TOWARD THE RATIONAL DESIGN OF BETTER ANTIVIRALS: THE DEVELOPMENT OF cGAMP AS AN HIV-1 ANTI-RETROVIRAL AND THE GENETIC SURVEILLANCE OF WNV EVOLUTION

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DEDICATION

To my parents, for all their love and sacrifice

TOWARD THE RATIONAL DESIGN OF BETTER ANTIVIRALS: THE DEVELOPMENT OF cGAMP AS AN HIV-1 ANTI-RETROVIRAL AND THE GENETIC SURVEILLANCE OF WNV EVOLUTION

by

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TOWARD THE RATIONAL DESIGN OF BETTER ANTIVIRALS: THE DEVELOPMENT OF cGAMP AS AN HIV-1 ANTI-RETROVIRAL AND THE GENETIC SURVEILLANCE OF WNV EVOLUTION

Publication No. _____

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The University of Texas Southwestern Medical Center at Dallas, Graduation Year

Supervising Professors: Edward Wakeland and Nan Yan, PhD's

The innate immune response is the first line of defense against pathogens and thus represents the first hurdle viruses must overcome to cause severe disease in humans. Understanding the consequences of viral evolution can give insights to mechanisms of viral pathogenesis as well as the development of novel therapeutics. Here I studied two clinically important viruses: Human Immunodeficiency Virus (HIV) and West Nile Virus (WNV).

HIV-1 has evolved several mechanisms to evade immune detection by the cGAS-STING cytosolic DNA sensing pathway. A small cyclic di-nucleotide, cGAMP, activates the same pathway by directly binding STING. Treatment with cGAMP, delivered by ultra-pH sensitive nanoparticles or by liposomes, in human peripheral blood mononuclear cells (PBMCs) induced potent and long-acting protection against replication of several laboratory-

adapted and clinical HIV-1 isolates in contrast to the short-lasting effect of current anti-retroviral therapy (ARTs). These results present the first evidence for potentially developing cGAMP or other STING agonists as a long-acting antiretroviral immunotherapy.

West Nile Virus (WNV) is a mosquito-borne *Flavivirus* which was introduced to North America in 1999 and is currently the leading cause of viral encephalitis. The lack of specific therapeutics or human vaccines makes WNV an ongoing public health threat. Now endemic, WNV is steadily evolving, but the contribution of positively-selected mutations to human disease remains unclear. In 2012 the second largest outbreak of human West Nile disease occurred in the U.S., with one-third of the cases happening in Texas. The outbreak was associated with groups of WNV carrying positively-selected mutations. By sequencing WNV in Texas from 2012-2015, we show that positively-selected mutations in WNV mediate increased circulation and over-wintering in the environment, which may promote increases of human disease. Additionally, we show evidence that the WNV population is still evolving new alleles. These results advance our understanding of the impact of WNV evolution to human disease, and may afford insights to the evolution of other invading flaviviruses, such as Dengue and Zika virus.

Altogether, these results show that understanding the consequences of viral evolution can be harnessed towards overcoming challenges to the development of more effective therapeutics.

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PRIOR PUBLICATIONS

1. **Aroh, Chukwuemika**, Zhaohui Wang, Nicole Dobbs, Min Luo, Oladapo Abodunde, Zhijian Chen, Jinming Gao, and Nan Yan. “Innate immune activation by cGAMP-PC7A nanoparticles leads to potent and long-acting inhibition of HIV-1 replication.” (Manuscript submitted)

2. **Aroh, Chukwuemika**, Mary D’Anton, Chaoying Liang, Nan Yan, Edward Wakeland. “Positively-selected mutations of 2012 WNV promote circulation and over-wintering.” (Manuscript in prep)

3. Gaviria-Agudelo, Claudia, **Chukwuemika Aroh**, Naureen Tareen, Edward K. Wakeland, MinSoo Kim, and Lawson A. Copley. "Genomic Heterogeneity of Methicillin Resistant Staphylococcus Aureus Associated with Variation in Severity of Illness among Children with Acute Hematogenous Osteomyelitis." *PloS one* 10, no. 6 (2015): e0130415.

4. Gao, Daxing, Jiaxi Wu, You-Tong Wu, Fenghe Du, **Chukwuemika Aroh**, Nan Yan, Lijun Sun, and Zhijian J. Chen. "Cyclic GMP-AMP synthase is an innate immune sensor of HIV and other retroviruses." *Science* 341, no. 6148 (2013): 903-906.

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LIST OF DEFINITIONS

AIM2: Absent in Melanoma 2
APOBEC3G: Apolipoprotein B mRNA Editing Enzyme Catalytic Subunit 3G
ATP: Adenosine triphosphate
CPSF6: Cleavage and polyadenylation specificity factor subunit 6
CRISPR: Clustered regularly interspaced palindromic repeats
CXCL10: C-X-C motif chemokine 10 which is an interferon inducible protein
DNA: Deoxyribonucleic acid
DsRNA: double stranded RNA
EMCV: El Moro Canyon virus
GCSF: Granulocyte colony-stimulating factor, a growth factor
GTP: Guanosine triphosphate
HIV: Human Immunodeficiency Virus
HSV: Herpes Simplex Virus
HSV: Herpes Simplex virus
IFIT: Interferon-induced protein with tetratricopeptide repeats
IFITM: Interferon induced transmembrane protein
IFN: Interferon
IKK: IκB kinase
IL18: Interleukin 18, a cytokine
IRF: Interferon regulatory factor
ISG: Interferon stimulated gene
JEV: Japanese Encephalitis Virus
KSHV: Kaposi's sarcoma associated herpesvirus (KSHV)
LGP2: Laboratory of Genetics and Physiology 2
MIP-1α: Macrophage inflammatory protein 1-alpha, an interferon induced protein
MxA: also, known as Mx1 or MX dynamin like GTPase 1
Mydd88: Myeloid differentiation primary response gene 88
NFKβ: Nuclear factor kappa light chain enhancer of activated B cells
RISC: RNA-induced silencing complex
RNA: Ribonucleic acid
RSV: Respiratory syncytial virus
TBK1: Tank binding kinase 1
TNF: Tumor necrosis factor
TREX1: three prime exonuclease 1
TRIF: TIR-domain-containing adaptor-inducing interferon-β
VSV: Vesicular stomatitis virus
WNV: West Nile Virus

CHAPTER ONE: INTRODUCTION

Pattern recognition receptors regulate host immune response to infection

In vertebrates, the innate and adaptive immune systems have evolved to protect the host against microbial infections. As the first line of defense, the innate immune system utilizes limited germline-encoded receptors called pattern recognition receptors (PRRs) to recognize invading pathogens and initiate rapid host responses. The adaptive immune system, which takes longer to respond, complements the innate immune system with diverse somatically-rearranged receptors, and generates long-lived memory responses to infection. Though adaptive immune cells are composed of varied effector responses (plasma cells, T-helper cells, and cytotoxic cells), their activation requires PRR-dependent induction of co-stimulatory molecules and the secretion of cytokines and chemokines in the innate immune system (Pasare *Microbes and Infection* 2004).

PRRs bind conserved molecular structures known as pathogen-associated molecular patterns (PAMPs) and trigger antiviral interferon and proinflammatory responses (Yan *Nat. Immunol.* 2012). Activation of PRRs by foreign molecules— viral nucleic acids, components of bacterial and fungal cell walls, flagellar proteins, and others — promotes host survival. However, inadvertent activation of PRRs by host molecules or gain-of-function mutations can lead to autoimmune diseases such as systemic lupus erythematosus (Meylan *Nature* 2006, Marshak-Rothstein *Nat. Rev. Immunol.* 2006). Thus, depending on the disease context, therapeutic benefit can be gained by activating or suppressing PRR pathways.

Previously identified nucleic-acid sensing PRRs

Most microbial infections introduce nucleic acids into the host cell, and detection of these nucleic acids by PRRs is central to the induction of protective host immune responses (Burdette *Nat. Immunol.* 2013). Innate nucleic acid sensors are categorized based on their subcellular localization: either in the endosome or in the cytosol (**Figure 1**). Endosomal nucleic acid sensors include several TLR family proteins that are expressed mostly in immune cells, such as dendritic cells (DCs), macrophages, and B cells (Wu *Annu. Rev. Immunol* 2014). Cytosolic nucleic acid sensors include RIG-I-like receptors (RLRs) that detect RNA and cGAS and others that detect DNA. These cytosolic sensors are ubiquitously expressed, and have the advantage of detecting microbial DNA and RNA in most cell types (Wu*Annu.Rev.Immunol.*2014)(**Figure2**).

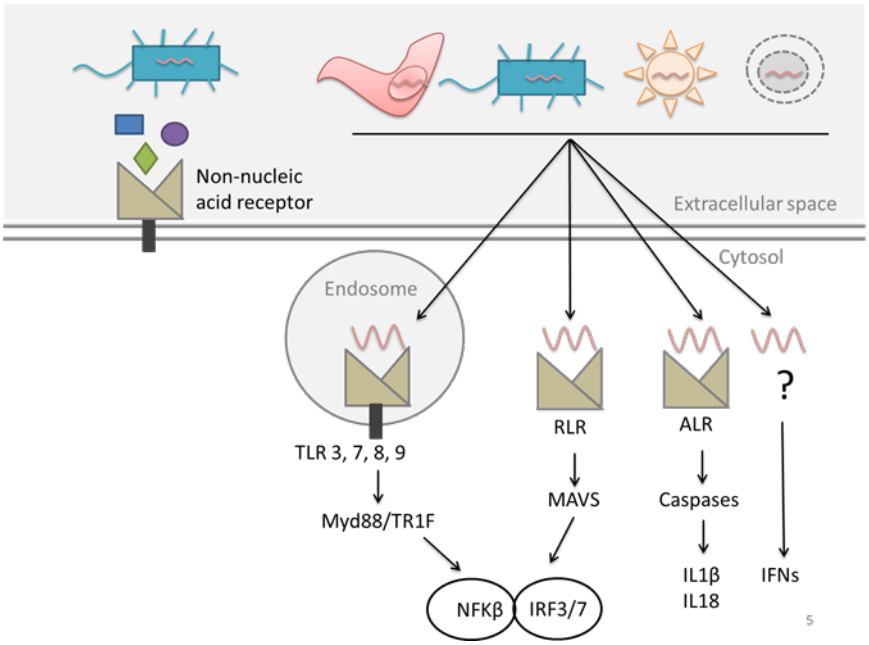

Figure 1: Shows nucleic acid sensing pathways both endosomal and cytosolic

Endosomal TLRs are type 1 transmembrane proteins with ectodomains for nucleic acid sensing, and a Toll/IL-1 receptor (TIR) domain protruding into the cytosol for downstream signaling. Four of the ten human TLRs are involved in nucleic acid sensing, including TLR3 that binds dsRNA, TLR7 and TLR8 that detect ssRNA, and TLR9 that detects unmethylated CpG DNA. These TLRs survey the lumen of endosomes for nucleic acids, once engaged, they activate a signal through the TIR domain that recruits distinct adaptor molecules. TLR7, TLR8, and TLR9 activation recruit the adaptor protein MyD88 whereas TLR3 recruits TRIF. MyD88 or TRIF subsequently activates the translocation of NF-κB and IRFs (IRF3 by MyD88 and IRF7 by TRIF) into the nucleus to induce type 1 interferons and proinflammatory cytokines. Endosomal TLRs are critical for host defense against bacteria, RNA viruses (such as VSV and WNV), and DNA viruses (such HSV) that may become internalized and recruited to endolysosomes (Kawai *Nat. Immunol.* 2010).

For the detection of intracellular pathogens that successfully invade the cytosol, especially in non-immune cells that the line mucosal surface and are thus most susceptible to microbial assaults, a distinct set of cytosolic nucleic acid sensors are required. RLRs are important for sensing RNA in the cytosol. RLRs family members include retinoic acid inducible gene-I (RIG-I), melanoma differentiation associated gene 5 (MDA5), and LGP2. All three proteins share RNA helicase domains with DExD/H motifs (Yan *Nat. Immunol.* 2012). In addition to the helicase domain, RIG-I contains a carboxyl terminal domain to bind viral RNA and two caspase-recruitment domains (CARDs) for signaling through mitochondrial antiviral signaling (MAVS) protein to activate IKK and IKK-like kinase TBK1. TBK1 phosphorylates IRF3 which dimerizes and translocates into the nucleus where,

in conjunction with NF-κB, it activates production of type I interferons and other antiviral proteins. MDA5, a sensor for long dsRNA, also signals through MAVS. LGP2 and is thought to negatively regulate RIG-I or MDA5 signaling, although the exact function is unclear. Cytosolic immune surveillance by RIG-I and MDA5 signaling is crucial for host defense against RNA viruses such as influenza, Sendai virus, hepatitis C virus and other flaviviruses (Wu *Annu. Rev. Immunol.* 2014).

The first described cytosolic DNA sensor is AIM2. AIM2 is a member of the PYHIN protein family with an N-terminal pyrin domain and an C-terminal HIN200 domain. AIM2 binds DNA with its pyrin domain, and signals through the HIN200 domain to the adaptor protein apoptosis-associated speck-like protein containing a CARD (ASC). ASC recruits and activates Caspase 1 to process pro IL-1β and pro IL-18 that produces the mature IL-1β and IL-18. AIM2-mediated inflammasome response to cytosolic DNA is crucial for host protection against bacterial pathogens such as *Francisella tularensis* and DNA viruses such as vaccinia virus (Rathinam *Nat. Immunol.* 2010)

The cGAS-STING cytosolic DNA sensing pathway

Cyclic GMP-AMP (cGAMP) synthase (cGAS) is a cytosolic DNA sensor with a nucleotidyltransferase domain and two major DNA binding domains (Chen *Nat. Immunol.* 2016). In the presence of DNA, cGAS binds the phosphate backbone, rather than any of the nucleotide bases of DNA, which explains its sequence independent recognition. After binding DNA, cGAS undergoes a conformational change out of its auto-inhibited state to catalyze the synthesis of 2'-3' cGAMP from GTP and ATP. cGAS can also activated by

guanine rich-single stranded DNA that form a Y-shaped structure but the structural mechanism for this activation is unknown (Herzner *Nat. Immunol.* 2015). DsRNA binds cGAS but does not mediate its activation perhaps due to its inability to open the active site of cGAS (Chen *Nat. Immunol.* 2016).

2'-3' cGAMP is a cyclic dinucleotide with unique 2'-5' and 3'-5' phosphodiester bonds. cGAMP functions as a second messenger that binds and activates the adaptor protein stimulator of interferon genes (STING), a transmembrane protein that resides on the endoplasmic reticulum (ER) (**Figure 2**). Bacterial cyclic dinucleotides (such as cyclic di-GMP, cyclic di-AMP, and 3'-3' cGAMP) are important regulators of many bacterial

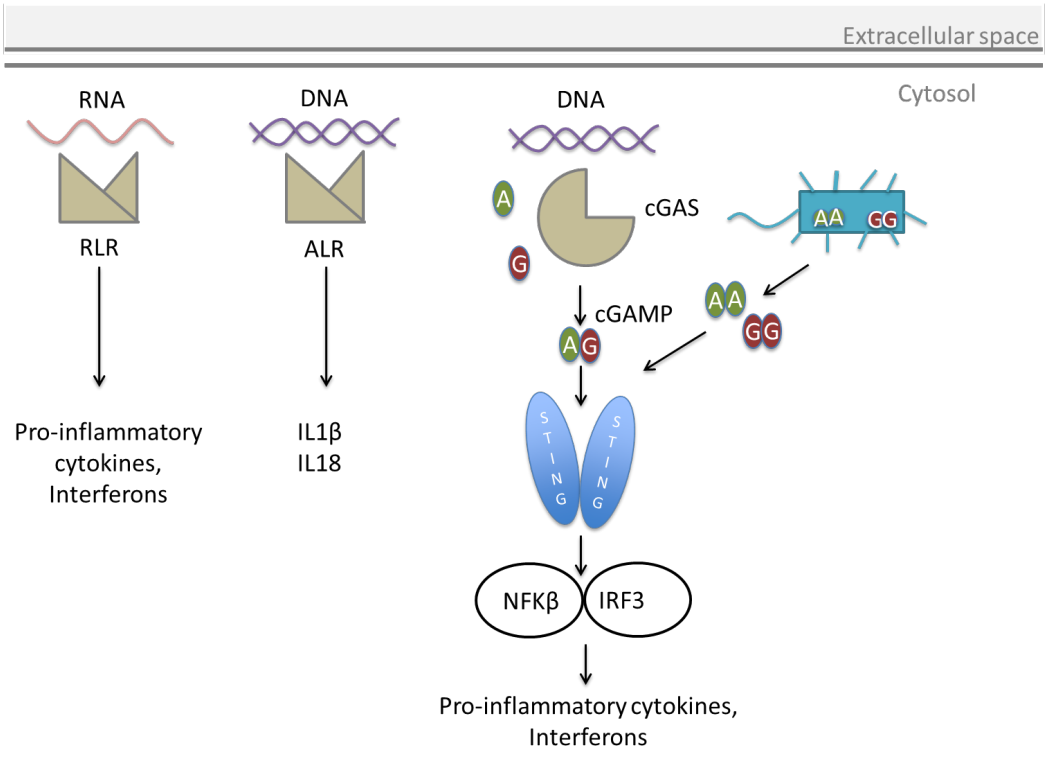

Figure 2: Shows cytosolic sensors and the newly discovered STING pathway

processes such as motility, biofilm formation and virulence (Tamayo *Annu. Rev. Microbiol.* 2007). Bacterial cyclic dinucleotides also bind and activate STING but at a lower affinity (4.9 μM for c-di-GMP versus 4nM for cGAMP) (Burdette *Nature* 2011). Recently it was shown that an oxidoreductase, Recon, is the predominant receptor for bacterial cyclic di-AMP and 3'-3' cGAMP to promote a proinflammatory and antibacterial state (McFarland *Immunity* 2017). Whether a similar receptor exists for cyclic di-GMP is unknown.

cGAMP binding triggers STING translocation from the ER to cytoplasmic vesicles where it recruits and activates TBK1 and IKK, that in turn activate IRF3 and NF-κB to initiate the transcription of proinflammatory cytokines and type I interferons such as IFNβ. The cGAS-STING pathway has been shown to play critical roles in the innate immune response to bacterial pathogens, DNA viruses, retroviruses, and host DNA (Chen *Nat. Immunol.* 2016). Activation of the cGAS-STING pathway by host DNA has been implicated in autoimmune and autoinflammatory diseases such as Aicardi–Goutières syndrome and SAVI (STING associated vasculopathy with onset in infancy) as well as tumor immunity ((Hasan *Nat. Immunol.* 2013, Pokatayev *J. Exp. Med.* 2016, Crow *Nat. Rev. Immunol.* 2015, Liu *NEJM* 2014, Deng *Immunity* 2014, Demaria *PNAS* 2015, Wang *PNAS* 2017).

Type I interferons are central to host defense against viral infection

The induction of type I interferons is a hallmark of the innate immune response to viral infection. Interferons mediate antiviral, anti-proliferative, and immunomodulatory effects in innate and adaptive immune cells (Platanias *Nat. Rev. Immunol.* 2005). Type 1 interferon contains thirteen subtypes of IFNα, IFNβ, IFNε, IFNκ, and IFNω. Due to

considerable structural homology, all type I interferons bind to a heterodimeric IFNα/β receptor (composed of IFNAR1 and IFNAR2) on the cell surface. IFNAR1 is associated with tyrosine kinase 2 (TYK2) and IFNAR2 is associated with Janus activated kinase (JAK1) to mediate signaling.

After PRR induced signaling, type I interferons are secreted to the outside the cell where they bind to IFNAR in an autocrine or paracrine manner (**Figure 3**). Ligand binding of the ectodomain of IFNAR signals to the intracellular kinases leading to the activation of associated JAK proteins, which phosphorylates signal transducer and activator of transcription one and two (STAT1 and STAT2). STAT1 and STAT2 heterodimerize and form the IFN-stimulated gene factor 3 (ISGF3) complex that binds IFN-stimulated response elements (ISREs) in the nucleus to induce the transcription of hundreds of interferon stimulated genes (ISGs). ISGs are antiviral factors that directly inhibit viral replication (Platanias *Nat. Rev. Immunol.* 2005). For instance, IFITM family proteins inhibit the cytosolic entry of flaviviruses, IFIT family proteins inhibit the translation of viruses lacking 2'-O-methylation, MxA blocks viral transcription, APOBEC3G inhibits reverse transcription of retroviruses, and Tetherin blocks release of enveloped viruses (Yan *Nat. Immunol.* 2012). The induction of these antiviral factors collectively render activated cells resistant to viral infection and replication.

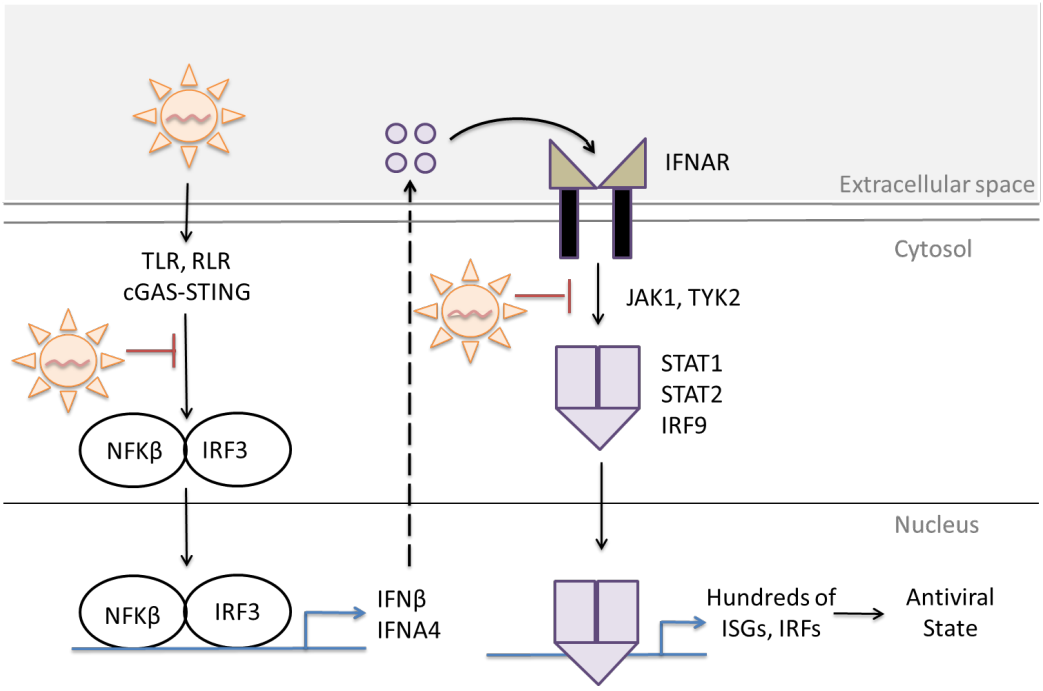

Figure 3: Shows the signaling cascade of Type 1 Interferons, the importance of which is indicated by the common inhibition of this pathway by pathogenic viruses

Due to the potency of the type I interferon response, viruses have evolved multiple ways to evade it. Some viruses antagonize PRRs directly such as KSHV inhibition of cGAS (Wu *Cell Host Microb.* 2015). Others such as flaviviruses antagonize the IFNAR receptor (Lubick *Cell Host Microb.* 2015). In addition, retroviruses such as HIV-1 evade detection of viral DNA from cytoplasmic sensors by co-opting host factors, such as the cytosolic DNase TREX1 (Yan *Nat. Immunol.* 2010). All these evasion strategies highlight the importance of the interferon pathway and its therapeutic potential in the treatment of viral infection.

The challenge of viral evolution to drug development

Hypermutation is a common strategy for viruses to evade host restriction. For instance, HIV-1 is estimated to evolve one million times faster than the mammalian DNA (Li *Molec. Biol. Evol.* 1988, Lemey *AIDS Rev.* 2006). Some of the mutations are neutral or harmful, but other mutations confer advantageous properties to the virus.

In this dissertation, I study two important human pathogens, HIV-1 and WNV, both of which are transmitted to humans from animal reservoirs (Rambaut *Nature* 2004, Pesko and Ebel *Infect. Genet. Evol.*2012). Derived from simian immunodeficiency virus in Chimpanzees (Gao *Nature* 1999), HIV-1 overcame the species barrier in part through specific mutations such as those that enable HIV-1 to antagonize antiviral factors like Tetherin (Sauter *Cell Host Microb.* 2009) and evasion of the innate immune response (Hasan *Frontiers Microbiol.* 2014). Current antiretroviral therapy can successfully control HIV infections, but these drug treatments induce the emergence of drug resistance mutations (Clavel and Hance *NEJM* 2004). WNV is the most geographically distributed arbovirus (Kramer *Annu. Rev. Entmol.* 2008). Introduced in 1999, WNV is now the leading cause of viral encephalitis in North America (Davis *Annals of Neurol.* 2006). Though adaptive mutations have been identified in circulating WNV strains from recent years, their role in mediating outbreaks of human disease is unknown. Currently there are no specific therapeutics or vaccine for WNV.

My central hypothesis in this dissertation is that by understanding the consequences of viral evolution on host pathogen interactions, we hope to gain critical molecular insights that can be harnessed toward more effective antiviral therapeutics against these and other important viral pathogens.

CHAPTER TWO: MATERIALS AND METHODS

FOR CHAPTER THREE: DEVELOPMENT OF CGAMP AS AN HIV-1 ANTI-RETROVIRAL

Cells and viruses

PBMC from anonymous healthy donors were isolated from blood purchased from Carter Bloodcare, a local blood bank, usually on the same day of blood draw. Peripheral immune cells were isolated using Ficoll Plaque Centrifugation at 1700rpm for 30mins and lysis of contaminating red blood cells were removed by resuspension of PBMC in ACK lysis buffer (Gibco) for 15mins on ice. PBMCs were maintained without stimulation in RPMI 1640 medium supplemented with 10% (vol/vol) FBS and 2 mM L-glutamine (Sigma-Aldrich), 10 mM HEPES (Sigma-Aldrich), 1 mM sodium pyruvate (Sigma-Aldrich), 100 U/ml penicillin (Sigma-Aldrich), and 100 mg/ml streptomycin (Sigma-Aldrich) at 37°C and 5% CO_2. Consenting HIV+ patients were recruited per IRB approved protocols and their PBMC were similarly isolated and cultured as described above.

Cell viability was determined using Fixable Red Dead Cell stain kit (Life technologies) using 0.5ul dye to stain cells in 1ml PBS for 5mins in the dark. These were then washed twice with PBS and viability was analyzed by flow cytometry on a FACS Calibur machine. As a positive control for dead cells, PBMC were boiled at 95º C for 20mins and stained with the same procedure to determine the peak of dead cells.

CD4+ T cells, B cells, and Monocytes were positively selected using Miltenyi Biotec human microbeads against CD4+ (130-045-101), CD19+ (130-050-301), CD14+ (130-050-

201) per manufacturer's recommendations. The labeled cells typically had purity over 90%. For depletion experiments the same isolation protocol was used but now the flow through PBMC were taken, we regularly achieved over 95% depletion for Monocyte and B-cells.

HIV strains (BaL, IIIB) were propagated on U937 cell lines, concentrated using Amicon Ultra-15 centrifugal filter units (Millipore) and tittered by PERT assay described below. HIV Lai strains were obtained from NIH AIDS reagent program (Cat. #2522), amplified in PBMC, and concentrated using LentiX concentrator (Clontech) per manufacturer's recommendation.

HIV-1 titer PERT assay

As was previously described (Vermeire *PLOS ONE* 2012, Pizzato *J Virol Methods* 2009), this assay uses reverse transcriptase activity in the sample to power an RTqPCR reaction, with the quantity of accumulated product serving as a proxy for viral concentration. Viral particles in the supernatant were lyzed by mixing 1:1 with lysis buffer for 10mins at room temperature. The reaction was quenched by adding nuclease free water (nine times lysate volume) to lysate. This diluted lysate (6ul) was added to a MicroAmp fast 96-well reaction plate (Applied Biosystems) along with 1:10 diluted Roche MS2 RNA (1ul), 10uM Sigma MS2 Fwd (1ul) & Rev primers (1ul), 1:10 diluted Invitrogen RNaseout (1ul), and Biorad Sybr green (10ul) for each sample. All dilutions were done in nuclease free water (Ambion). The sequences for the MS2 primers are included in the **Table 1**. These samples are then vortexed and spun down, prior to running on a AB 7500 Fast Real Time PCR System using the following program: (1) 42^0 20min. (2) 95^o 5min. (3) 95^o 3sec (4) 60^o 30sec

and plate read. (5) repeat from step 3, 39 times. To normalize measurements across different plates aliquots from one HIV-1 stock were included in all plates and its measurement was used to divide the values in the experimental conditions.

Syntheses of PEG-*b*-PR block copolymers

PEG-*b*-PR copolymers were synthesized by atom transfer radical polymerization (ATRP) following similar procedures previously reported (14). The dye free copolymers were used in polymer characterizations. PEG-*b*-PC7A is used as an example to illustrate the procedure. First, C7A-MA (1.48 g, 7 mmol), PMDETA (21 μL, 0.1 mmol), and MeO-PEG_{114}-Br (0.5 g, 0.1 mmol) were charged into a polymerization tube. Then a mixture of 2-propanol (2 mL) and DMF (2 mL) was added to dissolve the monomer and initiator. After three cycles of freeze-pump-thaw to remove the oxygen, CuBr (14 mg, 0.1 mmol) was added into the polymerization tube under nitrogen atmosphere, and the tube was sealed *in vacuo*. The polymerization was carried out at 40 °C for 10 hours. After polymerization, the reaction mixture was diluted with 10 mL THF, and passed through a neutral Al_2O_3 column to remove the catalyst. The THF solvent was removed by rotavapor. The residue was dialyzed in distilled water and lyophilized to obtain a white powder. After synthesis, the polymers were characterized by ^{1}H NMR and gel permeation chromatography (GPC).

Preparation of micelle nanoparticles

cGAMP loaded micelles were prepared following a solvent evaporation method. In the example of PEG-b-PC7A, both the copolymer (10 mg) and cGAMP (1 mg) were firstly

dissolved in 1 mL methanol and then added into 4 mL distilled water dropwise under sonication. The mixture was filtered 4 times to remove THF using the micro-ultrafiltration system (MW = 100 KD). The filtrate was collected and cGAMP was quantified by UV-Vis spectrometer at 258nm. After micelle formation, the nanoparticles were characterized by dynamic light scattering (DLS, Malvern MicroV model, He-Ne laser, λ= 632 nm) for hydrodynamic diameter (Dh). The control unloaded nanoparticles were prepared with same protocol as above, except the addition of cGAMP into the methanol.

To evaluate the loading stability, the micelle nanoparticles were stored in 4^0C for 1 week. The size and cGAMP encapsulation efficiency were measured by DLS and ultrafiltration method, respectively.

Viability Assay

Cell viability were determined using Fixable Red Dead Cell stain kit (Life technologies). Cell viability was measured by adding 0.5ul of dye to stain washed cells in 1ml PBS for 5mins in the dark. Stained cells were washed twice with 1ml PBS and ran immediately on FACS Calibur. Alternatively, stained and washed samples were fixed using 4% PFA for 10mins in room temperature. Fixed cells were then washed and re-suspended with 200ul PBS prior to refrigeration. Stored samples should be run within one to two weeks for best results.

As a positive control for dead cells, PBMC was boiled at 95°C or higher for 20mins and simultaneously stained with the same procedure as the remaining samples to determine

the peak of dead cells. Unstained PBMC was used as a control for live cells to ensure whole peak of live cells are contained within the graph.

RT-qPCR, milliplex and western blots

Cytokine mRNA was extracted from cells using TR1 Reagent (Sigma-Aldrich) and cDNA was generated using iSCRIPT master mix (Biorad). QPCR was run using specific primers (**Table 1**) and normalized relative to *Gapdh* mRNA. HIV patient cytokine mRNA profiles were determined using Prime PCR custom plates (Biorad) using their recommended primers against our genes of interest. Proteins of select cytokines in the culture supernatant were collected and measured using Luminex xMAP technology and a human Milliplex kit (Millipore).

For western blots, primary antibodies used were Rabbit anti-TBK (Cell signaling), Rabbit anti-IRF3 (Cell signaling), Rabbit anti-STING (Cell signaling), Rabbit anti-Phospho TBK (Ser172) Clone D52c2 (Cell signaling), Rabbit anti-HMGB2 (Abcam), and Mouse anti-Tubulin (Sigma-Aldrich) according to blotting procedures previously described (Dobbs *Cell Host Microbe* 2015).

Statistical analysis

Statistical significance was determined using a two-tailed student T-test analysis available on Graphpad Prism 6. P-values lower than 0.05 were considered significant.

FOR CHAPTER FOUR: GENETIC SURVEILLANCE OF WNV EVOLUTION

Sample collection, RNA extraction and next generation sequencing:

WNV RNA or mosquito pool lysates were obtained through a collaboration with the Texas State Health Department. RNA was extracted from lysates using Qiagen Qia Amp kit, followed by overlapping PCR, gel extraction of PCR amplicon, then sonication into short 2-300bp fragments to be used for RNAseq library prep through genomics core. Samples were sequenced using Illumina Hiseq sequencer using 50bp single-end reads.

Phylogenic Trees and Bioinformatic analyses

Reads were imported into CLCBio genomics workbench, which has a suite of tools for bioinformatic analyses. The general analysis pipeline is as follows: reads were mapped unto NCBI recommended reference genome (NC_009942), consensus reads were extracted, then these were mapped again to reference genome. This second mapping was in turn used for calling mutations using the quality variant detection tool. Consensus reads were also used to generate alignments, along with reference sequences either downloaded directly from NCBI from the workbench or imported via a fasta text file. The alignments were used for phylogenetic analyses using Neighbor Joining and 10,000 bootstrap methods.

WNV propagation and titration:

To propagate, concentrate and titrate viruses; slight modification of previously published protocols (D'Brien *Curr Prot Microbiol* 2013) was used. Typically, Vero cells were used for propagation and titration. For propagation, WNV was use to infect one or two

confluent T75 flasks of Vero cells at moi of 0.1 if the titer of virus was known. When unknown, 1-3ul of virus were used to infect Vero cells. Both procedures yielded high concentration stock after 40hpi and there was no need for further concentration. Viruses were frozen down in aliquots and tittered after one thaw cycle using plaque assay. For plague assay, I used the following alterations to the previously described methods: Vero cells were plated at $1.5x10^6$ cells per well in a six well plate, used three rather than a one hour viral adsorption as this increases sensitivity by lowering threshold of assay, after absorption I incubated the virus for three days for plaques to grow to size enabling proper counting. All work with replication competent WNV was done in a BSL3 facility.

Viral infection:

Our bird cell-line QT6 was purchased from ATCC and grown in the recommended media. These were infected at moi 0.1 in triplicates per condition over several days. To measure viral replication, 100ul aliquots of the supernatants were obtained and frozen at -80 till the rest of the experiment were finished prior to running plaque assays. At each timepoint, the volume of media removed was replaced with 100ul of fresh media.

PBMC from healthy donors were isolated from obtained from local blood bank (Carter Bloodcare) using ficoll plague centrifugation. Monocytes were isolated from PBMC and used to differentiate macrophage cells and dendritic cells using MCSF or GMCSF/IL4 respectively. Myeloid cells were infected at moi of 1.0 and RNA was collected by adding Trizol directly to cells.

Data Analysis

RNA from infected cells were isolated from Trizol and cDNA generated using iSCRIPT (Biorad). The cDNA was then used for qPCR detection of IFNB, CXCL10, and WNV RNA on Biorad's CFX connect thermal cycler (**Table 1**). Graphs were generated using Graphpad Prism. Student T-test analysis were also performed using Graphpad Prism.

Table 1: Primer sequence list

Gene		Sequence		
GAPDH	Fwd	GCA AAT TCC ATG GCA CCG T		
	Rev	TCG CCC CAC TTG ATT TTG G		
IFNB	Fwd	CAA GTG TCT CCT CCA AAT TGC TCT C		
	Rev	TCT CCT CAG GGA TGT CAA AGT TCA T		
IFIT1	Fwd	CTG CCT ATC GCC TGG ATG GCT TTA A		
	Rev	CTG TGA GGA CAT GTT GGC TAG AGC T		
CXCL10	Fwd	GCA TTA GTA ATC AAC CTG TTA ATC C		
	Rev	TCC TTG CTA ACT GCT TTC AGT AAA T		
IL6	Fwd	AGG AGA AGA TTC CAA AGA TGT AGC		
	Rev	CTC TTG TTA CAT GTC TCC TTT CTC		
IL8	Fwd	TCT TGG CAG CCT TCC TGA TTT CTG C		
	Rev	ATA ATT TCT GTG TTG GCG CAG TGT G		
TNFa	Fwd	CCA GGG CTC CAG GCG GTG CTT GTT C		
	Rev	AAC ATG GGC TAC AGG CTT GTC ACT C		
WNV	Fwd	GGA CCT TGT AAA GTT CCT ATC TCG		
	Rev	AGG GTT GAC AGT GAC CAA TC		
MS2	Fwd	TCC TGC TCA ACT TCC TGT CGA G		
	Rev	CAC AGG TCA AAC CTC CTA GGA ATG		

CHAPTER THREE: DEVELOPMENT OF cGAMP AS AN HIV-1 ANTIRETROVIRAL

Innate immune activation by cGAMP-PC7A nanoparticles leads to potent and long-acting inhibition of HIV-1 replication

BACKGROUND

Human cells detect invading pathogens with pattern recognition receptors (PRRs) that activate the production of type I interferons (IFN-Is). Type I IFN initiates immediate cell-intrinsic antiviral response to contain the pathogen as well as induces adaptive immune response to achieve long-term protection (Yan *Nat. Immunol.* 2012). To counteract this immune surveillance, many viruses have evolved strategies to evade innate immune detection. We showed previously that HIV-1 exploits host DNase TREX1 to prevent cytosolic DNA sensing, and *Trex1*-defeciency allows accumulation of HIV-1 reverse transcribed DNA to be detected by the cGAS-STING pathway (Yan *Nat. Immunol.* 2010, Gao *Science* 2013). TREX1 knockdown in vaginal tissue explant and in humanized mice also result in elevated IFN-I response to HIV-1 infection and reduced viral replication (Wheeler *Cell Rep.* 2016). Other studies in recent years have also uncovered a multi-faceted mechanism of HIV-1 immune evasion, by exploiting additional host proteins such as SAMHD1 and CPSF6, in a variety of immune cell types (Hasan *Front. Microbiol.* 2014). Overcoming HIV immune evasion allows robust sensing of HIV-1 in CD4 T cells, macrophages and mDCs, maturation of infected mDCs, production of IFN, and activation of T cells *in vitro* (Hasan *Front. Microbiol.* 2014, Li *Science* 2013). A striking common scheme of many of these HIV-1 evasion mechanisms is to prevent activation of the cGAS-

STING pathway, indicating the importance of this pathway in immune detection of HIV-1 and potential therapeutic opportunities for exploiting this pathway.

Upon DNA binding in the cytosol, cGAS is activated and produces cyclic dinucleotide cGAMP. cGAMP then binds to the adaptor protein STING on the ER, which recruits kinase TBK1 and transcription factor IRF3 leading to activation of type I IFN and inflammatory responses. cGAMP has unique properties of being a small molecule (dinucleotide) and a potent activator of STING signaling, and it has been demonstrated as a potent adjuvant to elicit antibody and T cell responses in mice (Li *Science* 2013)). In clinical settings (e.g. cancer), PRR agonists are becoming preferred therapy compared with IFN, thanks to their superior pharmacokinetics, ability to induce other immunoregulatory cytokines in addition to IFNs and better toxicity profiles (Parker *Nat. Rev. Cancer* 2016).

Although some cells can take up free cGAMP and cGAMP analogues in vivo (Li *Science* 2013, Wang *PNAS* 2016, Corrales *Cell. Rep.* 2015), delivery of naked cGAMP is less efficient compared to liposomes, likely due to the inherent dual negative charges of cGAMP and the presence of an extracellular enzyme that cleaves cGAMP (Li *Nat. Chem. Biol.* 2014). Multiple administration of cGAMP are needed to achieve anti-tumor effect in animal models. cGAMP also has never been evaluated as an anti-retroviral therapy. Recently, we have developed a series of ultra-pH sensitive nanoparticles (UPS-NP) with tunable, exquisitely sharp pH response arising from catastrophic phase transitions during pH triggered self-assembly of amphiphilic block copolymers (Li *Nat. Comm.* 2016). These nanoparticles can serve as carriers for therapeutic cargos and achieve cytosolic delivery of protein antigens for the activation of tumor-specific T cells (Luo et al Nature Nanotechnology, in press).

Here, we evaluated the use of multiple UPS-NP compositions to enhance cytosolic delivery of cGAMP into cells, and discovered that cGAMP-PC7A NP elicits potent and long-acting protection against multiple HIV-1 isolates in human peripheral blood mononuclear cells (PBMCs). We also elucidated the mechanism of cGAMP-PC7A NP mediated immune protection and demonstrated its efficacy in PBMCs isolated from HIV-1 positive individuals.

RESULTS

Formulation and characterization of cGAMP-UPS NP

Cytosolic delivery of cGAMP is challenging due to its membrane impermeable property because of dual negative charges. Here, we utilized the UPS-NP with exquisite pH response to the endo-lysosomal environment to deliver cGAMP into cells (**Figure 4A** and **Table 2**). UPS-NPs allowed for a highly efficient and stable loading of cGAMP (**Table 3**), potentially exploiting the synergy of hydrophobic interactions (i.e. guanidine and adenine bases with alkyl groups on the tertiary amines) and internal salt-bridge (i.e. cGAMP phosphates to ammonium groups in the UPS). The resulting cGAMP-NPs achieved high drug loading and stable encapsulation (no cGAMP release over 7 days) for all NPs (**Table 3**). At blood pH, UPS-NPs were present as self-assembled micelles with a diameter of 30-70 nm and a spherical morphology (**Figure 4B** and **C**). Micelle dissociation into unimer at pH below pKa resulted in efficient cGAMP release.

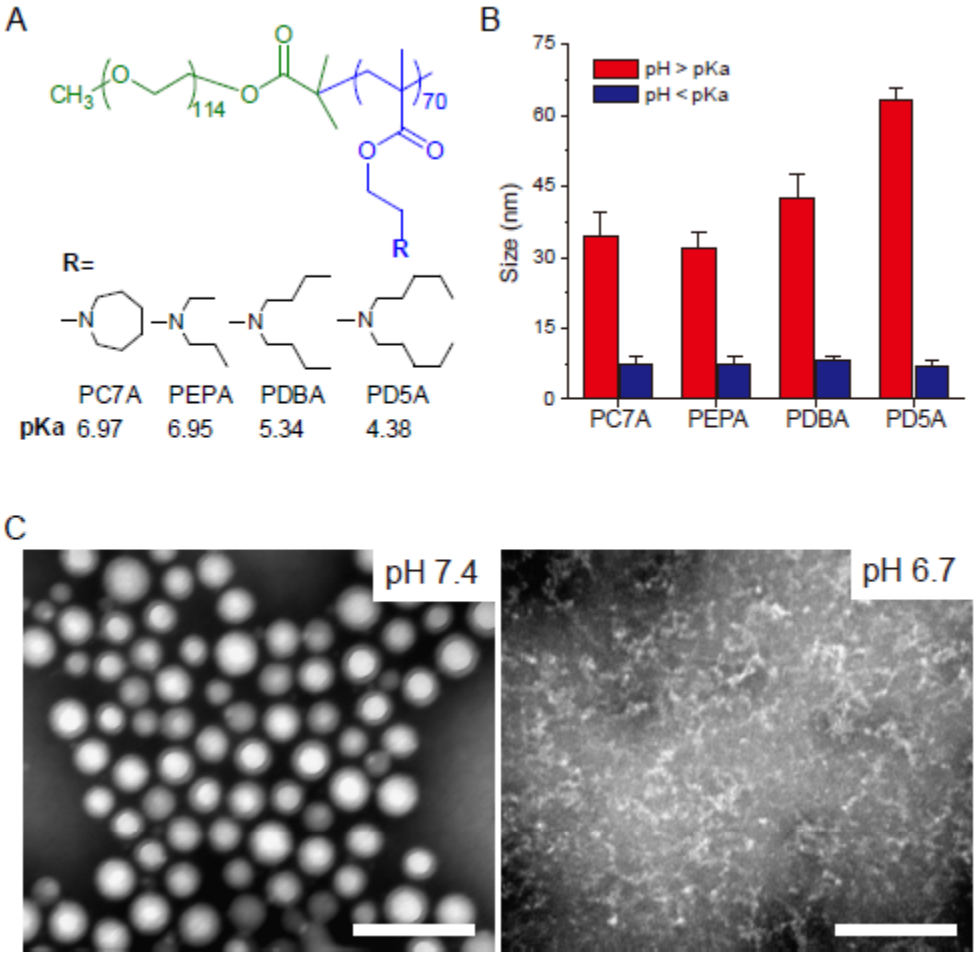

Figure 4. Preparation and characterization of cGAMP loaded micelle nanoparticles.
(A) Structures of the ultra-pH-sensitive copolymers. (B) Size changes of cGAMP loaded micelle nanoparticles in buffers with pH above or below pKa (n = 3). (C) Transmission electron micrographs images of cGAMP loaded PEG-b-PC7A nanoparticles at pH 7.4 and 6.7. Scale bar = 100 nm. This figure was generated by Zhaohui Wang from Gao Lab.

Table 2. Chemical compositions and physical properties of UPS nanoparticles.

Polymer	Monomer	Mn (kDa) [a]	PDI [a]	pKa [b]	D_h (nm) [c]
PC7A	C7A-MA	20.9	1.29	6.97	30.1±7.1
PEPA	EPA-MA	20.2	1.12	6.95	28.6±2.0
PDBA	DBA-MA	22.1	1.12	5.34	45.6±9.6
PD5A	D5A-MA	21.1	1.17	4.38	59.8±3.5

[a]Number-averaged molecular weight (Mn) and polydispersity index (PDI) were determined by GPC using THF as the eluent; [b]pKa was determined by pH titration of polymer solutions using 4 M NaOH. [c]Size was measured using dynamic light scattering, mean ± s.d. Data was generated by Zhaohui Wang from Gao Lab

Table 3. Characterization of cGAMP loaded UPS nanoparticles.

Nanoparticles	Time 0		1 week	
	D_h (nm) [a]	EE% [b]	D_h (nm)	EE%
cGAMP-PC7A NP	34.5±5.0	69.7	32.8±5.5	69.5
cGAMP-PEPA NP	31.8±3.4	33.4	32.7±3.3	33.4
cGAMP-PDBA NP	42.3±5.3	26.3	43.7±6.9	26.2
cGAMP-PD5A NP	63.3±2.4	38.5	64.6±4.1	38.5

[a]Size was measured using dynamic light scattering, mean ± s.d. [b]Encapsulation efficiency was measured by ultrafiltration method. Data was generated by Zhaohui Wang from Gao Lab

cGAMP delivered by nanoparticle or liposome elicits potent inhibition of clinical HIV-1 isolates in human PBMCs.

To determine whether activation of STING signaling by cGAMP-NPs could induce protective immunity against HIV-1 infection, we performed the HIV-1 spreading assay using human PBMCs isolated from healthy donors. We first infected PBMCs with a replication competent HIV-BaL strain in bulk for 3 days. Then, infected PBMCs were washed (to remove free viruses) and split into several conditions where we added uninfected naïve PBMCs from the same donor ('re-seeding') to allow HIV spreading (**Figure 5A**). Different treatments were administered at the point of 're-seeding' (Day 0). We then followed the growth of HIV-1 by measuring HIV-1 reverse transcriptase (RTase) activity in the media (see **Methods**). HIV-BaL titer increased steadily in PBMCs after re-seeding for approximately two weeks before starting to decline. As a positive control, RTase inhibitor nevirapine completely inhibited HIV-BaL spreading in PBMCs (**Figure 5B**). When we compared cGAMP-NPs releasing at different pHs ranging from 4.5 to 6.9, only cGAMP-PC7A NP (pH 6.9) inhibited HIV-BaL spreading (**Figure 5B**). Neither PC7A NP alone nor naked cGAMP induced detectable protective immunity against HIV-BaL replication. We also found that cGAMP-PC7A NP (we call cGAMP-NP below) induced protection against HIV-BaL in a dose dependent manner in PBMCs isolated from multiple donors (**Figure 5C, 5D**). cGAMP-NP did not induce appreciable cytotoxicity in PBMCs at the dose needed for full protection (**Figure 6A**). We also tried to deliver cGAMP complexed with liposomes (Lipofectamine 2000), and observed similar dose-dependent protection against HIV-BaL in PBMCs without cytotoxicity (**Figure 6B, 6C**), although a much higher amount of cGAMP-Lipo is needed to achieve similar level of protection as cGAMP-NP. We also compared cGAMP-NP and cGAMP-Lipo in THP-1 cells and found that cGAMP-NP is much more

potent at eliciting IFN and ISGs compared to cGAMP-Lipo (**Figure 6D**). HIV-BaL is an M-tropic R5 strain. We next infected PBMCs with two other T-tropic X4 strains of HIV-1, IIIB and LAI. cGAMP-NP inhibited spreading of both strains in PBMCs (**Figure 5E, 5F**). Together, we conclude that intracellular delivery of cGAMP can induce strong protection against replication competent HIV-1 spreading in PBMCs, and that cGAMP-mediated protection can achieve the same potency as RTase inhibitors in vitro. Our data also suggest that controlled early release of cGAMP by PC7A NP (compared to other NPs and liposomes) is advantageous for inducing robust protective immunity against HIV-1 replication.

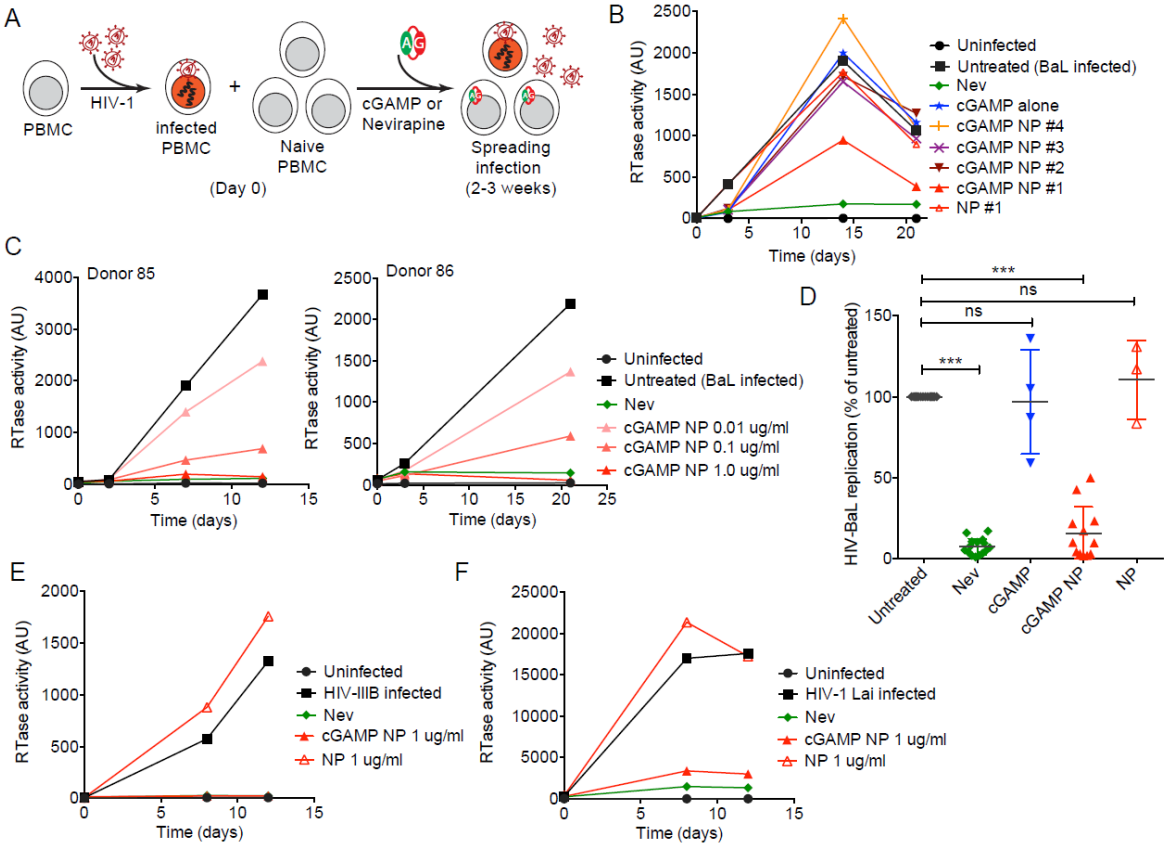

Figure 5. cGAMP delivered by nanoparticle or liposome elicits potent inhibition of HIV-1 in human PBMCs.
(A) A schematic diagram of HIV-1 spreading assay in PBMCs. (B) HIV-BaL replication in PBMCs treated with indicated reagents (right). NP, nanoparticle. Nev, nevirapine (4 μM, same throughout). (C) HIV-BaL replication in PBMCs treated with increasing amounts of cGAMP-NPs. Two separate donors are shown. (D) HIV-BaL replication in PBMCs treated with indicated reagents (bottom). cGAMP-NP was used at 1-4 μg/ml. Each data point indicates an individual donor. (E, F) HIV-IIIB (E) or HIV-LAI (F) replication in PBMCs treated with Nev or cGAMP-NP. Assay performed as illustrated in A. Data are representative of at least three independent experiments with independent donors. ***, $p<0.005$. ns, not significant. Student's t-test. Error bar, SEM.

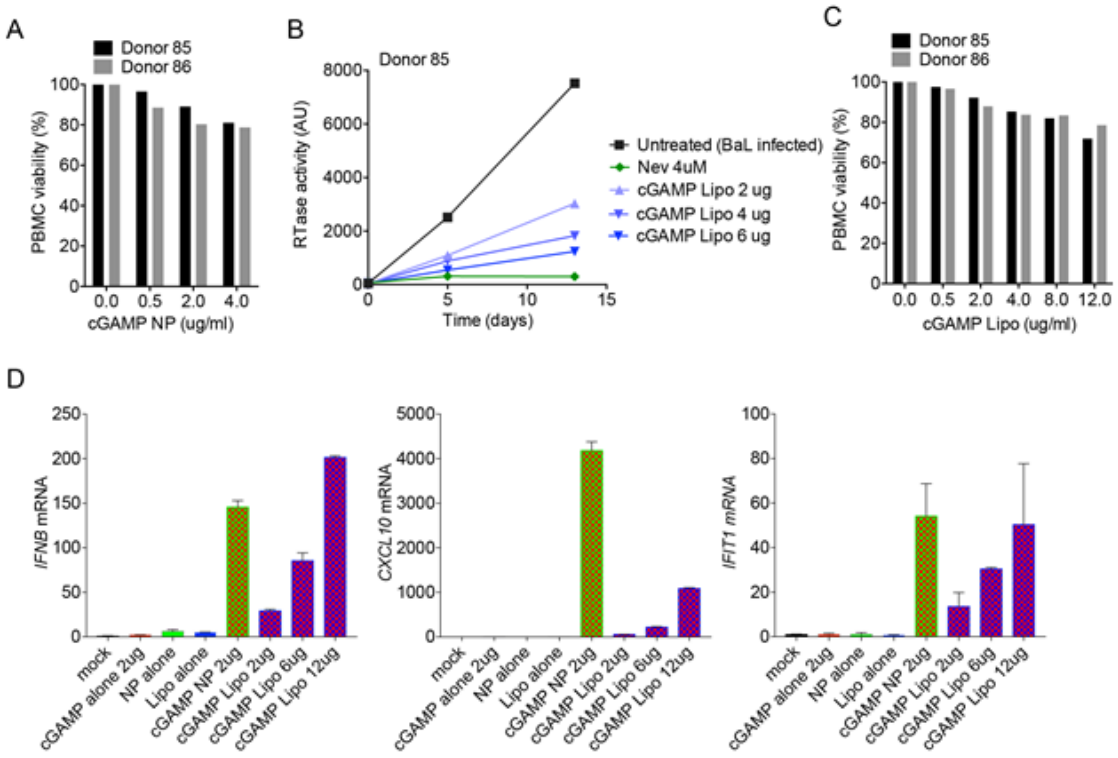

Figure 6. cGAMP NP is more potent than cGAMP Lipo. (A) Viability of PBMCs after cGAMP-NP treatment. Healthy donor PBMCs were treated with indicated amount of cGAMP-NP for 72 hours. Cell viability was measured using Live/Dead fluorescent staining. (B) HIV-BaL replication in PBMCs treated with increasing amount of cGAMP complexed with Lipofectmine 2000. One representative donor is shown. (C) Viability of PBMCs after cGAMP-Lipo treatment. Assay performed as in A. (D) Quantitative RT-PCR analysis of *IFNB*, *CXCL10*, and *IFIT1* in THP-1 cells treated with indicated conditions below for 5 hours. Data are representative for at least two independent experiments. Error bar, SEM.

cGAMP-induced protective immunity against HIV-1 is mediated through type I IFN signaling

We next compared cGAMP-NP to TLR3 agonist poly(I:C), TLR7/8 agonist R848, and TLR9 agonist ODN in the HIV-1 spreading assay. Many of these TLR agonists induce potent inflammatory responses. We confirmed that all adjuvants induced potent immune activation of their cognate pathways in PBMCs by RT-qPCR array, with cGAMP-NP mediating stronger IFN response and TLR agonists mediating stronger inflammatory response (**Figures 7A**). We found that none of the TLR agonist elicited detectable protection against HIV-BaL replication in PBMCs (**Figure 7B**). To determine whether cGAMP-induced protection is mediated through soluble factors, we next compared cGAMP-NP to extracellularly versus intracellularly delivered poly(I:C) (complexed with Lipofectamine, activates the cytosolic RIG-like receptor (RLR) pathway) in the 'conditioned media' experiment (**Figure 7C**). We first treated PBMCs with cGAMP-NP, poly(I:C) alone or poly(I:C)-Lipo, washed cells extensively 24 h later to remove excess agonists that were not taken up by cells. We then incubated stimulated PBMCs in fresh media for 48 hours to

collect secreted soluble factors. We added the conditioned media together with HIV-BaL to fresh PBMCs to examine whether they can confer protection against HIV-BaL replication. Conditioned media from cGAMP-NP-treated cells strongly inhibited HIV-BaL replication, suggesting that soluble factors are largely responsible for cGAMP-NP-mediated protection (**Figure 7D**). Treatment of naïve PBMC with cGAMP-NP showed that it is a robust activator of interferons and pro-inflammatory cytokines as compared to poly (I:C) and poly (I:C)-Lipo (**Figure 7E**). Media from poly(I:C)-treated cells did not confer any protection, and media from poly(I:C)-Lipo-treated cells conferred only partial protection. These data suggest that activation of cytosolic nucleic acid sensing pathway (e.g. the RLR and cGAS-STING pathways) are better (comparing to TLR pathways) at inducing protective immunity against HIV-1 replication in PBMCs.

We next investigated the role of IFN-I signaling in cGAMP-mediated protection against HIV-1 replication. Both recombinant IFNα and cGAMP-NP induced potent protection against HIV-BaL spreading in PBMCs, and in both cases the protection was completely reversed by an IFNα/β receptor (IFNAR) inhibitor, B18R, suggesting that the IFN signaling pathway plays a key role in cGAMP-mediated protective immunity (**Figure 7F**).

cGAMP-induced protective immunity in human PBMCs is dependent on monocytes

We next tried to determine the immune cell type in PBMCs that is responsible for cGAMP-induced protection against HIV-1 replication. We first characterized the signaling kinetics activated by cGAMP in positive selected immune cell populations. cGAMP induced

robust IFNβ expression in CD19+ B cells and CD14+ monocytes during the first 12 hours followed by gradual decline, likely due to cell-intrinsic mechanisms to prevent excessive IFN activation (**Figure 8A**). In contrast, CD4+ T cells presented a much slower kinetics of IFNβ expression after cGAMP stimulation, with IFNβ mRNA becoming detectable after 6 h and continued to increase up to 24 h. All immune cell types express similar levels of endogenous STING, TBK1 and IRF3 proteins (**Figure 8B**). HIV-1 replicates in CD4+ T cells in PBMCs. We next depleted B cells or monocytes with magnetic beads from whole PBMCs and evaluated HIV-1 replication with and without cGAMP-stimulation (**Figure 8C-8G**). We found that cGAMP-mediated protection was completely lost in monocyte-depleted PBMCs, whereas B cell depletion had little or no effect (**Figure 8C**). We confirmed the depletion by FACS (**Figure 8F**). We also analyzed STING signaling activation in PBMCs or monocytes by immunoblots. The kinetics of TBK1 phosphorylation in both cell cultures are similar, consistent with monocytes being the primary mediators of cGAMP-induced protection against HIV-1.

We next compared cGAMP to another STING agonist, c-di-GMP, at stimulating immune gene expression in PBMCs and a monocyte cell line THP-1 cells. cGAMP binds to STING at a much higher affinity compared to c-di-GMP (Zhang *Mol. Cell* 2013). Though both cyclic dinucleotides stimulate the same pathway (**Figure 9A),** we found that cGAMP also induced a more robust immune response compared to c-di-GMP in multiple PBMCs and THP-1 cells (**Figure 9B**). Collectively, these data suggest that monocytes are the key immune cell population in PBMCs that are mediating cGAMP-induced protection against

HIV-1 replication. Our data also demonstrate that mammalian cGAMP is more potent than bacterial cyclic dinucleotides at stimulating immune response in PBMCs.

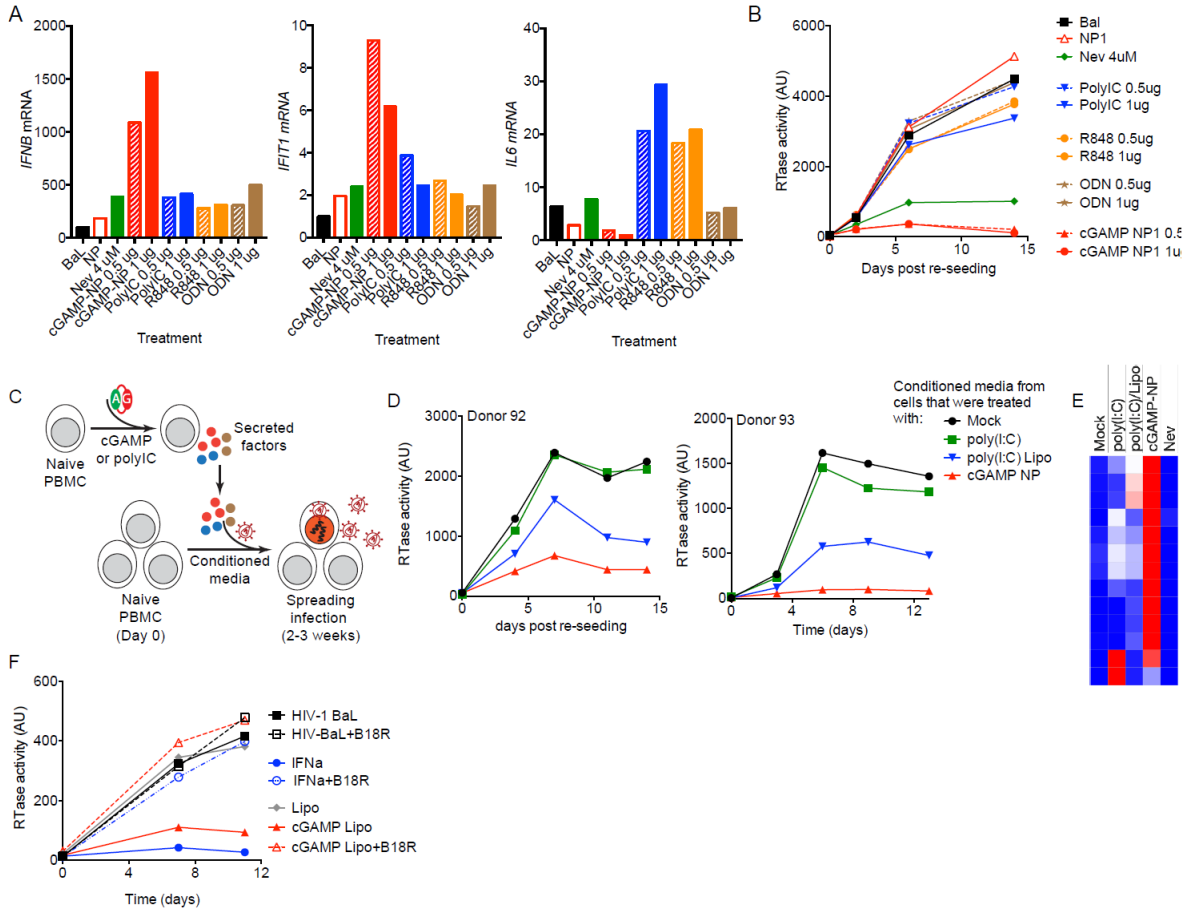

Figure 7. cGAMP-induces protective immunity is mediated through type I IFN signaling.
(A) Quantitative RT-PCR analysis of *IFNB*, *IFIT1* and *IL6* mRNA in HIV-BaL infected PBMCs treated with indicated reagents for 16 days (bottom). (B) HIV-BaL replication in PBMCs treated with cGAMP-NP or TLR agonists. (C) A schematic diagram of 'conditioned media' assay used in D. (D) HIV-BaL replication in PBMCs. Conditioned media from first collected from PBMCs treated with indicated reagents (right) for two days, then, these media (containing soluble factors) were mixed with HIV-BaL to infect fresh PBMCs. (E) A heat map of secreted cytokines levels after cGAMP or poly(I:C) treatment in D. (F) B18R inhibits cGAMP-induced protection against HIV-BaL. HIV-1 spreading assay was performed as in Figure 1. Indicated reagents were added at day 0. Data are representative of at least three independent experiments with independent donors.

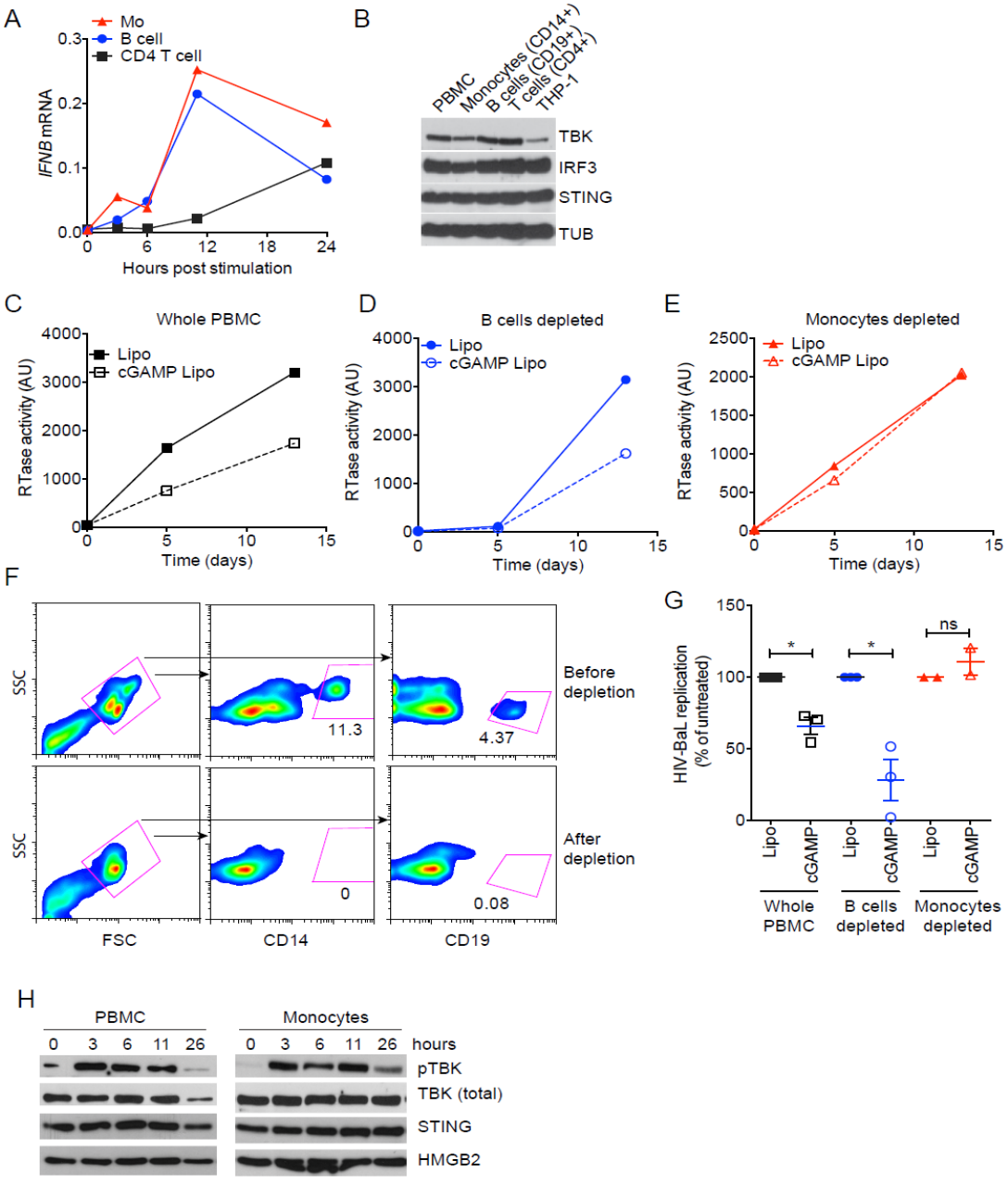
A
Mo
B cell
CD4 T cell
IFNB mRNA
Hours post stimulation
B
PBMC
Monocytes (CD14+)
B cells (CD19+)
T cells (CD4+)
THP-1
TBK
IRF3
STING
TUB
C
Whole PBMC
Lipo
cGAMP Lipo
RTase activity (AU)
Time (days)
D
B cells depleted
E
Monocytes depleted
F
SSC
FSC
CD14
CD19
Before depletion
After depletion
11.3
4.37
0
0.08
G
HIV-BaL replication (% of untreated)
ns
Whole PBMC
B cells depleted
Monocytes depleted
H
PBMC
Monocytes
hours
pTBK
TBK (total)
STING
HMGB2

Figure 8. cGAMP-induced protective immunity in PBMCs is dependent on monocytes.
(A) Quantitative RT-PCR analysis of *IFNB* mRNA in indicated cell types. Monocyte, B and CD4+ T cells were isolated from PBMCs by positive selection. Cells were then treated with cGAMP-Lipo (4 μg/ml) and *IFNB* expression was measured at indicated times. (B) Immunoblots analysis of STING, TBK1, IRF3 protein expression in whole PBMCs or sorted individual cell populations. (C-G) HIV-BaL replication in whole PBMCs (C) or B-cell-depleted (D) or monocyte-depleted (E) PBMCs. A representative donor is shown. Representative FACS plots confirming B cell (CD19) or monocyte (CD14) depletion is shown in F. Summary from multiple donors are shown in G. (D) Immunoblots analysis of STING signaling activation kinetics (phosphorylation of TBK1) in whole PBMCs or monocytes. Cells were treated with 2 μg/ml cGAMP-Lipo for indicated times (top). Data are representative of at least three independent experiments with independent donors. *, $p<0.05$. ns, not significant. Student's t-test. Error bar, SEM.

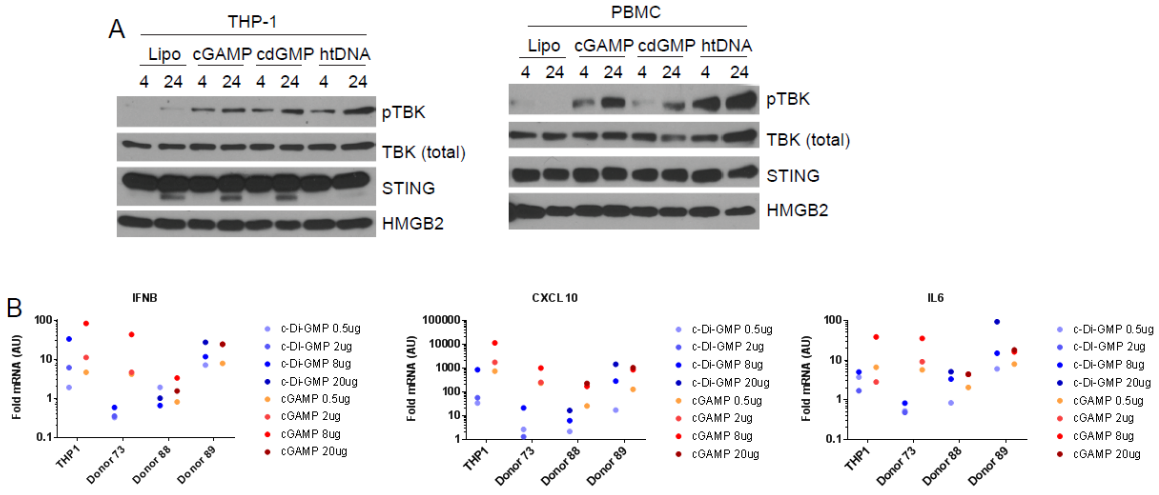

Figure 9. cGAMP is more potent than c-di-GMP at stimulating immune gene expression in PBMCs.
(A) Immunoblots and (B) quantitative RT-PCR analysis of STING signaling activation in THP1 and PBMCs by cGAMP (2 μg/ml), c-di-GMP (2 μg/ml) or htDNA (1 μg/ml) for Immunoblot analysis and several doses for qRT-PCR. Cells were treated with the indicated ligand for 4, 7 or 24 hours as indicated (4 and 24 h for immunoblots, 7 h for qRT-PCR). Data are representative of at least two independent experiments.

cGAMP-NP elicits long-acting protection against HIV-1

Two major challenges facing current antiretroviral therapy (ART) are patient adherence and drug resistance. Most antiretroviral regimens require daily dosing of a combination of antiretroviral drugs, and lapses in treatment often lead to rapid rebound of viral load. Thus, developing long-acting ARTs represents one major need for HIV/AIDS. To determine whether cGAMP-NP can elicit long-acting protection against HIV-1 replication by activating sustained immune responses, we performed the extended HIV-1 spreading assay interrupted by a 'media wash' that simulates treatment interruption and clearance of the initial antiretroviral drug (**Figure 10A**). The first stage of the assay is identical to the HIV-1 spreading assay described above, where HIV-BaL infected PBMCs were co-cultured with naïve PBMCs in the presence or absence of various treatments. After 7 days, we washed the cells and changed fresh media (to remove drugs and viruses), at which time we also added more naïve PBMCs and followed HIV-1 spreading for three more weeks. We found that both nevirapine and cGAMP NP protected against HIV-1 replication before treatment interruption ('media wash', **Figure 10B**). After treatment interruption, viral load in both untreated and nevirapine-treated samples increased quickly, rising to the level similar to the untreated condition before treatment interruption (**Figure 10B**). This rapid viral load rebound after ART treatment interruption closely resembles the clinical observation of HIV-1 infected patients experiencing a lapse in treatment. Remarkably, cGAMP-treated samples remained protective against HIV-BaL replication up to 3 weeks after 'media wash' in PBMCs isolated from multiple healthy donors (**Figure 10B, 10C**). We also measured cytokines produced

during 'long-acting' experiments. HIV-1 infection with and without Nev treatment in PBMCs produced several inflammatory cytokines (e.g. IL6, IL8, GCSF, TNFα), but not IFNs (IFNα2, IFNγ) in the media (**Figure 10D**). In contrast, HIV-1 infected PBMCs treated cGAMP-NP produced IFN and inflammatory cytokines. IFNα2 and MIP-1α (an ISG) are also continuous being produced at high levels in cGAMP-NP treated cells more than two weeks after the single treatment (**Figure 10E**). We also found that co-administration of B18R abolished cGAMP-NP-mediated long acting protection (**Figure 10F**) and no evidence of excessive cytotoxicity during the entire course of cGAMP treatment in multiple donors. These data suggest that cGAMP induces durable protective immune response in PBMCs against HIV-1 replication that is largely dependent on IFN-I. These data also demonstrate exciting potential for further development of cGAMP-NP as a PRR-based replacement for IFN therapy and a long-acting antiretroviral drug or a candidate drug for pre-exposure prophylaxis (PrEP).

cGAMP-NP stimulates protective immune response that inhibits HIV-1 spreading in HIV patient PBMCs ex vivo

We next evaluated cGAMP-NP in HIV patient PBMCs ex vivo. We recruited HIV-positive individuals who have detectable viral load (RNA copy > 50/mL) and CD4 T cells (count >100/mL), treatment naïve or treatment experienced but have not been on any antiretroviral medication for a minimum of 30 consecutive days prior to specimen collection. We first measured basal immune gene expression in PBMCs isolated from four HIV-positive patients and three healthy controls and how they respond to cGAMP-NP stimulation. All

healthy control and HIV-positive patients PBMCs express low levels of IFN genes (e.g. IFNα4, IFNβ1), ISGs (e.g. CXCL10, IFIT1, OASL, etc.) and inflammatory genes (e.g. TNFβ, IL6, IL8) (**Figure 11A**). cGAMP-NP induced comparable immune gene expression profiles in healthy control and HIV patient PBMCs (**Figure 11B**).

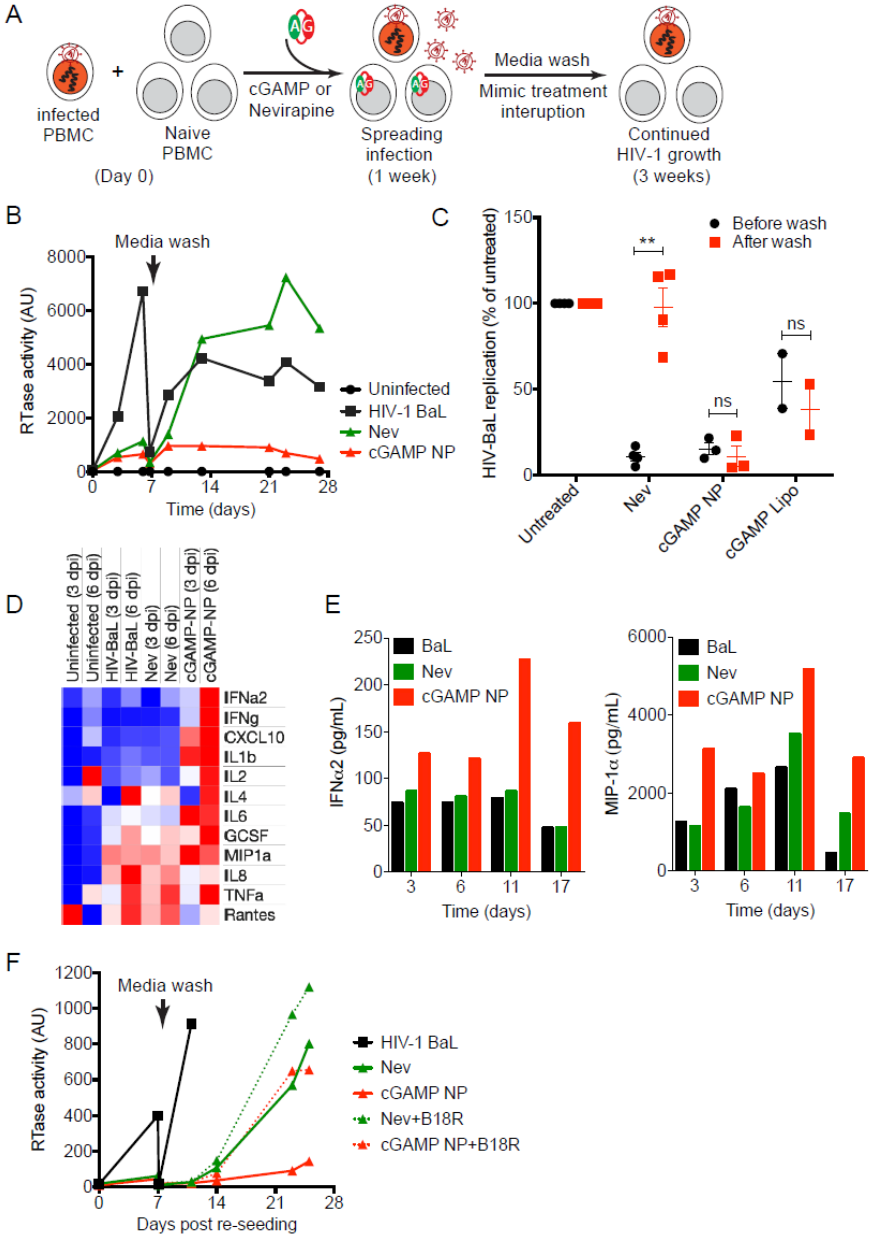

Figure 10. cGAMP-NP elicits long-acting protection against HIV-1.
(A) A schematic diagram of modified HIV-1 spreading assay to include treatment interruption after 1 week ('long-acting' experiment). (B, C) HIV-BaL replication in PBMCs treated with Nev (4 uM) or cGAMP-NP (2 μg/mL). Cells were washed with fresh media at day 7, and viral replication was continually monitored till day 27. One representative donor is shown in B. Summary from multiple donors is shown in C. (D) A heap map of secreted cytokine levels in the media. Treatments are shown on the top and cytokines are shown on the right. (E) IFNα2 and MIP-1α expression in the media from a representative 'long-acting' experiment. (F) HIV-BaL replication in PBMCs treated with Nev (4 uM) or cGAMP-NP (2 μg/mL) with or without B18R (500 ng/mL). **, $p<0.01$. ns, not significant. Student's t-test. Error bar, SEM.

We expect a substantial amount of HIV patient PBMCs to be latently infected, and would produce HIV-1 after PHA/IL-2 activation. New infection or HIV-1 spreading may be limited in these PBMC. We activated HIV patient PBMCs with PHA and IL-2 for 7 days to stimulate HIV-1 replication. In two cases, we observed substantial HIV-1 production in the media compared to non-activated PBMC from the same donor (**Figure 11C**). We next added cGAMP-NP, nevirapine or nevirapine + raltegravir at day 7 post re-activation, and monitored HIV-1 replication for up to 14 days. Nevirapine alone or with raltegravir reduced HIV-1 replication by 27-28% and 13-30%, respectively in patient 2, 0-15% and 18-56% respectively in patient 3 (**Figure 11D**). Since these two inhibitors only inhibit early stage of the HIV-1 life cycle, inhibitory effect by these two drugs indicates the extent of new infection that could occur in these ex vivo settings. cGAMP-NP inhibited 37-52% in patient 2 and 8-30% in patient 3 (**Figure 11D**). We were not able to use these clinical HIV-1 isolates to infect healthy donor PBMCs in the spreading assay due to extreme low virus titer. None of the treatment induced excessive cytotoxicity (data not shown). These data suggest that

cGAMP-NP is effective at eliminating new infections in HIV patient PBMCs, at least similar to the current combination ART. Given the distinct antiviral mechanism and long-acting benefit through stimulating the innate immune response, cGAMP-NP could be a desirable candidate as a new category of PRR-based long-acting ART.

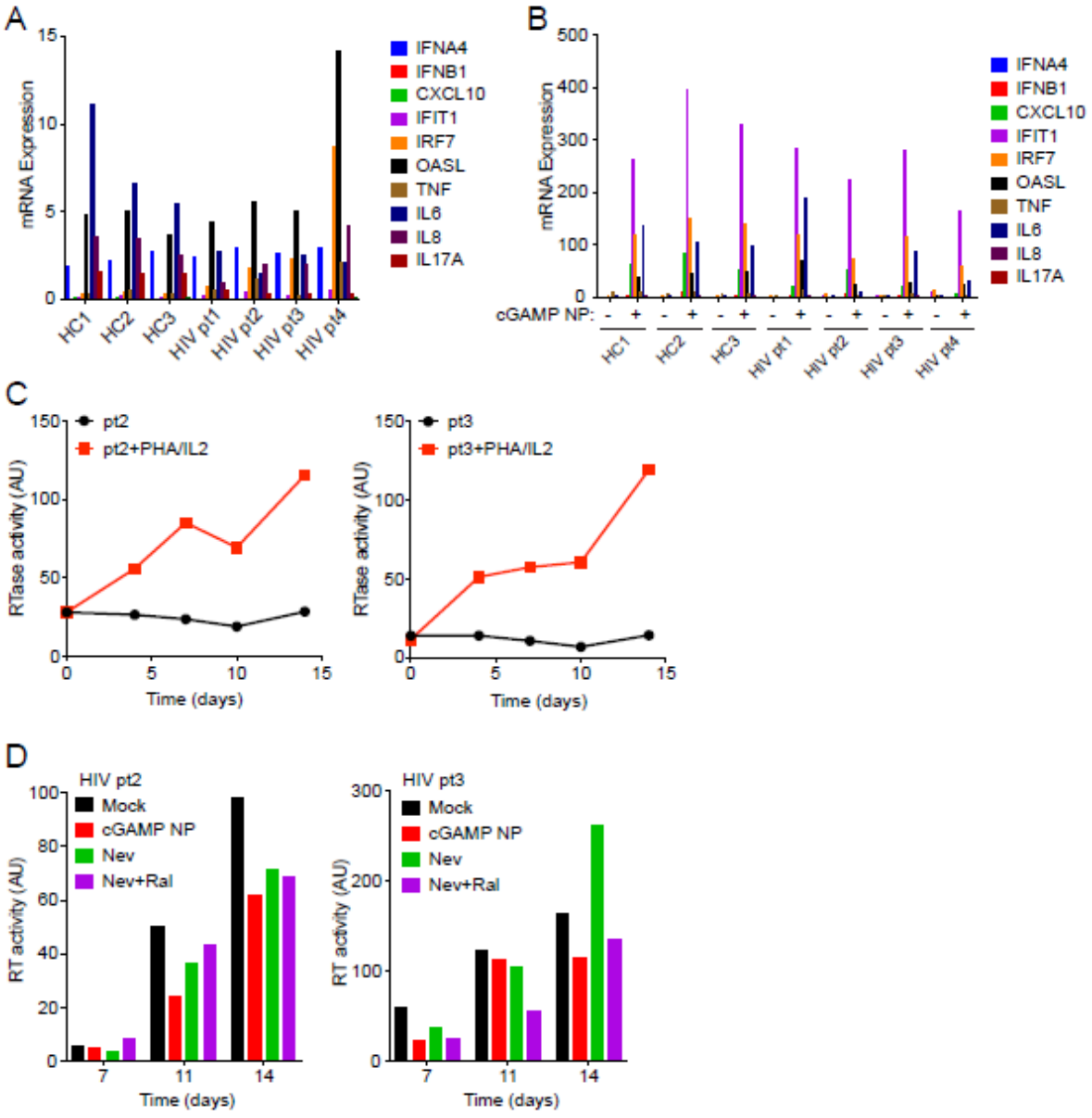

Figure 11. cGAMP-NP stimulates immune response and inhibits HIV-1 spreading in HIV-positive patient PBMCs ex vivo.
(A, B) Quantitative RT-PCR array analysis of indicated immune genes in PBMCs isolated from healthy or HIV-positive donors. A shows basal level expression in untreated PBMCs. B shows gene expression before and after 2 μg/ml cGAMP-NP treatment for 24 hours. (C) HIV-1 replication in patients PBMCs ex vivo after re-activation. (D) HIV-1 replication in patients PBMCs ex vivo after re-activation and indicated treatment.

DISCUSSION

Recent advance in understanding HIV-1 immune evasion mechanisms highlighted the importance of the cytosolic DNA sensing cGAS-STING pathway in host immune response to HIV-1 infection. The same signaling pathway can be triggered by a small molecule intermediate cGAMP that directly activates STING. We showed here that cGAMP NP induced potent protection against clinical HIV-1 isolates in PBMCs. Intracellular delivery of cGAMP is critical for inducing the protective immune response, as demonstrated by the lack of protection conferred by naked cGAMP. Among the nanoparticles we tested, PC7A NP conferred significantly improved protection over other NP compositions as well as lipofectamine. In addition to the optimal pH transition (6.9) that target the early endosomal pH for cGAMP release and the cyclic amine structure that facilitates the membrane disruption for cytosolic release (Wang *Angew. Chem. Int. Ed. Engl.* 2017), the direct binding of PC7A to the cytosolic domain of STING may also contribute to STING activation in addition to cGAMP. Indeed, recent studies in cGAS-/- knockout animals showed the cGAS-independent STING activation by PC7A NP alone in mice (Luo et al Nature Nanotechnology, in press), although with a lesser magnitude compared to cGAMP. Although

we did observe protection in human PBMCs by PC7A NP alone, the combined cytosolic delivery of cGAMP and cGAS-independent STING activation by PC7A confer a potential synergistic activation of STING to achieve maximal protection against HIV-1 replication.

As STING is a critical mediator of IFN production, STING agonists such as cGAMP and other cyclic dinucleotides are being developed as vaccine adjuvant to elicit potent immune response. STING signaling also elicits a strong anti-tumor response by boosting host immune recognition of tumor antigens (Li *Science* 2013, Corrales *Cell Rep*. 2015, Woo *Annu. Rev. Immunol*, Fu *Sci. Transl. Med*. 2015). Intratumoral or intramuscular (Wang et al., PNAS, 2017) injection of STING agonists such as cGAMP has shown remarkable therapeutic activity in mouse models. Our study extends the utility of cGAMP by demonstrating the therapeutic benefit of using cGAMP directly (without an antigen) as a potent and long-acting ART against HIV-1 replication. The PC7A NP formulation for cGAMP delivery could also be applied to enhance cGAMP-mediated anti-tumor immunity. The mechanism of antiviral activity for cGAMP is drastically different from traditional ARTs or long-acting (slow release) ART formulations. cGAMP-NP induces a broad and long lasting protective immunity, which could potentially overcome two major challenges faced by current antiretroviral therapy, namely drug resistance and patient adherence. Although we did not test how cGAMP-NP elicits adaptive immune response to HIV-1 infection in vivo, evidence from vaccine and cancer studies showed that cGAMP can also induce potent B cell and T cell mediated adaptive immune response in mice (Li *Science* 2013, Corrales *Cell Rep*. 2015).

We defined that cGAMP-NP-mediated protective immunity in PBMCs is mediated by monocytes and type I IFN. As PRR-based agonists are becoming more favorable therapeutics than recombinant IFN with less toxicity and better immune response profile, our study presents an important proof-of-principle for harnessing cGAMP-mediated innate immune response for HIV-1 therapy. The concern for cGAMP-NP triggering systemic autoimmunity can be at least partially mitigated by local administration as what have done for many other PRR-based vaccine adjuvants that induce potent immune responses. We envision cGAMP-NP as a novel class of immunotherapy that can be used for treating HIV and cancer. As more STING agonists and formulations entering clinical trials for anti-tumor therapy, we expect many of the concerns of adverse effects being addressed, and more exciting opportunities for parallel development of these reagents for HIV-1 ART. Another exciting property of cGAMP-NP and other STING agonists is its potential as a dual reagent, either as a pre-exposure prophylaxis (PrEP) or long-acting ART when used alone, or as an adjuvant to use in combination with an HIV-1 antigen vaccine, both of which fulfill important current needs for a functional HIV cure.

CHAPTER FOUR: GENETIC SURVEILLANCE OF WNV

Positively-selected mutations of 2012 WNV promote circulation and overwintering

BACKGROUND

West Nile Virus (WNV) is a neurotropic *Flavivirus* and the most widely distributed arbovirus (Diamond *Nat. Rev. Microbiol.* 2013, Kramer *Annu. Rev. Entomol.* 2008). As a member of the *Flaviviridae* family and shares many properties, like genome structure and replication lifecycle, with other mosquito borne flaviviruses such as Japanese Encephalitis Virus, Dengue, and Zika virus. WNV enters host cells through endocytosis after binding an unknown receptor on the cell surface. Fusion of the viral envelope with the endosomal membrane to deliver the RNA genome (**Figure 12A**) into the cytosol (Suthar *Nat. Rev. Microbiol.* 2013). A polyprotein is subsequently translated from the RNA genome and processed by host and viral proteases to generate three structural proteins (Capsid C, premembrane prM, and envelope E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5) (Pesko and Ebel *Infect. Genet. Evol.* 2012). Structural proteins are incorporated into the mature virion. Non-structural proteins play many important roles including facilitating viral replication and immune evasion (**Figure 12B)**. Following virion assembly, the mature viral particle is transported through the secretory pathway and released by exocytosis. The heavy reliance on host pathways for flaviviral replication implicates possibilities of therapeutically targeting the host to inhibit flavivirus replications (Zhang *Nature* 2016).

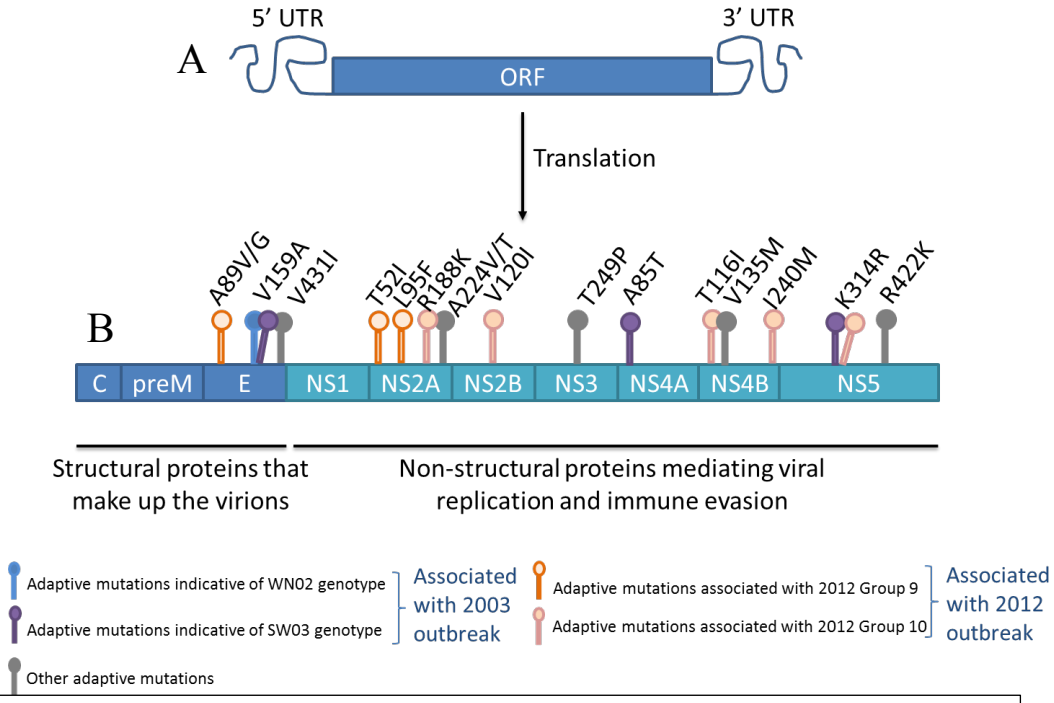

Figure 12: (A) Shows general organization of WNV genome which is translated in host cells such as myeloid cells to generate a single polyprotein which is cleaved into the various viral proteins shown. (B) Shows respective positions of amino-acid changing mutations predicted to mediate adaptive changes in WNV.

WNV is naturally maintained in an enzootic cycle between *Culex* mosquitoes and birds, and is transmitted to humans and other vertebrates as dead-end hosts (Reisen *Viruses* 2013). Because viral infection of humans is insufficient to lead to subsequent transmissions, selective pressure from the immune system in mosquitoes and birds predominately impacts WNV evolution and transmission (Lazear *Curr. Opin. Virol.* 2015). Generally, WNV gains genetic diversity through infection of mosquitoes. Purifying selection of WNV in birds also ensures a high fitness of the virus (Pesko and Ebal *Infect. Genet. Evol.* 2012). Birds are also important in the spreading of WNV. Migratory birds are believed to mediate long-distance transport agents of the virus

between continents (Peterson *Vector Borne Zoonotic Dis.* 2003). Resident birds, such as House Sparrows and American Robins, act as amplifying hosts and may be more important for the spread of virus across within countries (Komar *Vector Borne Zoonotic Dis.* 2001, Hayes *Emerg. Infect. Dis.* 2005, Komar *Am. J. Trop. Med. Hyg.* 2005, Rappole *Vector Borne Zoonotic Dis.* 2006).

Incidentally, WNV infects humans through the bite of an infected mosquito. Upon entering the skin, the virus replicates in keratinocytes, neutrophils, and dendritic and subsequently disseminated through the blood stream to peripheral tissues such as the spleen (Samuel *J. Virol.* 2006). At peripheral tissues, WNV undergoes additional replication in macrophages, and is either cleared by the immune response or invades the brain (Lazear *Curr. Opin. Virol.* 2015). The ability of WNV in peripheral tissues to invade the brain distinguishes virulent from avirulent strains. Both categories of WNV strains remain highly neurotoxic when injected directly into the brains of mice (Beasley *Virol.* 2002). Approximately 80% of WNV infections in humans are asymptomatic, and much of the remaining 20% results in mild symptoms such as fever or rashes. In approximately 1% of cases, WNV causes severe neuro-invasive disease such as meningitis and encephalitis, which can lead to death (Gray *Internat. J. Gen. Med.* 2014). Old age (over 60yrs) and immunodeficiency are associated with increased risk for symptomatic disease (O'Leary *Vector-borne and zoonotic diseases* 2002). In the United States alone, WNV has led to 3-5 million infections and over 43,000 human cases of West Nile disease (Suthar *Curr. Opin. Virol.* 2014, Grinev *Plos Negl. Trop. Dis.* 2016).

Currently there are no specific antivirals or vaccines available for WNV making it an ongoing public health threat.

There are two main lineages (lineage I and Lineage II) of WNV that cause disease in humans. Lineage II WNV was associated with mild disease but since 2010 has been associated with outbreaks of nuero-invasive disease in Europe (Papa *Emerg. Infect. Dis.* 2011). Most neuro-invasive WNV typically belong to lineage I, which is divided geographically into three sub-clades namely lineage Ia, lineage Ib and lineage Ic. Lineage Ib and Ic are primarily found in Australia and India respectively. In contrast, Lineage Ia is found worldwide in Africa, Europe, and the Americas (Gray *Internat. J. Gen. Med.* 2014). WNV was introduced into North America in 1999 (Lanciotti *Science* 1999), and quickly spread throughout continental United States and the western hemisphere (Peterson *Vector Borne Zoonotic Dis.* 2003). This introduction and spread of WNV provided a unique opportunity to prospectively observe the evolution of an exotic RNA virus to a naive ecosystem (Pesko and Ebel *Infect. Genet. Evol.* 2012). Genetic surveillance suggests that WNV population in the US is predominantly under purifying or negative selection (Bertolotti *Virol.* 2007, McMullen *Emerg. Infect. Dis.* 2011, Grinev *Plos Negl. Pathog.* 2016). Nonetheless, a few nucleotide positions in the WNV genome have been identified as undergoing positive selection and those nucleotide positions are often associated with outbreaks of human disease (**Figure 13B**). The largest outbreak of human West Nile disease occurred in 2003, and it was implicated with the discovery of E-V159A, NS4A-A85T, and NS5-K314R mutations. These mutations define the two dominant genotypes still co-circulating in North America, with E-V159A being

characteristic of West Nile 2002 (WN02) and NS4A-A85T or ND5-K314R indicative of Southwest/West Nile 2003 (SW03) genotype (McMullen *Emerg. Infect. Dis.* 2011) (**Table 4**). The E-V159 position is polymorphic in Old World WNV (WNV in Europe and Africa) but only in North America did an Alanine mutation arise, suggesting the effect of the mutation might be specific to the ecology of North America (Duggal *Negl. Trop. Dis*. 2014). Consistently, it was shown that WNV with the E-V159A mutation had shorter extrinsic incubation time in mosquitoes enabling faster transmission than NY99, the founding genotype (Ebel *Am. J. Trop. Med. Hyg.* 2004, Davis *Virol.* 2005). It is also suggested that the E-V159A mutation influences a nearby conserved glycosylation motif at position 154-156, which is implicated in neuro-invasiveness and virulence in mice and young chicks (Beasley *J. Virol.* 2005). The SW03 mutations (NS4A-A85T and NS5-K314R) are also polymorphic but their functions are unclear (Duggal *Plos Negl. Trop. Dis.* 2014). Adaptive mutations are thought to contribute to human disease either by increasing transmission efficiency or increased virulence in humans (Pesko and Ebel *Infect. Genet. Evol.* 2012, Duggal *Am. J. Trop. Med. Hyg.* 2013).

The second largest outbreak of WNV occurred in 2012. Positively-selected mutations were identified in sub-lineages of North American WNV, or groups, that were transmitted to several Midwestern and Southern states in the United States. These positively-selected mutations were predicted to confer adaptive changes but their function remain unknown (Grinev *Plos. Negl. Pathog.* 2016). Nationwide, the outbreak was associated with over 5,674 cases of human disease, more than one third of the cases occurred in Texas alone. Sequencing of Texas WNV strains recovered between 2002-

2012 detected a novel introduction of WNV groups with (Groups 9 and 10) and without (Groups 8) positively-selected mutations (Mann *Emerg. Infect. Dis.* 2013, Duggal *Am. J. Trop. Med. Hyg.* 2013). Genetic studies failed to associate any genetic variants to the outbreak, and thus the record outbreak was attributed to favorable environmental conditions such as higher than average temperatures (Duggal *Am. J. Trop. Med. Hyg.* 2013). However, in subsequent years after the 2012 outbreak the overall disease incidence reduced compared to 2012 but the disease incidence is still three-fold higher compared to the years before the 2012 outbreak (an average of 2283 versus 952 cases). Similar annual temperature in 2013-2014 (average 52.49^{o}F) as compared to 2008-2011 (52.71 ^{o}F). These observations suggested that other determinants beside temperature also contributed to the greater incidence of disease in 2013-2015.

To understand the impact of newly introduced groups of WNV in human diseases in Texas, we collected WNV from multiple counties from 2012 until 2015 and characterized them genetically and phenotypically. Our results suggest that WNV with positively-selected mutations (NS2A-R188K and NS4B-I240M) confer advantages in circulation and persistence in Texas which may have contributed to the higher than average incidence of disease in 2013-2015 in those years despite mild temperatures.

RESULTS**:**

Identification of Group 11 WNV as a newly introduced WNV group without positively-selected mutations

Sub-clusters within North American lineage 1a WNV are referred to as groups or nodes (Mann *Emerg. Infect. Dis.* 2013, Grinev *Plos Negl. Pathog.* 2016). Except for Groups 5 and 6 which belong to SW03 genotype, all other groups belong to WN02 genotype (**Table 4**). During the 2012 outbreak, Group 9 and 10 were identified as associated with the outbreak in multiple Midwestern and Southern states. The discovery of similar isolates across a broad

Table 4: Definition of major WNV genotypes in North America.

Genotype name (Abbreviation)	Initially isolated	Notes	References
East Coast Genotype (NY99)	New York and East Coast in 1999 and early 2000's	Epidemic WNV introduced from Europe or Africa, associated with high neuroinvasive potential in mice, currently extinct in U.S	Lanciotti *Science* 1999,
North American/West Nile 2002 (WN02)	Texas from 2001-2003	Evolved from and displaced NY99, signature mutation is E-V159A, said to be enable more rapid transmission in mosquitoes than NY99, still circulates today	Beasley *Virology* 2003; Davis *Virology* 2005
Southwest/West Nile 2003 (SW03)	Arizona and Mexico in 2003	Evolved from WN02 strains, defined by E-V159A and NS4A-A85T or NS5-K314R, along with WN02 still circulates today	McMullen *Emerg Infect Dis* 2011
Southeast Coastal Texas	Houston, Texas in 2002	Attenuated genotype that is believed to have become extinct after two years, associated with reduced neuro-invasinesness in mice	Davis *Virology* 2004

geographic area suggested that closely related genetic variants were spread over the Atlantic, Mississippi and Central bird flyways, but not the Pacific. WNV isolates from California and Arizona, located along the Pacific-bird flyway, were distinct from this

pattern and California and Arizona specimens clustered only with local circulating clades (Grinev *Plos Negl. Trop. Dis.* 2016). Moreover, positively-selected mutations were identified in Groups 9 and 10 (**Figure 13**) (Duggal *Am. J. Trop. Med. Hyg.* 2013, Grinev *Plos Negl. Trop. Dis.* 2016). In Texas, several studies identified the introduction of new WNV strains into multiple counties, namely Dallas Co., Collin Co., Montgomery Co., and Harris Co. (Duggal *Am. J. Trop. Med. Hyg.* 2013, Mann *Emerg. Infect. Dis.* 2013). Group 8, was also newly introduced into Texas but was only identified in Harris Co. within Texas. Group 8 WNV is defined by the NS3-E180D mutation, and it lacks other mutations predicted to be undergoing positive selection (**Figure 13**). Prior to the introduction of these groups of viruses into Texas in 2008-2011, the average number of human cases in Texas was 73 but after their introduction in 2013-2015 the average number of cases in Texas increased three-fold to 279, despite similar temperatures (**Figure 14A-14B)**. To understand the impact of these newly introduced WNV in human disease in Texas we collected and sequenced 74 WNV environmental isolates from multiple counties in Texas from 2012-2015.

The summary of our sequencing data is shown (in **Figure 15)** using a phylogenetic tree generated by neighbor joining and 10,000 bootstrapping method. In this analysis, we also included all previously published groups of Texas WNV (groups 1-7) and all 2012 WNV sequences. Our phylogenetic analysis reproduced previous reports suggesting that newly introduced Groups 8, 9, and 10 are independent clusters distinct from local circulating clades in Texas (Groups 1-7) (Duggal *Am. J. Trop. Med. Hyg.* 2013, Mann *Emerg. Infect. Dis.* 2013). Between 2012-2015 Group 9 and 10 isolates in

Texas continuously retained a subset of positively-selected mutations described previously, an NS2A mutation (L95F) for Group 9 and two mutations (NS2A-R188K and NS4B-I240M) for Group 10 WNV (**Figure 13**).

In addition to these previously identified Groups, we identified a novel Group of WNV in Dallas and Denton Co. Texas which we called Group 11. These strains were strongly related to one isolated strain identified in Harris Co. in 2012 (Mann *Emerg. Infect. Dis*. 2013) with 100% bootstrap support and the entire cluster was moderately related (78% bootstrap support) to a 2008 New York isolate (**Figure 15B**). Three non-synonymous mutations distinguished Group 11 strains (E-A51T, NS4B-N26T, and NS5-H42Y) from other groups but none were in positions known to be undergoing positive selection (**Figure 13**). Thus, our results suggest that at least four groups of WNV were introduced into Texas by 2012, two groups with (Groups 9 and 10) and two groups without (Groups 8 and 11) positively-selected mutations.

WNV Group 9 and 10 are widely circulated and persisted continually in Texas by overwintering

Groups 9 and 10 are associated with positively-selected mutations but their function are unknown (Grinev *Plos Negl. Trop. Dis*. 2016). To determine the effect of positively-selected mutations in circulation and persistence of WNV, we analyzed the distribution of Texas WNV from 2012 to 2015. Our results showed striking differences in the circulation and persistence of WNV with and without positively-selected mutations. In 2012, our sequences and those of others suggested that Groups 9 and 10 had wide

circulation in Texas as being present in nearly every county sampled (**Figure 16A-16B**). This was also consistent with the wide distribution of Group 9 and 10 nation-wide (Grinev *Plos Negl. Trop. Dis.* 2016). In contrast Group 8 and 11 WNV had circulation only in Harris County, and were not identified in adjacent counties such as Montgomery County (**Figure 16A**).

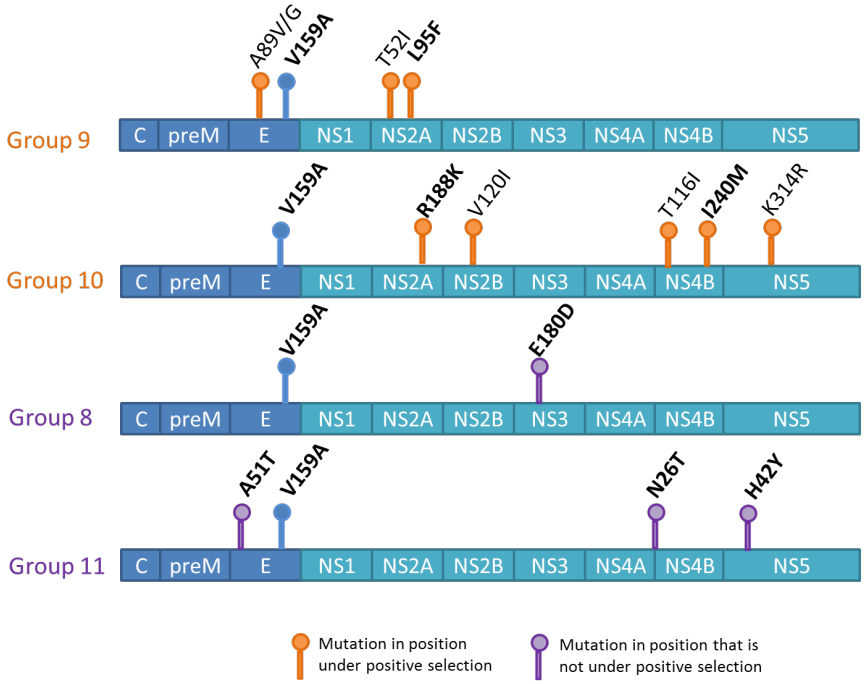

Figure 13: Shows the positions of mutations that characterize groups of WNV with positively-selected mutations (groups 9 and 10) and those without (groups 8 and 11). In Bold are mutations that were regularly identified in Texas WNV.

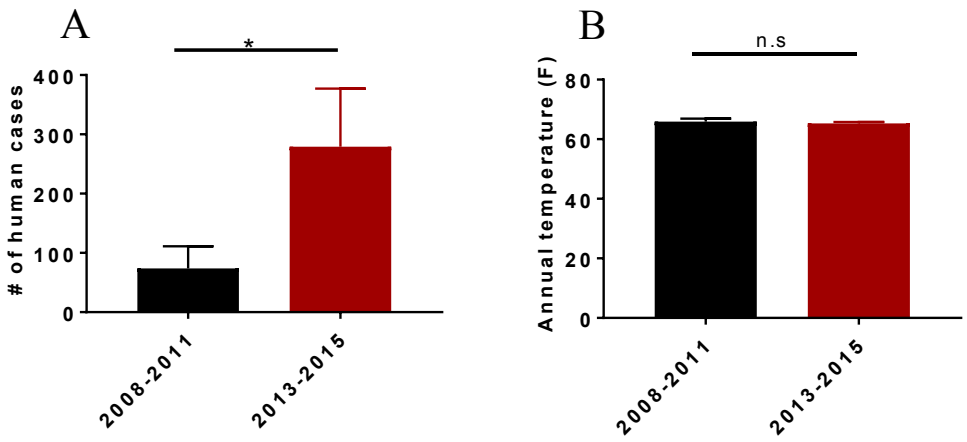

Figure 14: (A) Shows a 3.7-fold increase in the incidence of human West Nile disease cases in Texas after the outbreak than before the outbreak. This increases was independent of differences in temperature (B). "*"= $P<0.05$ as determined by student t-test. Error bars=Standard deviation

One potential effect of adaptive mutations is to increase the likelihood of perpetuation in different transmission cycles (Pesko and Ebel *Infect Genet Evol* 2012) which would allow viruses to overwinter. To determine the ability of these groups to persist in Texas we analyzed WNV from 2013-2015. Group 9 and 10 were identified in all three years, and Group 10 WNV showed better persistence than Group 9, indicating that specific mutations in Group 10 are important for persisting in the Texas environment (**Figure 16A**). In contrast, Group 8 and 11 isolates were not identified in any county in Texas sampled in 2014 (**Figure 17B**) including Dallas Co. where these isolates were most frequent in 2013 (**Figure 17B**), suggesting that Group 8 and 11 have a reduced ability to overwinter.

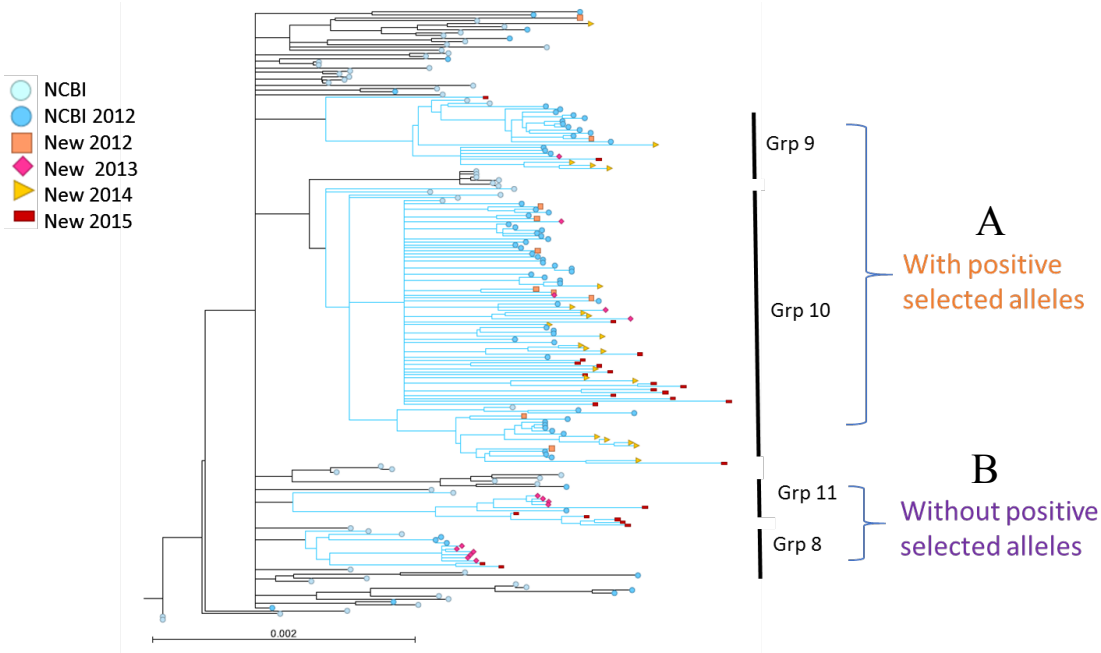

Figure 15: Phylogenetic tree summarizing sequencing data from over 70 WNV isolates collected from Texas 2012-2015. Tree was generated with neighbor-joining and 10,000 bootstrap methods, shown here is the resulting tree with a 50% bootstrap threshold. Blue colored branches identify the clades of WNV strains (A) carrying positively selected alleles and (B) shows groups without positively-selected mutations. Local strains of WNV previously identified in Texas, including groups 1-7, are depicted with black colored branches.

Over-wintering strains should accumulate mutations over time and thus should increase the nucleotide diversity. To examine the nucleotide diversity of Group 9 and 10 strains, we identified counties where at least two representative isolates were found in multiple years. In both Dallas and Montgomery Co., we observed significant increases in the mean pairwise diversity of Group 10 WNV over time. Between 2012 and 2015, nucleotide diversity of Group 10 isolates increased two-fold in Dallas Co. (**Figure 18A**) and four-fold in Montgomery Co. (**Figure 18B**). These results are consistent with Group 10 strains persisting by overwintering. Taken together, these results suggest that WNV

groups with positively-selected mutations had better circulation and increased likelihood of over-wintering.

Group 8 and 11 WNV were repeatedly introduced into Texas between 2012-2015

In contrast to the persistence of Group 10 strains over multiple years at multiple sites in Texas, WNV strains without positively-selected mutations were not detected in 2014 but were found again in 2015. The return of these strains may reflect a re-introduction event from a distal reservoir via ongoing bird migration. We expect that newly introduced WNV should show temporal clustering that is distinct from previous years. Phylogenetic analysis could provide evidence distinguishing re-introduction from over-wintering, thus we would expect newly introduced WNV strains to be closely related and have low nucleotide divergence, while over-wintering strains should show no temporal clustering, intermixed with previous years and more heterogeneous with high nucleotide diversity.

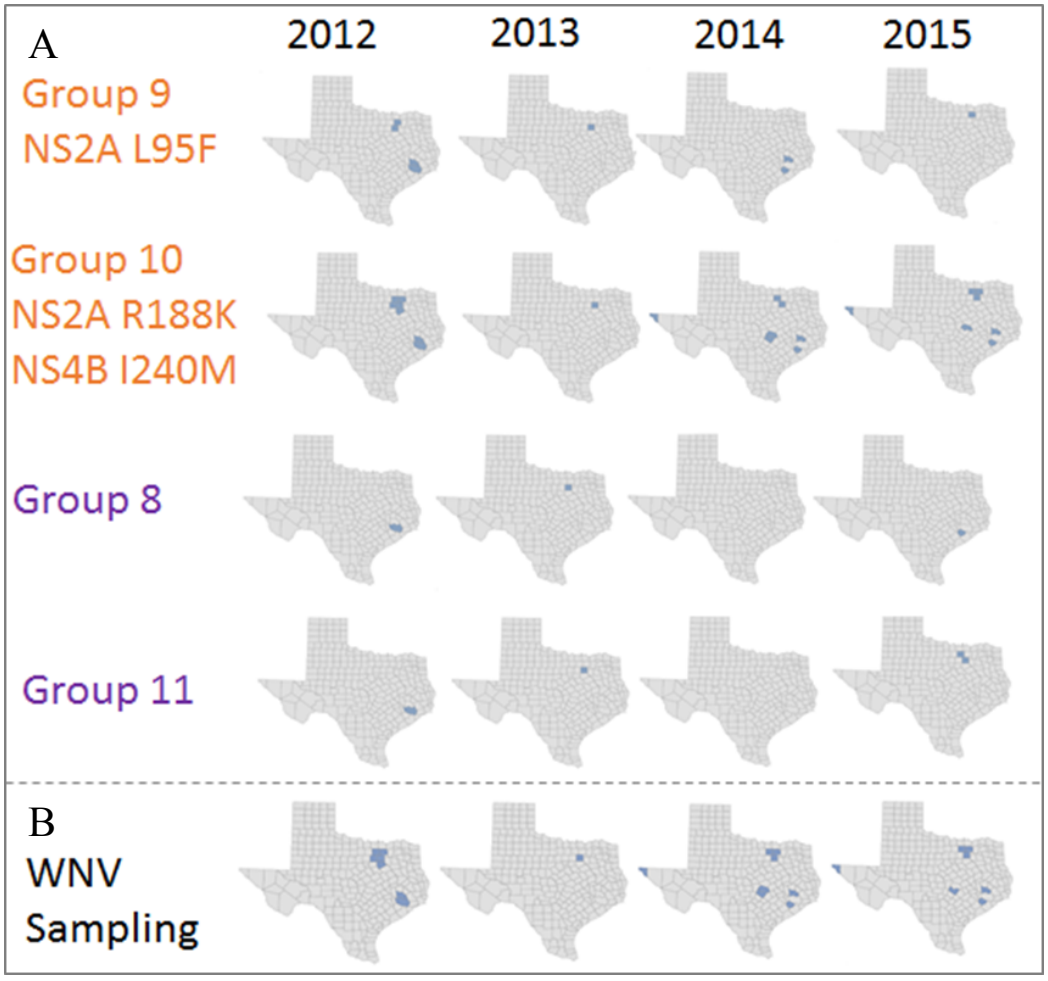

Figure 16: (A) Shows the counties in which isolates of different WNV groups were identified from 2012-2015. Under groups 9 and 10 are the positively-selected mutations found in all isolates of the group which may mediate their respective phenotypes. (B) shows the Texas counties that were sampled. The data for 2012 includes the results of previous reports but 2013-2015 sequencing are all newly done in this study.

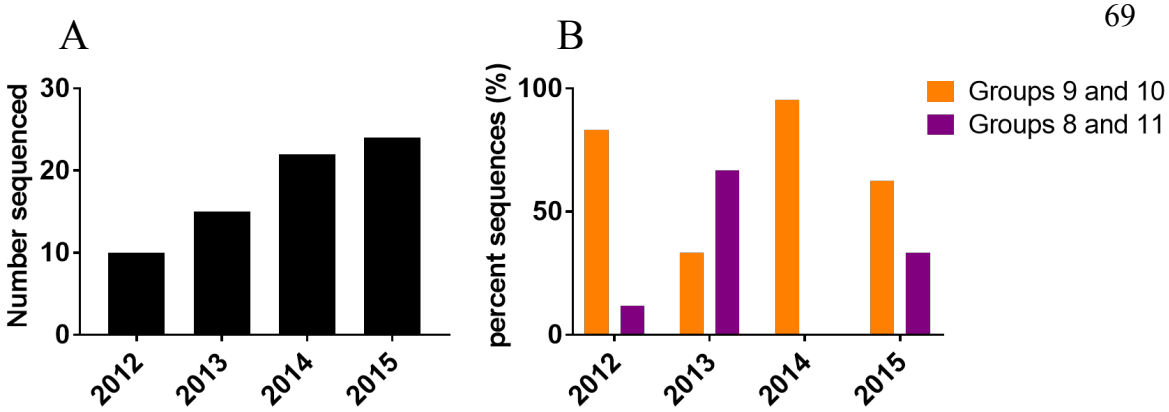

Figure 17: (A) Shows the number of WNV samples from Texas successfully sequenced in each year and (B) shows the frequency of those isolates that belong to WNV groups with (groups 9 and 10) and without (groups 8 and 11) positively selected mutations.

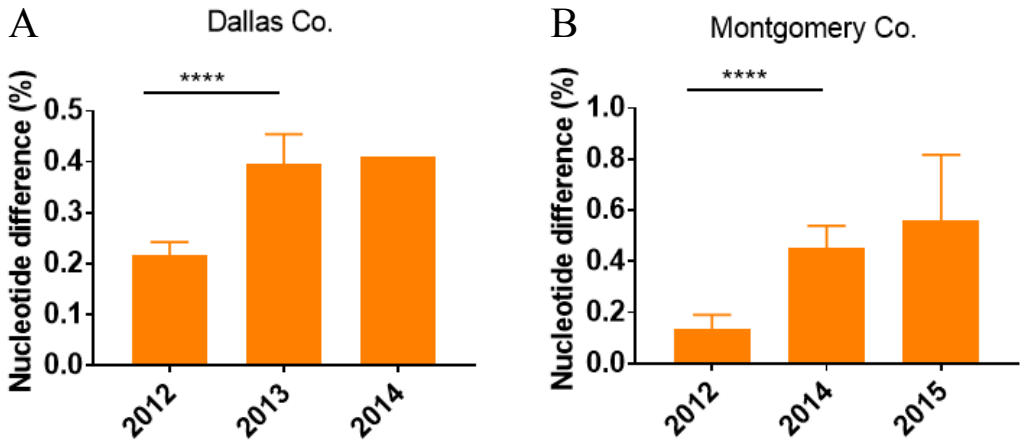

Figure 18: (A) Shows increasing nucleotide divergence over time in group 10 WNV found in Dallas (A) and Montgomery counties (B). "****" = P<0.0001 as determined by student-t test analysis. Error bars=standard deviation.

Phylogenetic analyses showed that Group 10 WNV from 2012-2015 were intermixed with no temporal clustering (**Figure 19A**). In contrast Group 8 and Group 11 showed evidence of well supported (>90% bootstrap support) temporal clustering in 2013 and 2015 (**Figure 19B**). In 2015, we identified one Group 11 isolate that clustered with

2012 and 2013 strains but most (5/6) clustered distinctly. We next measured the mean pairwise diversity in the various groups within the same county to normalize for potential impact of different ecologies in different counties (Duggal *Am. J. Trop. Med. Hyg.* 2013). Consistently, we observed high nucleotide diversity in Group 10 WNV but low nucleotide diversity in matched Groups 8 and 11 WNV (**Figure 20A-20B**). The low nucleotide diversity in Group 8 and 11 WNV in 2012, 2013, and 2015 indicate that these isolates represent distinct introduction events.

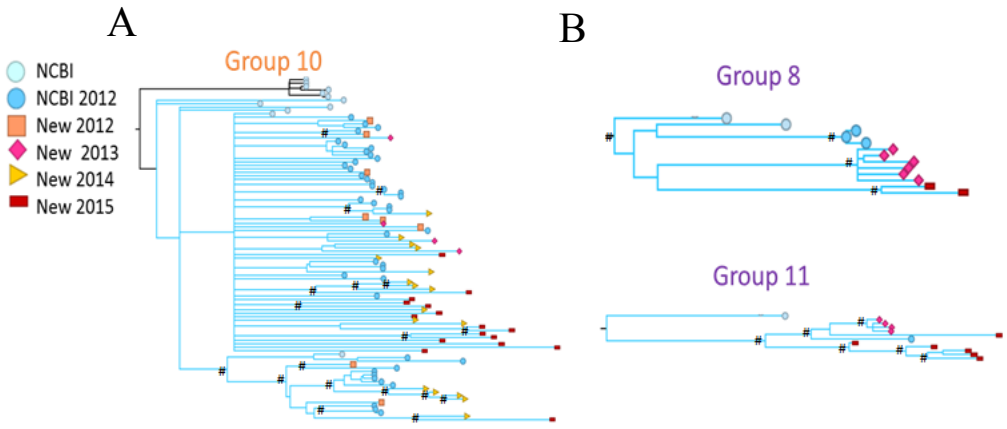

Figure 19: (A) Shows sub-clades of group 10 WNV from our sequencing and those of others from 2012-2015, there was no temporal clustering present. (B) Shows Group 8 and 11 WNV from 2012-2015 where isolates clustered temporally as indicative of new introductions. "#" identifies nodes with over 90% bootstrap support.

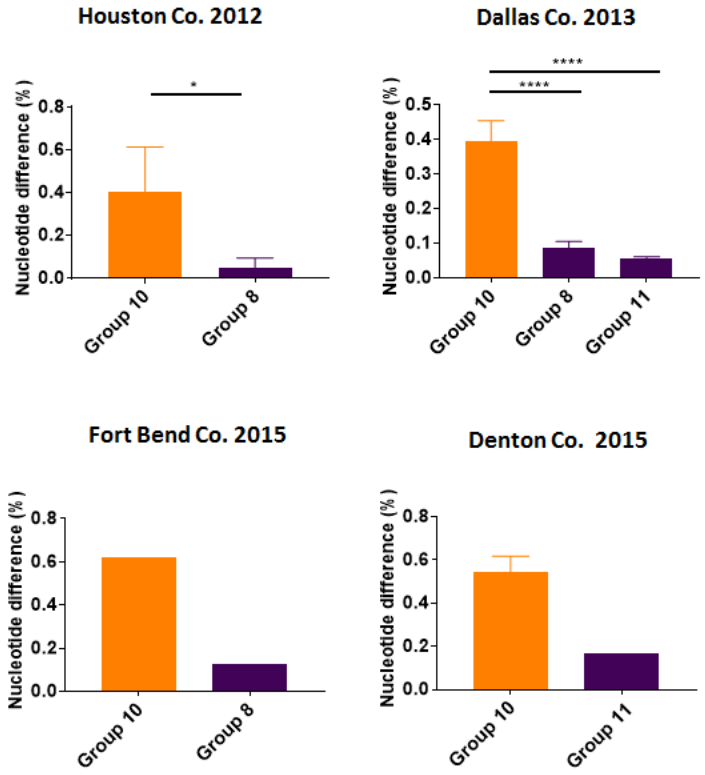

Figure 20: (A) Shows comparisons of the nucleotide divergence of group 10 strains versus those of groups 8 or 11 matched for time and place. Statistical analysis could not be done for 2015 (B) due to lack of sufficient samples in one or both groups, however the pattern is similar as in previous years. "*"= $p<0.05$ and "****"= $p<0.0001$ as determined by student t-test. Error bar= standard deviation.

Next, we tried to determine the potential source for these incoming strains. Since all 2012 WNV were associated with northeast strains, we added a 2008-2015 dataset of WNV from New York to our phylogenetic analysis in an attempt to find more recent sources than the 2006-2008 strains identified in prior studies (Mann *Emerg. Infect. Dis.* 2013). We observed that isolates from New York were present in all Groups except for Group 11. In Group 8, we found that 2011 and 2013 isolates from New York were more closely related (with over 90% bootstrap support) to Texas 2013 and 2015 WNV than to

Texas strains of the preceding years (**Figure 21A**). If Texas WNV in 2013 had a source in or near New York, there would be synonymous mutations shared with New York strains and not present in prior Texas strains. When we compared the mutations in New York, Harris Co. Texas, and Dallas Co. Texas, we identified nine synonymous mutations shared between New York and Dallas Co. Group 8 WNV that was not present in Harris Co (**Figure 21B**). We also identified unique mutations in 2011 New York and 2013 Dallas Co. (**Figure 21B**) Group 8 indicating that the divergence of these two populations from their most recent common ancestor preceded 2011. Taken together our results suggest that Groups 8 and 11 WNV were repeatedly introduced into Texas, perhaps from strains in the Northeast, while Group 10 persisted after a single introduction.

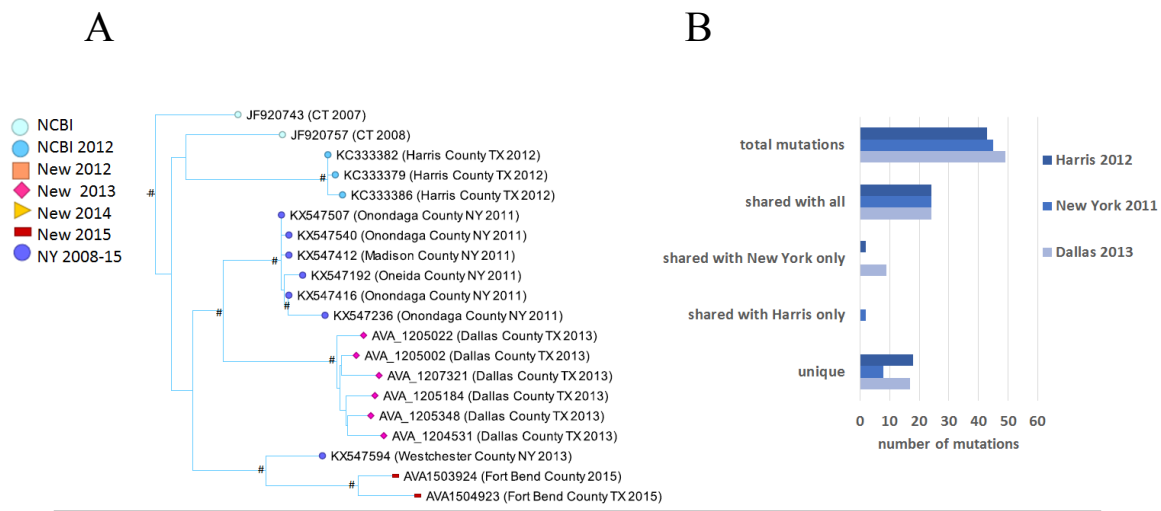

Figure 21: (A) Shows several group 8 isolates from New York that are more related to Texas group 8 strains in 2013 and 2015 than strains from previous years. (B) Shows comparisons of synonymous and non-synonymous mutations across three populations of group 8 WNV. "#" indicates nodes with >90% bootstrap support.

Group 8 and 10 WNV show differential ability to replicate and induce type 1 interferon response than NY99 in human myeloid cells

Our sequencing analysis suggested that Group 8 strains have a reduced ability to persist in the environment. We next expanded four Group 10 isolates and one Group 8 isolate, and compared their replication in non-immune cell lines and primary human myeloid cells.

We first performed multi-step growth curves in Vero and QT6, a quail cell line, and we observed no differences in the replication comparing Group 8 to Group 10 WNV or to a reference strain used in previous studies (Keller *J. Virol.* 2006) (**Figure 22A-22B**). Next, we infected human monocyte-derived macrophage (MDMs) and monocyte-derived dendritic cells (MDDCs) which mimic key innate immune cells that would respond to WNV infection in humans. At 4 hours post infection (h.p.i), we observed low but equal amounts of WNV RNA for all strains indicating comparable multiplicity of infection (m.o.i). At 17 h.p.i, we observed a strong increase in WNV RNA and increased expression of IFNB and CXCL10 in both MDMs and MDDCs (**Figure 23A-23B**). Interestingly, we observed that Group 10 isolates (8920 and 7393) showed 3-fold increase in viral RNA replication in MDMs. These two Group 10 isolates carry the same positively-selected mutations (NS2A-R188K and NS4B-I2513M) except for one differentiating mutation in the NS1 region. Both Group 10 isolates induced increased IFN mRNA expression compared to NY99 strain by 24 h, but one isolate induced decreased CXCL10 (an ISG) expression (**Figure 23A**). The same ability to suppress the induction of CXCL10 was shared by Group 8 WNV (5587), and the replicative advantage of both

positively selected WNV was not observed with the DC infection (**Figure 23B**). Together, these data suggest that both Group 8 and 10 WNV are fit and not intrinsically attenuated which is often associated with reduced viral replication and increased induction of IFNs of interferons and ISGs (Daffis *J. Virol.* 2011).

DISCUSSION:

The WNV population is predominantly under purifying selection indicating that non-synonymous mutations tend to be deleterious (Pesko and Ebel *Infect. Genet. Evol.* 2012; Grinev *Plos Negl. Trop. Dis.* 2016). However, a few examples of adaptive mutations have been described and associated with specific outbreaks of human disease. The most recent outbreak of WNV was 2012 which was associated with the broad geographic spread of Group 9 and Group 10 WNV. The role of positively-selected mutations identified in these groups is unknown and their contribution to human disease is unclear (Duggal *Am. J. Trop. Med. Hyg.* 2013, Grinev *Plos Negl. Trop. Dis.* 2016). Three groups of WNV (8,9, and 10) were previously reported to be newly introduced into Texas (Mann *Emerg. Infect. Dis.* 2013). We identified here a new invading cluster of WNV that we called Group 11. To gain insight into the effect of positively-selected mutations, we compared WNV groups, with and without mutations undergoing positive selection.

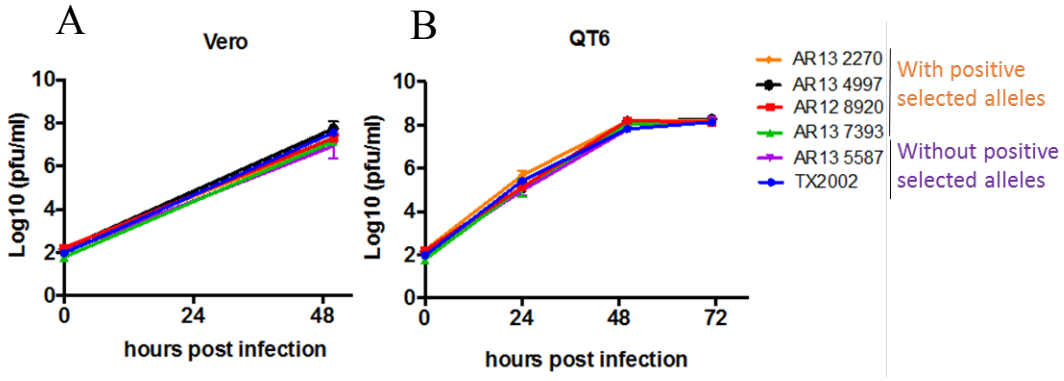

Figure 22: Environmental isolates were expanded twice in Vero cells to generate high titre stocks (>10^7 pfu/ml). Subsequently we identified isolates belonging to group 10 and group 8 WNV (AR13 5587) through sequencing. These in addition to TX2002, a standard reference strain for WN02 genotype, was used to infect Vero cells (A) and a quail cell-line, QT6 (B), at moi of 0.1. Media was collected over two to three days and frozen at -80. After the experiment, plaque assays were used to measure viral replication.

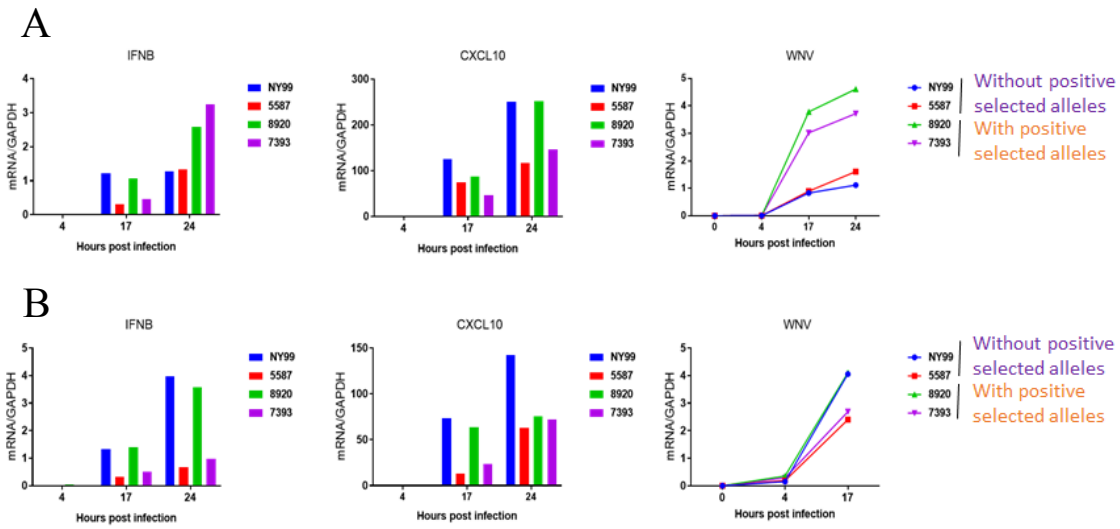

Figure 23: WNV group 10 isolates (8920 and 7393) and WNV group 8 isolate (5587) were used to infect human primary myeloid cells and RNA was collected at indicated timepoints to measure immune response and viral RNA in monocyte-derived macrophages (MDMs) (A) or monocyte-derived dendritic cells (MDDC) (B).

From 2012-2015, WNV Group 10 showed the widest circulation and were found in nearly every county sampled from 2012-2015 in both our sequencing and that of other researchers. Group 9 WNV also had wide circulation, but only in 2012. In contrast, both groups 8 and 11had limited circulation when identified. The circulation of these strains in Texas is consistent with their circulation nationally (**Figure 24**). Group 9 and Group 10 WNV were identified in multiple Northeastern, Midwestern, and Southern States while Group 8 and Group 11 WNV were only identified in Texas (Grinev *Plos Negl. Trop. Dis.* 2016). The broad distribution of positively selected WNV suggests that these mutations may impact their ability to be transmitted in birds along the Atlantic, Mississippi, and Central bird flyways. Consistently, a recent article showed that amongst several WNV tested from East Coast, WN02, and SW03 genotypes (**Table 4**), two isolates both bearing NS2A-R188K mutations induced the highest viremia in House Sparrows (Duggal *Plos Negl. Trop. Dis.* 2014), a resident bird population competent for WNV, which may promote their rapid spread.

We also observed that Group 10 WNV, more than any other group, persisted throughout the years sampled (**Figure 24**). This persistence is most likely due to overwintering for two reasons: first, there was no evidence of temporal clustering that indicate new introduction events; Secondly, we detected evidence of genetic drift in Group 10 isolates over time in two counties. In contrast, Group 8 and Group 11 did not persist in Texas but were repeatedly introduced at least on three occasions between 2012-2015. This is also supported by the low diversity of incoming strains and the temporal clustering of new strains. We also saw that the potential source of these incoming Group

8 strains in 2013 and 2015 may be in the Northeast, perhaps in New York. We did not find strains related to Group 11 WNV amongst the 2008-2015 New York isolates; perhaps more sampling of other states in the northeast is needed. These differences between Group 9/10 and Groups 8/11 suggest that positively-selected mutations may promote rapid spread, increased likelihood for persistence, but also hinders regular transmission. These observations are consistent with reports showing the NS2A-R188K mutation associated with significantly increased mortality in inoculated house sparrows (Duggal *Plos Negl. Trop. Dis.* 2014). If Group 10 WNV acquire high toxicity in resident birds typically needed for transmission, this would prevent subsequent transmissions by decreasing bird population or else inducing potent immune responses in surviving hosts that might impede subsequent infection. Further studies are needed to determine the specific role of NS2A-R188K mutation on WNV pathogenesis in birds as have been done with NS3-T249P mutation which increases mortality of WNV in American crows (Brault *Nat. Genet.* 2007).

The role of viral genetic mutations on human disease outbreaks is unclear. Prior studies detected no association between genetic factors and magnitude of disease in 2012 (Duggal *Am. J. Trop. Med. Hyg.* 2013, Grinev *Plos Negl. Trop. Dis.* 2016). Instead it was suggested that environmental factors such as higher than average temperatures played a major role (Duggal *Am. J. Trop. Med. Hyg.* 2013). Our study adds insight by showing evidence of the potential contribution of viral genetic mutations. By considering the interplay of temperature and presence of WNV with positively-selected mutations, we could see a three-fold increase in human cases in Texas from 2013-2016 compared to

2008-2011 despite similar annual temperatures. Our sequencing data suggested only Group 10 WNV had both wide circulation and persistence throughout Texas. Since Group 10 WNV were not detected in Texas prior to the 2012 outbreak, their presence after the outbreak might explain the increased number of cases. While the role of temperature is appreciated in WNV epidemiology, our study suggests that viral genetic mutations also contribute to disease outbreaks. However, the absence of any major differences between these groups when replicating in human cells indicates that increases of human disease is not directly caused by greater virulence of Group 10 WNV but instead on its wider circulation (**Figure 24**).

Lastly, we discovered the presence of the same mutations (NS3-R493G, NS3-I494T, Y27H) emerging independently in multiple Groups. Initially they were found in Group 9 and 10 isolates in 2014, and later they emerged in groups 8 and 11 in 2015 (**Table 5**). These mutations were well supported with high coverage, and blasting a 20-base sequence selection containing the various mutations found no identical WNV match in the NCBI database. Given the suggested novelty of these mutations and their independent evolution in multiple lineages it will be interesting to see whether they are retained in sequences of future TX WNV, as well as to determine whether they may confer adaptive advantages. Sequencing of contemporary WNV in other states would also be necessary to determine if this mutation is specific to Texas, and if so what might be driving its selection.

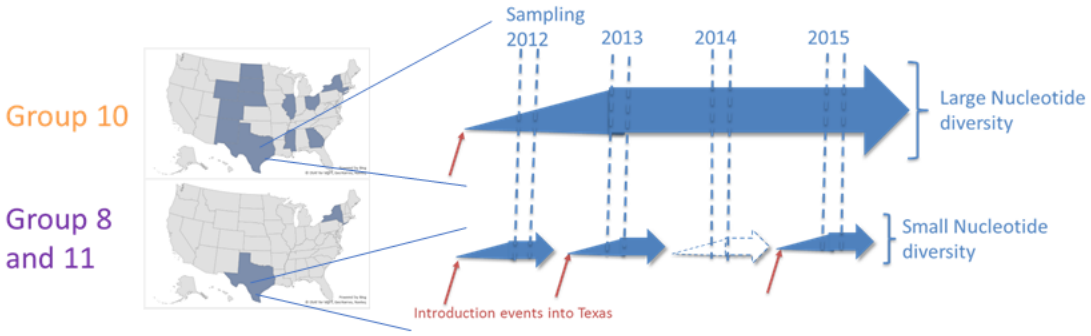

Figure 24: Graphical summary of the study showing that positive selected mutations in Group 10 are associated with increased circulation and persistence by overwintering, which may promote human disease.

Table 5: Novel mutations common to Texas WNV

Year	Group	NS3-R493G (G->A pos. 6089)	NS3-I494T (T->C, pos. 6092)	NS5-Y27H (T->C, pos. 7759)
2014	9	3/4	3/4	1/4
2014	10	9/16	8/16	14/16
2015	8	1/2	1/2	2/2
2015	10	11/16	11/16	15/16
2015	11	2/6	2/6	5/6

Shows number of isolates of each group and year that have each mutation of interest.

CHAPTER SIX: CONCLUSION AND PERSPECTIVES

Toward better therapeutics for HIV

Though no vaccine is yet approved for HIV-1, the widespread use of ARTs has halted and begun to reverse the global spread of HIV-1. According to the world health organization, between 2000-2014 the number of new infections dropped by 35% to 2.1 million and the number of HIV-1 related death fell by 24% to 1.1 million. These statistics reflect the tremendous success but also the persistent challenges to current anti-retroviral treatments.

Despite their success, the current ARTs have at least three limitations. The first is the short-lasting nature requiring the daily treatments. Secondly, the high adherence required of patients to be maintained for the rest of their lives to remain healthy. The last limitation is the presence of drug resistance mutations in patients necessitates the development of new drugs thus over 40 ARTs have been approved for HIV (FDA.org). These limitations indicated the need for long-acting ARTs.

Attempts to address the problems of the current therapies are being made with the development of long-acting ARTs. These are formulations of poorly soluble compounds formulated with crystalline nanoparticles with high enough potency that slow release can still confer protection. Due to these dosing requirements and other physicochemical properties most current once-daily ARTs cannot be reformulated into long acting ARTs (Spreen *Curr Opin HIV AIDS* 2013) necessitating a long and costly process of developing new ARTs suitable for formulation as long-acting ARTs. Currently only two drugs show promise as potential long-acting ARTs, a reverse transcriptase inhibitor (TMC278) and

an integrase inhibitor (GSK1265744). These are currently past phase I trials which suggested that these long acting ARTs can be used once a month. Late stage trials are yet ongoing but results presented at CROI 2016 appear promising (Aidsmap.com). Though these ARTs would help address the problem of patient adherence if approved; the cost of such treatments (83% increase over first line ART regimen), the threat of viral drug resistance, and the need for developing more long-acting ARTs remain considerable challenges (Ross *Clin. Infect. Dis.* 2015).

An important goal in long-acting anti-retrovirals development is for use as a pre-exposure prophylaxis (PreP) (Boffito *Drugs* 2014). In my dissertation, I showed that cGAMP nanoparticle induces robust and long-acting inhibition of viral replication dependent on monocyte derived type 1 IFNs (**Figure 25**). These results were the first demonstration of the potential of cGAMP to be used as an anti-retroviral. Nonetheless, several questions remain to be addressed in future studies. Firstly, the dependence of cGAMP-induced protection on IFN raises potential challenges when proposing cGAMP for prophylactic use. One recognized deficiency of IFN treatment is its associated toxicity. Interestingly, observations from cancer studies suggest that the use of agonists may be more advantageous than recombinant IFN protein. TLR agonists are associated with mild toxicity and sustained efficacy in different cancer settings such as brain neoplasms (Aranda *Oncoimmunology* 2014). The increased efficacy of PRR agonists may be due to improved pharmacokinetics or their ability to induce other immunoregulatory cytokines (Parker *Nat Rev Cancer* 2016). I demonstrated that cGAMP does not induce robust toxicity profile. Given the ability of IRF3 to induce IFN-independent ISGs in

addition to type 1 interferons there might be differences in the profile of immune responses triggered by cGAMP versus recombinant IFN. To test this, RNAseq should be run on cGAMP and IFN treated cells. Additionally, comparisons between cGAMP and recombinant IFN induced toxicity are needed in vivo. These studies might be done in naïve mice treated with cGAMP and IFN. Measurements of weight and cell populations in the blood can be used to detect anorexia and lymphopenia which are commonly associated with IFN toxicity (Parker *Nat Rev Cancer* 2016).

Using a spreading assay with half of the cells already infected, we demonstrated that cGAMP treatment prevents the spreading of HIV to naïve cells. It remains to be shown, what part of the viral lifecycle this block, or blocks, might take place. Another question that needs to be addressed is whether cGAMP pretreatment renders cells non-permissive to direct viral infection from free floating virions. If so, how long does this protection last? This question can be addressed in vitro by pretreating naïve cells and then infecting them with HIV. Measurements can be done to quantify effects of treatments in viral DNA in the cytosol or integrated into host genome. It would also be important to show cGAMP mediated protection from HIV infection in vivo. This might be done in humanized mice pretreated with cGAMP prophylactically and then infected with HIV (Denton *Plos Med.* 2008). This study would address the question of whether cGAMP inhibits infection. It will also address the question of how cGAMP protection compares with anti-retrovirals in a prophylactic model. With these and other studies, cGAMP can be demonstrated to be a great candidate for long-acting PreP.

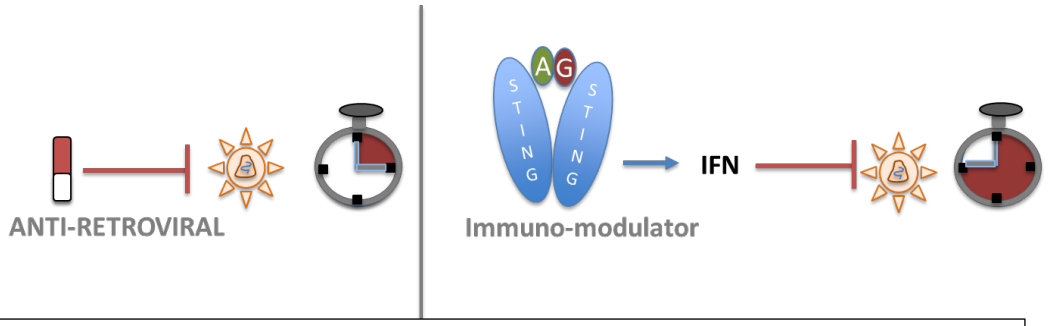

Figure 25: A Graphical summary of data from HIV studies

Toward better therapeutics for Flaviviruses

WNV is a member of the *Flaviviridae* family that includes important human pathogens such as Japanese Encephalitis Virus (JEV), St Louis Encephalitis Virus (SLEV), Dengue Virus, and Zika Virus. Flaviviruses are important emerging diseases causing several hundred million infections yearly. For instance, it is estimated that Dengue causes over 100 million infections each year (Bhatt *Nature* 2013). Additionally, flaviviruses have recently had a massive increase in global distribution exposing emerging viruses to naïve and susceptible populations. For instance, in 1999 WNV was introduced to Western Hemisphere and in 2002-2003 caused the largest outbreak of West Nile disease (total of 14,000 cases in two years). Similarly, in 2013 Zika virus was introduced into Brazil and in 2015 caused an unprecedented outbreak with over 30,000 cases (Rodrigues Faria *Science* 2016). Currently, there are no specific antivirals for flaviviruses and no approved human vaccines for WNV and Zika (Cohen *Science* 2016). Given the annual burden of flaviviruses and the increasing global spread of once geographically isolated strains, there is an escalating need to understand the interplay of

viral evolution and human disease. Such insights may help overcome current challenges to vaccine development.

In the case of WNV, a major challenge to the development of specific therapeutics is finding enough patients with neuro-invasive disease to hold clinical trials (Gray *Intern J Gen Med* 2014). Apart from an outbreak year, the number of patients with neuro-invasive disease is insufficient to hold clinical trials even at multiple sites. A recent instance of this occurred with a WNV neutralizing antibody (MGAWN1) with promising preclinical results. One treatment with MGAWN1 protected 90% of infected mice even when given five days after infection (Oliphant *Nat Med* 2005, Diamond *Antivir Res* 2009). After completing safety trials in 2008 the sponsoring company, MacroGenics, initiated a phase 2 trial in 2009 but terminated the trial in 2010 because of insufficient patient number (13 recruited out of 120) (Clinicaltrial.gov). Unfortunately, the second largest outbreak of WNV disease occurred in 2012. Thus, a major hindrance to vaccine development is the current inability to predict outbreaks in advance.

Finding predictors of WNV outbreaks has been an area of much interest since it might help public health officials efficiently mobilize interventions (DeFelice *Nat Comm* 2017). Several attempts (Shaman *J Med Entmol* 2005, Ruiz 2010, Reisen *J Med Entmol* 2006) have been made based on mathematical modeling of diverse factors but these efforts are hindered by the complex interplay of environmental, anthropic, and viral factors required for successful transmission (Reisen *Annu. Rev. Entomol* 2010). An important indicator of outbreaks of human disease is mosquito vector index. Mosquito vector index is an accurate measurement that factors in the abundance of mosquitoes and

the percent of these infected, thus the higher the index the higher the risk of spillover into humans (Chung *JAMA* 2013). Currently, in Dallas County a vector index over 0.5 in June or July is used as a predictor of an epidemic. However, the forewarning given by the vector index is only predictive of the same transmission season. More recent efforts based on mosquito vector index can give an additional 6-9 weeks of forewarning (DeFelice *Nat. Comm.* 2017), which is still not enough time to begin the process of starting a clinical trial.

A better strategy may be to identify WNV positively-selected mutations that mediate increased high incidences of human disease. Comparing incidences of human neuro-invasive disease in multiple states before and after the 2012 outbreak suggested a role for positive-selected mutations in human disease. Temperature is thought to have played a dominant role in the size of WNV outbreak for instance the 2012 outbreak was ascribed to above average temperatures (3^{o}C over annual average) (Duggal *Am. J. Trop. Med. Hyg.* 2013). In 2013-2014, temperatures in the U.S dropped to similar levels as in 2008-2011. Interestingly, despite similar temperatures before and after the outbreak there was increased incidence of disease in the U.S. In my dissertation, I showed that in Texas this was mainly due to the introduction and persistence of Group 10 WNV. Group 10 WNV were the most frequently identified strains throughout multiple Mid-western and Southern states in 2012, so it may also be the cause for the overall increase of cases nationally from 2013-2015 (Grinev *Plos Negl. Trop. Dis.* 2016). However, this remains to be confirmed with new sequencing in other states. If Group 10 were also found to be more persistent in other states, this would support a model in which both viral genetics

and high temperature can contribute to human disease (**Figure 26**). If true, the detection of well-characterized positively selected alleles in circulating WNV can be a useful warning of increased incidence of disease in the following years.

Much is still unknown about positively selected alleles, so it remains necessary to continue surveillance studies in a similar pattern as was used in this dissertation (**Figure 27**). Next generation sequencing technologies now make it possible to sequence large numbers (>60) of WNV genomes in a single run. The relative ease with which WNV genomes can be sequenced should increase our ability to track the circulation of WNV strains in the U.S. Such studies are important because the ability of WNV to circulate widely is often correlated with large outbreaks. For instance, both the 2003 and 2012 outbreaks were associated with the widespread distribution of WNV with positively selected mutations. Interestingly, my study suggests the circulation of WNV with positively selected alleles does not occur repeatedly; the cause of this pattern remains unclear.

Birds are often implicated as the mediators of WNV spread. WNV infects over 300 species of birds but it remains unclear which species of birds are most important for WNV circulation. We observed that WNV without positively-selected mutations were repeatedly introduced into Texas from the Northeast perhaps by birds. If this inference is true, there might be traceable routes for the spreading of these viruses across multiple states. By identifying routes of WNV circulation and correlating them to migratory bird routes may help identify the bird species mediating WNV transmission. Continued

surveillance of WNV in multiple states is needed to shed light on these important questions.

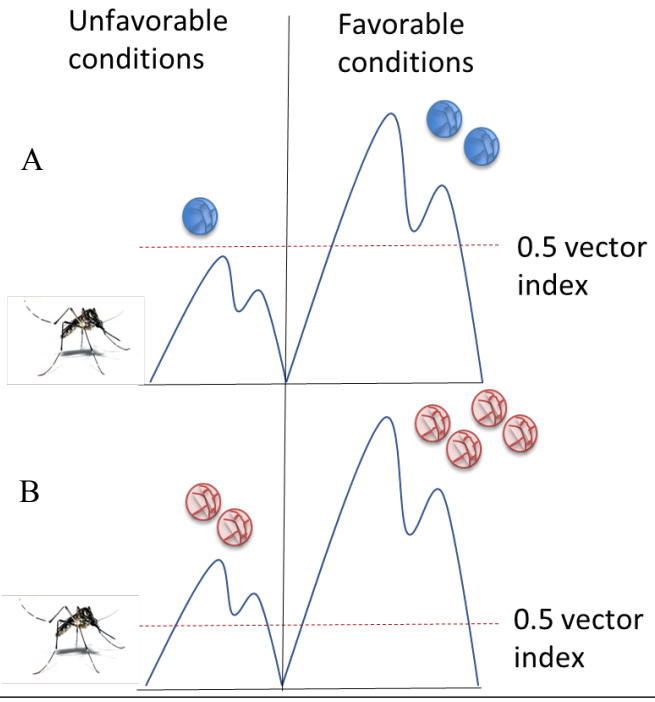

Figure 26: My model on the contribution of viral genetics to magnitude of disease, with (A) indicating a virus without adaptive alleles and (B) depicting WNV with adaptive alleles. Blue lines indicate mosquito vector index, which is predictive of outbreak size if above 0.5. Favorable conditions can refer to higher than average temperatures or other environmental conditions.

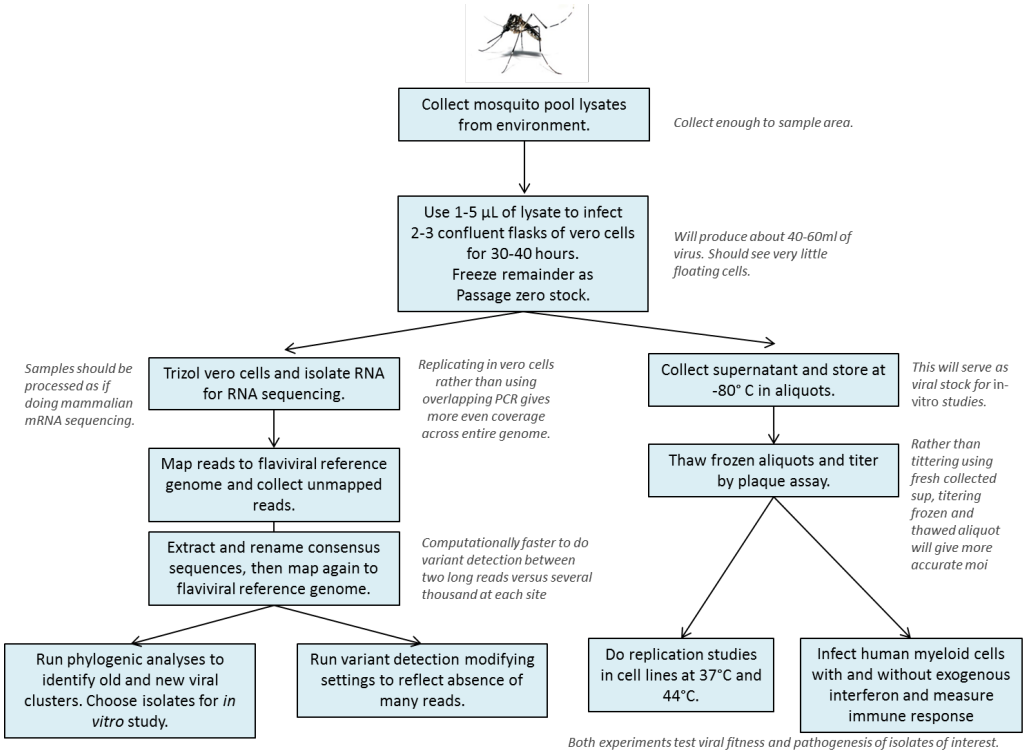

Figure 27: Simplified pipeline for genetic and phenotypic surveillance of mosquito-borne pathogens

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