and wallabies, although in urban areas possums, horses and possibly birds and flying foxes play a certain role (120, 121).

Table 1: Table of alphaviruses with disease and geographic distribution. Alphaviruses are distributed globally and an important cause of encephalitis and/or arthritis in humans. RRV and CHIKV are emerging and are able to cause massive epidemics. Source Rulli et al. 2007 (122).

Alphavirus	Disease caused	Geographic distribution
Chikungunya(CHIK)	Arthropathy	Africa, Asia
Mayaro (MAY)	Arthropathy	South America
O'nyong-nyong (ONN)	Arthropathy	Africa
Igbo Ora	Arthropathy	Africa
Ross River (RR)	Arthropathy	Australia, Oceana
Sindbis Ockelbo	Arthropathy	Scandinavia
Sindbis Babanki	Arthropathy	West Africa
Barmah Forest (BF)	Arthropathy	Australia
Semliki Forest (SFV)	Systemic febrile illness	Africa, Euracia
Venezuelan equine encephalitis (VEE)	Systemic febrile illness	South and Central America
Everglades (EEE)	Systemic febrile illness	Florida, USA
Mucambo (MUC)	Systemic febrile illness	Brazil, Peru
Tonate (TON)	Systemic febrile illness	South America
Eastern equine encephalitis (EEE)	Encephalitis	North and South America
Western equine encephalitis (WEE)	Encephalitis	North and South America
Highlands J (HJ)	Encephalitis	USA

1.4.2. Genome and virion replication

RRV is a small, enveloped, positive-sense single-stranded RNA virus (123). The genome is approximately 11.9kb in length and consists of two separate open reading frames: non-structural and structural (124, 125). The 5' end of the RRV RNA genome (7.4kb) encodes four non-structural proteins (nsP1 to nsP4) involved in genomic replication and mRNA synthesis. The 3' end (3.8kb) encodes six proteins, which consist of the capsid protein, the three envelope glycoproteins E1 to E3 and the small 6K/transframe hydrophobic protein (Figure 6) (112, 115, 121, 125).

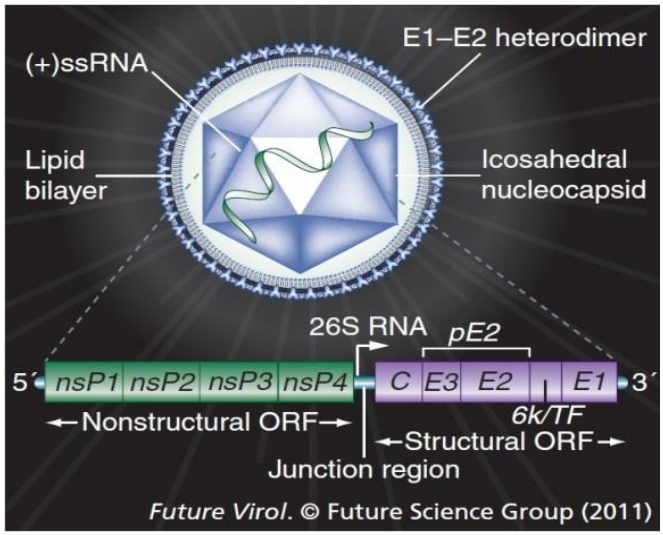


Figure 6: The structural and genomic features of alphaviruses; ORF – Open reading frame, ssRNA – single stranded RNA. Source Foo et al. 2011 (112).

RNA viruses such as RRV generally show great genetic flexibility and relatively high mutation rates (126). During the process of entering a susceptible host cell by an alphavirus particle, such as RRV, the viral E2 glycoprotein is the conduit of receptor interaction (127). The entry into a host cell is based on clathrin-dependent endocytosis. Subsequent acidification of the endosomes leads to the destabilisation of the E1-E2 heterodimers and triggers membrane fusion of the virus. Thus, the viral genome can be released into the cytoplasm, where replication occurs (112, 127, 128).

1.4.3. Epidemiology

Alphaviruses, such as RRV, and many other zoonotic or multi-host viruses have been labelled as (re-)emerging viruses and are widespread around the globe (Figure 7) (129-131). Due to an increase in frequencies of epidemics and emergence, these viruses are notifiable and remain a major concern to public health, costing the Australian health system at least USD 10 million annually (132). Human demographics and behaviour (e.g. population growth, global travels etc.), environmental and ecological changes (e.g. increasing urbanisation and agriculture), but also clinical attentiveness, laboratory facilities and epidemic surveillance systems are all factors that impact the emergence of such

diseases (133, 134). A major player in the global spread of viral diseases in the last decade is the *Aedes* species, particularly the Asian tiger mosquito *Aedes albopictus.* Global shipment of goods contributes to spread and the permanent introduction of the vector to new territories (135). For example endogenous cases of vector-borne diseases including West Nile fever, CHIKV, and Dengue are frequently being reported in Europe, which highlights the increased risk for tropical diseases for the European population(136).

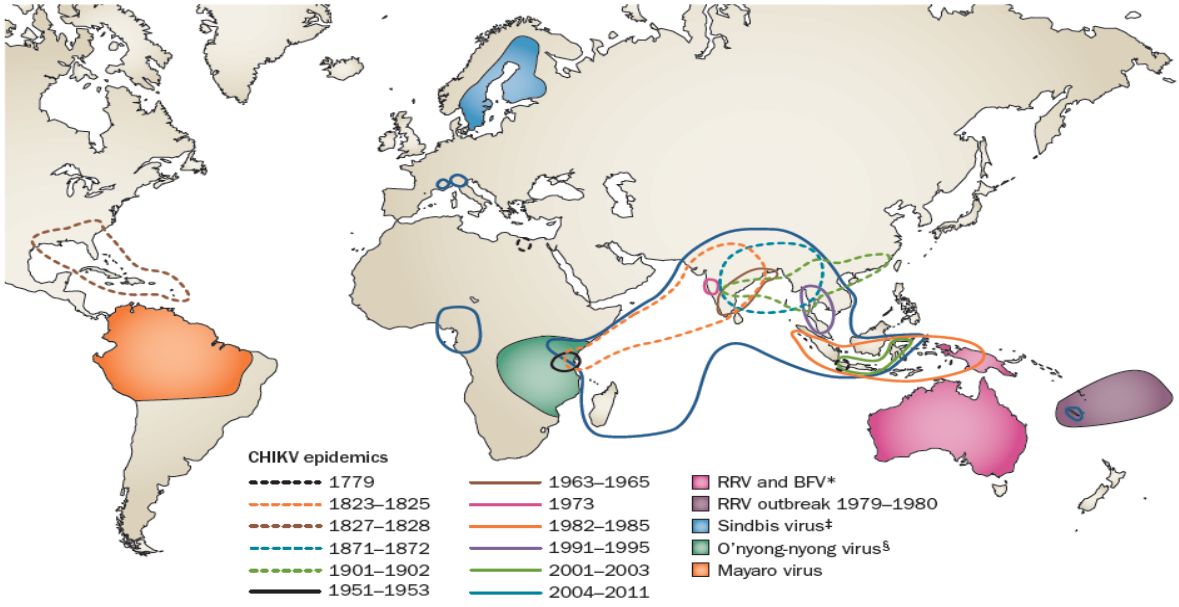


Figure 7: Locations are shown for arthritogenic alphaviruses and large outbreaks of CHIKV disease. Dashed lines represent epidemics prior to 1902 and were initially classified as dengue, but likely to have been CHIKV. Locations of BFV and RRV overlap. Source Suhrbier 2012 (116).

The first outbreak of an RRV epidemic was reported in 1928 in New South Wales, where the nameless disease was described as “epidemic polyarthritis”. The virus was later isolated for the first time in 1959 from an *Aedes vigilax* mosquito near the Ross river in Townsville, Queensland, hence the name Ross River virus (120, 137, 138). RRV is endemic and enzootic throughout Australia and was also found in Papua New Guinea and other western and central South Pacific islands (139-141). RRV was responsible for large explosive epidemics with an estimated number of cases over 60,000 that occurred on isolated Western Pacific Islands in the years 1979-1980. During these outbreaks up to 40-50% of the local population were infected with the virus and epidemiological studies suggested that the virus was maintained primarily in a man-mosquito-man cycle (115, 117,

137, 141, 142). In Australia, RRV is the most common arbovirus with a mean of 4,661 reported cases per year and a yearly incidence of 7-41 RRV infections per population of 100,000 between 1993 and 2015 and has recently surged with 9,557 reported cases in 2015 (143). The rate of RRV disease is generally four times higher in tropical populations than in subtropical populations and follows a seasonal fluctuation, with higher number of cases in the wet season when mosquito infestations are at their peak (117, 123, 144). Climate change followed by environmental changes is suggested to extend the range and activity of RRV and other mosquito-borne infections in the future (145-147). A spread of RRV into urban areas has also been proposed by contributing factors including urbanisation. Changes in agricultural practices (e.g. building dams and irrigation) and expanding residential and industrial developments have all increased the exposure risk of humans to infected mosquitoes (129, 130, 148).

1.4.4. Clinical disease

With nearly 5,000 reported cases annually, RRV is the most common and most important public health arbovirus in Australia. Although infections are not fatal, there is a considerable morbidity associated with debilitating polyarthritis as the main symptom (117, 149, 150). The major characteristics of RRV disease in human are various combinations of arthralgias (joint pains) and arthritis, involving joints of the extremities, myalgia (muscle pains), lethargy, a maculopapular rash, headache and fever (138, 151). RRV disease commonly occurs in adults between 25-44 years of age and affects males and females equally. The age-specific risk for disease with RRV differs supposably because of age-related discrepancy of the immune response to infection (123). The incubation period varies between 5 and 15 days, but can be as long as 21 days or as short as 3 days (123, 144). The onset of RRV disease can appear sudden and severe, but generally resolves progressively over 3 to 6 months (148, 151, 152). In the acute phase, joint pains, such as wrists, knees, ankles, fingers, elbows, neck, shoulders and back, are present in 95% of patients and are

symmetrical (151, 153, 154). These pains can range from tenderness with minor restriction of movements to severe redness, heat and swelling (122, 123). Joint pains are accompanied by fever, myalgia and joint swelling which occur in 50-60% of patients (Table 2). Other symptoms include tiredness, which occurs in over 90% of patients, or headaches and depression, which are experienced in 45-50% of patients. Rash develops in 50-75% of patients, is generally maculopapular (but also vesicular or purpuric), appears predominantly on the limbs and trunk and usually does not last more than 10 days (Figure 8) (120, 121, 123, 154). Atypical symptoms were further described such as photophobia, neck stiffness and sore throat (122). Recent studies revealed bone lesions in joints of patients infected with the related CHIKV, which provided evidence that alphavirus-induced disease can result in bone pathologies (155). Acute ear redness has been described with a prevalence of 25% during an CHIKV outbreak in 2008 in Malaysia, thus ear chondritis could be considered as a sensitive diagnostic criterion of the acute stage of CHIKV disease (156).

Table 2: Frequency of symptoms and sign of RRV disease.

Symptoms/Signs	**Frequency (%)**	**Reference**
Joint pains	95	(151, 153, 154)
Joint swelling and Myalgia	50-60	(153)
Fever	50-60	(151, 154)
Tiredness	90	(151, 154)
Rash	40-60	(151, 154)
Headache and depression	45-60	(151, 154)

Studies form the 90's proposed the possibility of chronic manifestation of RRV disease with reports of arthralgia, tiredness and depression persisting years after diagnosis (151, 154). In a cohort study, these symptoms lasting for longer than 3-6 months were nearly all diagnosed with other conditions that accounted for the long-term disease, including osteoarthritis, chronic RA and depression. However, RRV disease lasted over a year in 2% of patients without any confounding conditions (152). It was later proposed that RRV disease

is caused by immunopathologic inflammatory response targeting virus persisting in synovial macrophages (157). Furthermore, a cohort study on the related alphavirus CHIKV also reported chronic incapacitating arthralgia and arthritis, where CHIKV was found in perivascular synovial macrophages in a patient 18 months p.i. (158). In recent years, bone lesions of CHIKV-infected patients have been reported (155), providing evidence that alphavirus-induced disease can result in bone pathologies. MRI findings showed joint effusion, bony erosion, marrow oedema, synovial thickening, tendinitis and tenosynovitis. Thus, there is evidence that CHIKV can induce chronic inflammatory erosive arthritis, which has implications for the management of the infection, such as long term strategies which target chronic inflammation after acute symptoms of virus infections have disappeared. Furthermore, studies have linked CHIKV to the development of unspecific post-viral arthritis, RA, seronegative spondylitis and other non-inflammatory musculoskeletal complaints like persistent arthralgia (159). Taken together, these conditions represent a significant burden of disease, affect the quality of life and weigh in on direct and indirect economic loss, with considerable financial impacts on health care systems.

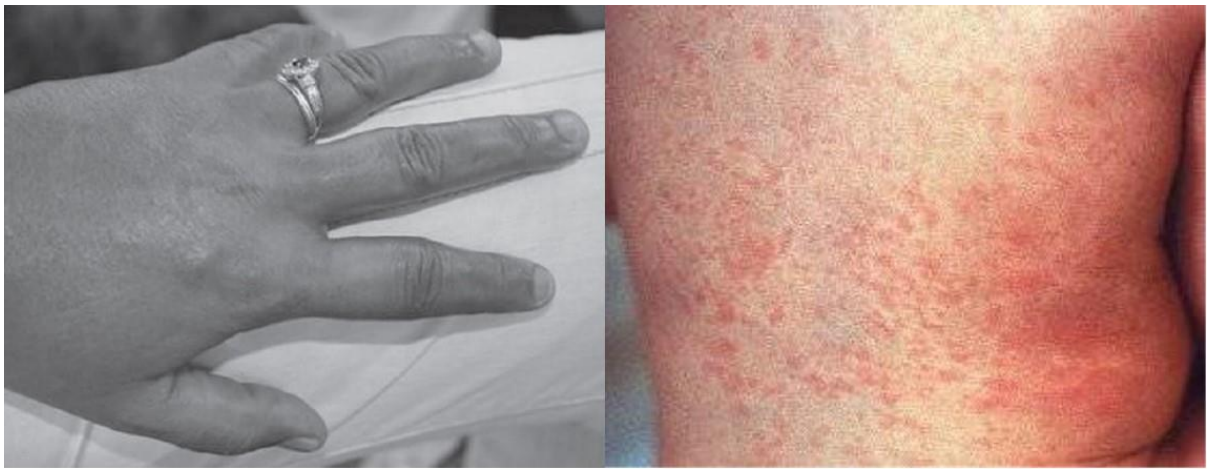


Figure 8: Swelling of hand during acute CHIKV disease and maculopapular rash. Source Hunter et al. 2013 (117) and University of Maryland 2014 (160).

1.4.5. Animal models

Animal models that mimic human disease, are invaluable tools used in the investigation of disease pathogenesis and in the identification of both mechanisms and

factors responsible for the initiation and progression of human disease. The use of a mouse model for RRV-induced disease has helped to unravel the pathobiology of infection and to identify new drugs to ameliorate disease. RRV is one of the most extensively studied old world alphavirus in small animal models, in which the mouse was utilised in the majority of studies (161). The first RRV animal model, developed by Lidbury *et al*., demonstrated that 14-21 day old outbred mice, subcutaneously (s.c.) inoculated with the T48 strain of RRV, were susceptible to RRV disease, characterized by limb weakness, muscle wasting and weight loss (162). The disease of mice correlated closely to the signs seen in the arthritogenic disease of human RRV infection, which provided a novel model to examine the mechanisms of RRV-induced arthritogenic disease. Furthermore, the treatment of mice before infection with macrophage-toxic agents completely prevented RRV-induced muscle inflammation in this model. This suggested a key role for the innate immune response in the development of RRV-induced disease (162). The model was further developed in 24 day old C57BL/6 mice, where bone, joint and skeletal muscle tissues of the hind limbs were the primary targets of RRV infection. Disease signs were observed by 4-5 days p.i., peaked at 10 days p.i. and resolved by 20 days p.i. Infected mice showed severe disease signs such as loss of hind limb gripping, hind limb dragging and a lack of weight gain. Titres of infectious virus were detected at 24 to 48 hours p.i. Inflammatory macrophages, natural killer (NK) cells, $CD4^+$ and $CD8^+$ T lymphocytes were the major components of virus-induced inflammatory response (163). The detection of RRV in multiple bone and joint-associated tissues, such as synovial tissue, periosteum, tendons and ligaments is consistent with observations of RRV-infected humans (164). The large areas of RRV-infected muscle fibres found in the hind limb skeletal muscle tissue in this mouse model could account for the myalgia experienced by 60% of patients diagnosed with RRV disease (153). In addition, both NK cells and macrophages have been detected within synovial exudates from the knees of RRV-infected patients, as well as $CD4^+$ and $CD8^+$ T lymphocytes which have been found in synovial tissue sections from knee joint biopsies (165). In recent years many studies have set their focus on bone loss induced by arthritogenic alphaviruses such as RRV

and CHIKV, after evidence of bone lesions in patients suffering from these viruses (155). The RRV mouse models of RRV disease offers a valuable tool to investigate alphavirus-induced bone loss, as the histological findings resemble human arthritogenic alphavirus disease (166) (Figure 9).

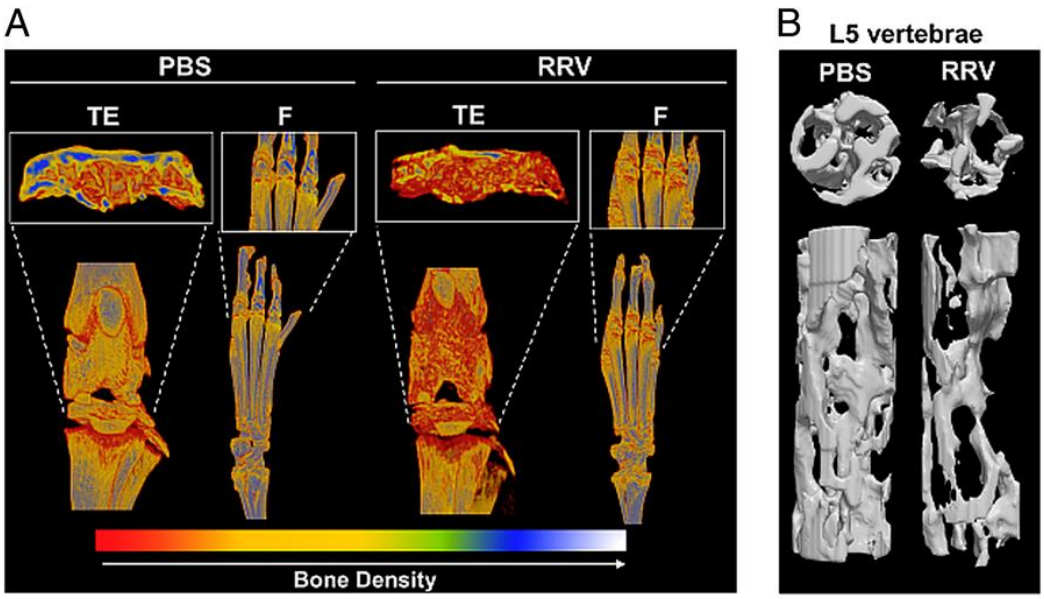


Figure 9: Bone phenotype of RRV-infected C57BL/6 mice. µCT surface reconstruction performed in (A) hind limbs and (B) L5 vertebrae of mock- and RRV-infected WT mice (day 15 p.i.). Source Chen et al. 2014 (166)

1.4.6. Pathogenesis

Host factors - cellular

The development of animal models has led to the discovery of a number of cellular and soluble factors that play a key role in disease initiation and progression. One of the most important findings has been the recognition of the central role of the innate immune system in alphavirus-induced arthritogenic disease (163, 167). Initially, RRV replicates at the site of infection, in cells which are likely to be dendritic (DC) and Langerhans cells (163, 168, 169). From there the virus spreads through the blood stream and proceeds to replicate in skeletal muscle, joint and bone tissues, where the host tissues become penetrated inflammatory infiltrates, mainly macrophages, but also NK cells and $CD4^+$ and $CD8^+$ T cells (Figure 10) (163). In the original RRV mouse model, the macrophages and monocytes infiltration into infected tissues and joints and their crucial role in the development of RRV disease was

shown using macrophage-toxic agents such as silica, carrageenan or liposomes containing clodronate. The gradual clearance of macrophages from damaged tissue correlated with recovery from disease (162). Extensive disruption of striated muscle fibres was associated with the monocyte/macrophage infiltrate, where tissues from infected macrophage-depleted mice exhibited no detectable damage (170). Further RRV mouse studies have also demonstrated that RRV inflammation of joint, bone and skeletal muscle tissue is mostly regulated by the innate immune system (163). This was achieved by using C57BL/6J RAG-1-/- mice, which lack functional T and B lymphocytes. These mice, when infected with RRV, showed similar disease signs to wild-type mice, including failure to gain weight, severe symmetrical hind limb dysfunction and infiltration of macrophages and NK cells. This observation suggested that the adaptive immune response does not play a critical role in the development of RRV disease. However, recent studies have shown that virus titres were higher in muscle tissue but not bone tissue at days 14 and 21 in mice depleted of CD8 T cells (171). Furthermore, T cells contributed to the control of RRV infection in the absence of B cells and antibodies, which indicates a T-cell specific role of the adaptive immune system.

Recent findings have shown the important impact of tissue repairing M2 macrophages on viral titres and pathology in musculoskeletal tissues (172). Severe damage to musculoskeletal tissue of RRV-infected mice promotes a wound-healing mechanism characterised by M2 macrophages. High arginase 1 levels are expressed by the musculoskeletal inflammatory tissues and the present macrophages, leading to the activation of M2 macrophages, which show immunosuppressive activity. The use of a mouse model lacking arginase 1 in neutrophils and macrophages demonstrated dramatically decreased viral loads and improved pathology in RRV disease compared with wild type mice. In accordance with these findings is a new study, where deletion of arginase 1 reduced virus titres and disease severity in RRV-infected mice. Furthermore, when CD4 and CD8 T cells were depleted in Arginase 1 deleted mice, virus loads were restored (173). Conclusively, arginase 1 producing myeloid cells inhibited virus specific T-cells in the

inflamed and infected musculoskeletal tissue, which proves an involvement of not just the innate immune system, but also the adaptive immune system, in fighting alphavirus disease.

DCs have been shown to be an important early target cell for mosquito-borne viruses, capable of producing high levels of IFN-α/β and are thought to be the major producer of cytokines in response to viruses (174-176). In addition, RRV envelope glycans were shown to contribute to IFN production in myeloid DCs (169). Mosquito-cell-derived RRV exhibited enhanced infection of primary myeloid DCs compared to mammalian-cell-derived RRV. Furthermore, mosquito-cell-derived RRV only induced poor IFN induction in comparison to mammalian-cell-derived RRV (177). This seems to be an important mechanism how RRV overcomes the strong, myeloid DCs induced, IFN-system. As the IFN system plays an essential role in the early control of viral infections (178), the viruses' ability to avoid IFN responses by DCs could be a strategy to successfully make the transition between arthropod vector and mammalian host.

Interestingly, in mice studies of the new world Eastern equine encephalitis virus, infection of osteoblasts (OB) was observed with clinical features such as abnormal bone characterised by loss of OB, reduced osteoid production and cartilage hypertrophy (179). This is remarkable as new world alphaviruses are known for their ability to cause encephalitis as opposed to arthritogenic disease. Recent studies have shown the importance of OBs as a niche for viral replication with the ability to produce high levels of inflammatory cytokines *in vitro* for RRV and CHIKV (180, 181). It was shown that primary human OBs were able to induce osteoclastogenesis in response to RRV infection *in vitro* by staining for tartrate-resistant acid phosphatase positive (TRAP+) osteoclast-like cells. The underlying mechanism was determined to be the receptor activator of nuclear factor kappa-B (NF-κB) ligand (RANKL)/osteoprotegerin (OPG) system (182), which is generally disrupted in synovial fluids of RRV patients as well as in the RRV mouse model (180).

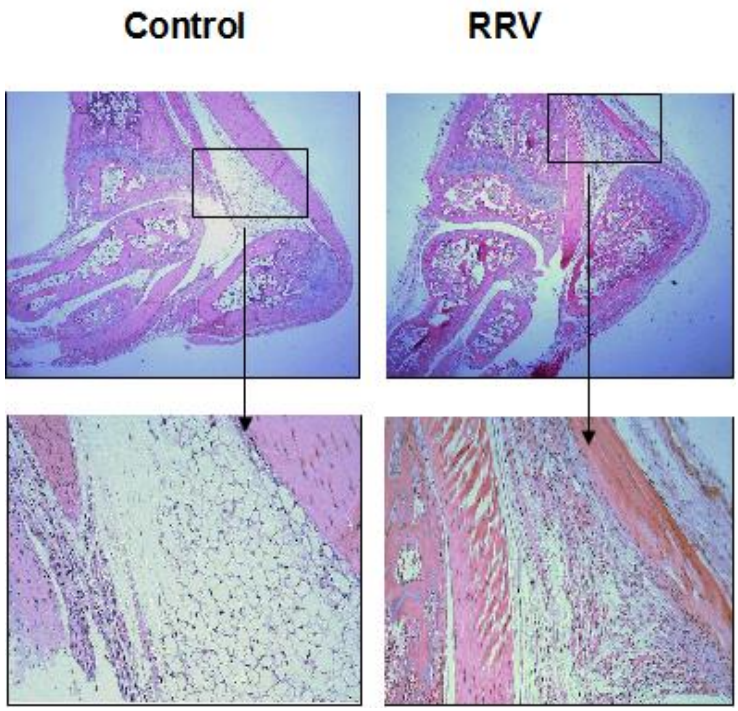


Figure 10: H&E staining of histopathology of the synovium of a mock and RRV-infected mouse ankle. Damaged striated muscle fibres and cellular infiltration was observed in the synovium. Image kindly provided by NE Rulli.

Host factors – cytokines, chemokines and complement

The participation of cytokines and chemokines in RRV infection has been examined in animal models as well as in RRV-infected patients. IFN-γ, TNF, CCL-2 and reactive nitrogen intermediates were detected in the synovial fluid of patients with RRV-induced polyarthritis (170). Gene expression of TNF, CCL-2, IL-1β and IFN-γ levels were significantly higher in muscle and joint tissues in RRV-infected mice (75, 170). In RRV-infected macrophage depleted mice, TNF, CCL-2 and IFN-γ protein levels were dramatically reduced in comparison to mice without macrophage depletion (170), which indicates a correlation between these cytokines and the severity of disease. In addition, RRV-infected macrophages and synovial fibroblasts secrete CCL-2 and IL-8 *in vitro*. The expression of CCL-2 was consistent with the predominant monocyte infiltration found in the synovia of RRV patients, since CCL-2 recruits and activates monocytes and macrophages (183). Thus, there is evidence that these soluble factors enhance the immunopathogenesis of RRV disease (170). In a retroperspective study on laboratory confirmed cases of CHIKV, high levels of IL-1β and IL-6, which are both endogenous pyrogens, were detected. Statistical

analysis showed that an increase in IL-1β and IL-6 were associated with disease severity and can therefore be used as biomarkers (184). IL-1β was also suspected to mediate the development of abrupt and persistent arthralgia since this cytokine is involved in the immunopathogenesis of many arthritic pathologies such as RA. In addition, IL-6 was linked to the development of bone erosion in arthritogenic alphaviral disease in mice (180).

Another pro-inflammatory cytokine associated with macrophage infiltration is the macrophage migration inhibitory factor (MIF). MIF has been identified to be a pivotal regulator of innate immune response and is an important cytokine in the pathogenesis of RA, where MIF is responsible for elevated IL-1, TNF, IL-6, IL-8, cyclooxygenase-2, phospholipase A2 and matrix metalloproteinases (185, 186). In a RRV-induced arthritis mouse model, MIF levels were elevated in serum and tissues with corresponding severe inflammation and tissue damage. MIF-deficient mice ($MIF^{-/-}$), as well as wild type mice treated with MIF antagonists, developed mild disease accompanied by reduced inflammatory infiltrates and muscle destruction in tissues, despite similar viral titres. In addition, adding MIF into RRV-infected $MIF^{-/-}$ mice induced RRV disease (187). In follow up experiments with CD74 knock-out mice, the cell surface receptor of MIF, mild clinical features and low levels of tissue damage were observed in the RRV mouse model. Leukocyte infiltration, in particular inflammatory monocytes and NK cells, were substantially reduced in the tissues of $CD74^{-/-}$ mice. However, the production of pro-inflammatory cytokines and chemokines was not decreased. These mechanistic studies could be valuable in a clinical context, as alphaviral infection with RRV or CHIKV resulted in a dose-dependent upregulation of CD74 expression in human peripheral blood mononuclear cells and serum MIF levels were significantly elevated in patients (188).

TNF is another pro-inflammatory cytokine, which is upregulated during alphaviral infections, where it is expressed by activated macrophages and lymphocytes, but to a lesser extent by NK cells and epithelial cells. Macrophage-depleted mice had dramatically reduced levels of TNF following RRV infection compared to wild type mice. TNF plays an important role in the viral-induced immunopathogenesis via NF-κB and activating protein 1 activated

production of pro-inflammatory cytokines (170). Neutralisation of TNF showed reduced development of severe RRV disease and blocking NF-κB activity ameliorated RRV inflammatory disease and tissue damage in a mouse model. However, TNF is also an important factor in antiviral immunity and its inhibition with anti-TNF agents has been associated with an enhanced risk for herpes zoster in patients treated for RA (189). In a different experiment, the blockage of TNF with the commercial available drug Enbrel® (Etanercept) in RRV-infected mice resulted in dramatic disease exacerbation, increased virus titres and death in 100% of the mice (190). Consequently, TNF may have critical antiviral activity and thus anti-TNF therapy is not recommended for the treatment of alphavirus-induced arthritogenic disease. IFN-α/β are important cytokines in the early control of virus replication by the innate immune system (191). DCs rapidly express IFN-α/β following various infections and in return undergo maturation in response to IFN-α/β. This stimulation potently enhances immune responses, which indicates that IFN-α/β links the signalling between innate and adaptive immunity. Furthermore, studies on the related alphavirus Western equine encephalitis virus demonstrated that IFN-α/β increased antiviral activity and cytoprotective responses in a neuronal cell line model (192). In follow up experiments of the Enbrel-study it was shown that IFN-α/-β levels were greatly reduced or undetectable in mice infected with RRV and treated with Enbrel. Thus, anti-TNF dampened host anti-viral immunity by inhibiting the production of IFN-α/-β. The high potency of Enbrel is therefore likely due to dual inhibition of the synergistic partners TNF and IFN-α/-β (193). TNF has also been demonstrated to be an important contributor of vascular leakage and shock in Dengue virus disease and is thought to act similar in CHIKV disease (194, 195). TNF was heavily elevated in a CHIKV mouse model with compromised IFN-α/β response.

The development of alphaviral disease has been shown to depend on numerous soluble factors. The complement system for instance, which is a complex system of pathways comprising over 30 different proteins with the main function of eliminating invading organisms, can be activated through the lectin pathway (161). A recently discovered important factor in alphavirus-induced disease is the mannose binding lectin (MBL). Within

the host complement system, the MBL pathway, but not the classical or alternative complement activation pathways, is essential for the development of RRV-induced disease (196). This pathway is centralised around the action of the protein complement component 3 (C3), which has been shown to have a crucial role contributing to the destructive phase of the inflammatory disease and triggering severe inflammatory arthritis and myositis (197). In RRV mouse studies, C3 activation products, such as the complement receptor 3 (CR3) ligand iC3b, have been found at the sites of RRV-induced inflammation. C3 was suspected to be responsible for the tissue destruction phase of RRV-induced inflammatory disease (167). The role of CR3 in the pathogenesis of RRV disease was supported by an animal model using mice deficient in functional CR3 (CD11b-/-) that showed substantially reduced disease signs and tissue destruction when infected with RRV (197). MBL-deficient mice showed reduced disease, tissue damage and complement deposition compared to wild-type mice. These findings correlate to the serum levels of human patients diagnosed with RRV disease, which had elevated serum MBL levels compared to healthy controls (196). Furthermore, severity of disease was associated with higher MBL levels in the serum and synovial fluid in patients suffering RRV disease.

1.4.7. Current treatments

The use of animal models has a great impact on the identification of potential drug targets, which possibly leads to the development of effective anti-alphaviral therapeutics. At this moment, no licensed vaccines or specific anti-viral therapeutics are available on the market to prevent or treat arthritogenic alphaviral disease. Infections caused by RRV are restricted to symptomatic treatment, with simple analgesics like paracetamol and NSAID (123, 152). Oral corticosteroid therapy has also been used on treating symptoms of RRV disease (157). Although patients were generally satisfied with the clinical outcome of these treatments, there remain concerns as these drugs are not suitable for long term use due to possible side effects like osteonecrosis for corticosteroids or gastrointestinal issues for

NSAIDs. In theory, corticosteroids could make RRV disease worse by dampening the immune response, however, RRV patients in a small cohort study seemed to have benefited from corticosteroid treatment (123, 152, 157).

A different approach to relief alphavirus-induced rheumatic symptoms is the use of TNF blockers. In a limited study with patients suffering from alphaviral disease, in particular CHIKV disease, this has led to successful amelioration of the rheumatic symptoms (198). In accordance are results obtained from a RRV mouse model, where inhibition of the pro-inflammatory factors TNF, IFN-γ and CCL-2 with neutralising antibodies resulted in reduced severity of RRV disease (170). However, as stated previously, administration of Enbrel to mice infected with RRV resulted in exacerbation of myositis and arthritis as well as increased viral titres. Thus anti-TNF agents may be ill-advised during the acute phase of RRV disease (190). Another approach is to target NF-κB activity, which is linked to subsequent cytokine production and plays a role in RA in humans, as well as in animal models of inflammatory arthritis (199). This has been successfully studied in the RRV mouse model, where blocking NF-κB activity with sulfasalazine led to milder disease with less severe tissue destruction and lower levels of inflammatory cell recruitment compared to untreated mice (170). The novel drug bindarit, which is currently under development by the Italian pharmaceutical company Angelini, is a potent inhibitor of CCL-2 synthesis. In animal models of alphavirus-induced disease, bindarit was able to diminish cell infiltration and to lower production of NF-κB and TNF, which are both involved in mediating tissue damage (74, 75).

There is also hope for drug repurposing of the wide range of anti-rheumatic drugs which are being developed for RA to treat alphaviral arthritides. However, drugs that compromise viral immunity should be avoided during the acute phase of disease, as seen in the Enbrel study described above. In accordance is an approach with methotrexate to treat RRV disease in mice. After positive feedback from methotrexate treatment in humans during the 2005-2006 La Reunion outbreak (158), methotrexate was tested in a RRV mouse model. In that study, methotrexate treatment worsened RRV-induced musculoskeletal disease with

a significant influx of inflammatory cell infiltrates into the skeletal muscle tissue without affecting the levels of pro-inflammatory cytokines and chemokines. The viral load was increased at early time points, which suggests that methotrexate treatment of acute RRV disease in mice provides no therapeutic benefit (200). In recent studies focusing on alphavirus-induced bone loss, several approaches have been shown promising as potential treatment options. In an animal model of RRV-induced bone loss, antibodies of IL-6 prevented disease shown as reduced bone volume increment measured with μCT (166). Similarly, in another study using the pre-clinical compound bindarit, RRV and CHIKV-induced bone loss was prevented in mice (201). In a different approach targeting cartilage destruction with a novel glycosaminoglycan-like molecule, pentosane polysulfate successfully reduced cartilage destruction and inflammatory disease in animal models of RRV and CHIKV (202).

1.4.8. Vaccine

In a passive immunisation study with human immunoglobulins tested in mice, the administration of anti-CHIKV antibodies exhibited a high *in vitro* neutralizing activity and a powerful prophylactic and therapeutic efficacy against CHIKV infection *in vivo* (203). This approach could help in preventing infections of neonates born to viraemic mothers by administering antibodies at birth. Passive immunisation could also be recommended for exposed heavily immunocompromised patients or even be used prophylactically during an epidemic. Nevertheless, the administration of antibodies before endogenous antibody production is likely to be impractical, because disease outbreaks are often sudden and affect large parts of the population. Thus the wide administration of antibodies and their production could be limiting factors.

Several CHIKV vaccine candidates have been developed since the 1980s, but none has turned out to be practical for the vaccination of populations in endemic regions (204). Inactivated virus, deoxyribonucleic acid (DNA) and virus-like particle vaccines offer major

advantages in safety, but require multiple dose application and can be cost intensive in manufacturing. Live-attenuated vaccines offer rapid, single-dose protection and long-lived immunity, but can be unstable in traditional attenuation approaches, leading to adverse events following administration (205). Live-attenuated vaccines can also be capable of infecting mosquitoes, which is a major safety concern for their use in non-endemic regions when there is a potential for the reversion of attenuating point mutations (206). However, a recent study of two live-attenuated vaccine candidates based on the insertion of a picornavirus internal ribosome entry site sequence into the genome of CHIKV in macaques, the animals showed no signs of disease and the vaccine was highly immunogenic after single dose application (207).

An adjuvanted, inactivated whole-virus RRV vaccine tested in phase I/II human trials was reported to be highly immunogenic in RRV-naïve adults and well tolerated at all dose levels (208). Human vaccinee sera were administered to mice before RRV challenge and protected them from viraemia. Antibody-induced enhancement of infection did not occur in mice and vaccine-induced antibodies were partially cross-protective against infection with the related alphavirus CHIKV (208, 209). Cross-protection has also been shown for CHIKV in another study where mice that had previously been infected with RRV or treated with anti-RRV antibodies did not induce disease (210). Phase III human trials of the vaccine candidate have been completed and the results have recently been published. The inactivated RRV vaccine was well tolerated and immunogenic among 2,000 healthy adults and no arthritis resembling RRV disease was reported among the vaccinated individuals (211). In a novel vaccine approach, a virion lacking the 6k proteins were shown to cause milder disease than wild-type virus, with viral titres being reduced in infected mice. Immunisation with the 6k mutant resulted in a reduced viral load and accelerated viral elimination upon secondary infection with wild-type RRV or CHIKV (212). The phase III vaccine candidate seems to be a very promising prospect under development. However, major hurdles for the commercial development and ultimate deployment of vaccines against arthritogenic RRV exist, such as a small market size and the fact that disease is normally

self-limiting. However, there are currently four vaccine candidates undergoing clinical trials for the related alphavirus CHIKV; with live-attenuated, virus-like particles, inactivated whole virus and live-vectored approaches (213). This is mainly due to great efforts by pharmaceutical companies after the rise of CHIKV over the last decade, with transmission reported in more than 100 countries worldwide and millions of cases each year.

1.4.9. IL-1 cytokine family and anakinra

Interleukin 1

The cytokine IL-1 affects nearly every tissue and organ system and is considered a "prototype" of the pro-inflammatory cytokines. IL-1 is also considered as the "alarm" cytokine that acts in a variety of defence mechanisms, immunologic and hematologic responses in particular (214-216). It increases body temperature and induces the expression of various genes and the synthesis of several proteins such as acute phase proteins and chemoattractants for lymphocytes. In this manner IL-1 can induce acute and chronic inflammatory disease. RA, osteoarthritis, type II diabetes, gout/pseudogout or hereditary disease such as Cryopyrin-associated periodic syndrome (CAPS) are all examples of diseases linked to IL-1 induced inflammation (214). In autoimmune disease (e.g. RA, Crohn's disease) blocking TNF can be highly effective, where in autoinflammatory disease (e.g. Hereditary disease CAPS, gout/pseudogout, type II diabetes) the release of IL-1 is elevated and its inhibition has been successful in ameliorating disease. In addition, bone is highly sensitive to IL-1, which is involved in the regulation of both bone formation and bone resorption. IL-1 has also been identified as osteoclast activating factor and participates in various steps of osteoclast development *in vitro* (217). OBs have to be present in order to obtain mature osteoclasts, the only cell type able to perform bone resorption, through the RANKL system (218). IL-1, IL-6 and TNF have bone actions to promote osteoporosis and subchondral bone resorption, where they act on OBs to promote RANKL expression and consequently induce osteoclastogenesis (Figure 11) (219). RANKL acts through its receptor

RANK and is essential in bone resorption. The ratio between RANKL and its natural inhibitor, the decoy receptor OPG, is an index of osteoclastic stimulus and plays a crucial role in the skeletal deterioration in RA (220, 221). RANKL has also been demonstrated to upregulate the expression of IL-1 receptor (IL-1R) (222). Furthermore, IL-1 can stimulate RANK directly, which makes this cytokine especially interesting as a target in the prevention of bone loss (217). Interestingly, inhibition of both IL-1 and TNF prevented the pathologic increase in serum RANKL and local bone loss in ankles of two rat arthritis models (223). This adds up with the fact that IL-1 is a downstream mediator of TNF-induced disease and has been shown to be downregulated spontaneously in RA patients with anti-TNF antibody treatment (224, 225).

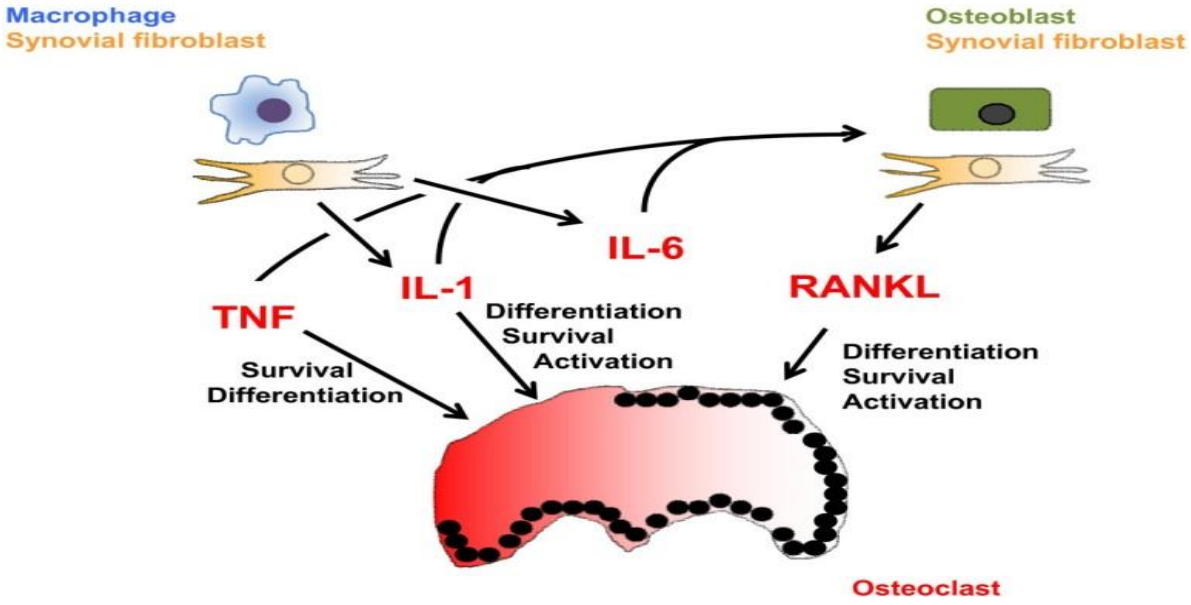


Figure 11: Cytokines activating osteoclastogenesis in RA. TNF, IL-1 and IL-6 upregulate expression of osteoblasts and synovial fibroblasts. RANKL mediates differentiation, survival and activation of osteoclasts. TNF, produced by fibroblasts and macrophages, promotes differentiation and survival of osteoclasts. IL-1 supports differentiation, survival and activation of osteoclasts. IL-6 promotes osteoclastogenesis indirectly. IL-6 is largely produced by fibroblasts and macrophages and enhances the expression of RANKL. Source Braun et al. 2011 (226).

IL-1 family and receptors

The IL-1 family consists of 11 members (227) and includes the 3 structurally related polypeptides: IL-1α, IL-1β and IL-1R antagonist (IL-1RA) (228), which genes are similar and located close to each other in the human chromosome (229, 230). They all act on the same

receptors, whereas IL-1RA does not induce any intracellular response and is therefore considered as a natural IL-1 antagonist (231). Thus, IL-1RA as well as the recombinant form anakinra are competitive IL-1R antagonists with very high affinity in the picomolar range (232, 233). Both IL-1α and IL-1β are synthesised as precursors. Where pro-IL-1β is biologically inactive, IL-1α can induce a response in its pro and mature form (234). IL-1 binds to three different receptors, IL-1RI, IL-1RII, IL-1RAcP, which all can be found either as membrane-bound or soluble proteins expressed on all cells except red blood cells (235). IL-1RI binding induces the recruitment of IL-1RAcP and downstream cell signalling, whereas IL-1RII does not transduce any intracellular signal and thus can act as a regulatory decoy receptor (236). IL-1α/-β are primarily synthesised by activated monocytes and macrophages (228, 237). However, IL-1α remains associated with the plasma membrane and mainly functions by stimulating cell-cell interactions (238), whereas IL-1β is the predominant secreted form (239). IL-1β is primarily a product of monocytes and macrophages, but can also be released by DCs as well as B-lymphocytes, NK cells and OBs (180, 181, 240). Unlike IL-1α, the IL-1β precursor is not present in health where circulating human blood monocytes or bone marrow cells do not express mRNA for IL-1β continuously. On the other hand, endothelial cells, skin keratinocytes, fibroblasts and epithelial cells contain constitutive IL-1α as precursors as well as mRNA (240).

Anakinra (Kineret®)

The biopharmaceutical Kineret® with its active ingredient anakinra is a 17 kDa recombinant protein homologues to the natural occurring IL-1R antagonist (IL-1RA) (241). Kineret® has been approved by the FDA for the use against RA since 2001 (242). Daily s.c. injections of 100 mg reduce the signs and symptoms of active disease and help to prevent progression of disease while relieving pain and discomfort (243-246) Unlike TNF-blockers, which are commonly used in the treatment of RA, anakinra does not increase the risk for opportunistic infections such as tuberculosis (247). Anakinra has been demonstrated to be safe and well tolerated in a diverse population of patients with RA in a large and placebo

controlled safety study. No infections were linked to opportunistic microorganisms or resulted in death (248). Anakinra is rapidly excreted by the kidney and IL-1Rs are readily generated each day. This can lead to low blood levels of anakinra within 24 hours, thus daily injections are necessary (235).

1.5. Thesis objective

This thesis investigates therapeutic approaches in two models of viral inflammatory disease: 1) lung inflammation in mice infected with IAV, and 2) musculoskeletal disease following infection with an arthritogenic alphavirus. These models are well established and are highly suited for testing novel therapeutics for the corresponding human diseases. The objective is to test drugs under development and to re-purpose drugs already in use for other diseases with the goal of identifying new treatments for infections by alphaviruses, IAV and other pathogens. In addition, an IAV surveillance study in swine from the Southeastern USA and a high-throughput screening (HTS) on host cell miRNAs for IAV (H7N9) have been conducted.

Chapter 2 - Molecular Surveillance and Preliminary Subtyping of Influenza A Viruses in Swine, Southeastern United States, Summer 2014

Manuscript in preparation

Statement of Contribution to Co-authored Published Paper

This chapter includes a co-authored paper. The bibliographic details of the co-authored paper, including all authors, are:

Stefan Wolf*, Constantinos S. Kyriakis*, Les P. Jones, Byoung-Shik Shim, Jarod M. Hanson, Ming Zhang and Ralph A. Tripp

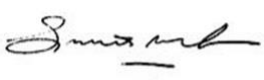
* Co-first authors

My contribution to the paper involved:

Substantial contributions to acquisition of data, analysis and interpretation of data, including the provision of data, the complete analysis and presentation of data into publishable format.

Stefan Wolf Gold Coast, 18.08.16

Corresponding author of paper: Prof. Ralph Tripp Gold Coast, 18.08.16



Supervisor: Prof. Suresh Mahalingam Gold Coast, 18.08.16

Preface

Influenza A viruses are among the leading pathogens, causing epidemics of respiratory disease in humans, resulting the death of more than five hundred thousand people annually and the hospitalization of many more. The ability of influenza A viruses to cross species barriers and infect new hosts, or reassort with circulating viruses has raised public health concerns about the possibility of novel viruses emerging in animals and then crossing to humans. The 2009 H1N1 pandemic virus (pmH1N1) that emerged through the reassortant of previously known swine influenza A viruses highlighted the role of pigs in ecology of influenza A viruses and the importance of the species for the protection of public health. Swine and human influenza viruses have a parallel history since the co-occurring of respiratory disease in the 1918-19 Spanish Influenza pandemic, when outbreaks of acute respiratory disease were recorded in pigs and their human cohorts.

Molecular Surveillance and Preliminary Subtyping of Influenza A Viruses in Swine, Southeastern United States, Summer 2014

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Abstract

Swine influenza A virus (IAV) causes a widespread debilitating respiratory disease with morbidity, mortality and substantial economic impact to the industry. Furthermore, swine are considered as “mixing vessels” linked to the generation of pandemic influenza viruses due to their susceptibility to both avian and human IAVs. Currently, three IAV subtypes (H1N1, H3N2 and H1N2) circulate in swine in North America. They are of diverse genetic origin, frequently combining gene segments from avian and/or human viruses. The objectives of this study were to investigate the prevalence of IAV in commercial swine herds between June and August of 2014, identify and characterize viruses that circulate in the population. A total of 1,878 oral fluid samples were collected from pigs of all ages from 201 commercial farms located in North and South Carolina. RNA was extracted from each sample and screened with M-gene Real-Time PCR. Positive samples were subtyped using a multiplex Real-Time RT-PCR. Sixty-eight oral fluid samples from 35 farms were found positive in the M-gene PCR. The overall rate of IAV-positive samples was 3.6%, comparable to other active surveillance studies conducted in the past. However, on a herd level, the percentage of IAV-

positivity was significantly higher reaching 17.4%. Fifty-six viruses were subtyped, while 12 were partly subtyped or not subtyped at all. The majority of the IAVs subtyped had an H1 hemmaglutinin, indicating a significant higher prevalence over H3 viruses, which may have important implications for the vaccination strategies followed by swine producers.

Introduction

Influenza A viruses (IAVs) are members of the *Orthomyxovirdae* family. They are enveloped, single stranded ribonucleic acid (RNA) viruses with a segmented genome (1). This constitutes of 8 RNA segments encoding between 11 and 13 proteins, including the two transmembrane “spike-like” glycoproteins: the hemagglutinin (HA) and the neuraminidase (NA). The HA and NA are of particular interest because they facilitate the entry and release of the virus into and out of the host cell and they are the primary targets recognized by the immune system after infection and vaccination (2, 3) . They are also the two proteins that determine the subtype of the IAV. The significant genetic and antigenic changes that may result in virus alteration in IAV, occur in the HA and NA genes. The six remaining gene segments, known as the “internal protein genes”, encode the matrix protein (M), the Non-Structural protein and the ribonucleoprotein complexes, which consist of the nucleoprotein, and three polymerases (4).

IAVs are among the leading pathogens, causing epidemics of respiratory disease in humans, resulting in the death of more than five hundred thousand people annually and the hospitalization of many more (5). The natural reservoir of IAVs lays in the wild migratory waterfowl population, but the range of species that may be infected includes domesticated poultry, and mammals, from humans and swine to felines, horses, dogs and aquatic mammals (6, 7). The ability of IAVs to cross species barriers and infect new hosts, or reassort with circulating viruses, has raised public health concerns about the possibility of novel viruses emerging in animals and then crossing to humans. The 2009 H1N1 pandemic virus (pmH1N1), which emerged through the reassortment of previously known swine IAVs, highlighted the role of pigs in the ecology of IAVs and their importance for the protection of public health (8). Swine and human influenza viruses have a parallel history since the co-occurrence of respiratory disease in the 1918-19 Spanish Influenza pandemic, when outbreaks of acute respiratory disease were recorded in pigs and their human cohorts (9, 10).

Furthermore, influenza in swine is one of the most important viral diseases of pigs themselves, with a heavy economic burden to the pork industry. In the US alone, the annual cost of swine influenza is estimated between $360 million and $1 billion (11, 12). The clinical manifestation of the disease in pigs may range from asymptomatic to widespread respiratory disease characterized by abdominal breathing, coughing, sneezing, fever, anorexia and lethargy (13). Three subtypes of IAVs are found in pigs worldwide; H1N1, H3N2 and H1N2 (8, 14-17). The epidemiology of IAVs in swine in North America has become very complex over the last two decades with the same three strains circulating in pigs, but including gene segments of diverse genetic and antigenic origin, which complicates diagnosis and prevention (18, 19). Until the late 1990s, only one subtype of single origin IAV was found in swine in North America, the H1N1 virus, which is directly descending from the 1918-19 outbreak. This virus was known as the “classical swine” H1N1 virus. However, since 1998, a variety of viruses have been found, baring genes of human and avian influenza origins. These viruses resulted from single, double or triple reassortant events (20, 21). H1N1, H3N2 and H1N2 viruses with HA and NA of human origin and different combinations of internal genes have established themselves in the swine population and since been circulating with varying success. The so-called “triple reassortant cassette” that includes internal protein genes of human, avian and classical swine, became established as the dominant backbone of IAVs in pigs, with different combinations of HA and NA proteins (21). Since 2009, with the introduction of the reassortant pmH1N1 virus to pigs, further reassortment events have been recorded both in North America and Europe (22, 23). While the pmH1N1 has not been able to become established in the pig population, reassortant viruses that include one or more genes of pandemic origin are frequently isolated. In 2012-13 the introduction of human seasonal H3 gene in swine-origin NA and internal protein gene backbone was identified and followed up (24).

Taking into account the complexity of IAV epidemiology in pigs and the importance of constantly monitoring the situation, we proceeded to conduct a long-term surveillance study, initially focusing on the Southeastern United States and specifically in North Carolina. Pig

production in the US is characterized by the fact that there is a disproportionate number of sow farms in North Carolina compared to other pork producing states (25). From there, weaned piglets, at the age of 21 to 28 days, are shipped to the Midwest, where they are raised until they are lead to consumption at six months of age. Through transportation of the piglets to finishing farms in different states, IAVs travel and when the piglets mix, they may reassort. Thus, the Southeastern region of the US plays a key role in the epidemiology of IAVs. Here we report the results of our initial active surveillance-screening of IAVs in pigs and the subtypes of viruses we were able to identify in the Summer of 2014. The aim of this study was to set up a baseline for our knowledge of the various IAV strains circulating in the area, to sequence these viruses and to identify known or unknown reassortment events.

Materials and Methods

Sample collection

Between June and August 2014, we conducted active surveillance in swine farms in the states of North and South Carolina. Unlike passive surveillance, when only diseased pigs are sampled, in active surveillance clinical specimens are screened for influenza on a regular time basis regardless of their health status. Overall, 1,878 oral fluid samples from 201 farms were processed. Oral fluids are sampled by hanging a cotton rope in a pen of pigs, allowing the animals to chew on it and then by collecting fluid that has accumulated in the rope. Thus, each sample is not from an individual animal, but from multiple pigs, which is an advantage of this sampling method, because it allows for faster and more efficient detection of influenza from a particular herd (26). Although oral samples contain contaminants, such as feces and feed and are not the ideal medium for virus isolation, they have been proven to be efficient for pathogen screening in pigs (27).

Influenza A Virus screening

RNA was extracted by the RNAzol RT method (28). Briefly, 200µl of each clinical specimen was mixed with 500µl of an acid guanidinium thiocyanate-based commercial product (RNAzol® RT, Molecular Research Center, Inc. USA) and 200µl of molecular grade water (Thermo Fisher Scientific, Waltham USA). Following centrifugation at 12,000g for 15 minutes, 700µl of supernatant were transferred in a new tube and centrifuged for 10 minutes at the same speed. RNA was isolated after washing once with 100% isopropanol and twice with 75% ethanol. The RNA was re-suspended in 20µl molecular grade water.

Initial IAV screening was conducted by real-time reverse transcription PCR (RRT-PCR) selective for the M-gene. Sequences for the primers and probe were based on the assay described by Spackman and coworkers (2002) (29) and Richt and coworkers (2004) (30). For each reaction, 0.5 pmol of each primer and 0.1µM of probe were combined with 6.75µl of OneStep RRT-PCR Master Mix (Taqman® Fast Virus OneStep Master Mix, Thermo Fisher Scientific, USA) and 2µl of each sample containing 200ng of RNA. Molecular grade water was added to reach a total volume of 25µl. RRT-PCR was carried out using the Stratagene Mx3005P real-time PCR system (Agilent Technologies, Santa Clara USA). The reverse transcription (RT) step was set at 50°C for 20 minutes, followed by 10 minutes of RT inactivation at 95°C. The PCR cycling protocol included 40 cycles of 95°C for 10 seconds and 50°C for 20 seconds. Ct values <32 were considered positive. RRT-PCR positive samples were subtyped by multiplex RRT-PCR.

IAV subtyping

The multiplex RRT-PCR for swine IAV subtyping was based on the method described by Zhang and Harmon (2014) (31). Two separate RRT-PCR reactions were performed; one for the HA and another for the NA type. The VetMAXTM-Gold SIV Detection Kit (Life Technologies, USA) was used, which included H1H3 and N1N2 primer-probe mixes. In summary, 6µl of OneStep RRT-PCR Master Mix (Taqman® Fast Virus OneStep Master Mix,

Thermo Fisher Scientific, USA) and 8µl of sample of RNA were combined with 1µl of H1H3 or N1N2 primer-probe mix and 10µl molecular grade water to reach a volume of 25µl. The thermocycler was set at following cycles: one RT step at 50°C for 20 minutes, 10 minutes of RT inactivation at 95°C and 40 cycles of 95°C for 15 seconds and 60°C for 45 seconds. Ct values between 24 and 30 in the VIC channel were regarded as positive for H1 and N1 and in the FAM channel for H3 and N2.

Results

Of the 1,878 oral fluid samples examined, 68 (3.6%) were positive by the M-gene RRT-PCR. These samples came from 35 out of 201 farms, indicating a circulation of IAVs in 17.4% of the herds examined.

Table 1 shows the subtypes of swine IAVs identified by multiplex RRT-PCR. The majority of the samples tested, 56 out of 68 were successfully subtyped, while six sampled were partially subtyped. Neither the HA or the NA was identified in another 6 samples. No H3N1 virus was identified. The main IAV subtype identified was H1N1 (35.3%), while H1N2 and H3N2 viruses were also detected at a rate of 23.5% and 16.2%, respectively. Interestingly, in 5 samples there was evidence for a mixed infected with two or more subtypes of IAV, which potentially gives ground for reassortment events.

Discussion

While only 3.6% of the samples examined were found positive for IAV, on herd level we found a high prevalence of infection, indicating that at any given time, even during the summer months, IAVs are circulating in 17.4% of the farms. While clinical manifestation of influenza in swine has been recorded primarily from October to April, the virus may be present all-year round, as it has been reported in previous studies (14, 15, 32). The

percentage of positive samples, while it may appear low, is comparable with other IAV active surveillance studies conducted in swine (15, 19).

An important issue addressed in this study is the practical application of oral fluids as a sample for monitoring influenza or other swine pathogens. While we successfully detected and subtyped SIV with RRT-PRC, we failed to isolate virus from RRT-PRC positive samples in cell cultures. This may have been partly attributed to the fact that the samples were collected for routine Porcine Respiratory and Reproductive Syndrome Virus monitoring by the swine producer we collaborated with and were stored at 4°C for at least three weeks before they arrived at our laboratory. Furthermore, oral fluid samples contain environmental contaminants, such as urine, feces and feed, but also saliva, which may inactivate virus. However, according to both our experience from consequent testing and other studies indicating that virus can be isolated at a high percentage rate (>75%) when processing of oral fluid samples is being initiated within 48 hours of collection (26, 27). Thus, oral fluid samples are a proper material for swine virus monitoring and isolation as long as it is handled in an adequate and timely matter. However, performing next generation sequencing for IAV whole genome sequencing directly from oral fluid samples may be problematic as the isolated material contains RNA from swine cells, bacteria, fungi and other viruses.

Our preliminary subtyping data showed a strong prevalence of H1 IAVs over H3 viruses. Specifically, 47 out of 60 samples that were fully subtyped or only partially subtyped at the level of HA, were identified as containing H1 IAV. On the other side, only 16 samples contained an H3 virus, while mixed infections containing an H1N1 and an H3N2 virus were found in 3 samples. The prevalence of H1 and H3 subtypes is not in accordance with the epidemiological picture observed in other studies conducted for other swine-producing areas of the USA, such as the Midwestern part of the country, where about one third of swine IAVs isolated bare an H3 HA (19, 33, 34).

Consequently, the findings of this study are of significant importance, since commercial swine IAV vaccines are either bivalent, containing and H1N1 and an H3N2 strain or trivalent, containing H1N1, H3N2 and H1N2 viruses (35, 36). Further genetic investigation is required

in order to clarify the exact classification of the H1 HA found in this study, such as next generation sequencing. This will allow the suggestion of alternative vaccine strategies, adjusted to regional differences in the epidemiology of swine IAVs.

Finally, the multiplex method used in this investigation for subtyping swine IAVs was able to identify 56 out of 68 M-gene positive samples, while it either partly identified or did not identify identify at all the remaining 12. These samples had a Ct value ≥ 28, indicating a correlation between low Ct value and successful sub typing by this method.

Acknowledgments

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Table 1: Swine influenza A viruses identified by multiplex RRT-PCR.

	Number of samples (percent)
Single infection:	
H1N1	24 (35.3)
H3N2	11 (16.2)
H1N2	16 (23.5)
Mixed infection:	
H1N1 and H3N2	3 (4.4)
H1N1 and H1N2	2 (2.9)
Partial subtype:	
H1Nx	2 (2.9)
H3Nx	2 (2.9)
HxN1	1 (1.5)
HxN2	1 (1.5)
No subtype identified:	6 (8.8)

Chapter 3 - MicroRNA Regulation of Human Genes Essential for Influenza A (H7N9) Replication

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Statement of Contribution to Co-authored Published Paper

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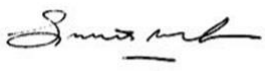
My contribution to the paper involved:

Substantial contributions to conception and design, acquisition of data, analysis and interpretation of data in validation studies. The work included the provision of data, the complete analysis and presentation of data into publishable format and providing direction on the scope and structure of the analysis.

Stefan Wolf Gold Coast, 18.08.16

Corresponding author of paper: Prof. Ralph Tripp Gold Coast, 18.08.16



Supervisor: Prof. Suresh Mahalingam Gold Coast, 18.08.16

Preface

The previous chapter focused on the importance of surveillance of influenza A viruses in swine for the detection of strains that reassert and potentially cause pandemic in humans. Novel avian influenza A (H7N9) virus with pandemic potential and high mortality has recently emerged in China in 2013. No vaccine is currently available and the use of antivirals is complicated due the frequent emergence of drug resistant strains. Thus, there is an imminent need to identify new drug targets for therapeutic intervention.

In the current study, a high-throughput screening (HTS) assay was performed using microRNA (miRNA) inhibitors to identify new host miRNA targets that reduce influenza H7N9 replication in human respiratory (A549) cells. Validation studies lead to a top hit, hsa-miR-664a-3p, that had potent antiviral effects in reducing H7N9 replication (TCID50 titers) by two logs. In silico pathway analysis revealed that this microRNA targeted the LIF and NEK7 genes with effects on pro-inflammatory factors. In follow up studies using siRNAs, anti-viral properties were shown for LIF. Furthermore, inhibition of hsa-miR-664a-3p also reduced virus replication of pandemic influenza A strains H1N1 and H3N2.

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RESEARCH ARTICLE

MicroRNA Regulation of Human Genes Essential for Influenza A (H7N9) Replication

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Abstract

Influenza A viruses are important pathogens of humans and animals. While seasonal influenza viruses infect humans every year, occasionally animal-origin viruses emerge to cause pandemics with significantly higher morbidity and mortality rates. In March 2013, the public health authorities of China reported three cases of laboratory confirmed human infection with avian influenza A (H7N9) virus, and subsequently there have been many cases reported across South East Asia and recently in North America. Most patients experience severe respiratory illness, and morbidity with mortality rates near 40%. No vaccine is currently available and the use of antivirals is complicated due the frequent emergence of drug resistant strains. Thus, there is an imminent need to identify new drug targets for therapeutic intervention. In the current study, a high-throughput screening (HTS) assay was performed using microRNA (miRNA) inhibitors to identify new host miRNA targets that reduce influenza H7N9 replication in human respiratory (A549) cells. Validation studies lead to a top hit, hsa-miR-664a-3p, that had potent antiviral effects in reducing H7N9 replication ($TCID_{50}$ titers) by two logs. *In silico* pathway analysis revealed that this microRNA targeted the LIF and NEK7 genes with effects on pro-inflammatory factors. In follow up studies using siRNAs, anti-viral properties were shown for LIF. Furthermore, inhibition of hsa-miR-664a-3p also reduced virus replication of pandemic influenza A strains H1N1 and H3N2.

Introduction

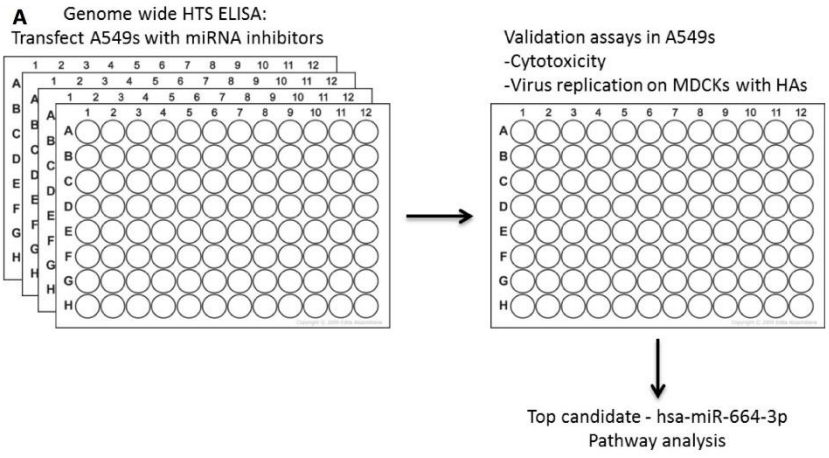
Influenza virus is still a serious global health threat affecting humans, wildlife and agricultural species. Human infection with avian influenza A H7N9 virus (H7N9) were first reported in China in March 2013 [1] Most of the infections are believed to have resulted from exposure to infected poultry or contaminated environments, as H7N9 viruses have been found in poultry in China. While some mild illnesses in humans infected with H7N9 has been reported, most patients experienced severe respiratory illness, such as pneumonia (97.3%) and acute respiratory distress syndrome (71.2%), leading to high rates of intensive care unit admissions [2]. Human mortality attributed to influenza H7N9 is over 38% with 175 deaths from 450 confirmed cases within a 20-month period [3]. No evidence of sustained human-to-human transmission of H7N9 has been recorded; however, there was some evidence for limited person-to

person spread under rare circumstances [4]. H7N9 began in China, but now has rapidly spread to other countries [5]. Recently, the first documented case of H7N9 in humans was reported for North America in Canada [6]

No vaccine is currently available for H7N9 [7]. There are several drugs available for the treatment of influenza infections including the M2 ion channel inhibitors amantadine and rimantadine, and the neuraminidase inhibitors, zanamivir and oseltamivir [8, 9]. Early treatment with these antiviral drugs has been shown to reduce the duration of symptoms and time to recovery, however, the use of antiviral drugs is complicated by the emergence of drug resistant viruses [10, 11]. Consequently, oseltamivir-resistant H7N9 strains have already been described in recent reports from Taiwan [12]. In addition, the use of antiviral drugs may have an effect on population vulnerability due to lack of seroconversion, as well as driving drug resistance among circulating strains [13]. To prevent the spread of infection, new drug and vaccine development is needed. However, difficulties include a lack of understanding of the host factors required for replication, and unusual mutations that occur in the virus that differ from other avian influenza viruses [14].

Linking high-throughput screening (HTS) with RNA interference (RNAi) allows for the rapid discovery of the molecular basis of disease pathogenesis, and the identification of potential pathways for the development of safe and effective treatments. Recent advances in our understanding of RNAi have allowed for genome-wide screens to determine and validate the host cell genes that may are required for influenza virus replication [15] Small interfering RNA (siRNA) can be readily developed to target viral or host genes, and have been successfully applied in disease intervention approaches. For example, siRNA targeting respiratory syncytial virus has been efficacious for silencing virus replication [16]. In the case of influenza, inhibiting the host gene CAMK2B prevented virus replication *in vitro* [17], and knocking down trypsin also inhibited virus replication and apoptosis [18]. In a siRNA screen of 481 human protease genes in A549 cells, 5 genes, ADAMTS7, CPE, DPP3, MST1 and PRSS12, were identified as essential for influenza virus replication [19]. Another siRNA screen of 720 human protein kinase genes (HPK), 17 HPKs were validated as essential for influenza A replication [20]. In both screens vital genes for influenza A replication were found that affect multiple host pathways and that are regulated by microRNAs (miRNAs) induced during infection.

In addition to host gene involvement during viral infection, the magnitude and tempo of host gene expression is governed by factors such as miRNA. miRNAs have been used to validate the impact of host genes on virus replication and have been used as therapeutics [21] with the ability to negatively affect influenza replication [22]. Thus, host miRNAs have a role in host gene expression in response to influenza infection. miRNAs are small noncoding single-stranded RNA molecules composed of 18–23 nucleotides which regulate gene expression in eukaryotes. The miRNA family is a global regulatory network controlling homeostasis, inflammatory responses affecting immunity and disease pathogenesis [23]. miRNAs are involved in the degradation of cytokine transcripts and modulate the expression of negative regulators of cytokine expression and signaling pathways [24]. Paired analysis of miRNA inhibitors and mimics enables gain and loss of function studies for a given miRNA. miRNA can not only be used as a tool for screening, but also as a therapeutic itself. The drug miravirsen is an anti-miRNA drug candidate currently in late stage clinical evaluation for treatment of hepatitis C virus (HCV) infections. Miravirsen is thought to work mainly by hybridizing to mature miR-122 and blocking its interaction with HCV RNA [25]. In this study, a genome wide HTS was performed to identify target miRNAs as countermeasures of H7N9 replication. Several pro-viral host miRNA's were identified which are important for H7N9 replication. In validation studies, the most promising target, hsa-miR-664a-3p (miR-664), was further investigated and the effects on downstream genes, Leukemia inhibitory factor (LIF) and NIMA-related kinase 7 (NEK7), were explored (Fig 1A).



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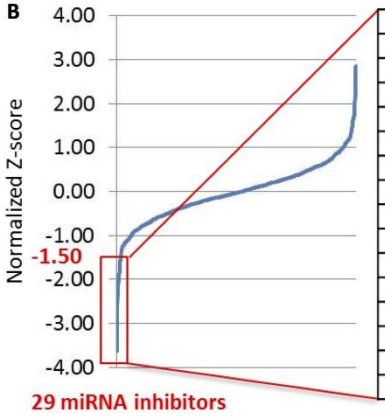

miRNA inhibitors	Z-score	miRNA inhibitors	Z-score
hsa-miR-328	-3.24	hsa-miR-762	-1.92
hsa-miR-3912	-2.63	hsa-miR-548b-3p	-1.90
hsa-miR-214	-2.57	hsa-miR-921	-1.89
hsa-miR-4286	-2.27	hsa-miR-4281	-1.89
hsa-miR-664	-2.25	hsa-miR-212	-1.83
hsa-miR-1244	-2.25	hsa-miR-1249	-1.83
hsa-miR-494	-2.24	hsa-miR-504	-1.81
hsa-miR-3911	-2.22	hsa-miR-3176	-1.78
hsa-miR-200a	-2.16	hsa-miR-3619	-1.70
hsa-miR-510	-2.14	hsa-miR-933	-1.63
hsa-miR-662	-2.01	hsa-miR-3673	-1.62
hsa-miR-4308	-1.99	hsa-miR-711	-1.58
hsa-miR-136	-1.96	hsa-miR-522	-1.56
hsa-miR-1972	-1.94	hsa-miR-524-3p	-1.52
hsa-miR-3913	-1.94	-	-

Fig 1. Workflow from HTS to pathway analysis of miRNA top hits. (A) A549 cells were reverse-transfected with a library of 1,200 miRNA mimics at a final concentration of 20 nM. Non-targeting miRNA mimic controls and siRNAs (siNP) to countermeasure H7N9 replication were used as negative and positive controls, respectively. 48 h after miRNA transfection, the cells were infected with H7N9 at MOI of 0.01 for 24 h. 24 h after infection, the plates were fixed and analyzed by ELISA. 10 top hits from HTS ELISA were validated in virus replication studies. Top hit has-miR-664a-3p was then investigated for pathway analysis and downstream target effects. (B) A plot of normalized Z-score values calculated based on the ELISA results showing a wide range of virus replication modulation. All miRNAs with a Z-score < -1.5 were subjected to further validation.

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Results

Genome wide high-throughput screen of miRNAs that govern influenza H7N9 replication

A human genome wide HTS was performed in A549 cells using miRNA inhibitors to reveal possible host targets that are important for H7N9 replication. Z-score (the number of standard

Table 1. List of miRNA top hits from HTS with z-scores.

miRNA inhibitors Z-scores*			
miR-136	-3.62	miR-664	-2.25
miR-328	-3.24	miR-1244	-2.25
miR-3912	-2.63	miR-3911	-2.22
miR-380	-2.62	miR-510	-2.14
miR-4286	-2.27	miR-662	-2.01

* *Z-score*: the number of standard deviations from the mean. A negative z-score represents the pro-viral character of the corresponding miRNA. A more negative z-score reflects a greater importance of the miRNA for virus.

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deviations from the mean) was used to normalize data to provide explicit information on the strength of each miRNA inhibitor relative to the rest of the sample distribution. A negative z-score reveals inhibitors that decrease virus replication, where a positive z-score reflects miRNA inhibitors that increase virus replication. All miRNAs inhibitors having a z-score lower than -1.50, 29 in total, were investigated (Fig 1B). Cytotoxicity assays were performed to rule out miRNA inhibitors with a toxic effect on cells (data not shown). Finally, 10 miRNA top hits with the most negative z-scores were identified (Table 1) for validation studies.

Validation of top 10 miRNA inhibitors that reduce influenza A H7N9 replication

To validate the top 10 miRNAs from the HTS, A549 cells were transfected (25 nM) with miRNA inhibitors or mimics, and then infected (MOI 0.01) with H7N9. Supernatants and cell monolayers were processed to investigate virus replication. Cellomics ArrayScan (Thermo-Scientific) was used to count the number of infected cells by using fluorescent viral nucleoprotein (NP)-staining combined with DAPI (4',6-diamidino-2-phenylindole) staining of the nuclei. Approximately 40% of cells were infected in the non-transfected control (NTC) (Fig 2A). Reduction of H7N9 infectivity, as predicted from HTS, was confirmed in validation studies for 8 miRNAs. Inhibition of H7N9 replication by miRNA-664 was confirmed where infectivity of H7N9 was reduced by 27% (Fig 2B). siNP, which targets the viral nucleoprotein (NP), was used as positve control for the inhibition of H7N9 replication. siNP reduced replication of H7N9 by 63%. Negative controls having minimal sequence identity were used for all miRNA inhibitors and mimics to differentiate between specific and non-specific effects, and the negative transfection controls had no impact on virus replication.

The pro-viral properties of miR-664 were confirmed as the inhibitor of miR-664, miR664i, significantly ($p<0.05$) reduced virus replication by nearly 2 logs. siNP, as the positive control, significantly ($p<0.05$) reduced H7N9 replication by 1 log. Negative transfection controls had no impact on virus replication. Together, miR664i revealed the most potent antagonizing effect on H7N9 replication and was therefore selected to investigate downstream events and to perform host pathway analysis. Other potent miRNAs regulating H7N9 replication, e.g. hsa-miR-1244 and hsa-miR-4286, were not further investigated in this study, but is the subject of a burgeoning study.

Identification of miRNA gene targets during influenza A H7N9 infection

To determine whether miR-664 inhibitor, miR664i, targets influenza A (H7N9) or impairs virus replication by affecting the host cell machinery, a BLAST (Basic Local Alignment Search

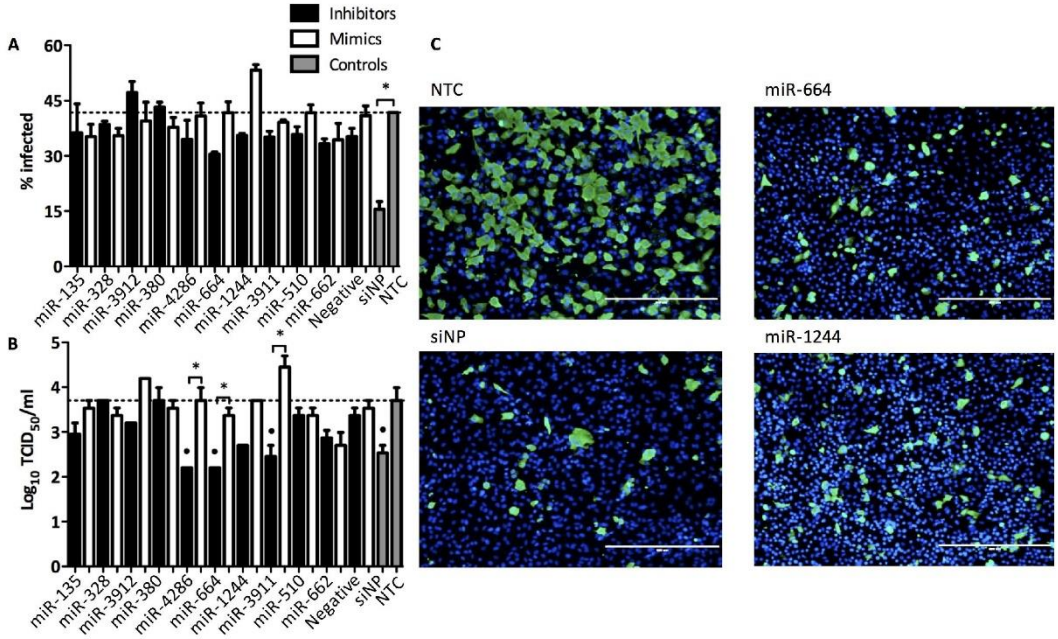

Fig 2. Validation of miRNA inhibitor top hits that reduce H7N9 replication. (A-B) A549 were transfected with miRNA inhibitors and mimics at 25 nM for 48 h and then infected with H7N9 at MOI 0.01 for 48 h. Cells were fixed with 10% formalin and stained for viral NP and counterstained with DAPI. Stained cells were visualized and analyzed using Cellomics ArrayScan high content imaging system. NTC; Non-transfected control, siNP; Positive control (A) Percentage of infected cells was graphed. The data are from 3 replicate wells +/- SEM. *p<0.05. (B) Supernatants from transfected and infected A549 cells were titrated on MDCK cells. The data are from 3 replicate wells ± SEM. *p<0.05, °p<0.05 compared to positive. (C) Representative fluorescent microscope images were taken with EVOS FL imaging system (Life Technologies). Scale bar, 400 μm. NP, green; DAPI (nuclei), blue.

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Tool) search was performed to compare miR-664 and H7N9 virus microRNA seed sites. No noteworthy microRNA seed sites in the H7N9 sequence were found (fludb.org), so it is unlikely the miRNA inhibitor is directly targeting viral gene expression. Thus, miR-664 may be a pro-viral miRNA, required by the virus to downregulate downstream target genes which are important for viral defense mechanisms by the host cell. Pathway analysis using Ingenuity Pathway Analysis (IPA) was used to explore downstream targets of miR-664. A data set was created from the targetscan.org database using the miRNA target scan option in IPA. Since there was no experimental data available for miR-664, results were filtered for targets with highly-predicted confidence for an interaction with miR-664. The IPA target scan produced a pathway for 8 highly predicted targets of miR-664 and their predictive interactions (Fig 3).

Pathways of the predicted target genes for miR-664 are not well characterized; however, some are known to have ubiquitous functions in the cell (Table 2). Specific focus was set on molecules LIF and NEK7. LIF and NEK7 may be linked to regulatory functions in cytokine pathways such as the pro-inflammatory cytokines interleukin-6 (IL-6) and IL-12a and the chemokine CXCL10 (Table 3). Reports from H7N9-infected humans have shown significant upregulation of IL-6 and CXCL10 compared to healthy controls [2, 26–29]. In a study on mice, IL-6

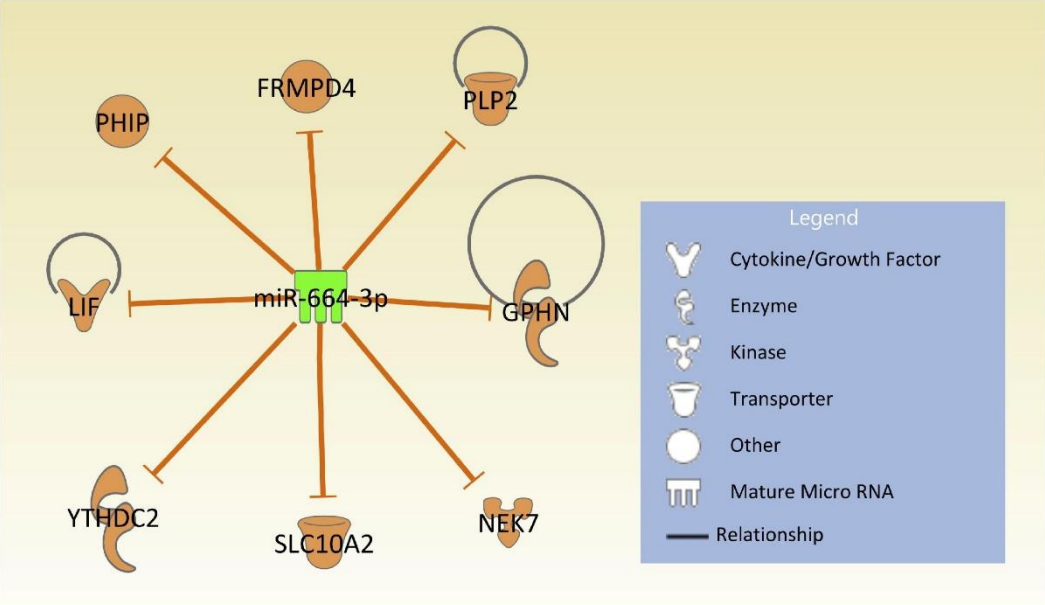

Fig 3. Eight highly predicted target genes of miR-664. Ingenuity pathway analysis was used to determine target genes of miR-664. Only predicted molecules with high confidence are displayed. Molecules are part of different proteins such as cytokines, enzymes, kinases, transporters or as other if the function is unknown. Orange arrows show inhibitory effects of miR-664 on target genes. Pathway diagram was created using Qiagen IPA Path Designer.

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was shown to be a main driver of lung pathology [30]. An interesting finding was that the expression of IL-12a was upregulated with other avian influenza viruses, but not in cells infected with H7N9 [31]. Therefore, roles of LIF and NEK7 in the pro-viral effect of miR-664 were further investigated.

Table 2. Highly predicted target molecules/genes for miR-664 by IPA software.

Symbol	Pathway
FRMPD4	Unknown
GPHN	GABA Receptor Signaling, Molybdenum Cofactor Biosynthesis
LIF	ERK5 Signaling, Hematopoiesis from Pluripotent Stem Cells, Hepatic Cholestasis, HMGB1 Signaling, Mouse Embryonic Stem Cell Pluripotency, Role of NANOG in Mammalian Embryonic Stem Cell Pluripotency, Role of NFAT in Cardiac Hypertrophy, Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses
NEK7	Unknown
PHIP	Unknown
PLP2	Unknown
SLC10A2	FXR/RXR Activation, Hepatic Cholestasis
YTHDC2	Unknown

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Table 3. Regulatory relationship of LIF and NEK7.

Molecule	Family	Regulates	Regulated by
LIF	Cytokine	STAT3, SOCS3, POMC, Erk1/2, IL6ST, STAT1, LIFR, SOCS1, IL-6*, FGF5, FOS, Alp, T, KDR, POU5F1	lipopolysaccharide, TNF, IL-1β, progesterone, TGFB1, IL-2, Il-1, dexamethasone, LPP, MAPK8, Lethal toxin, TP53, AGT, phorbol myristate acetate, Tgf beta
NEK7	Kinase	EDN1, RGCC, LMO4, IL-12A*, U90926, ACPP, ISG15, PTGS2, CXCL10*	TLR4, PLK, triamcinolone acetonide

* pro-inflammatory cytokines and chemokines

doi:10.1371/journal.pone.0155104.t003

Regulation of miR-664 expression and host gene targets during Influenza H7N9 infection

RT-qPCR was used to investigate the gene expression levels of miR-664 and predicted host target genes, LIF and NEK7, during infection with H7N9. miR-664 was upregulated nearly 10-fold ($p<0.05$) in A549 cells infected (MOI = 0.1) with H7N9 at 24 h pi (hours post-infection) compared to non-infected A549 cells (Fig 4). Importantly, potential target genes LIF ($p<0.01$) and NEK7 ($p<0.05$) were significantly downregulated, indicating a correlation between miR-664 and the target genes predicted by IPA.

Follow up studies aimed to investigate whether there was a direct effect of miR-664 inhibitor, miR664i, on the gene expression levels of the target genes during infection with H7N9. A549 cells were transfected with miR664i prior to infection with H7N9. LIF and NEK7 gene expression was downregulated during H7N9 infection compared to non-infected control cells as previously observed (Figs 4 and 5). LIF and NEK7 gene expression levels were rescued when the cells had been transfected with miR664i prior to infection (Fig 5). Thus, miR-664 may have a direct impact on target genes LIF and NE7 and their expression levels during influenza A H7N9 infection.

Target gene knock-down identifies antiviral properties

Small interfering RNAs targeting LIF (siLIF) or NEK7 (siNEK7) were used to investigate the role of target genes LIF and NEK7 on virus replication (Fig 6). A549 cells were transfected

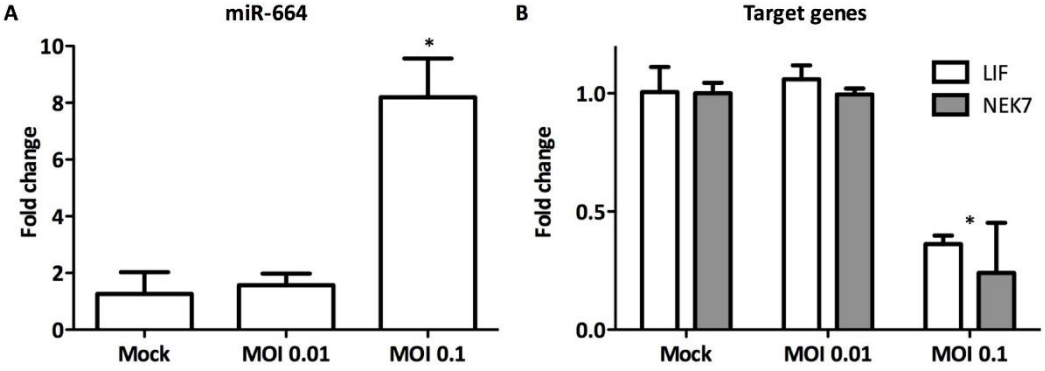

Fig 4. Effect of influenza A (H7N9) on gene expression levels of miR-664 and target genes. A549 cells were infected with H7N9 at MOI 0.01 and 0.1 for 24 h. RNA was extracted for gene expression analysis of A) LIF and NEK7, and B) miR-664 using RT-qPCR. Expression is normalized to 18S and relative to non-infected cells. Data are from 3 replicate wells ± SEM. *p<0.05.

doi:10.1371/journal.pone.0155104.g004

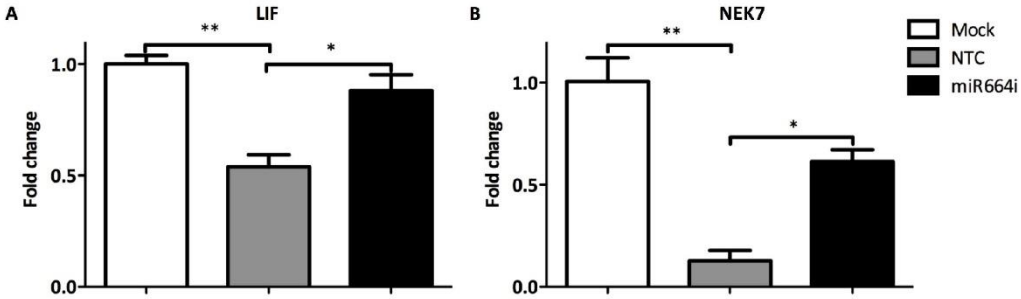

Fig 5. Impact of miR664i on expression of target genes during influenza H7N9 infection. A549 were transfected with miR664i and then infected with H7N9 at MOI = 0.1 for 24 h. Gene expression of target genes LIF and NEK7 were measured with qRT-PCR to evaluate the impact of miR664i on target molecule expression during H7N9 infection. Gene expression was normalized to 18S and relative to non-infected cells. (3 replicate wells) ± SEM. *p<0.05, **p<0.01.

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(50 mM) with siRNA against LIF and NEK for 24 h and then the A549 cells were infected (MOI = 0.1) with A/Anhui (H7N9), A/Ca (H1N1) and A/Phi (H3N2) to determine the role of these genes in influenza A virus replication. Knock-down of LIF increased virus titers for all three influenza A strains investigated, indicating an important role of LIF in virus defense. Knocking down NEK7 did not impair virus replication, and thus this target gene may not be a critical factor in defense against influenza A viruses (data not shown). All siRNAs had prior been evaluated for their efficacy in downregulating the specific mRNA by RT-qPCR (S1 Fig). These results demonstrate that combining HTS and RNAi can lead to the rapid discovery of host cellular targets and the underlying pathways that participate in virus replication.

miR664i reduces the replication of pandemic influenza A strains A/Ca and A/Phi

In order to investigate potential broad-spectrum antiviral effects of miR664i, A549 cells were transfected with miR664i at 25 nM for 48 h and subsequently infected with with A/Ca (H1N1) at MOI 0.1 and A/Phi at MOI 0.05 for 48 h. miR664i reduced both cell infectivity and virus titers for both virus strains investigated (Fig 7) by almost 2 logs $TCID_{50}$. Targeting the host cell machinery to countermeasure influenza A H7N9 helped to discover a miRNA with broad pro-viral characteristics. Inhibition of miR664 limited the replication of various influenza A strains,

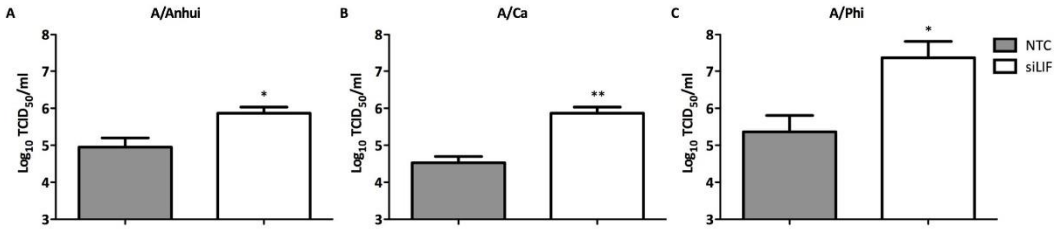

Fig 6. Knock-down of LIF enhances the replication of influenza A. A549 cells were transfected with siRNA against LIF 50 nM for 48 h. Cells were infected with A) A/Ca (H1N1) at MOI 0.1, B) A/Phi (H3N2) at MOI 0.05 and A/Anhui (H7N9) at MOI 0.01. At 48 h pi, supernatants were collected for virus titration in MDCK cells. (3 replicate wells) ± SEM. *p<0.05, **p<0.01.

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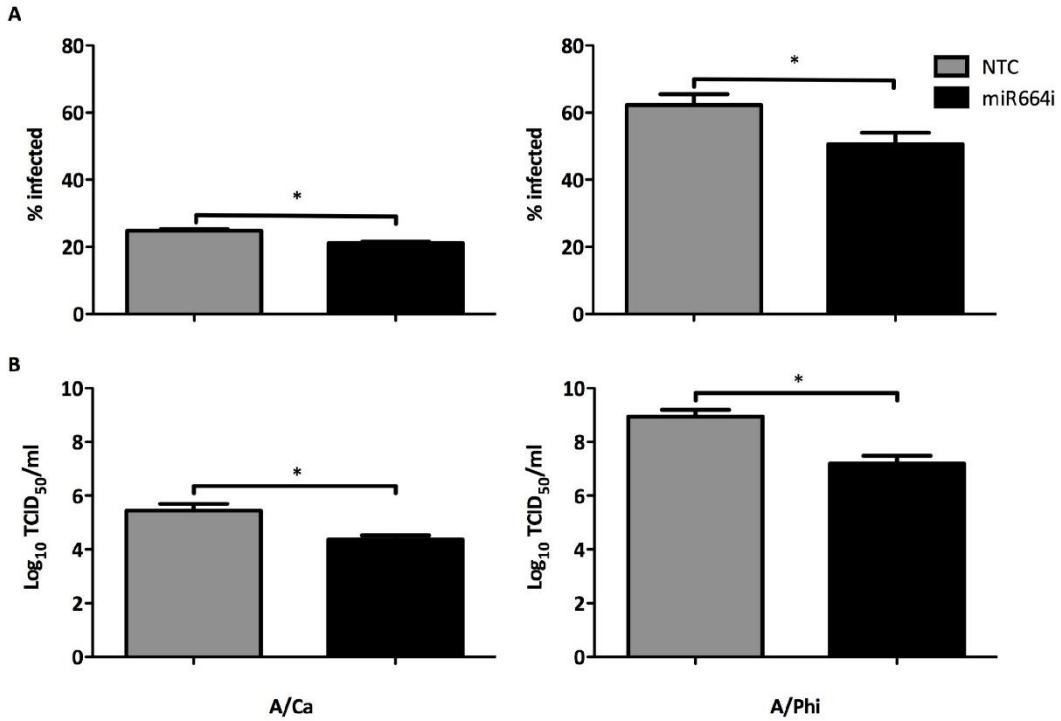

Fig 7. miR664i reduces the replication of other influenza A strains. (A-B) A549 cells were transfected with miR664i at 25 nM for 48 h. Cells were infected with A/Ca (H1N1) at MOI 0.1 and A/Phi at MOI 0.05 for 48 h. NTC; Non-transfected control (A) Cells were fixed with 10% formalin and stained for viral NP and counterstained with DAPI. Percentage of infected cells was measured with Cellomics ArrayScan and graphed. Data are from 3 replicate wells +/- SEM. *p<0.05. (B) Supernatants from transfected and infected A549 cells were titrated on MDCK cell. $TCID_{50}$ values were calculated and graphed. (3 replicate wells) ± SEM. *p<0.05.

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including H7N9 and the pandemic H1N1 and H3N2, without putting evolutionary pressure on the virus which could potentially lead to resistant mutations.

Discussion

To our knowledge this is the first report on a miRNA-based antiviral therapeutic that counter-measures H7N9 replication *in vitro*. miR664i, an inhibitor of host cell miRNA miR-664, showed potent antiviral effects inhibiting H7N9 replication by two logs ($TCID_{50}$ titers) in A549 cells. A549 cells are a good proxy to establish and characterize an *in vitro* model of Type II respiratory alveolar epithelium, and as α2,3-linked or α2,6-linked sialic acids in principle dictate influenza virus susceptibility, A549 cells are highly susceptible to influenza infection and have been widely used as a cell culture model to study influenza A for almost two decades [32]. We anticipate examining the effects of miR664i on influenza A replication in BEAS2B cells or differentiated NHBE cells. In addition, we aim to investigate the antiviral potential of miR664i in animal models. There have been numerous successful attempts to increase potency

and stability and to reduce off-target effects of RNAi therapeutics in recent years [33]. Locked-nucleid acid (LNA) modification can improve the serum stability, where the design of short hairpin RNA (shRNA) can be used as potent RNAi triggers. Lipid nanoparticles as carriers and adenovirus-mediated RNAi against viral infections have also successfully been applied [34, 35]. *In vivo* studies showed a reduction of hepatitis B virus (HBV) when mice were injected with LNA-modified RNAi therapeutics [36]. The most prominent use of LNA technology is the clinical candidate miravirsen, where monthly subcutaneously application can downregulate miR-122 and subsequently reduce HCV replication [37, 38]. In future studies, we will implement some of these techniques to investigate the potential of miR664i to countermeasure influenza A replication in a mouse model.

In a recent publication, MIR2911, a broad spectrum antiviral miRNA suppressed replication of H1N1, H5N1 and H7N9 influenza virus in *vitro* and *in vivo* [39]. MIR2911 targeted the PB2 and NS1 gene of influenza A, and considerably inhibited their protein expression. However, MIR2911 was ineffective against mutant influenza viruses in which the MIR2911-binding nucleotide sequences were altered. In contrast, miR664i does not target the virus directly and the antiviral effects may be directed through intracellular mechanisms in the host cell. One advantage of targeting the host cell miRNA, instead of the virus directly is the lower probability of the virus evolving mutations that would potentially lead to resistance against the therapeutic. In these studies, which examined mechanisms underlying the antiviral actions of miR664i, upregulation of miR-664 was observed during H7N9 infection of A549 cells. This is in accordance with a previous study investigating the expression of miRNAs in blood serum samples from H7N9-infected patients [40]. There, miR-664 expression was shown to be highly upregulated compared to healthy controls. Further, in a microarray screen of human blood serum from pandemic H1N1-infected patients and of H1N1-infected cell lines, miR-664 has been shown to be upregulated during infection [41]. However, whether the virus directly controls the upregulation of miR-664 in the cell, or if the virus takes advantage of the cells unbeneficial upregulation of miR-664 remains unclear.

The two molecules LIF (leukemia inhibitors factor), and NEK7 (NIMA-related kinase 7), were highly predicted target genes for miR-664based on the targetscan.org database. Gene expression studies showed that while miR-664 was upregulated during H7N9 infection, target genes LIF and NEK7 were downregulated. When miR664i was transfected prior to infection, expression levels of target genes were subsequently normalized. Thus, the inhibitory effect of miR-664 on its target genes during influenza infection may facilitate virus replication (Fig 8). A similar effect has been shown for miR-466I, which inhibits the host antiviral innate immune response against Sendai virus by targeting interferon (IFN)-α expression *in vitro* [42]. Unlike miR-466, no seed sites for miR-664 can be found in any of the human IFN genes. Therefore, a direct action of miR-664 IFN gene expression is not expected. However, it is possible that miR-664 can regulate virus-induced IFN production and secretion indirectly through other molecules in the IFN induction pathway. Furthermore, ongoing studies also aimed to address whether IFN regulates miR-664 expression in infected and non-infected cells. As these examples suggest, upregulation of specific miRNAs during virus infections can be harmful for the host. Nevertheless, several reports have shown that downregulation of other miRNAs can also result in increased virus replication.

In studies examining the furin-dependent proteolytic activation of highly pathogenic influenza H5 and H7 viruses, the miR-24 response was shown to strongly decrease both furin messenger RNA (mRNA) levels and intracellular furin activity in A549 cells [43]. Cells transfected with miR-24 mimics showed a decrease of H5N1 infectious virions and a complete block of H5N1 virus spread that was not observed in cells infected with H1N1 virus. This suggests that

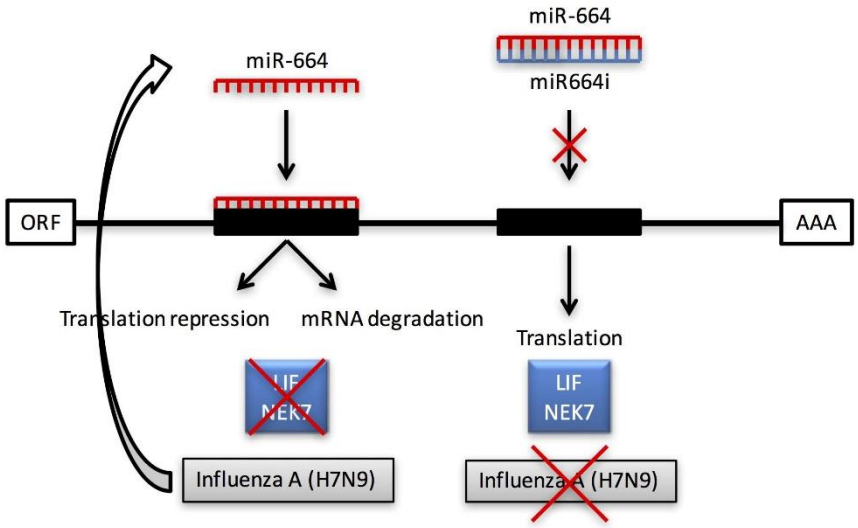

Fig 8. A model for miR-664-mediated regulation of influenza A H7N9 virus infection. miR-664 is upregulated during the course of influenza A infection of A549 cells, which inhibits the expression of target genes of LIF and NEK7. When an inhibitor of miR-664 is used, such as miR664i, molecules LIF and NEK7 are expressed normally, which counteracts the replication of influenza A.

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a virus-specific downregulation of furin-directed miRNAs such as miR-24 may represent a viral regulatory mechanism to govern the production of infectious virions.

To investigate the underlying mechanisms of miR-664 during influenza replication, knock-down studies using siRNA targeting LIF and NEK7 were performed. Knocking down NEK7 had no impact on virus replication, which suggests a non-relevant role of this particular kinase during influenza infection However, knocking down LIF increased virus replication in various influenza A strains. This suggests that miR-664 may downregulate LIF, which may be an important factor in immune defense against influenza A. Whether there are other factors involved and what happens downstream of LIF has yet to be determined. The importance of LIF in antiviral defense has been reported in previous publications [44]. *In vivo* studies were performed with LIF knock-out mice that were infected with RSV. LIF knock-out mice yielded higher virus titers compared to control mice. Furthermore, mice treated with anti-LIF IgG developed enhanced RSV pathology observed with increased airspace protein content, apoptosis and airway hyperresponsiveness compared to control IgG treatment. Thus, LIF may be a critical factor in the antiviral defense against RSV and other respiratory diseases such as influenza A. Whether this is solely mediated by miR-664, or by other mechanisms, remains unknown and will need to be investigated. Furthermore, it remains unclear if virus infection and /or replication initiates the upregulation of miR-664 to avoid the antiviral effects of LIF, or if this mechanism is a non-beneficial reaction by the host cell during virus infection.

In conclusion, the current study reports the discovery of an important pro-viral miRNA that can be targeted by readily available miRNA inhibitor miR664i. The field of miRNA inhibitors is on the rise with many candidates currently being tested in clinical trials. The most clinically advanced example of a miRNA antagonist is the antiviral miravirsen which is currently

undergoing multiple clinical phase II studies against Hepatitis C (ClinicalTrials.gov identifier: NCT01200420, NCT02031133, NCT01872936, NCT01727934). Miravirsen targets the conserved host factor miR-122, which stimulates translation of HCV RNA. Thus, miR-122 is a promising target, as it is hoped that the virus would not be able to acquire resistance mutations to miravirsen. The promising antiviral candidate miR664i owns unique characteristics in the novel field of miRNA inhibitors as countermeasures against the potential pandemic and life-threatening infectious disease caused by H7N9. In addition, miR664i also shows great potential in treating pandemic circulating influenza A strains such as H1N1 and H3N2.

Materials and Methods

Cell lines and viruses

All *in vitro* experiments were performed under the guidance of the Animal Health Research Center (AHRC) in an approved biosafety level 3 laboratory with the use of a powered air-purifying respirator, according to "UGA Laboratory Safety Guidelines". Influenza virus A/Anhui/1/2013 (A/Anhui; H7N9) and A/Philippines/2/82-X79 (A/Phi; H3N2) were propagated in embryonated eggs. Influenza A/California/04/09 (A/Ca; H1N1) was propagated in Madin-Darby canine kidney (MDCK) cells (ATCC CCL-34). Virus was titrated on MDCK cells to determine $TCID_{50}$ values. The human type II pulmonary epithelial cell line A549 (ATCC CCL-185) was used for transfections and infections and cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM: HyClone GELifeSciences) supplemented with 5% heat-inactivated fetal bovine serum (FBS) (HyClone, Logan, UT) in a 37°C incubator with 5% CO_2. Virus propagation in embryonated eggs was carried out in strict accordance with the recommendations by the University of Georgia Institutional Animal Care and Use Committee (IACUC). The protocol was approved by the University of Georgia IACUC (A2013 06-016-Y3-A3 Title: NIAID Centers of Excellence for Influenza Research and Surveillance: Egg Usage).

High-throughput miRNA inhibitor transfection screening

The miRNA inhibitor screen was performed in A549 cells as shown in Fig 1A. miRNA inhibitors (Dharmacon, GELifeSciences) were reverse-transfected into A549 cells at a final concentration of 20 nM in 0.3% DharmaFect1 (Dharmacon, GELifeSciences) transfection reagent. Non-targeting miRNA inhibitor controls (Dharmacon, GELifeSciences) were used in all assays as negative controls and siRNAs targeting NP (siNP) was used as positive controls. Briefly, using a 96-well format, miRNA inhibitors were mixed with DharmaFect1 reagent in serum-free medium (Opti-MEM, GELifeSciences) and incubated at room temperature for 20 min. A549 cells were then added at 10,000 cells/well in DMEM supplemented with 10% FBS using Liqudator96 (Mettler Toledo). 48 h post-transfection, the cells were infected with H7N9 in 100 μl of DMEM supplemented with 2% FBS, at a MOI 0.1. 24 h pi, the cells were fixed with methanol acetone (80:20) for 15 min and stored until analysis by ELISA.

ELISA assay

The miRNA primary screen to identify host genes involved in down-regulating H7N9 virus replication in A549 cells was completed using a NP-specific ELISA assay. The fixed cells were incubated with a mouse anti-NP monoclonal antibody (ATCC H16-L10-4R5) at 37°C for 1 h and then incubated with an HRP-conjugated goat anti-mouse secondary (Invitrogen). Afterwards, the plates were washed with PBS followed by 15 min incubation with HRP-substrate solution (SureBlue TMB). H_2SO_4 stop solution was then added to the plates to terminate the reaction. The plates were scanned with a plate spectrophotometer at a wavelength of 450 nm.

The OD values of experimental wells were normalized to the control wells and converted to a Z-score to create a hierarchical list containing miRNAs in the entire screen according to methods described previously [20]. In this study, all hits with a Z-score $\leq$ -1.5 and cytotoxicity (ToxiLight™ bioassay kit; Lonza) below 5% were subject to further validation in A549 cells.

Validation assays

miRNA hairpin inhibitors and mimics and ON-TARGETplus siRNAs (Dharmacon, GE Life-Sciences) were reverse-transfected with 0.3% DharmaFect1 transfection reagent into A549 cells at a final concentration of 25 nM and 50 nM, respectively. Non-targeting miRNA inhibitor controls (Dharmacon, GELifeSciences) were used as negative controls and siRNAs targeting NP (siNP) was used as positive controls. Briefly, using a 96-well format, miRNA inhibitors and mimics and siRNAs were mixed with DharmaFect1 transfection reagent in serum-free medium (HBSS, Invitrogen) and incubated at room temperature for 20 min. A549 cells were then added at 10,000 cells/well in DMEM supplemented with 5% FBS. 48 h post-transfection, the cells were infected with influenza A viruses while the transfection medium was tested for cytotoxicity with a ToxiLight™ bioassay kit (Lonza). miRNA inhibitors and mimics exceeding a toxicity of 5% were excluded for further validation.

48 h pi, the cell monolayers were fixed with 10% buffered formalin and permeabilized with Triton X-100. Cells were stained with a mouse anti-NP monoclonal antibody (ATCC H16-L10-4R5) and subsequently with a secondary Alexa Fluor 488 anti-mouse antibody (Life Technologies) each for 1h at room temperature. Cells were counterstained with DAPI to quantify the number of infected cells with Cellomics ArrayScan (Thermo Scientific). Supernatants were used to determine $TCID_{50}$ titers on confluent MDCK cells. 10-fold serial dilutions were added to MDCK cells in a 96-well plate format and incubated for 72 h at 37°C and 5% CO_2. Supernatants from MDCK cells were then tested for hemagglutination with 0.5% turkey red blood cells. $TCID_{50}$ values were calculated using the Spearman Karber method [45, 46]. All infections were performed in the presence of 1 μg/mL (L-tosylamido-2-phenyl)ethyl chloromethyl ketone treated (TPCK) trypsin (Worthington Biochemical) in modified Eagle's medium (MEM) supplemented with 0.3% bovine serum albumin (Gibco, Life Technologies).

RNA isolation and qRT-PCR

Total RNA was isolated using TRIzol (Invitrogen Life Technologies). The concentration of total isolated RNA was measured by using a Microplate Spectrophotometer (Epoch, BioTek). qRT-PCR was used to validate mRNA and miRNA expression changes using the Stratagene Mx3005P real-time PCR system (Agilent Technologies). The reverse transcription reactions were performed using a miRNA 1st-Strand cDNA Synthesis Kit (Agilent Technologies) using 500 ng total RNA for each reaction. qRT-PCR was performed using the Brilliant III Ultra-Fast SYBR® Green QRT-PCR Master Mix (Agilent Technologies) to determine miRNA and mRNA expression, and data were normalized to 18S expression using the $2^{-\Delta\Delta Ct}$ method. Primer sequence for miR-664 was, 5'-TATTCATTTATCCCCAGCCTACA-3' (forward primer) and a universal reverse primer. Primers sequences for LIF were, 5'-ACAGAGCCTTT GCGTGAAAC-3' (forward primer) and 5'-TGGTCCACACCAGCAGATAA-3' (reverse primer). Primer sequences for NEK7 were, 5'-CACCTGTTCCTCAGTTCCAAC-3' (forward primer) and 5'-CTCCATCCAAGAGACAGGCTG-3' (reverse primer).

Statistical analysis

All experiments were performed in biological triplicates and technical duplicates and have been repeated at least twice. Data are expressed as means ± SEM. Differences between different

biological groups were compared by one-way analysis of variance (ANOVA). Individual differences between groups were tested by multiple comparison and analysis using the Tukey post-test. Pairs of groups were compared by Student's t-test (two tailed). P values for significance were set at 0.05, unless otherwise stated. All analysis was performed using Graphpad Prism Software (Version 5 for Windows).

Supporting Information

S1 Fig. siRNA successfully knocks down expression of targeted genes. A549 cells were transfected with siRNA against LIF and NEK7 at 50 nM for 48 h. Cells were infected with A/Ca (H1N1) at MOI 0.1. RNA was extracted for gene expression analysis of A) LIF and B) NEK7 using RT-qPCR. Expression is normalized to 18S and relative to non-infected cells. Data are from 3 replicate wells ± SEM. $^{*}p<0.05$, $^{***}p<0.001$.
(TIFF)

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Author Contributions

Conceived and designed the experiments: SW WW OP. Performed the experiments: SW CJ WW. Analyzed the data: SW WW. Contributed reagents/materials/analysis tools: RT. Wrote the paper: SW OP RT. Supported the work through collaboration: SM.

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Chapter 4 - Targeting the pro-inflammatory factor CCL2 (MCP-1) with bindarit for influenza A (H7N9) treatment

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This chapter includes a co-authored paper. The bibliographic details of the co-authored paper, including all authors, are:

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My contribution to the paper involved:

Substantial contributions to conception and design, acquisition of data, analysis and interpretation of data, including the provision of data, the complete analysis and presentation of data into publishable format and providing direction on the scope and structure of the analysis. The project was based on collaborative efforts to combine materials and methods from both groups involved.

Stefan Wolf Gold Coast, 18.08.16

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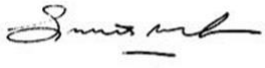

Supervisor: Prof. Suresh Mahalingam Gold Coast, 18.08.16

Preface

The previous two chapters focused on the importance of influenza A surveillance programs and the discovery of novel antiviral host targets to combat the burden of influenza A virus disease. Due to increased resistance among circulating and novel influenza A strains and the lack of specific vaccines against H7N9, there remains an imminent need for drug repurposing because the development of novel antivirals and specific vaccines will require many years of preclinical and clinical studies before their availability for clinical use. Following human influenza A (H7N9) infection, there is excessive production of pro-inflammatory factors CCL2, IL-6, IL-8, IFN-α, IFN-γ, IP-10, MIG, and MIP-1β has been shown to contribute to fatal disease outcome in mice. In the current study, the potent inhibitor of CCL2-synthesis bindarit, was examined as a countermeasure against influenza A H7N9-induced inflammation in a mouse model. However, bindarit treatment of mice infected with H7N9 resulted in increased weight loss, virus titer, cellular infiltration into the lungs, and pro-inflammatory cytokines. Interestingly, bindarit was previously shown to successfully reduce disease in other animal models of viral infection, without dampening viral clearance. Consequently, the results suggest that bindarit might be ill-advised in the treatment of influenza A (H7N9) disease.

Targeting the pro-inflammatory factor CCL2 (MCP-1) with Bindarit for influenza A (H7N9) treatment

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Abstract: 237 words

Abstract

Influenza A viruses are important human and animal pathogens. Seasonal influenza viruses infect humans every year, and occasionally zoonotic viruses emerge to cause pandemics/epidemics with significantly higher morbidity and mortality rates. In March 2013, public health authorities in China reported three cases of laboratory confirmed human infection with avian influenza A (H7N9) virus, and there have been several cases reported across South East Asia and recently in North America. Most patients experience severe respiratory illness, with mortality rates near 40%. No vaccine is currently available and the use of antivirals is complicated due the frequent emergence of drug resistant strains. Thus, there is an imminent need to identify new drugs for therapeutic intervention and disease control. In humans, following H7N9 infection, there is excessive expression of pro-inflammatory factors CCL2, IL-6, IL-8, IFNα, IFN-γ, IP-10, MIG, and MIP-1β which has been shown to contribute to fatal disease outcomes in mouse models of infection. In the current study, the potent inhibitor of CCL2 synthesis, Bindarit, was examined as a countermeasure for H7N9-induced inflammation in a mouse model. Bindarit treatment of mice did not have any substantial therapeutic efficacy in H7N9 infection.Consequently, the results suggest that Bindarit may be ill-advised in the treatment of influenza H7N9 infection.

Summary statement

While influenza A vaccination is the first and best way to prevent influenza illness, prophylactic and/or therapeutic drug treatment to prevent replication and or disease is an important option for influenza virus infection in an ill patient with clinical signs and symptoms compatible with influenza. Antiviral treatment also can be considered for any previously healthy, symptomatic outpatient not at high risk with confirmed or suspected influenza on the basis of clinical judgment, and if treatment can be initiated within 48 hours of illness onset. Bindarit, a small-molecule indazolic derivative with anti-inflammatory properties has been reported to reduce disease signs in animal models of inflammatory disease, includingmice

infected with viruses such as chikungunya virus. Bindarit has not been previously tested for emerging highly pathogenic strains like H7N9 viruses. Unfortunately, Bindarit treatment of mice did not have therapeutic efficacy in H7N9 infection. Consequently, the results suggest that Bindarit treatment might be ill-advised for the treatment of influenza A (H7N9) disease infection.

Introduction

Despite readily available vaccines and therapeutics for circulating strains, influenza virus remains a serious global health threat affecting humans, wildlife, and agricultural species. Avian influenza A H7N9 virus (H7N9) infections in humans were first reported in China in March 2013 [1]. Most infections are believed to have resulted from exposure to infected poultry or contaminated environments, as H7N9 viruses have been found in poultry in China. Infected children typically suffer only mild disease [2], whereas elderly patients are generally more severely afflicted [3]. This suggests that there is minimal heterologous immunity from previous influenza infections. Most patients infected with H7N9 experienced severe respiratory illness, such as pneumonia (97.3%) and acute respiratory distress syndrome (71.2%), leading to high rates of intensive care unit admissions [4]. The mortality rate attributed to influenza H7N9 infection in human is >38%, with 175 deaths from 450 confirmed cases reported within a 20-month period [5]. No evidence of sustained human-to-human transmission of H7N9 has been recorded. However, there is some evidence of limited person-to-person spread under rare circumstances [6]. After human H7N9 infections were first reported in China, the virus spread rapidly to other countries [7]. Recently, the first case of human H7N9 infected in North America was documented in Canada [8].

No specific vaccine is currently available for H7N9 [9]. There are several antivirals available for the treatment of influenza infections including the M2 ion channel inhibitors amantadine and rimantadine, and the neuraminidase inhibitors zanamivir and oseltamivir [10, 11]. Early treatment with these antiviral drugs reduces the duration of symptoms and recovery time. However, the use of antiviral drugs is complicated by the emergence of drug resistant viruses [12, 13], and oseltamivir-resistant H7N9 strains have recently been described in Taiwan [14]. In addition, the use of antiviral drugs may affect population vulnerability due to lack of seroconversion, as well as driving drug resistance among circulating strains [15]. To prevent the spread of infection, development of new drugs and vaccines is urgently needed. The development of new drugs and vaccines against H7N9 may take many years. Drug

repurposing, or the use of clinical drug candidates, may help to overcome this lengthy process of development and reduce the impact of. H7N9 disease results in a potent immune response believed to contribute to tissue destruction and pathology [16]. The host's immune response towards H7N9 infection has not been fully characterized, which is an issue for the discovery of novel therapeutics and target identification for drug repurposing.

In studies of fatal H7N9 infections in humans, there is evidence of immune pathological changes caused by a heightened innate immune response [16]. Greatly elevated cytokine and chemokine levels are associated with severe lung damage and a compromised airway [4]. Vigorous proliferation of H7N9 in the lower respiratory tract causes excessive activation of the innate immune response [16]. This leads to production of inflammatory mediators, many expressed by intrapulmonary macrophages and alveolar cells [16]. Some of the major histological characteristics of H7N9 infection include diffuse alveolar damage, hyaline membrane and fibroproliferation in the lung, and spotty necrosis in the liver [16]. Histological observations have shown a depletion of T lymphocytes, a fluctuating numbers of neutrophils, and highly abundant and activated macrophages which are characteristic of H7N9 infection in the alveoli [16]. In addition, high levels of intrapulmonary inflammatory mediators, such as the interferon gamma-induced protein 10 (IP-10) and IL-6 have been detected [16]. Plasma levels of IL-6 and IL-8 are sharply upregulated, whereas IL-10, macrophage inflammatory protein-1β (MIP-1β), and interferon-γ (IFN-γ) are increased to intermediate levels [4]. In contrast, IL-1β, TNFα, and MIP-1α are found only at minimal concentrations. Whether the greatly elevated cytokine and chemokine levels cause or contribute to severe H7N9 disease, or if they simply correlate with inflammation and pathology, have yet to be determined [4]. However, the upregulation of pro-inflammatory cytokines and chemokines such as the monocyte chemoattractant protein-1 (MCP-1/CCL2), interleukin-6 (IL-6), IL-8, IFN-α, IP-10, MIG, and MIP-1β, was described in H7N9-infected patients with lung injury and severe pneumonia [2]. The cytokine levels in C57BL/6 and BALB/c mice infected with H7N9 (A/Anhui/A/2013 strain) were compared. C57BL/6 mice exhibited more severe lung injury, slower recovery from lung damage, less effective viral

clearance, higher levels of CCL2, IL-6, and IL1β, and lower levels of TNFα and IFN-γ than BALB/c mice. These data suggest that TNFα and IFNγ may help to suppress viral gene expression and increase viral clearance, while CCL2 and IL-6 may contribute to lung injury during H7N9 disease [17].

The focus of this study was to assess the effects of drug inhibition of the pro-inflammatory factor CCL2, which may have a pathogenic role during H7N9 disease. Bindarit represents a novel class of inhibitor that reduces CCL2 synthesis [18]. Bindarit has been successfully used to alleviate virus-induced inflammation in several animal models of disease. For example, Bindarit was efficacious in mouse models of Chikungunya and Ross River virus infections, where it was shown to ameliorate infections and disease [19, 20]. Furthermore, Bindarit was able to reduce arthritic inflammation without showing any detrimental effect on virus clearance in these animal models of alphavirus infection [21]. Therefore, Bindarit's activity to reduce CCL2 level in a mouse model of H7N9 disease was evaluated. Bindarit has an innovative mechanism of action; it selectively inhibits cytokine/chemokine production, particularly monocyte chemotactic proteins. CCL2 is further a critical mediator of neuroinflammation in myriad disease states, including multiple sclerosis [22], human immunodeficiency virus (HIV)-1-induced encephalitis [23], Guillain-Barré syndrome [24], Alzheimer's disease [25], ischemia [26], neurotrauma [27], epilepsy [28], neurogenic hypertension [29], and alcoholism [30]. Bindarit has also been studied as a therapeutic intervention for these diseases. A substantial and encouraging set of clinical tolerability data in different CCL-2 dependent illnesses with more than 600 subjects, healthy volunteers and patients, demonstrated Bindarit safety of up to a maximum dose of 2400 mg/day for as long as 6 months and suggests Bindarit's potential to be beneficial in a range of diseases [31].

Thus, this study aimed to explore the role of CCL2 in H7N9 disease and the potential of Bindarit as a countermeasure against H7N9-induced pathology in a mouse model. Intriguingly, the survival rate of Bindarit-treated mice was comparable to that of non-treated mice, while weight loss, cellular infiltration, and viral titers were considerably increased with

Bindarit treatment. Thus, the use of Bindarit as a therapeutic to treat H7N9 disease seems ill-advised.

Results

Bindarit treatment reduces *CCL2* gene expression in lung epithelial cells after influenza A infection

To evaluate the effectiveness of Bindarit in reducing CCL2 production, lung epithelial cells were infected (MOI=0.1) with A/Ca (H1N1) virus and simultaneously treated with Bindarit. RT-qPCR was used to investigate the effect of Bindarit on *CCL2* gene expression during infection with A/Ca (H1N1), a representative, currently circulating influenza A subtype, in a human epithelial cell line (A549). CCL2 was considerably upregulated by 3.21 fold ($p<0.01$) in A/Ca (H1N1)-infected A549 cells compared to mock-infected controls at 24 h pi (Fig 1). When A549 cells were treated with Bindarit (100 μM), *CCL2* gene expression was significantly reduced to a level comparable to mock-infected controls. ± SEM. **$p<0.01$.

Bindarit treatment does not protect mice from lethal influenza A (H7N9) infection

The ability of Bindarit to protect from lethal H7N9 infection was investigated in a mouse model. Mice were infected with a lethal dose of H7N9 and treated with Bindarit (70 mg/kg) twice daily starting at day 1 pi. Mice were monitored for weight loss and survival. Bindarit treatment had no impact on weight loss or survival of mice. Mice in both groups lost body weight to a similar extent, reaching a 25% reduction in body weight by day 5 (Fig 2A). Mortality typically occurred between day 5 and 10 pi for untreated mice and between day 7 and 9 for Bindarit-treated mice (Fig 2B). No significant ($p<0.1$) difference was observed in the survival rate between the two groups.

Bindarit treatment does not affect lung pathology in mice infected with a lethal dose of H7N9

The effect of Bindarit on lung inflammation and pathology was investigated in mice infected with H7N9. Mice were intranasally infected with a lethal dose of H7N9 (10^5 PFU) and orally treated with Bindarit (70 mg/kg) twice daily starting at day 1 pi. Mice were sacrificed at day 4 pi and lungs were collected for histopathology. Lungs in both groups, mock- and Bindarit-treated, showed moderate to severe necrotic bronchitis and bronchiolitis. The peribronchiolar and perivascular infiltration was mild to moderate for both groups and animals showed mild to severe alveolitis (Fig 3 C and D). Two mock-treated mice infected with H7N9 developed hemorrhage, a feature that was not observed in Bindarit-treated animals. Taken together, there were no substantial changes in histopathology when mice were orally treated with Bindarit (70 mg/kg). All mice showed a lung score of 3 (Table 1).

Bindarit treatment was associated with increased cellular infiltration into the lungs after lethal influenza A (H7N9) infection.

To investigate the effects of Bindarit on pulmonary cellular infiltration, flow cytometry was performed using the bronchoalveolar lavage (BAL) fluid from infected mice. A significant ($p<0.001$) increase in total number of leukocytes was observed in mice orally treated with Bindarit (70 mg/kg) after lethal infection with H7N9 at day 4 pi (Fig 4A). Interestingly, the number of alveolar macrophages was increased after Bindarit treatment (Fig 4B), despite its known ability to reduce production of the monocyte attractant factor MCP-1/CCL2. Furthermore, the influx of eosinophils and CD8+ T-cells was also increased in Bindarit-treated animals (Fig 4C and F).

Bindarit-treatment was associated with weight loss in mice after sub-lethal infection withH7N9

No differences in weight loss were observed between mock- and Bindarit-treated mice after intranasal lethal H7N9 infection (10^5 PFU). The lethal challenge may have been overwhelming and therefore the effect of Bindarit miniscule. Therefore, a study using a sub-lethal dose of virus was performed. Mice were infected with a sub-lethal dose of H7N9 and orally treated with Bindarit (70 mg/kg) twice daily starting at day 1 pi. Mice in both groups lost body weight until day 4 pi. However, on days 5, 6, and 8, Bindarit-treated mice showed a considerable increase in weight loss compared to mock-treated control mice (Fig 5A).

Bindarit treatment led to increased lung viral titers after sub-lethal H7N9 infection

In order to investigate the role of CCL2 in viral clearance when mice were treated with Bindarit during H7N9 disease, pulmonary virus titers were evaluated. Viral titer was measured with RT-qPCR from 5 ng of total RNA from homogenized lung samples using primers and probe specific to the viral M gene. Mice orally-treated with Bindarit (70 mg/kg) had higher lung virus titers compared to mock-treated control mice at day 8 pi (Fig 5B). The virus titer was approximately 1 log higher in the bindarit-treated group.

Bindarit treatment did not alter pro-inflammatory cytokines gene expression after sub-lethal H7N9 infection

The effect of Bindarit on the expression of pro-inflammatory cytokines in the lungs was investigated to show the effectiveness of oral Bindarit treatment. RT-qPCR was used to measure the expression of pro-inflammatory cytokine genes *Il6*, *Ifng*, and *Ccl2* in the lungs at day 8 pi. *Il6* and *Ifng* were of particular interest as IL-6 has been linked to tissue destruction in mice infected with influenza A [17] and IFNγ is known for its protective role against influenza A disease [32]. Bindarit did not appreciably modulate the level of these pro-

inflammatory cytokines. The effect of oral Bindarit treatment on the expression of *Ccl2* and other cytokines in the lungs appeared to be minimal (Fig 6).

Bindarit treatment moderately affected pro-inflammatory cytokine levels after sub-lethal H7N9 infection

To confirm the RT-qPCR results, a Luminex ELISA was used to measure the pro-inflammatory cytokines IL-6, IL-15, CCL2, RANTES, and TNF in the BAL at day 8 pi (Fig 7A-E). The IL-15 protein level was significantly ($p<0.05$) increased when H7N9-infected mice were treated with Bindarit. Bindarit treatment did not appreciably change the level of other pro-inflammatory cytokines. Bindarit oral treatment also had minimal effect on cytokine expression levels in the lungs. Interestingly, CCL2 protein level was not reduced, but appeared enhanced after treatment with Bindarit.

Bindarit treatment did not alter the cellular influx after sub-lethal influenza A (H7N9) infection

To attempt to determine the underlying mechanism of increased weight loss in Bindarit-treated mice, cellular infiltration into the BAL was investigated using flow cytometry. Total numbers of leukocytes as well as macrophages, eosinophils, and T cells were not significantly ($p<0.01$) increased after Bindarit treatment, but were slightly higher than those in mock-treated mice (Fig 8). The effect of Bindarit treatment on cellular infiltration appeared to be weaker after sub-lethal infection than after lethal infection. Interestingly, the number of alveolar macrophages did not change after treatment with Bindarit, despite its known ability to reduce production of monocyte attractant MCP-1/CCL2 in other models [18].

Discussion

The severe disease in mice infected with influenza A H7N9 is associated with a 'cytokine storm' characterised by upregulation of pro-inflammatory cytokines and chemokines such as CCL2, IL-6, IL-8, IFN-α, IFNγ, IP-10, MIG, and MIP-1β. A similar cytokine storm is thought to contribute to lung injury and severe pneumonia in H7N9-infected patients [2]. Mouse studies suggested that TNFα and IFNγ may help to suppress viral gene expression and increase viral clearance, while CCL2 and IL-6 may contribute to lung injury during H7N9 disease [17]. Therefore, this study aimed to investigate the role of CCL2 in the context of influenza A (H7N9) disease by using the potent CCL2 synthesis inhibitor Bindarit. Treatment of H7N9-infected mice with Bindarit enhanced some aspects of disease including increased virus titers, weight loss, and cellular infiltration in the BAL. These results suggest that CCL2 has an antiviral role against H7N9 replication; thus, therapeutic approaches targeting CCL2 may be ill advised for the treatment of H7N9 influenza infection. This is the first study evaluating the effects of CCL2 inhibitors for the treatment of influenza-induced disease and the results suggest that this class of drugs may not be suitable for treatment of severe influenza infections.

CCL2 is upregulated in several viral diseases in humans, such as HIV, hepatitis C virus, several herpes viruses, Japanes encephalitis virus and respiratory syncytial virus and has been considered as a biomarker linked to disease severity in HIV [33]. Furthermore, CCL2 has been linked to inflammation and tissue damage in human disease [33, 34, 35]. In animal studies of various inflammatory diseases, Bindarit was effective in reducing CCL2 production *in vitro* and *in vivo* and successfully alleviated CCL2-driven diseases such as arthritis, encephalomyelitis and prostate and breast cancers [36, 37, 38]. In animal models of arthritogenic alphavirus disease, Bindarit reduced disease symptoms such as clinical score, cellular infiltration of muscle tissues, and bone loss but had no effect on virus clearance [19, 20, 21]. These studies indicate that Bindarit may be potentially used in the treatment of virus-induced inflammation. Furthermore, Bindarit reduces inflammation and ameliorates disease in a

mouse model of autoimmune encephalomyelitis [37], which mimics many aspects of Guillain-Barre syndrome [39], indicating that CCL2 inhibitors could potentially be beneficial in preventing exacerbated Guillain-Barre syndrome. However, CCL2 may have dual roles in antiviral defense, mediating both protective and pathogenic functions. For example, in a study evaluating the role of CCL2 using an animal model of HIV, CCL2 receptor (CCR2) knockout mice showed increased virus titers and disease [35]. In a different study on CHIKV a similar importance for the CCR2 was found. CCR2 deficiency promoted exacerbated chronic erosive neutrophil dominated CHIKV-induced arthritis in mice [40].

CCL2 is highly upregulated in patients suffering from H7N9 influenza infection and has been linked to lung injury in mouse models [2]. However, in this study, when H7N9-infected mice were treated with Bindarit, mice exhibited heightened disease signs as demonstrated by an increase in weight loss, pro-inflammatory factors, cellular infiltration, and virus titers. Thus, blocking CCL2 dampened viral clearance and was associated with upregulation of pro-inflammatory cytokines and cellular infiltration. In earlier studies examining the effects of anti-CCL2 antibodies on influenza A disease, mice exhibited enhanced pneumonitis compared to non-treated animals, despite reduced numbers of cellular infiltrates such as leukocytes, macrophages, and neutrophils in the lungs [41]. Furthermore, infection of CCL2 knockout mice with a non-lethal dose of a mouse adapted strain of influenza A resulted in a profound increase in weight loss, elevated viral loads and pro-inflammatory cytokines, and enhanced leukocyte recruitment into the infected lungs compared to wild-type mice [42]. Interestingly, in that study, pro-inflammatory cytokines such as TNFa, IL-6, and IFNγ were enhanced, but cellular infiltrates into the lungs were reduced [42]. However, one limitation of that study was the analysis of the cellular infiltrate in full lung homogenate as opposed to BAL, which may have influenced the outcome of the study. In the current study, we observed an increase in pro-inflammatory cytokine expression and cellular infiltration in the BAL.

Interestingly, *Ccl2* gene expression was not reduced in the lungs after oral treatment with Bindarit, despite its known capability in reducing CCL2 synthesis *in vitro* and *in vivo*

from earlier studies by other groups [18]. Oral administration of Bindarit may be a limitation in the treatment of pneumonia, as there are difficulties in reaching therapeutic concentrations of drugs in the lungs when administered by this route [43]. It remains a possibility that oral delivery of Bindarit was not completely effective or led to a systemic reduction of CCL2 production that contributed to disease enhancement. In future studies this limitation will be addressed by intranasal administration of bindarit, which may increase the concentration of the drug in the lungs. In addition, IL-15 was highly upregulated in the BAL of Bindarit-treated H7N9-infected mice. IL-15 has recently been described as a critical factor in the pathogenesis of influenza A in mice with virus-induced acute lung injury [44]. Whether there is a link between increased IL-15 production in the lung and a systemic inhibition of CCL2 synthesis remains a subject of further studies.

Various approaches have been investigated for the treatment of H7N9 infection in the recent years. Treatment with corticosteroids was evaluated, but it led to increased mortality in patients suffering from acute H7N9 infection [45]. Due to increased drug-resistance among circulating and novel influenza A strains and the lack of specific vaccines against H7N9, there remains an imminent need for drug repurposing because the development of novel antivirals and specific vaccines will require many years of preclinical and clinical studies before their availability for clinical use.

Materials and Methods

Cell cultures and influenza virus stock

All *in vivo* experiments were performed under the guidance of the Institutional Animal Care and Use Committee (IACUC) and Animal Resources at the Animal Health Research Center (AHRC), which has approved biosafety level 2 and 3 laboratories. Influenza virus A/Anhui/1/2013 (A/Anhui; H7N9) was propagated in embryonated chicken eggs. Influenza A/California/04/09 (A/Ca; H1N1) was propagated in Madin-Darby canine kidney (MDCK)

cells (ATCC CCL-34). Viruses were titrated on MDCK cells as described previously [46]. The human type II respiratory epithelial cell line A549 (ATCC CCL-185) was maintained in Dulbecco's Modified Eagle's Medium (DMEM; HyClone, Logan UT) supplemented with 5% heat-inactivated fetal bovine serum (FBS; HyClone) in a 37°C incubator with 5% CO_2. Virus propagation in embryonated eggs was carried out in strict accordance with the recommendations by the University of Georgia IACUC. The protocol was approved by the University of Georgia IACUC.

In vitro influenza infection and Bindarit treatment

Bindarit (2-Methyl-2-[[1-(phenylmethyl)-1H-indazol-3-yl]methoxy]propanoic acid) was synthesized by Chemlin (Nanjing, China). Bindarit was dissolved in ultrapure water (Thermo Fisher Scientific, Waltham MA). A549 cells were grown to 80% confluency in a 48-well plate and infected with A/Ca (H1N1) at multiplicity of infection (MOI) of 0.1. After infection, Bindarit was added to the wells at a concentration of 100 μM. After 24 h, cells were collected in TRIzol (Thermo Fisher) for total RNA purification. Gene expression was assessed using RT-qPCR. All infections were performed in the presence of 1 μg/mL (L-tosylamido-2-phenyl)ethyl chloro-methyl ketone (TPCK)-treated trypsin (Worthington Biochemical, Lakewood NJ) in modified Eagle's medium (MEM) supplemented with 0.3% bovine serum albumin (Thermo Fisher Scientific).

In vivo influenza infection and Bindarit treatment

BALB/c female mice (6-to-8 weeks old) were obtained from the National Cancer Institute. All experiments and procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Georgia. All experiments were performed with 5-7 mice per group and repeated independently at least twice. Bindarit was dissolved in 0.1% methylcellulose (Thermo Fisher Scientific). To evaluate the effect of Bindarit on disease burden, Bindarit was administered using oral gavage twice daily at 70 mg/kg starting

at day 1 pi. Mice were inoculated intranasally with influenza virus strain A/Anhui/1/13 (H7N9) with either a lethal dose (10^5 PFU) or a sub-lethal dose ($10^{2.7}$ PFU) of H7N9. BAL fluid was collected in PBS to determine cell number with flow cytometry and cytokine levels using multiplex ELISA (enzyme-linked immunosorbent assay). Lungs were collected in 10% buffered formalin for histopathological analyses or homogenized in serum-free DMEM and processed with TRIzol for RNA extraction and subsequent RT-qPCR.

RNA isolation and RT-qPCR

Total RNA was isolated using TRIzol as previously described [47]. Briefly, the concentration of total RNA was measured using a microplate spectrophotometer (Epoch; BioTek, Winooski VT). RT-qPCR was used to validate mRNA expression changes and virus load using the Stratagene Mx3005P real-time PCR system (Agilent Technologies, Santa Clara CA). The reverse transcription reactions were performed using the SuperScript VILO cDNA Synthesis Kit (Thermo Fisher Scientific) and 1000 ng total RNA for each reaction. qPCR was performed using the GoTaq Green Master Mix (Promega, Madison WI) to determine mRNA levels, and data were normalized to 18S expression using the $2^{-\Delta\Delta Ct}$ method [48]. Primer sequences for *CCL2* were: 5'-GAACACACTCAGCGCAGTTA-3' (forward primer) and 5'-CACCCACCCTCTCTTTGATTAC-3' (reverse primer). The virus load was determined using 5 ng of total RNA with a TaqMan® Fast Virus 1-Step Master Mix (Thermo Fisher Scientific). The standard curve was produced using an M gene plasmid [49].

Histopathological evaluation

Lungs from infected mice were harvested at 4 days pi, perfused with 10% buffered formalin, and fixed in 10% buffered formalin. The sections were embedded in paraffin, cut into 5 µm sections, and stained with hematoxylin and eosin. The sections were evaluated using light microscopy. A histological score for each lung was determined according to the following criteria: 0 = no lung abnormality; 1 = <10% of airways inflamed; 2 = 10-30% of

airways inflamed; 3 = 30-50% of airways inflamed and 4 = >50% of airways inflamed [50]. The slides were evaluated by a pathologist without prior knowledge of the infection and treatment status.

BAL collection and quantification of cytokines

Eight days pi, mice were sacrificed and tracheotomy was performed. The mouse lungs were flushed with 1 ml of PBS, and the retained BAL was centrifuged at 400×g for 5 min at 4°C. The recovered supernatants were collected and stored at -80°C until assessed for cytokine concentration, and the cell pellet were resuspended in 200 µL of 10% buffered formalin. Total cell numbers were counted using a hemocytometer. Cytokines in BAL supernatants were quantified with the Luminex® xMAP™ system using a MILLIPLEX MAP mouse cytokine immunoassay (MCYTOMAG-70K; Millipore, Billerica CA) according to the manufacturer protocol. Briefly, beads coupled with anti-CCL2, anti-IL-6, anti-IFN-γ, anti-RANTES, anti-IL-15 and anti-TNF monoclonal antibodies were sonicated, mixed, and diluted 1:50 in assay buffer. For the assay, 25 µL of beads were mixed with 25 µL of PBS, 25 µL of assay buffer, and 25 µL of BAL supernatant, and incubated overnight at 4°C. After washing, beads were incubated with biotinylated detection antibodies for 1 h and the reaction mixture was then incubated with streptavidin-phycoerythrin (PE) conjugate for 30 min at room temperature, washed, and resuspended in PBS. The assay was analyzed on a Luminex 200 instrument (Luminex Corporation, Austin, TX) using the Luminex xPONENT 3.1 software.

Flow cytometry

For flow cytometry analysis, cell suspensions were incubated in FACS staining buffer (PBS containing 1% BSA) and subsequently stained for 30 min at 4°C with an optimized concentration of antibodies (BD Bioscience, Franklin Lakes NJ): PE-conjugated anti-CD3, PerCPCy5.5-conjugated anti-CD8, PE Cy7-conjugated anti-CD4, PerCPCy5.5-conjugated anti-CD45, APC-conjugated anti-CD11c, and PE-conjugated anti-SiglecF to determine cell

types in the BAL. Cells were acquired on an LSRII flow cytometer (BD Bioscience) and the data were analyzed using the FlowJo software (v 7.6.5) (Ashland OR). Based on surface marker expression, six different cell types were identified: $CD45^+$ (total leukocytes), $CD45^+SiglecF^+CD11c^{low}$ (eosinophils), $CD45^+SiglecF^+CD11c^{high}$ (alveolar macrophages), $CD45^+CD3^+$ (total T cells), CD4 T cells ($CD45^+CD3^+CD4^+$), and CD8 T cells ($CD45^+CD3^+CD8^+$).

Statistical analysis

All experiments were performed in triplicate and the experiment independently repeated at least twice. Data are expressed as mean ± SEM. Differences between groups were determined by one-way analysis of variance (ANOVA). Individual differences between groups were tested by multiple comparison and analysis using the Tukey post-test. Pairs of groups were compared by student's t-test (two tailed). P values of <0.05 were considered significant. All analysis was performed using Graphpad Prism Software (La Jolla CA).

Acknowledgements

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Conflict of interest

The authors declare no conflict of interest.

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Figure Legends

Fig 1: Bindarit reduces the gene expression of *CCL2* in influenza A infected cells. A549 cells were infected with A/Ca (H1N1) at MOI 0.1 and subsequently treated with Bindarit at 100 μM. At 24 h pi, RNA was extracted for host gene expression analysis of *CCL2* using RT-qPCR. Expression was normalized to 18s and compared to non-infected cells. Data are from three independent replicates using 3 replicates/well.

Fig 2: Effect of Bindarit-treatment on weight loss and survival rate. Mice were infected intranasally with a lethal dose ($10\times LD_{50}$) of A/Anhui (H7N9). Mice were then treated with either Bindarit or vehicle starting at day 1 pi. Mice were monitored for weight loss (A) and survival (B). Time points represent days pi. Data are from seven mice per group ± SEM.

Fig 3: Effect of Bindarit on histopathological changes following H7N9 infection. Mice were infected intranasally with a lethal dose ($10\times LD_{50}$) of H7N9 or PBS. Mice were then treated with either Bindarit or vehicle starting at day 1 pi. At day 4 pi, mice were sacrificed and lungs collected for histopathological analysis. Mock-infected methylcellulose (A) and

Bindarit-treated (B). Viable epithelial cells line the bronchioles and there is no exudate within the lumen. No peribronchiolar or perivascular infiltrations are present and the parenchyma has no changes. H7N9-infected methylcellulose (C) and Bindarit-treated (D). There is diffuse necrosis and loss of the bronchiolar epithelium with luminal necrotic cellular debris lining the denuded wall. A mild to moderate peribronchiolar and perivascular infiltration of mostly lymphocytes is also present. Parenchymal changes are mild to moderate with mild thickening of the alveolar septa and a few inflammatory cells in alveoli. B= Bronchiole, V=Vessel, P= Parenchyma

Fig 4: Effect of Bindarit on cellular infiltration after influenza A (H7N9) infection. Mice were infected intranasally with a lethal dose ($10\times LD_{50}$) of A/Anhui (H7N9) or PBS. Mice were then treated with either Bindarit or vehicle starting at day 1 pi. At day 5 pi, mice were sacrificed and BAL fluids were collected for analysis with flow cytometry. Data are presented as the number of specific type of cells per million total cells. Data are from five mice per group ± SEM. *p<0.05, **p<0.01, ***p<0.001.

Fig 5: Effect of Bindarit treatment on weight loss and virus titer after H7N9 infection. Mice were infected intranasally with an intranasal sub-lethal dose of H7N9 ($10^{2.7}$ PFU) or PBS. Mice were then orally treated with either Bindarit or vehicle starting at day 1 pi. Mice were monitored for weight loss until day 8 pi (A), when mice were sacrificed and lungs were collected for virus titer analysis with RT-qPCR (B). Virus titers were determined per 5 ng of total RNA extracted from lung homogenates. Time points represent days pi. Data are from five to six mice per group ± SEM. *p<0.05, **p<0.01.

Fig 6: Effect of Bindarit on pro-inflammatory cytokine gene expression following H7N9 infection. Mice were intranasally infected with a sub-lethal dose of H7N9 ($10^{2.7}$ PFU) or PBS. Mice were then treated with either Bindarit or vehicle starting at day 1 pi. On day 8 pi, mice were sacrificed and lungs were collected for gene expression analysis for *Ccl2* (A),

Il6 (B), and *Ifng* (C) with RT-qPCR. Expression was normalized to 18S and relative to non-infected mice. Data are from five mice per group ± SEM. *p<0.05, **p<0.01.

Fig 7: Effect of Bindarit on protein level of pro-inflammatory cytokines after influenza A (H7N9) infection. Mice were infected intranasally with a sub-lethal dose of A/Anhui (H7N9) or PBS. Mice were then treated with either Bindarit or vehicle starting at day 1 pi. On day 8 pi, mice were sacrificed and BAL was collected for protein expression analysis of CCL2 (A), IL-6 (B), RANTES (C), IL-15 (D), and TNFα (E) with a multiplex ELISA. Data are from five mice per group ± SEM. *p<0.05.

Fig 8: Effect of Bindarit on pulmonary cell infiltrates after H7N9 infection. Mice were intranasally infected with a sub-lethal dose of H7N9 ($10^{2.7}$ PFU) or PBS. Mice were then treated with either Bindarit or vehicle starting on day 1 pi. On day 8 pi mice were sacrificed and BAL fluids collected for analysis with flow cytometry. Data are presented as the number of specific type of cells per million total cells. Data are from five mice per group ± SEM. *p<0.05.

Tables

Table 1. Lung scores and observations of lesions after lethal H7N9 infection in mice.

Group	Lung score	Bronchitis /bronchiolitis	Peribronchial/ perivascular infiltration	Alveolitis	Additional observation
H7N9+vehicle	3	severe necrosis	moderate	mild	
	3	moderate necrosis	mild	moderate	
	3	moderate necrosis	mild	severe	hemorrhage
	3	severe necrosis	moderate	moderate	hemorrhage
H7N9+Bindarit	3	moderate necrosis	moderate	moderate	

	3	moderate necrosis	moderate	moderate	
	3	moderate necrosis	moderate	moderate	

Figures

Figure 1

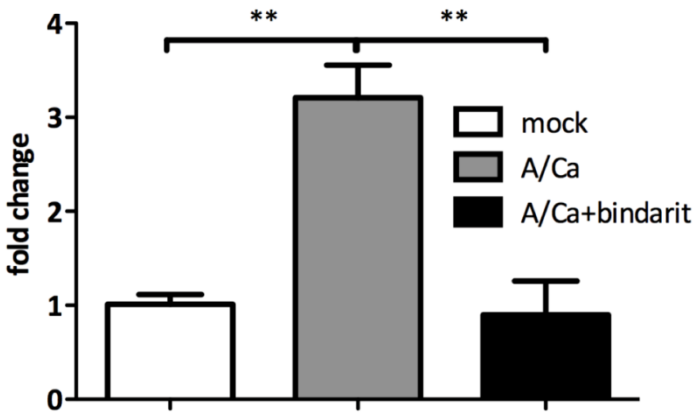


Figure 2

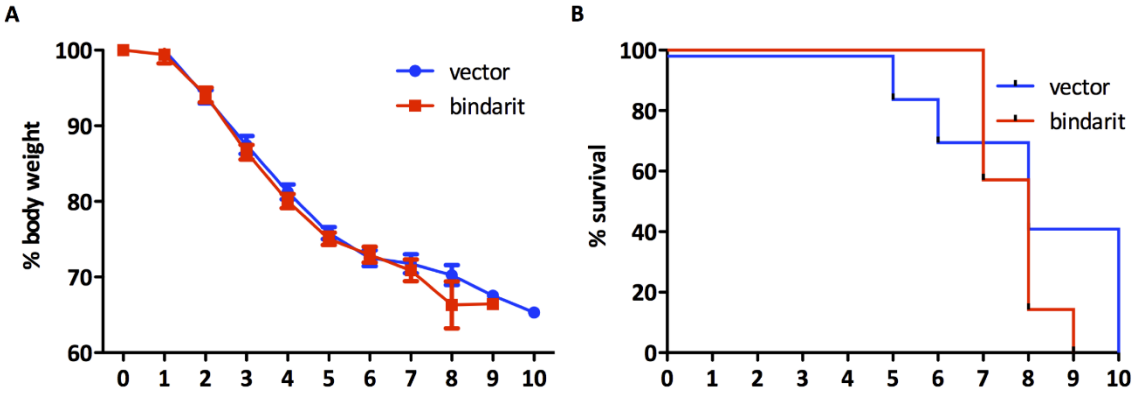


Figure 3

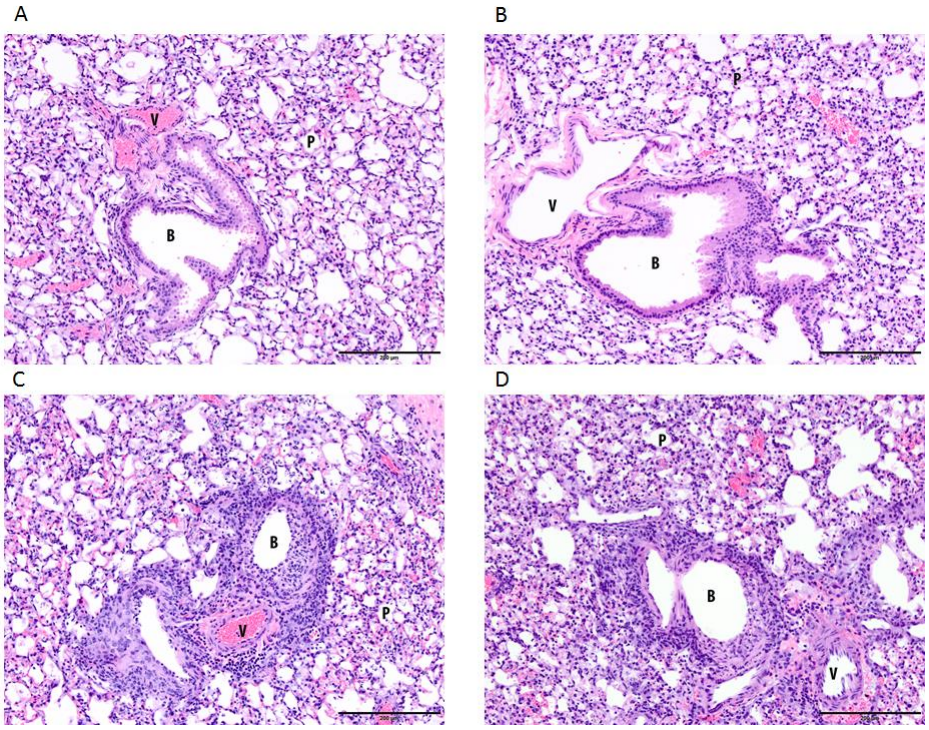


Figure 4

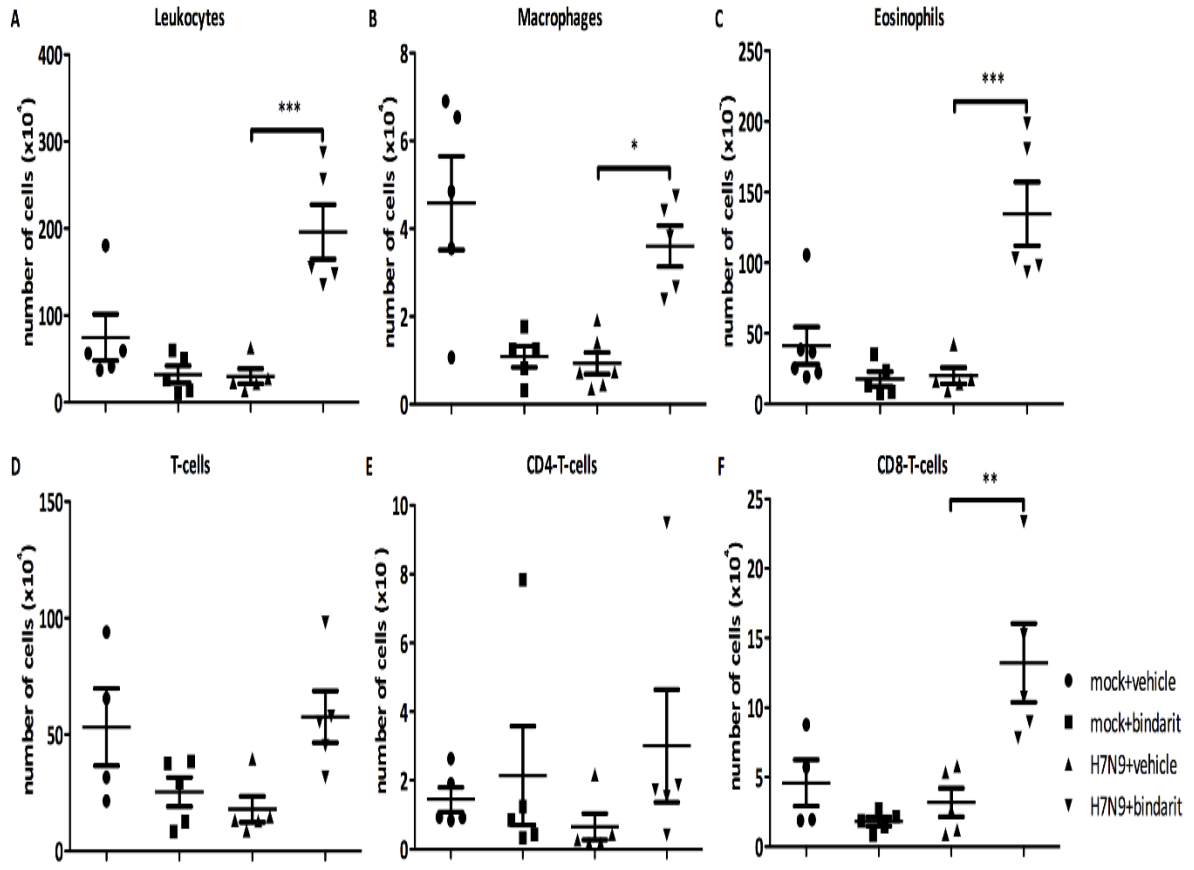


Figure 5

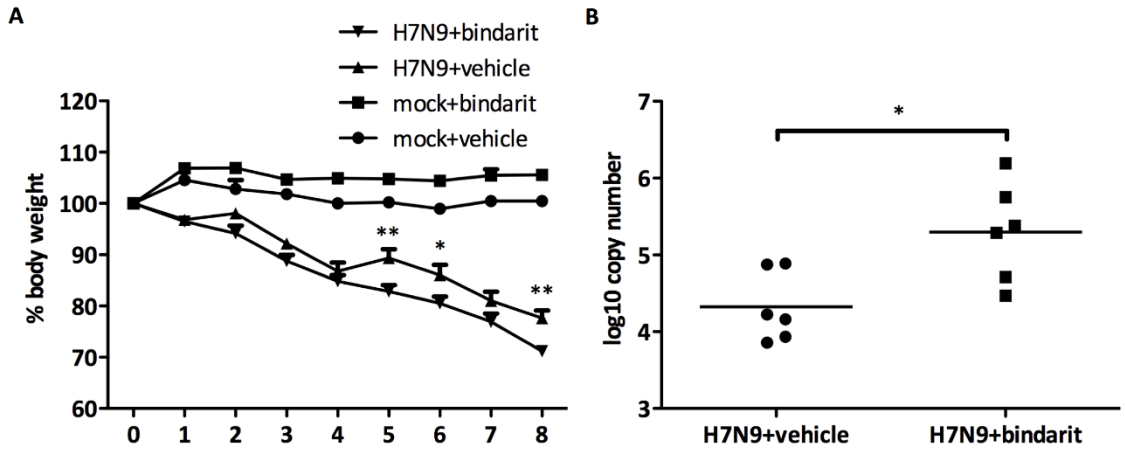


Figure 6

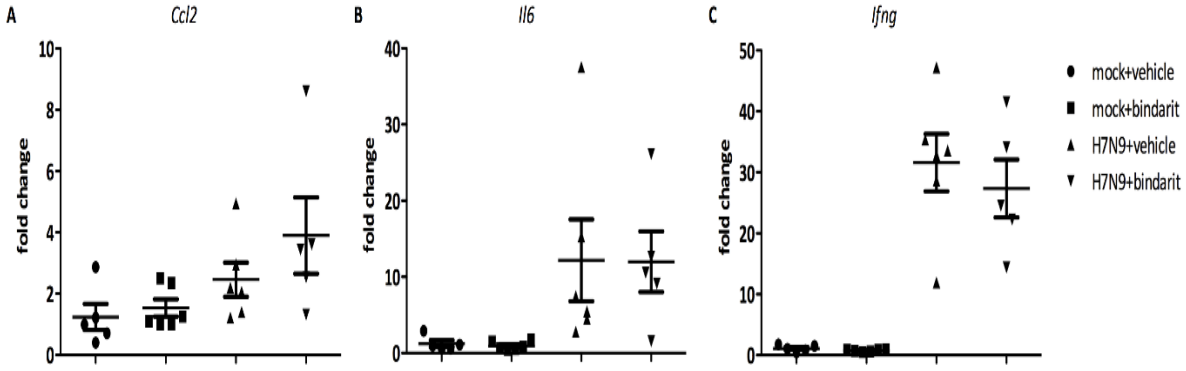


Figure 7

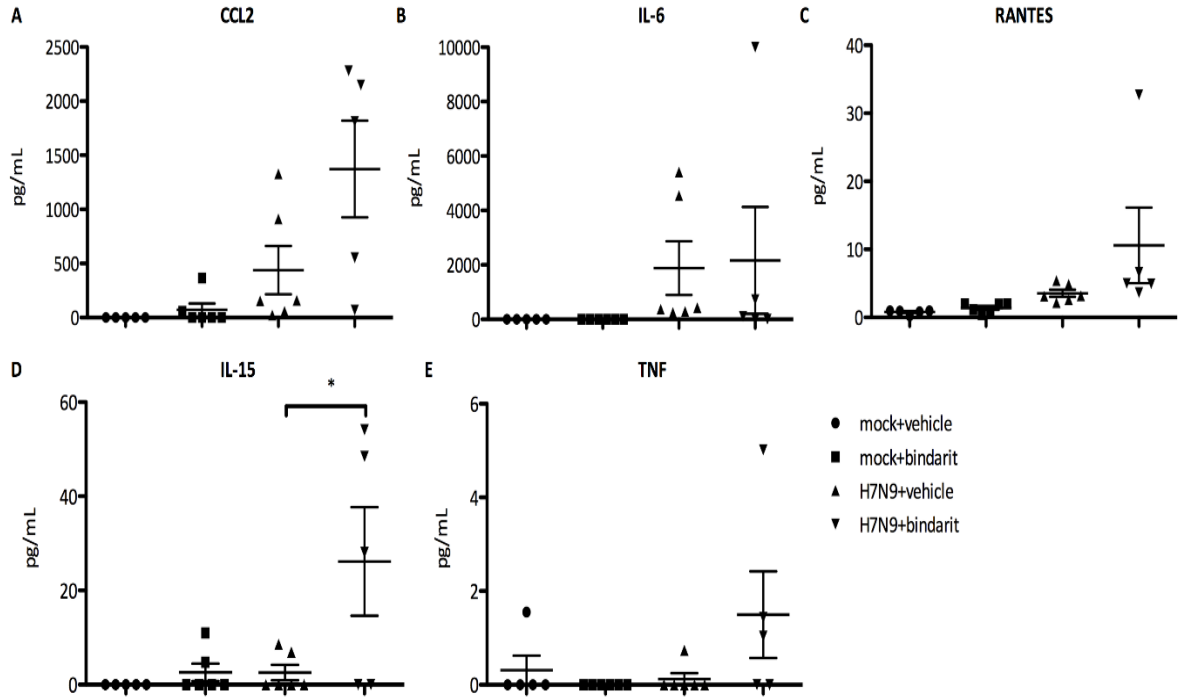
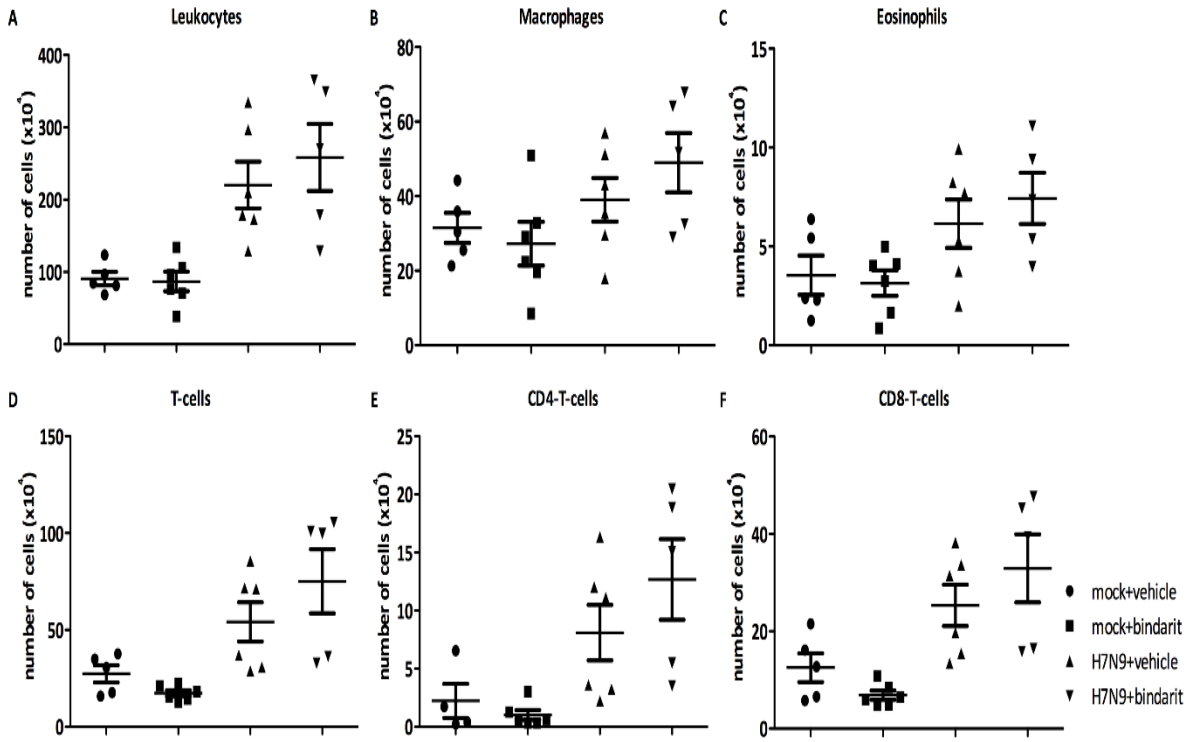


Figure 8


A
Leukocytes
B
Macrophages
C
Eosinophils
D
T-cells
E
CD4-T-cells
F
CD8-T-cells
number of cells (x10⁶)
mock+vehicle
mock+bindarit
H7N9+vehicle
H7N9+bindarit

Chapter 5 - Inhibition of IL-1 signaling reduces bone loss in arthritogenic alphavirus disease

Manuscript in preparation for publication in Arthritis & Rheumatology: Brief reports

Statement of Contribution to Co-authored Published Paper

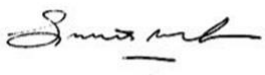
This chapter includes a co-authored paper. The bibliographic details of the co-authored paper, including all authors, are:

Stefan Wolf (MSc), Lara Herrero (PhD), Andreas Surhbier (PhD) and Suresh Mahalingam (PhD)

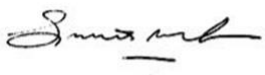
My contribution to the paper involved:

Substantial contributions to conception and design, acquisition of data, analysis and interpretation of data, including the provision of data, the complete analysis and presentation of data into publishable format and providing direction on the scope and structure of the analysis.

Stefan Wolf Gold Coast, 18.08.16



Corresponding author of paper: Prof. SureshMahalingam Gold Coast, 18.08.16



Supervisor: Prof. Suresh Mahalingam Gold Coast, 18.08.16

Preface

The first two chapters focused on the importance of influenza A surveillance programs and the discovery of novel antiviral host targets to combat the burden of influenza A virus disease. In the third chapter, a drug repurposing approach for the treatment of influenza A (H7N9) disease was investigated. In this chapter, the focus is on drug repurposing in the context of arthritogenic alphavirus disease in an animal model of Ross River virus disease. Arthritogenic alphaviruses, such as Ross River virus (RRV), chikungunya virus (CHIKV), Sindbis virus (SINV), Barmah Forest virus, o'nyong-nyong virus and Mayaro virus, cause sporadic, sometimes large, outbreaks worldwide. These viruses particularly affect joints of the extremities and can lead to debilitating and potentially chronic arthritis/arthralgia. The host's innate immune response plays a crucial role in inducing elevated levels of various pro-inflammatory host factors, leading to tissue destruction and bone loss in the joints.

Anakinra demonstrated a significant effect on arthritogenic alphavirus disease by reducing RRV-induced bone loss in the mouse model. In histological analysis of the knee joint, treatment with anakinra reduced epiphyseal growth plate thinning, the loss of epiphyseal bone volume and osteoclastogenesis in the tibia. Importantly, blocking the IL-1 receptor with anakinra did not enhance other clinical signs including disease score, cachexia or viremia.

Inhibition of IL-1 signaling reduces bone loss in arthritogenic alphavirus disease

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Abstract

Objective. Arthritogenic alphaviruses, such as Ross River virus (RRV), chikungunya virus (CHIKV), Sindbis virus (SINV), Barmah Forest virus, o'nyong-nyong virus and Mayaro virus, cause sporadic, sometimes large, outbreaks worldwide. These viruses particularly affect joints of the extremities and can lead to debilitating and potentially chronic arthritis/arthralgia. The host's innate immune response plays a crucial role in inducing elevated levels of various pro-inflammatory host factors, leading to tissue destruction and bone loss in the joints. This study was designed to test a novel approach for the treatment of RRV- -induced arthritis using the IL-1 signaling inhibitor anakinra in mice.

Methods. Mice were infected with RRV and treated with anakinra. Weight gain and disease score was measured, tissue viral titers were determined, and histological changes in muscle and joint tissues were assessed.

Results. Anakinra demonstrated a significant effect on arthritogenic alphavirus disease by reducing RRV-induced bone loss in the mouse model. In histological analysis of the knee joint, treatment with anakinra reduced epiphyseal growth plate thinning, the loss of epiphyseal bone volume and osteoclastogenesis in the tibia. Importantly, blocking the IL-1 receptor with anakinra did not enhance other clinical signs including disease score, cachexia or viremia.

Conclusion. The safety profile of anakinra as a widely used therapeutic in patients diagnosed with RA, together with our experimental data, suggests the potential use of anakinra for patients affected by arthritogenic alphavirus disease, in particular those who already suffer from pre-existing medical conditions such as RA.

Introduction

Mosquito-borne alphaviruses such as Ross River virus (RRV), chikungunya virus (CHIKV), Sindbis virus (SINV), Barmah Forest, o'nyong-nyong virus and Mayaro virus virus cause sporadic, frequently large outbreaks of rheumatic disease worldwide (1, 2). Over the last decade, CHIKV has raised special attention after an outbreak with up to 6.5 million cases occurred in India and surrounding islands. This epidemic began in Kenya, spread across the Indian Ocean to India and later to South East Asia and the Pacific Ocean, affecting nearly 40 countries including imported cases to several European countries, the USA and Japan (1, 3). Two hotspots of this epidemic were Grande Comore Island and La Reunion in 2005-2006 with an attack rate of 50% and 38% of the population, respectively. The emergence of CHIKV has recently reached new levels, as an epidemic is currently affecting the Caribbean, reaching areas in Brazil, Mexico and even Florida, USA, with an estimated 1.6 million cases (2, 3). The worldwide spread of arthritogenic alphaviruses may be attributed to the expansion of their main vectors, the mosquitoes of the *Aedes* and *Culex* genus, which is fueled by global trade and travel (2, 4).

RRV and other alphaviruses cause disease manifesting as polyarthritis/arthralgia, accompanied by myalgia, fever and often rash (1). RRV-induced arthritis commonly affects the ankles, knees and peripheral joints in a symmetrical fashion. Hallmarks include incapacitating joint pain and polyarthralgias with a level of disability comparable to rheumatoid arthritis (RA), osteoarthritis or lyme disease (1, 5). The overlapping clinical and immunological features highlight the need for rheumatologists to consider arthritogenic alphavirus infection when evaluating patients with new, symmetrical polyarthritis (6). The onset of arthritogenic alphavirus disease can be sudden and debilitating, and despite commonly self-limiting, can evolve into a chronic disease stage (1, 7, 8). There are currently no specific antivirals or vaccines against alphavirus-induced disease available. Thus, the current primary therapy consists of non-steroidal anti-inflammatory drugs, which only offer partial pain relief (5). Since the 2005/2006 CHIKV outbreak, extensive research has been

undertaken, resulting in several vaccines undergoing clinical evaluation. The first formalin-inactivated CHIKV vaccine had been prepared in 1967, however there are still no effective vaccines available to prevent disease today. Hence, therapy is still limited to supportive care as antivirals are yet in different pre-clinical stages of testing or development.

In a meta-analysis on 18 studies amongst 5,702 CHIKV patients, at least 25% of CHIKV cases developed chronic inflammatory rheumatism (34% for most representative studies with at least 200 patients) and 14% chronic arthritis (9). Levels of pro-inflammatory cytokines, such as interleukin-1β (IL-1β), IL-6, tumor necrosis factor (TNF) and monocyte chemotactic protein-1 (MCP-1/CCL2), have been shown to be elevated in the sera of human CHIKV patients (7, 10). For example, Il-6 and MCP-1/CCL2 have been linked to high viral load, whereas IL-6 and IL-1β have been reported to be biomarkers for CHIKV severity (11). Consistently, these factors are upregulated in arthritogenic alphavirus disease in mice and are being investigated as the underlying mechanisms in which the innate immune system combats arthritogenic alphavirus disease and in turn causes inflammation in infected tissues (7).

In recent years, bone lesions of CHIKV-infected patients have been reported (8), providing evidence that alphavirus-induced disease can result in bone pathologies. MRI findings showed joint effusion, bony erosion, marrow oedema, synovial thickening, tendinitis and tenosynovitis. There is evidence that CHIK arthritis can induce chronic inflammatory erosive arthritis, which has implications for management of the infection. Furthermore, studies have linked CHIKV to the development of unspecific post-viral arthritis, RA, seronegative spondylitis and other non-inflammatory musculoskeletal complaints like persistent arthralgia (9). Taken together, these conditions affect the quality of life and weigh in on direct and indirect economic loss, which represent a significant burden of disease, with considerable financial impacts on health care systems. In physiological conditions, a balance between bone-forming osteoblasts and bone-absorbing osteoclasts is maintained. In the case of RA and alphavirus-induced arthritis, pro-inflammatory cytokines such IL-1β, IL-6,

TNF and MCP-1/CCL2 can disturb this balance, leading to increased osteoclast activity and bone loss (12, 13). Serum levels of RRV patients showed elevated levels of tartrate-resistant acid phosphatase (TRAP)5b, a serum marker for bone resorption and RANKL/OPG disruption in the synovial fluid (13). Recent work has shown that inhibiting IL-6 or MCP-1/CCL2 can reduce alphavirus-induced bone loss in mice (13, 14). The aim of this study was to look at the impact of IL-1 signaling in the context of alphavirus-induced bone loss. Thus, mice were infected with RRV and subsequently treated with the IL-1 receptor antagonist anakinra (Kineret®), which is a commonly used therapeutic in the treatment of structural joint damage in patients suffering from RA (15, 16). Anakinra successfully reduced RRV-induced bone loss in our mouse model. Thus, this study presents a readily available therapeutic with great potential in the treatment of arthritogenic disease caused by alphaviruses such as RRV and CHIKV.

Materials and Methods

Virus. Stocks of T48 strain of RRV (RRV-T48) were generated from the full- length T48 cDNA clone. RRV titers were determined by plaque assay on Vero cells as described previously (17).

Mice and treatment. C57BL/6 wildtype mice were obtained from the Animal Resources Centre and bred in-house. The 21-days-old male and female mice, of equal distribution, were inoculated subcutaneously in the thorax below the right forelimb with 10^4 pfu of RRV-T48 diluted in PBS. Mice were monitored daily for diet and well-being and were weighed and scored for disease signs every 24 h as described previously (17). All animal experiments were approved by the Animal Ethics Committee of Griffith University (GLY/10/13/AEC). Mice were injected intraperitoneally with anakinra (Kineret®, Swedish Orphan Biovitrium; 150 µg per mouse) or PBS in a 50 µL volume. Treatment was performed daily and commenced on the day of RRV infection.

Histology. Mice were euthanized, hind limbs collected, fixed in 4% (wt/vol) paraformaldehyde (PFA), decalcified in 14% EDTA, and embedded in paraffin. Five micrometer sections were dewaxed, rehydrated, and stained with hematoxylin and eosin (H&E), toluidine blue and TRAP. Histomorphometrical analysis and quantification of growth plate width (in μm) and epiphyseal bone volume (% **B**one **V**olume/**T**issue **V**olume) was performed using OsteoMeasure (Osteometrics). Bone loss was examined by three parameters: Growth plate width, epiphyseal bone volume and osteoclastogenesis (TRAP).

Statistical analysis. Data on weight and disease score differences between groups were analyzed by two-way analysis of variance, followed by Bonferroni adjustment for multiple comparisons. Data on difference in virus titers between experimental groups were assessed by unpaired 2-tailed *t*-test. Statistical analyses were performed using GraphPad Prism software version 5.0f. *P*-values less than 0.05 were considered significant.

Results

Manipulation of the inflammatory response during viral infections potentially reduces inflammation, but may also dampen viral clearance (18, 19). Thus, we investigated whether anakinra can be used safely to treat rheumatic alphavirus disease, without enhancing disease score, cachexia or virus titers.

RRV-infected, anakinra-treated and PBS-treated mice exhibited similar disease score and cachexia, which reached statistical significant difference compared to mock-infected mice on days 5 and 6 post-infection (p.i)., respectively ($p<0.05$) (Figure 1A/B). Mock-infected, anakinra-treated control mice showed no significant difference in disease score and weight gain compared to mock-infected, PBS-treated animals (Figure 1A/B). To investigate the impact of anakinra treatment on viral clearance, virus titers were determined in the ankle, femur, quadriceps and serum at day 1, 3 and 10 after infection. No significant difference was observed between anakinra-treated and PBS-treated animals in any of the

tissues investigated at any of the time points (Figure 1C-F), suggesting that inhibition of IL-1 signaling did not affect viral clearance.

MRI of patients infected with arthritogenic alphavirus disease have shown bone lesions, which delivered evidence for these viruses to cause bone loss (8). In accordance, animal models of RRV-infected mice have recently shown to exhibit bone loss in the knee joints, characterized by reduced growth plate thickness, decremented epiphyseal bone volume and enhanced osteoclastogenesis (13). Thus we examined joint tissue samples histologically at peak disease (day 10 p.i.). As expected, RRV-infected, mock-treated mice showed apparent epiphyseal growth plate thinning, reduced bone volume of the tibial epiphysis and enhanced osteoclastogenesis, shown by TRAP-staining for osteoclast-like cells, compared to mock-infected control mice (Figure 2). However, RRV-infected, anakinra-treated mice showed reduced growth plate thinning and bone volume decrement in the tibial epiphysis and dampened osteoclastogenesis compared to RRV-infected, mock-treated mice (Figure 2). Growth plate and bone volume of the tibial epiphysis were significantly reduced in mice infected with RRV ($p<0.001$) compared to healthy mock-infected control mice, which was significantly reversed by anakinra treatment ($p<0.001$) (Figure 3). Furthermore, treatment with anakinra had no impact on the histology of mock-infected mice (data not shown). Interestingly, anakinra treatment reduced the number of cellular infiltrates in the quadriceps of RRV-infected mice, without altering disease score (data not shown). Further studies will be required to investigate the effects of anakinra on muscle tissues during RRV disease.

Discussion

These results suggest that IL-1 plays a critical role in the induction of bone loss in arthritogenic alphavirus disease, but may not be critical for antiviral defense. Thus, anakinra may be of great potential to prevent bone loss in patients suffering from alphaviral

arthritides. This is of special importance as there are currently no specific therapeutics available for the treatment of arthritogenic alphavirus disease. Drug repurposing may be inevitable in the fight against emerging alphaviral disease, since the relatively small market size and the cost for clinical development hinder the development of novel drugs.

This is the first study testing IL-1 receptor inhibitors for the treatment of a viral arthritis and suggests that this class of drugs may be of potential use for alphaviral disease. A study on the related SINV has also revealed a pathogenic role for IL-1, where IL-1β-deficient mice were resistant to fatal SINV encephalitis (20). In contrast, in studies on influenza-infected IL-1 receptor knock-out mice, preventing IL-1 signaling has led to disease exacerbation including elevated virus titers and mortality (19). In a virus-induced model for multiple sclerosis, IL-1 has been shown to play a pathogenic role, but also a crucial role for viral clearance (21). Earlier attempts to explore the possibilities of targeting RA related soluble factors in arthritogenic alphavirus disease, such as TNF, IL-6 and MCP-1/CCL-2 have shown variable outcomes (13, 14, 18). RRV-infected mice that were treated with an antirheumatic therapeutic, the TNF inhibitor etenarcept (Enbrel®), developed enhanced disease with subsequent resulting fatalities (18). Interestingly, there have also been successful attempts in reducing alphavirus-induced bone loss by targeting other known arthritis-inducing soluble factors such as IL-6 and MCP-1/CCL-2 (13, 14). Mice infected with RRV and subsequently treated with either IL-6 antibodies or the pre-clinical MCP-1/CCL2 synthesis inhibitor bindarit, developed reduced arthritogenic disease compared to non-treated mice. However, the application of antibodies and pre-clinical compounds against arthritogenic alphavirus disease in humans are limiting factors. Safety studies for clinical approval for human use require years of research and come at considerable cost. On the other hand, there has been an approach to target arthritogenic alphavirus disease by using a disease modifying osteoarthritis drug pentosane polysulfate (PPS), which successfully reduced cartilage damage in mice infected with RRV or CHIKV (22).

Even though arthritogenic alphaviruses are on the rise with CHIKV's worldwide emergence, which resulted in a big push for vaccines by pharmaceutical companies, the

availability of effective vaccines will be years away and treatments are needed now. Our work with repurposing drugs has enormous value in treating the condition. Thus, anakinra has a major advantage as a readily available therapeutic for drug repurposing due to the existence of human safety data from years of applications in RA patients. Furthermore, anakinra has not been found to be associated with the development of opportunistic infections (16), which may be of concern with other immunomodulatory drugs such as corticosteroids or TNF-inhibitors. Therefore, anakinra may provide a readily available treatment option until novel therapeutics are developed specifically against alphavirus-induced arthritis. Taken together, anakinra was able to reduce bone loss, where other drugs such as PPS reduce cartilage damage in mice, so there might be a potential of combinational therapy. Furthermore, even with vaccines being available in the future, there is still a need for therapeutics against the chronic stages of arthritogenic alphavirus disease, as vaccines may not be able to reverse exisitng symptoms. Thus, anakinra may be a safe treatment option of arthritogenic alphavirus disease, as it did not enhance disease signs or virus replication, but in turn acted as a crucial player in reducing bone loss in our mouse model of RRV-induced disease.

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Figure legends

Figure 1: Anakinra treatment may be safe during RRV disease.

RRV-infected, anakinra-treated mice did not show signs of enhanced disease such as disease score or reduced weight gain (Fig 1A-B). In virus replication studies, anakinra did not alter viral clearance in various tissues at different time points. Thus, treatment with anakinra may be safe during RRV infection. Data are from 5 biological replicates ± SEM. *p<0.05.

Figure 2: Anakinra treatment reduced bone loss in RRV-infected mice.

The knee joints of RRV-infected mice were paraffin embedded, sectioned and stained with H&E, toluidine blue and TRAP. Representative images from three mice per group are shown (20x). Treatment with anakinra visually reduced growth plate (gp) thinning and epiphyseal (ep) bone decrement in the proximal tibial epiphysis when joint tissues were stained for H&E and toluidine blue. In addition, TRAP-staining for osteoclastogenesis was reduced for mice treated with anakinra (arrow).

Figure 3: Anakinra treatment significantly reduced histological changes.

Histomorphological analysis of the growth plate and the epiphysis were performed using the OsteoMeasure quantification software. Growth plate thinning (in μm) and epiphyseal bone volume (BV/TV) was significantly reduced after anakinra treatment of RRV-infected mice. Data are from 3 biological replicates and technical duplicates ± SEM. ***p<0.001.

Figures

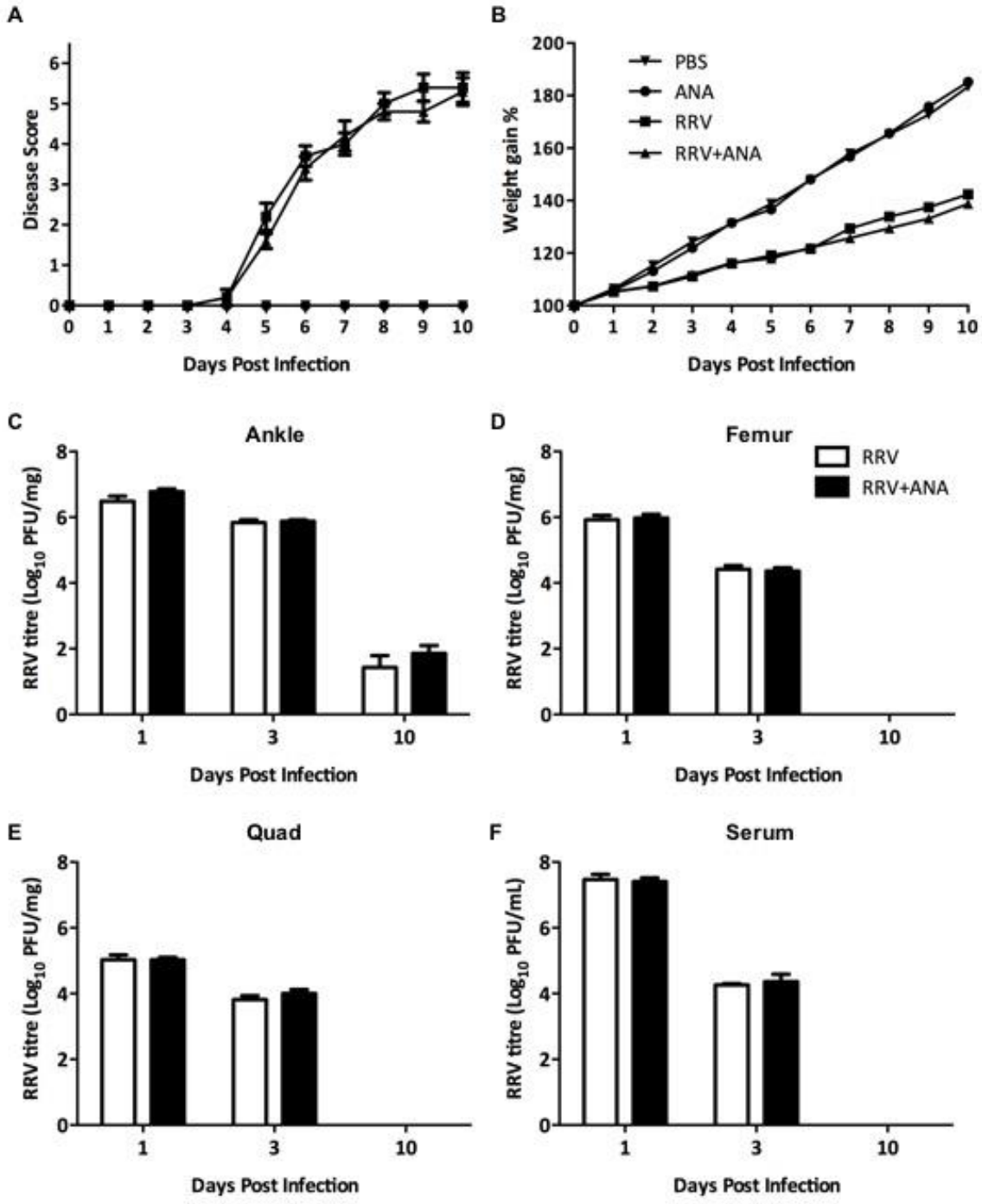

Figure 1: Scores, weight gain and virus titers

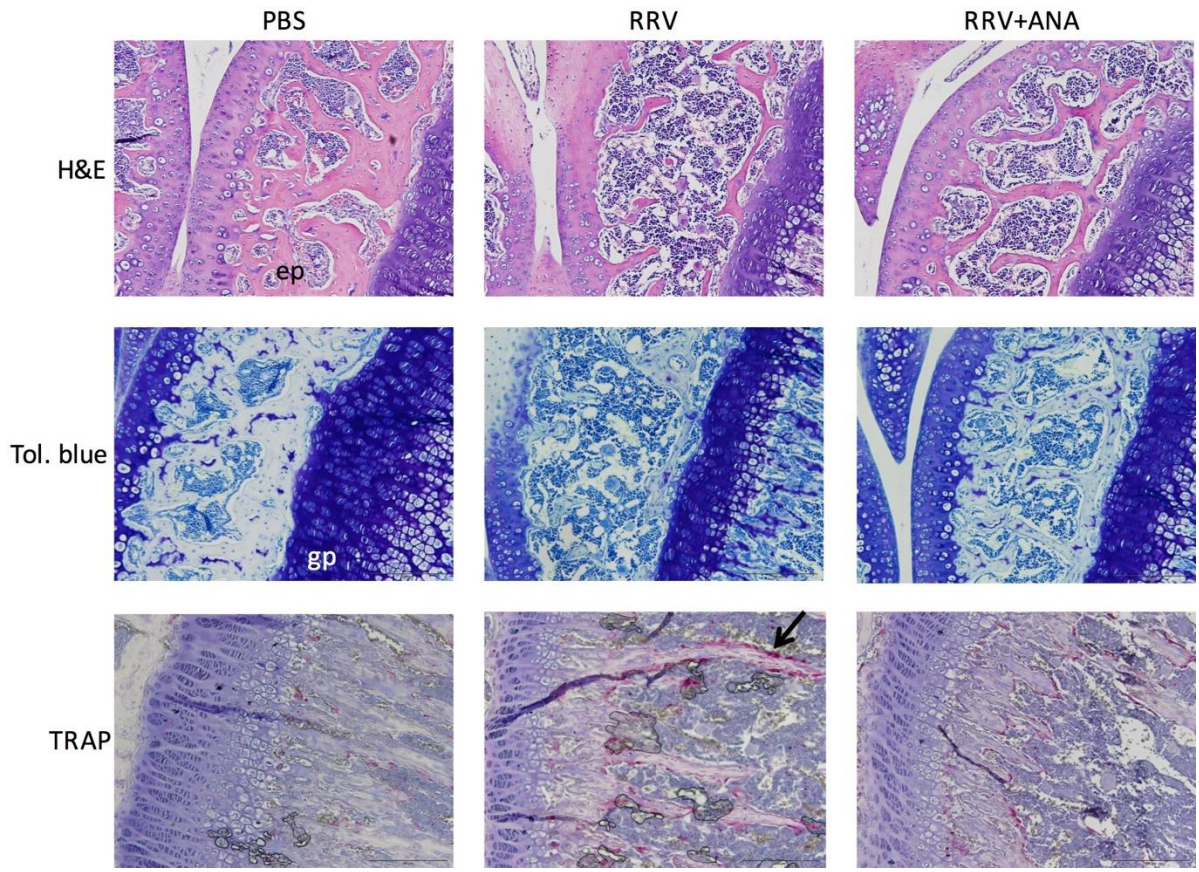

Figure 2: Histology of the knees with H&E, toluidine blue and TRAP staining

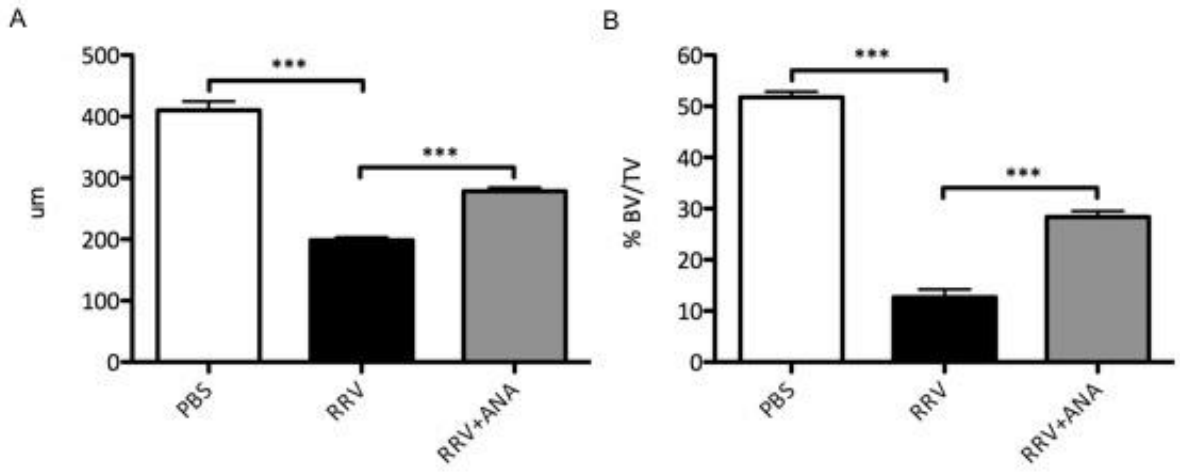

Figure 3: Histomorphometrical analysis

Chapter 6

6.1. Final discussion

IAVs are among the leading pathogens, causing epidemics of respiratory disease in humans, resulting in the death of more than five hundred thousand people annually and the hospitalisation of many more (249). The ability of IAVs to cross species barriers and infect new hosts, or reassort with circulating viruses, has raised public health concerns about the possibility of novel viruses emerging in animals and then crossing to humans. The 2009 H1N1 pandemic virus (pmH1N1), which emerged through the reassortment of previously known swine IAVs, highlighted the role of pigs in the ecology of IAVs and their importance for the protection of public health (250). Swine and human IAVs have a parallel history since the co-occurrence of respiratory disease in the 1918-19 Spanish Influenza pandemic, when outbreaks of acute respiratory disease were recorded in pigs and their human cohorts (251, 252). Taking into account the complexity of sIAV epidemiology in pigs and the importance of constantly monitoring the situation, we proceeded to conduct a long-term surveillance study, initially focusing on the Southeastern USA and specifically in North Carolina. Pig production in the USA is characterized by the fact that there is a disproportionate higher number of sow farms in North Carolina compared to other pork producing states (253). From there, weaned piglets, at the age of 21 to 28 days, are shipped to the Midwest, where they are raised until they are led to consumption at six months of age. Through transportation of the piglets to finishing farms in different states, swine IAVs travel and when the piglets mix, they may reassort. Thus, the Southeastern region of the USA plays a key role in the epidemiology of IAVs.

The first chapter of this PhD thesis includes the results of our initial active surveillance-screening of IAVs in pigs and the subtypes of viruses we were able to identify in the Summer of 2014. The aim of this study was to set a baseline for our knowledge of the various swine IAV strains circulating in the area, to sequence these viruses and to identify known or unknown reassortment events. While only 3.6% of the samples examined were

found positive for IAV, on herd level we found a high prevalence of infection, indicating that at any given time, even during the summer months, IAVs are circulating in 17.4% of the farms. While clinical manifestation of IAV in swine has been recorded primarily from October to April, the virus may be present all-year round, as it has been reported in previous studies (254-256). The percentage of positive samples, while it may appear low, is comparable with other IAV active surveillance studies conducted in swine (256, 257). However, according to both our experience from consequent testing and other studies, virus can be isolated at a high percentage rate (>75%) when processing of oral fluid samples is being initiated within 48 hours of collection (258, 259). Thus, oral fluid samples are a proper material for swine virus monitoring and isolation as long as they are handled in an adequate and timely matter. Our preliminary subtyping data showed a strong prevalence of H1 IAVs over H3 viruses. Specifically, 47 out of 60 samples that were fully subtyped or only partially subtyped at the level of HA, were identified as containing H1 IAV. On the other side, only 16 samples contained an H3 virus, while mixed infections containing an H1N1 and an H3N2 virus were found in 3 samples. The prevalence of H1 and H3 subtypes is not in accordance with the epidemiological picture observed in other studies conducted for different swine-producing areas in the USA, such as the Midwestern part of the country, where about one third of swine IAVs isolated bare an H3 HA (19, 33, 34). Finally, the multiplex method used in this investigation for subtyping swine IAVs was able to identify 56 out of 68 M-gene positive samples, while it either partly identified or did not identify at all the remaining 12. These samples had a Ct value ≥ 28, indicating a correlation between low Ct value and successful sub typing by this method.

IAV is a serious global health threat not only due to zoonotic reassortment in agricultural species such as swine, but also birds. Human infection with avian IAV (H7N9) were first reported in China in March 2013 (22). While some mild illnesses in humans infected with H7N9 has been reported, most patients experienced severe respiratory illness, such as pneumonia (97.3%) and acute respiratory distress syndrome (71.2%), leading to high rates of intensive care unit admissions (23). Human mortality attributed to IAV (H7N9)

has been over 38% with 650 confirmed cases since 2013 (24, 25). Recently, the first documented case of H7N9 in humans was reported for North America in Canada (27). No vaccine is currently available for H7N9 (56) and the use of antiviral drugs is complicated by the emergence of drug resistant viruses (52, 53). Consequently, oseltamivir-resistant H7N9 strains have already been described in recent reports from Taiwan (58). To prevent the spread and impact of infection, new drug and vaccine development is needed. However, difficulties include a lack of understanding of the host factors required for replication, and unusual mutations that occur in the virus that differ from other avian IAVs (57). Linking HTS with RNAi allows for the rapid discovery of the molecular basis of disease pathogenesis, and the identification of potential pathways for the development of safe and effective treatments. Recent advances in our understanding of RNAi have allowed for genome-wide screens to determine and validate the host cell genes that may be required for IAV replication (77). In addition to host gene involvement during viral infection, the magnitude and tempo of host gene expression is governed by factors such as miRNA. miRNAs have been used to validate the impact of host genes on virus replication and have been used as therapeutics (81) with the ability to negatively affect IAV replication (82). In our study, a genome wide HTS was performed to identify target miRNAs as countermeasures of H7N9 replication. Several pro-viral host miRNAs were identified which are important for H7N9 replication. In validation studies, the most promising target, hsa-miR-664a-3p (miR-664), was further investigated and the effects on downstream genes, Leukemia inhibitory factor (LIF) and NIMA-related kinase 7 (NEK7), were explored. To our knowledge this is the first report on a miRNA-based antiviral therapeutic that countermeasures H7N9 replication *in vitro*. miR664i, an inhibitor of host cell miRNA miR-664, showed potent antiviral effects inhibiting H7N9 replication by two logs ($TCID_{50}$ titers) in A549 cells. In a recent publication, MIR2911, a broad spectrum antiviral miRNA suppressed replication of H1N1, H5N1 and H7N9 influenza virus in *vitro* and *in vivo* (260). MIR2911 targeted the PB2 and NS1 gene of IAV, and considerably inhibited their protein expression. However, MIR2911 was ineffective against mutant IAVs in which the MIR2911-binding nucleotide sequences were altered. In contrast,

miR664i does not target the virus directly and the antiviral effects may be directed through intracellular mechanisms in the host cell. One advantage of targeting the host cell miRNA, instead of the virus directly is the lower probability of the virus evolving mutations that would potentially lead to resistance against the therapeutic. In addition, the two molecules LIF, and NEK7, were highly predicted target genes for miR-664 based on ingenuity pathway analysis (IPA) software. Gene expression studies showed that while miR-664 was upregulated during H7N9 infection, target genes LIF and NEK7 were downregulated. When miR664i was transfected prior to infection, expression levels of target genes were subsequently normalised. Thus, the inhibitory effect of miR-664 on its target genes during IAV infection may facilitate virus replication. To investigate the underlying mechanisms of miR-664 during IAV replication, knock-down studies using siRNA targeting LIF and NEK7 were performed. Knocking down NEK7 had no impact on virus replication, which suggests a non-relevant role of this particular kinase during IAV infection. However, knocking down LIF increased virus replication in various IAV strains. This suggests that miR-664 downregulates LIF, which may be an important factor in immune defence against IAV. Whether there are other factors involved and what happens downstream of LIF has yet to be determined. The importance of LIF in antiviral defence has been reported in previous publications (261). *In vivo* studies were performed with LIF knock-out mice that were infected with RSV. LIF knock-out mice yielded higher virus titres compared to control mice. Furthermore, mice treated with anti-LIF IgG developed enhanced RSV pathology observed with increased airspace protein content, apoptosis and airway hyperresponsiveness compared to control IgG treatment. Thus, LIF may be a critical factor in the antiviral defence against RSV and other respiratory diseases such as IAV.

While our HTS targeting miRNA during H7N9 infection has allowed us to discover a potent target to countermeasure virus replication and to describe underlying downstream mechanisms, the development of a novel drug such as miR664i still requires years of clinical testing. Due to increased resistance among circulating and novel IAV strains and the lack of specific vaccines against H7N9, there remains an urgent need for drug repurposing because

the development of novel antivirals and specific vaccines will require many years of preclinical and clinical studies before their availability for clinical use. H7N9 disease results in a potent immune response believed to contribute to tissue destruction and the resulting pathology (39). The host's immune response towards H7N9 infection has not been fully elucidated, which is an issue for the discovery of novel therapeutics and target identification for drug repurposing. In IAV (H7N9) disease, a cytokine storm, or the upregulation of pro-inflammatory cytokines and chemokines, such as CCL2, IL-6, IL-8, IFN-α, IFN-γ, IP-10, MIG, and MIP-1β, was reported in H7N9-infected patients with lung injury and severe pneumonia in (40). Various approaches have been investigated for the treatment of H7N9 infection in recent years. For example, treatment with corticosteroids was evaluated; however, they were shown to increase the mortality of human patients suffering from acute H7N9 infection (59). CCL2 has been shown to be upregulated in many viral diseases in humans and has been considered as a biomarker linked to disease severity (262). Furthermore, CCL2 has been linked to inflammation and subsequent tissue damage in human disease (262-264). In animal studies on various inflammatory diseases, bindarit has been shown to reduce CCL2 production *in vitro* and *in vivo*, and successfully alleviated disease (265-267). In animal models of arthritogenic alphavirus disease, bindarit was able to reduce disease symptoms such as clinical score, cellular infiltration of muscle tissues, and bone loss (74, 75, 201). In these studies, modulating the innate immune response by inhibiting CCL2 was shown to reduce disease signs without altering viral clearance. Thus, bindarit may be potentially used in the treatment of virus-induced inflammation. Furthermore, bindarit has been shown to reduce inflammation and ameliorate disease in an animal model of autoimmune encephalomyelitis (266), which mimics many aspects of Guillain-Barre syndrome (268), indicating that CCL2 inhibitors could potentially be beneficial against the increasing number of Guillain-Barre syndrome in areas affected by Zika virus infection. As mentioned, CCL2 inhibitors such as bindarit were shown to relief symptoms caused by virus infections. However, CCL2 may play dual roles in antiviral defense: a protective and a pathogenic role. For example, in a study evaluating the role of CCL2 using an animal model of HIV, CCL2

receptor (CCR2) knockout mice showed increased virus titres and disease (264). CCL2 has been shown to be highly upregulated in patients suffering from IAV (H7N9) disease and has been linked to lung injury in mouse models (40). However, in this study, when H7N9-infected mice were treated with bindarit, mice exhibited enhanced disease signs as demonstrated by an increase in weight loss, pro-inflammatory factors, cellular infiltration, and virus titres. Thus, blocking CCL2 dampened viral clearance and in turn caused the upregulation of pro-inflammatory cytokines and cellular infiltration. In earlier studies examining the effects of anti-CCL2 antibodies on IAV disease, mice exhibited enhanced pneumonitis compared to non-treated animals, despite reduced numbers of cellular infiltrates such as leukocytes, macrophages, and neutrophils in the lungs (269). Furthermore, in a study evaluating IAV disease in CCL2 knockout mice, infection with a non-lethal dose of a mouse adapted strain of IAV resulted in a profound increase in weight loss, elevated viral loads and pro-inflammatory cytokines, and reduced leukocyte recruitment into the infected lungs (270). Taken together, our results together with earlier knock-out attempts indicate that bindarit may be ill-advised against H7N9 infection.

In the fourth chapter of this PhD thesis, the focus was set on different single stranded RNA viruses, the alphaviruses. Even though they are unrelated to IAV, they share common features such as overreacting immune response towards the virus, an emerging prevalence worldwide and difficulties in treatment. Mosquito-borne alphaviruses such as RRV, CHIKV, SINV, BFV, ONNV and MAYV cause sporadic, frequently large outbreaks of rheumatic disease worldwide (116, 119). The onset of arthritogenic alphavirus disease can be sudden and debilitating, and despite commonly self-limiting, can evolve into a chronic disease stage (116, 155, 271). There are currently no specific antivirals or vaccines against alphavirus-induced disease available. Thus, the current primary therapy consists of non-steroidal anti-inflammatory drugs, which only offer partial pain relief (123). Since the 2005/2006 CHIKV outbreak, extensive research has been undertaken, resulting in several vaccines undergoing clinical evaluation. The first formalin-inactivated CHIKV vaccine had been prepared in 1967, however there are still no effective vaccines available to prevent disease today. Hence,

therapy is still limited to supportive care as antivirals are yet in different pre-clinical stages of testing or development. In recent years, bone lesions of CHIKV-infected patients have been reported (155), providing evidence that alphavirus-induced disease can result in bone pathologies. Taken together, these conditions affect the quality of life and weigh in on direct and indirect economic loss, which represent a significant burden of disease, with considerable financial impacts on health care systems. In physiological conditions, a balance between bone-forming OBs and bone-absorbing osteoclasts is maintained. In the case of RA and alphavirus-induced arthritis, pro-inflammatory cytokines such as IL-1β, IL-6, TNF and CCL2 can disturb this balance, leading to increased osteoclast activity and bone loss (166, 219). Recent work has shown that inhibiting IL-6 or CCL2 can reduce alphavirus-induced bone loss in mice (166, 201). The aim of this study was to look at the impact of IL-1 signalling in the context of alphavirus-induced bone loss. Thus, mice were infected with RRV and subsequently treated with the IL-1 receptor antagonist anakinra (Kineret®), which is a commonly used therapeutic in the treatment of structural joint damage in patients suffering from RA (272, 273). Anakinra successfully reduced RRV-induced bone loss in our mouse model. Thus, this study presents a readily available therapeutic with great potential in the treatment of arthritogenic disease caused by alphaviruses such as RRV and CHIKV. This is the first study testing IL-1 receptor inhibitors for the treatment of a viral arthritis and suggests that this class of drugs may be of potential use for alphaviral disease. A study on the related SINV has also revealed a pathogenic role for IL-1, where IL-1β-deficient mice were resistant to fatal SINV encephalitis (274). In contrast, in studies on IAV-infected IL-1 receptor knock-out mice, preventing IL-1 signalling has led to disease exacerbation including elevated virus titres and mortality (275). In a virus-induced model for multiple sclerosis, IL-1 has been shown to play a pathogenic role, but also a crucial role for viral clearance (276). Earlier attempts to explore the possibilities of targeting RA related soluble factors in arthritogenic alphavirus disease, such as TNF, IL-6 and CCL-2 have shown variable outcomes (166, 190, 201). RRV-infected mice that were treated with an antirheumatic therapeutic, the TNF

inhibitor etenarcept (Enbrel®), developed enhanced disease with subsequent resulting fatalities (190). Interestingly, there have also been successful attempts in reducing alphavirus-induced bone loss by targeting other known arthritis-inducing soluble factors such as IL-6 and CCL-2 (166, 201). Mice infected with RRV and subsequently treated with either IL-6 antibodies or the pre-clinical CCL2 synthesis inhibitor bindarit, developed reduced arthritogenic disease compared to non-treated mice. However, the application of antibodies and pre-clinical compounds against arthritogenic alphavirus disease in humans are limiting factors. Safety studies for clinical approval for human use require years of research and come at considerable cost. Even though arthritogenic alphaviruses are on the rise with CHIKV's worldwide emergence, which resulted in a big push for vaccines by pharmaceutical companies, the availability of effective vaccines will be years away and treatments are needed now. Furthermore, a novel vaccine is most likely important for patients suffering from chronic disease. Thus, our work with repurposing drugs has enormous value in treating the condition. Intriguingly, anakinra has a major advantage as a readily available therapeutic for drug repurposing due to the existence of human safety data from years of applications in RA patients. Furthermore, anakinra has not been found to be associated with the development of opportunistic infections (273), which may be of concern with other immunomodulatory drugs such as corticosteroids or TNF-inhibitors. Therefore, anakinra may provide a readily available treatment option until novel therapeutics are developed specifically against alphavirus-induced arthritis.

6.2. Conclusion

In our surveillance study on sIAV in swine in the Southeastern USA, the prevalence of H1 and H3 subtypes differs from the epidemiological picture observed in studies conducted for other swine-producing areas of the USA, such as the Midwestern part of the country, where about one third of swine IAVs isolated bare an H3 HA (19, 33, 34).

Consequently, the findings of this study are of significant importance, since commercial swine IAV vaccines are either bivalent, containing and H1N1 and an H3N2 strain or trivalent, containing H1N1, H3N2 and H1N2 viruses (35, 36). This is an important finding for monitoring IAV evolution and may be valuable for the development of future vaccines specifically for sows and piglets in the Southeastern USA.

In our HTS study on pro-viral miRNA in the context of the avian IAV (H7N9) infection, we report the discovery of an important pro-viral miRNA that can be targeted by readily available miRNA inhibitor miR664i. The field of miRNA inhibitors is on the rise with many candidates currently being tested in clinical trials. The most clinically advanced example of a miRNA antagonist is the antiviral miravirsen which is currently undergoing multiple clinical phase II studies against HCV (277). Miravirsen targets the conserved host factor miR-122, which stimulates translation of HCV RNA. Thus, miR-122 is a promising target, as it is hoped that the virus would not be able to acquire resistance mutations to miravirsen. The promising antiviral candidate miR664i owns unique characteristics in the novel field of miRNA inhibitors as countermeasures against the potential pandemic and life-threatening infectious disease caused by H7N9. In addition, miR664i also shows great potential in treating pandemic circulating IAV strains such as H1N1 and H3N2. Therefore, our study shows a rapid way of discovering novel targets for IAV replication, while unravelling underlying mechanisms of disease.

In our second study investigating disease mechanisms of H7N9 disease, we investigated the role of CCL2 in mice infected with H7N9. Mice studies suggested that TNF-α and IFN-γ may help to suppress viral gene expression and increase viral clearance, while CCL2 and IL-6 may contribute to lung injury during H7N9 disease (44). Therefore, this study was aimed to investigate the role of CCL2 in the context of IAV (H7N9) disease by using the potent CCL2 synthesis inhibitor bindarit. Oral treatment of H7N9-infected mice with bindarit resulted in enhanced disease including increased virus titres, weight loss, and cellular infiltration in the bronchoalveolar lavage (BAL). These results suggest that CCL2 plays a critical antiviral role against H7N9 replication; thus, therapeutic approach targeting CCL2

with bindarit may be ill-advised for the treatment of IAV (H7N9) infection. This is the first study evaluating the effects of CCL2 inhibitor for the treatment of IAV-induced disease and suggests that this class of drugs may need to be used cautiously for this disease group.

In the fourth chapter of this PhD thesis on novel approaches in the treatment of alphavirus disease, we investigated the role of IL-1 in the induction of bone loss in an animal model of RRV disease. Taken together, anakinra was able to reduce bone loss, without enhancing virus titres or cellular infiltration. These results suggest that IL-1 plays a critical role in the induction of bone loss in arthritogenic alphavirus disease, but may not be critical for antiviral defence. Thus, anakinra may be of great potential to prevent bone loss in patients suffering from alphaviral arthritides. This is of special importance as there are currently no specific therapeutics available for the treatment of arthritogenic alphavirus disease. Drug repurposing may be inevitable in the fight against emerging alphaviral disease, since the relatively small market size and the cost for clinical development hinder the development of novel drugs. Furthermore, even with vaccines being available in the future, there is still a need for therapeutics against the chronic stages of arthritogenic alphavirus disease, as vaccines may not be able to reverse existing symptoms. Thus, anakinra may be a safe treatment option of arthritogenic alphavirus disease, as it did not enhance disease signs or virus replication, but in turn acted as a crucial player in reducing bone loss in our mouse model of RRV-induced disease.

6.3. Future directions

The sIAV surveillance project is in the early stages and adequate techniques for detection and screening of HA subtypes have already been established, which will enable a constant and efficient surveillance of sIAV strains for many years. Further genetic investigation is required in order to clarify the exact classification of the H1 HA found in this study, such as next generation sequencing. This will allow the suggestion of alternative vaccine strategies, adjusted to regional differences in the epidemiology of swine IAVs.

Furthermore, classification with next generation sequencing will further allow us to monitor reassortment events and the introduction of human and avian IAV strains into swine and vice versa.

Our HTS and validation studies revealed an important pro-viral target and the potential of its inhibitor as a potent therapeutic. We anticipate examining the effects of miR664i on IAV replication in BEAS2B cells or differentiated NHBE cells in the future. In addition, we aim to investigate the antiviral potential of miR664i in animal models. In the meantime, other pro-viral miRNAs that have been found in the initial screening, such as miR-1244, can be investigated for their antiviral properties including the underlying mechanism. Furthermore, our screening approach with validation and analysis on the involvement of host cell mechanisms, can be applied to other IAV strains, or even unrelated viruses.

Our animal study on H7N9 aimed to investigate the importance of CCL2 in the context of disease. The outcome of disease with increased virus titres reflects an unfavourable role of bindarit in the treatment of influenza A (H7N9), which suggests that this class of drugs may need to be used cautiously for this disease group. A different route of application, e.g. intranasal delivery of the drug may be considered in future experiments and could potentially alter the outcome of the study. However, CCL2 has been shown to be an important factor in antiviral defence in earlier studies in the context of IAV, thus CCL2 may not be an ideal target for disease intervention. Other predicted factors in disease pathology and tissue destruction, such as IL-6, may be a better target to reduce the disease impact of H7N9 (44). This however requires further testing and exceeds the frame of this PhD thesis.

In our study on the treatment of alphavirus-induced bone loss, anakinra has shown to be a valuable tool to reduce disease in a mouse model of disease. To further strengthen our data and the impact of our finding, we are currently testing anakinra in a mouse model of CHIKV disease. Research on CHIKV disease is of international interest during the currently ongoing emergence of disease. Since anakinra has been shown beneficial in RRV-induced bone loss, it is likely to prevent bone loss in this related alphavirus with similar disease sign.

In the future, humans infected with arthritogenic alphavirus disease, especially patients suffering from chronic disease, could be treated in a clinical setting or as off-label treatment during an epidemic. However, if the evaluation of anakinra in our CHIKV model renders the drug Kineret beneficial in the fight against alphavirus-induced bone loss, we will consider approaching the manufacturer, Swedish Orphan Biovitrum, in order to request re-patenting for the indication “alphavirus-induced bone-loss”. Kineret has been used for decades in patients suffering from arthritis, thus safety studies are available. If Kineret could be proven beneficial in clinical efficacy studies, it could become the first potential drug specifically indicated for the treatment of alphavirus disease.

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