UNIVERSITY OF CALIFORNIA, SAN DIEGO

HIV Restriction Factors as TLR Signaling Modulators

A thesis submitted in partial satisfaction of the requirements for the degree Master of Science

in

Biology

by

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The Thesis of Yejin Kim is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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ABSTRACT OF THE THESIS

HIV Restriction Factors as TLR Signaling Modulators

by

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The complete cellular sensor pathway and effector involved in innate immune signaling pathways that response to HIV-1 infection is not clearly understand yet. The focus of global innate immune responses to HIV-1 infection program is to apply a comprehensive system-level understanding of these essential immediate virus-host responses is significantly interfere with the initial establishment of HIV infection. Our study is to understand the role of HIV restriction factors with the TLR9 stimulation. We hypothesized that the responsiveness of HIV-infected cells towards TLR9 stimulation is a requirement for HIV replication, and innate immune signaling pathway involve via TLR9 mediate pathway. From the study, we

identified CUL1, RNF31, and ARL8A act as positive regulators of innate immune signaling pathway via TLR9 receptors. By knowing the correlation between TLR9 and restriction factors, we switched the gear toward other TLR family member, TLR3. Interestingly, additional experiment conducted in our lab shows a significant number of the putative HIV restriction factors acted as strong negative regulators of TLR3 signaling, suggesting that additional evidence of a strong correlation between HIV replication and TLR families.

Introduction

Human Immunodeficiency Virus and Acquired Immunodeficiency Syndrome

In 2003, statistically about 40 million people in world are living with acquired immune deficiency syndrome or acquired immunodeficiency syndrome (AIDS) [1]. AIDS is a disease caused by gradual attack from the human immunodeficiency virus (HIV) [1]. HIV have been found in various sources such as saliva, tear, nervous system tissue, blood, semen and breast milk; however, HIV-1 spreads dominantly via heterosexual transmission[2]. Since heterosexual transmission is the dominant mode of HIV-1 spread worldwide, it is essential to understand the early innate immune response in the vaginal mucosa to prevent infection. The classical way of HIV infection induces strong anti-HIV T-cell response during the early infection, which suppresses the virus loads in plasma, yet this response would not clear the infection from sites including the mucosa. People with HIV infection live in average 10 years or longer without having any symptoms[3]. During asymptomatic period, host CD4+ T-cells are gradually eliminated by HIV-1, and finally the total number of CD4+ T-cell and host response decreased to the point where the immune system became too weak to fight off with infection and HIV infected persons are diagnosed as AIDS patients[3]. AIDS patients show a dramatic reduction of CD4 and T-cell in the blood, therefore host cell is no longer able to control replication resulting in increasing virus load, which become more susceptible to other diseases[4]. Now then, pathogens can infect the immunosuppressed host, resulting in morbidity and mortality. An important insight of immune deficiency is that the result of chronic activation of both the adaptive and innate immune system is not due to spontaneous effect of HIV infection[2].

According to the recent studies, there are compelling evidence suggest that first days and weeks after HIV infection are critical determinants that account for the failure of the later adaptive response to determine the later stage of infection and clear infection. Thus, deeper and clearer understanding of early innate response to HIV infection is critical key to find the solution for the AIDS.

Global Innate Immune Response to HIV Infection Program

The complete pathway of cellular sensors and effectors involved in the innate signaling and the role of those key components in early innate immune response is still remained unclear. Even though we know that interferon(IFN) and Toll-like Receptor(TLR) signaling pathways are involved in HIV-1 replication, however, the innate responses to HIV-1 infection have not clearly understand.

The purpose of the global innate immune response to HIV infection program is to answer the early question of early innate signaling pathway and cellular sensors and effectors by using a system biology approach and improve the understanding of HIV infection. By combining thirteen research groups different expertise arranged into six scientific projects and a scientific core allow us to make system-based approach, and this would provide deeper understanding of the complete repertoire of cellular sensors and effectors involved in the innate signaling pathway respond to HIV-1 infection (project 1), the rate-limiting components(project 6); the kinetic regulation of different steps of HIV-1 infection by their circuits (project 3); the role of these circuits in regulating primary myeloid and lymphoid cell activity that are relevant during HIV-1 infection (project 2 and 4); and the relationship between these circuit and the clinical outcome of infection(project5). This systematic study would allow us to have a better understanding of the immediate innate response to HIV

infection. Hopefully, by identifying novel genetic and networks involved in innate responses to HIV would allow us to identify unique characteristic associated with HIV infection.

Our laboratory participated in Project 2, and we hypothesized that several of the restriction factors identified from preliminary studies are important components of the host antiviral sensing machinery and/or of pathways downstream of this sensing machinery. There are four well-known families of molecules that sense viral pathogens that induce IFN and/or antiviral response, my study is focus on extracytoplasmic receptor Toll-like receptor (TLR). Since the interaction of TLR and restriction factors are not clearly understand, and how sensing pathway induces an antiviral response such as induction of IFN and/or IFN-stimulated genes (ISGs) , our goal is to understand the role of these restriction factors in early innate immunity, and their pathway. The essential restriction regulators identified will be followed up by investigators in Project 4 to determine the relevance in primary immune cell type. Moreover, Project 1, 4, and 6 will determine which restriction factors are induced by IFN signaling, and lastly, Project 3 will determine the effect of IFN treatment on the kinetics of different steps of HIV replication.

Preliminary studies

The preliminary studies identified host proteins essential for either early or late stages of HIV-1 replication by using three independent genome-wide RNAi screening. In addition, another data analyzed from gain-of function to test the effects of cDNA overexpression upon HIV-1 replication. Then the preliminary study team assimilated results from all different screens to initiate a systems-based analysis of HIV cellular restriction mechanisms. To further understand the functional role of the putative cellular restriction factors identified by screens, they compared the identified genes to datasets enriched for factors that participate in

innate immune signaling and responses. Interestingly, among 2370 factors found as HIV restriction factors also contained signaling components of TLR3, TLR7, TLR9, RIG-I, and NLR-mediated pathways. These analyses support that these potential HIV restriction factors identified by functional genomics approaches are highly involved in HIV innate response. However, the specific pathways and the role in host innate response have not yet identified.

Additional to the preliminary study, siRNA screen in Project 1 will address the issue of false negatives that occur through this approach. The Project 1 has been characterizing other host-pathogen protein-protein interaction involving factors encoded by HIV.

Innate Immune System

The innate response is an ancient defense system made up of functionally different subsystem that evolved to response to pathogens such as HIV. In general, the innate response is nonspecific first line of defense, which induces an interferon production; eliminate the replication of pathogens in early infection. If successful innate response occurred, then the production of cytokines and growth factors that are components of later specific acquired immune response is generated. The acquired response is primarily composed of B- and T-lymphocyte that are specific to antigen and long lasting effect. In contrast to the adaptive immune system, innate response does not provide long lasting immunity to the host.

Macrophages and dendritic cells are innate immune cells, which are capable to detect and destroy pathogens and results immune response by secreting cytokines and growth factors18. The interaction of some of these factors are significant in host innate responses, therefore, identifying these immediate virus-host responses are important to understand the

HIV infection. Furthermore, by identifying HIV-specific response will allow developing effective therapeutic approach and developing vaccine for HIV infections.

Early Innate Immune response relies on RAMP and PRR

To understand the early innate response, it is important to know that the immediate innate immune response relies on the recognition of conserved structure called pathogen associated molecular patterns(PAMPs) and pattern recognition receptors(PRRs). PAMPs also referred as a small molecular motif associated with group of pathogens and recognized by cell of the innate immune system. When PAMP interacts with PRR, the innate immune response activated to protect the host from infection.

PAMP is recognized by PRR presented on the cells of the innate immune system. The dominant form of PRRS of vertebrates include Toll-like receptors(TLRs), NOD-like receptors(NLRs), RIG-I-like helicases(RLH), and cytoplasmic DNA sensing pathways. These innate immunity receptors bind specific PAMPs directly or with help of cofactors. When PAMP binds with specific innate immunity receptor, an induction of several intracellular signaling events, including activation of the NF-kB and IRF pathways, and inflammatory response occurred. The activation of these innate response triggers the induction of short-lived cytokines and interferons, restrictors of pathogen infection, and long-lived adaptive immune responses are also resulted.

Potential PAMPs present in HIV are single-stranded RNA and a short double-stranded RNA region. In addition, cytoplasmic viral double stranded DNA and DNA/RNA intermediates are presented during the HIV life cycle. This suggested that nucleic acid sensing PRRs may be essential components of the innate immune responses resulted from

HIV infection. There is evidence where TLR signaling pathways regulated HIV infection. For instance, HIV-1 ssRNA can activate TLR7 and TLR8 signal pathway. Moreover, induction of TLR2 and TLR9 leads to an increase in HIV-1 gene expression and viral production, which points out the important correlation between HIV and TLR signaling.

Production of Interferon and IFN-induced HIV-1 Restriction Factors

The presence of pathogen cause host cells to produce interferons (IFNs) mainly from dendritic cells (DCs). IFN known as antiviral agent initiates the protective defenses of the immune system by allowing cells to communicate and increases amount of natural killer cells and macrophages, thus cell can resist upon the viral infection and used as a major component of the innate antiviral response. Interferon interferes with the viral replication within host cells, which includes IFN-alpha, IFN-beta, and Type II interferon (IFN-gamma). In parallel, the viral recognition by TLR-3 induces activation of the adaptor protein TIR-domain-containing adapter-inducing interferon-B (TRIF), which later induces phosphorylation and homo and hetero-dimerization of transcription factors known as Interferon Regulatory Factors (IRFs): IRF3 and IRF7. An adapter TRIF activates TLRs, and mediates two TLR-associated signaling cascades, where one is dependent upon a MyD88 adapter.

Many studies reveal that IFN is a key component of the innate antiviral responses and activity of IFN in control of HIV in vivo. For example, increasing IFN activity resulted after early infection, which is correlated with the decreasing viral titer and increase in CD4+ T cell numbers. It seems like IFN act in multiple steps in the HIV life cycle, and the effectiveness is depending on cell type, interferon type, depending on the virus.

Immune Response of HIV Infection

Most of HIV infection is caused by HIV-1, which is more virulent and infective form of HIV virus compared to HIV-2[5]. When specific lymphocytes such as T-cells, macrophages, and DCs in human immune system are attacked by HIV-1, it results in a change of an immune response such as hyperactivation or reduction of immune system[6]. Virus said to become latent, where host cells are no longer capable of detected by the immune system[3]. HIV-1 is a retroviridae, which transmits as a single stranded known as RNA genome[7]. HIV-1 forms a double-stranded DNA along with reverse transcriptase, and the viral DNA incorporated into the host nucleus with help of integrates and host co-factors[2]. New RNA and viral proteins are started to generate, then finally cause apoptosis of CD4 cells[5]. HIV infection leads an immediate innate response with antiviral factors, which constitutively expressed or are induced by innate signaling pathway.

Toll-like Receptors and Families

Currently, TLR family seems to be the most innate recognition system in vertebrates, which located on the plasma and intracellular membrane of DCs[9]. TLRs are named due to the structure similarity to Toll, which is a receptor originally known for developmental function in fruit fly Drosophila[19]. Most mammalian species have ten to fifteen types of TLR, and thirteen TLRs have been found in human and mice. TLRs are highly specific to the pathogens, and evolutionary conserved, therefore, unlikely to be eliminated nor modified by any mutation. The well-conserved structures in pathogens such as lipopolysaccharides (LPS), lipoproteins, lipopetides, double-standed RNA of viruses, or the unmethylated CpG islands of bacterial and viral DNA are seem to be recognized in TLRs, the list of TLRs and specific ligands are indicated in the table 1.

Table1. Types of TLRs and Ligands.

Toll-like Receptor(s)	Ligand(s)
TLR1/TLR2	Triacyl Lipopeptides
TLR2/TLR6	Diacyl Lipopeptides, Zymosan, LTA
TLR2	GPI anchors, Porins, Prosteglandin
TLR3	dsRNA
TLR4	LPS, F protein, HSP60, Fibronectin, Taxol
TLR5	Flagellin
TLR7	ssRNA
TLR8	ssRNA
TLR9	CpG DNA
TLR11	Profilin-like molecule

CpG Oligodeoxynucleotides

A short single-stranded synthetic DNA molecule composed of a cytosine and guanine is known as CpG oligodeoxynucleotides (CpG ODN) act as immunostimulants by producing Type I pro-inflammatory response[21]. A synthetic CpG ODN contains modified structure such as partially or completely phosphorothioated (PS) backbone with poly G tail at the 3' end, 5' end. The synthetic modification made on CpG protects the CpG ODN from the nuclease degradation and improves the cellular uptake compared to the ordinary structure. CpG ODN have been known as member of PAMPs, which recognized by the PRR, specifically by TLR9 that expressed only in B cells and plasmacytoid dendritic cells (pDCs). TLR9 stimulator CpG

ODNs are classified as following: 1)Type A CpG ODN have a phosphodiester located on the central CpG-containing palindromic motif addition to a phosphorothioated 3’ poly G tail. Type A induces mainly IFN-alpha production in pDCs but a weak stimulator in TLR-9-denpendent NF-kB signaling. 2)Type B have a full phosphorothioated backbone with CpG dinucleotide, which activates B cells yet produces a weak secretion of IFN-alpha. Type C CpG ODN is combined features of CpG A and CpG B, which produce strong IFN-alpha signal in both pDCs and B cells.

Toll-Like Receptor 9 Signaling Pathway

TLRs play an essential role in the innate immune response against pathogens by recognition of PAMP. Among TLRs, TLR9 is activated by unmethlyated CpG-DNA or its analog, synthetic oligonucletodies that contains CpG motif. The activation of TLR9 leads multiple downstream signaling pathways, which eventually leads the activation of nuclear factor-keppa-B (NF-kB), MyD88-dependent signaling cascades, mitogen-activated protein kinase (MAPK), and interferon regulatory factor (TNF)-alpha and IFN-gamma. According to recent studies TLR9 also plays essential role in the induction of the inflammatory responses. When TLR9 activated by CpG-DNA through a clathrin-dependent endocytic pathway, an adopter protein myeloid differentiation primary response gene 88 (MyD88) gets recruited and these interaction leads to activate the NF-kB.

Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB)

NF-kB is known as one of the transcription factors that play a central role of the transcription factors that plays a central role in inflammatory response induced by various stimuli such as stress, cytokine, free radicals, ultraviolet irradiation, bacterial, viral antigens.

The intranuclear translocation of activated NF-kB, which located in the cytosol inactive state, induces the expression of inflammatory cytokine genes involved in innate and adaptive immune regulation, inflammatory response, and etc. In addition to inflammation, NF-kB is also involved in many biological phenomena, including cell survival. When pathological conditions such as allergic and autoinflammatory diseases and malignancies occur, an abnormal activation of NF-kB is observed. Thus, impairments of NF-kB regulation have strong correlation with various diseases such as cancer, rheumatoid arthritis, atherosclerosis, inflammatory bowels disease, systemic inflammatory response syndrome, septic shock, and metabolic disease, including diabetes. In resting cells, NF-kB is inactive by bound to inhibitor proteins called inhibitors IkBs and resides in the cytoplasm. Upon stimuli by inflammatory cytokines or TLR ligands, the IKK(IkB kinase) complex, composed of IKK alpha, IKK beta, and NF-kB essential modulator(NEMO), which is also known as IKK gamma, is activates and phosphorylates specific Ser residues in IkBs. Phosphorylated IkBs are degraded in an ubiquitin-dependent manner, which releases NF-kB and allows it to translocation into the nucleus to induce the transcription target genes. NF-kB can be activated in two different pathways, the canonical and non-canonical.

The Ubiquitin System

The NF-kB activation pathway is strongly correlated with the ubiquitin conjugation pathway. The ubiquitination at lysine modifies the protein, which cause degradation by proteasome. The upstream signaling cascade is not clearly understood, however, the phosphorylation and ubiquitination reaction are catalyzed by three enzymes as follow: E1 ubiquitin-activating enzymes, E2 ubiquitin-conjugating enzymes, and E3 ubiquitin-protein ligases. These enzymes have different name based on their function, yet they work together

as one component. Target proteins are specifically recognized by E3 enzymes. First, E1 activate c-terminus of ubiquitin by using a large energy generated by hydrolysis reaction of ATP. Then E1 is now conjugated to the active site of cysteine residue by a reactive thioester bond. The ubiquitin then transfer to an active site of E2 cysteine. Finally, E3 selectively choose substrates and E2, and catalyzed ubiquitin transfer from E3 to mostly the NH2 group of Lys in target proteins. E3 plays significant roles in the spatiotemporal-specific recognition of the target proteins for ubiquitination.

The Linear ubiquitin Chain Assembly Complex (LUBAC)

The complete mechanism of polyubiquitin chains reaction has not clearly identified, however, many studies revealed that E2 enzyme plays important role in determining the type of polyubiquitin chain generated. The LUBAC identifies the specific substrate and the particular polyubiquitin chain. LUBAC is composed of HOIL-1L, HOIP and SHARPIN. LUBAC-mediated linear polyubiquitination is involved in NF-kB activation. LUBAC forms a complex with NEMO, and linearly polyubiquitinated NEMO seems to have potential role in activation of IKK. Thus, current concept for LUBAC-mediated NF-kB activation is as follow: upon stimulation ligands of some Toll-like receptors, LUBAC recognizes and linearly polyubiquitinates NEMO. The IKK activation and subsequent degradation of IkBalpha occur as following reaction. Finally, a free NFkB translocated into the nucleus and activates the transcription of target genes.

Role of the Skp, Cullin, F-box containing (SCF) complex

The ubiquitination of protein lead proteasomal degradation by using multi-protein E3 ubiquitin ligase complex called SCF complex. The role of E3 in the ubiquitination of proteins,

which involves in the cell cycle and other cellular protein for destruction is known. SCF complex is formed with three component as following: 1)F-box protein serves in formation of proteins independently of the complex and let proteins to attach the Skp1 component. 2)Skp1is bridges protein between F-box and cullin. 3)Cullin(CUL1), which also known as the major part of the SCF complex, allows the connection between the Skp1 and RBX1. RBX1 contains a zinc-binding domain call RING Ringer (RNF) to E2 allowing the transferral of the ubiquitin to a lysine residue of the target protein. The regulation of cell cycle in eukaryotes completed through synthesis and degradation or phosphorylation and dephosphorylation of cell-cycle-regulating proteins by controlling the transitions between G1/S and G2/M phases.

Small interfering RNA

Small interfering RNA (siRNA) is a double-stranded RNA molecule, which is composed of 20-25 base pairs in length. siRNA also known as short interfering RNA or silencing RNA due to its function in the RNA interference (RNAi) pathway. siRNA inhibits the expression of specific gene with complementary nucleotide sequence. The activity of siRNA first discovered from plant, yet synthetic version of siRNA could induce RNAi in mammalian cells. The dicer enzyme catalyzes the production of siRNA from long double stranded RNA and small hairpin RNA. siRNA is widely used in lab to validate gene function since by using siRNA, any gene can be knocked down with the introduction of synthesis siRNA in a complementary sequence.

Luciferase Assay

Numerous type of luciferase is used in a variety of organisms regulate their light production. Luciferase is widely used term, yet it is also known as an oxidative enzymes used in bioluminescence and contains a different feature from a photoprotein. The production of light from luciferase assay is tremendously useful in various parts of experiment, the firefly luciferase from firefly Photinus pyralis is widely use as a laboratory reagent. The chemical reaction catalyzes by firefly luciferase takes in two steps as follow:

Step1. Luciferin + ATP => Luciferyl Adenlate + PPi

Step2. Luciferyl adenylate + O2 => Oxyluciferin + AMP + light

The light is generated due formation of oxyluciferin in an electronically excited state. The excited oxyluciferin release a light in unit of photon while it returns to the ground state. Firefly luciferase is a monomeric 61kDa protein, catalyzes luciferin oxidation using ATP-Magnesium ion as a co-substrate. The light emitted when luciferase act on the appropriate luciferin substrate, light detecting apparatus such as luminometer or modified optical microscopes can measure the photon emission. Luciferase is highly useful in biological research to study the transcriptional activity in cells that are transfected with a genetic construct containing the luciferase gene under the control of a promoter of interest. Moreover, luciferase assay can be used to measure the cellular ATP concentration in cell or for kinase activity assays since it acts as an ATP sensor protein.

Hypothesis

According to the pre-existing studies indicating that TLR signaling pathway regulate HIV-1 infection; however, the complete innate signaling pathway with HIV-1 infection, rate-

limiting factors, relationship between these pathway and clinical outcome of infection and various questions are still not yet fully understand.

Our study is part of the program project team is based on assembly of thirteen research groups with a diverse expertise arranged into six projects to study immediate innate response due to HIV-1 infection and its innate signaling pathways. The goal of this study is to understand the complete cellular sensors and effectors involved in innate signaling mechanism response to HIV-1 infection. Each project has a different strength, thus, by sharing different information among these projects will help us to understand the genetic, biochemical and molecular level of the host innate response to HIV infection. Six projects are categorized as following: (1) mathematical modeling, system-based analysis, network mapping of the innate response to HIV infection (Project 1, 6), understand the function of HIV restriction factors in HIV replication and innate immune response (Project 2, 3), Project 4 validates the role of these factors in ex vivo primary human myeloid and lymphoid culture system, and lastly Project 6 focus on relevance of these molecular level study in clinical outcomes.

The preliminary study identify collection of 2,500 restriction factors are likely to regulate the innate response due to HIV-1 infection. By using this dataset, we can study early immune response to HIV infection primarily at the cellular level. HIV restriction factors are screened from genome-wide three independent RNAi screens, which found to be important host proteins in early or late stages of HIV-replication. In addition, gain-of function studies are completed with cDNA overexpression upon HIV-1 replication to complement these studies. Specifically, preliminary study identified restriction factors play critical role in innate immune signaling and responses. To collect the highly significant HIV-restriction factors,

preliminary studies compare their data with microarray based studies, and previously implicated HIV restriction factors.

Our study is concentrated on innate immunity and HIV restriction. However, how HIV interacts with these receptors is not clearly understands and results in production or reduction of antiviral response by generating IFN. There are four known families of sensing molecules of viral pathogens such as TLR, and lectin-like receptors(LLR), and two cytoplasmic receptors known as Nod-like receptors and the RIG-I-receptors. We hypothesize that several restriction factors are involved with the host antiviral sensing and/or the downstream pathways during HIV infection and their impact in the HIV antiviral response. We hypothesize that the attenuated responsiveness of HIV-infected cells towards TLR stimulation is requirement for HIV replication, thus restriction factors activate or inactivate TLR responses will categorized as essential restriction factors. Genome-wide small interfering RNA (siRNA) screening studies had been identified numerous late-stage HIV restriction factors, which we hypothesize that these restriction factors regulates TLR signaling pathway. We will first determine restriction factors act via the extracytoplasmic sensor TLRs and their downstream components. In addition, in this study we will focus on TLR9 among many TLR families.

In order to test out hypothesis we incorporated siRNA into 293T cells by reverse transfection, then stimulated with specific ligand for TLR9 to trigger the downstream response such as expression of NF-KB and luciferase expression to distinguish whether restriction factors are positively or negatively regulate the downstream signaling components.

Material and Method

a) Cell culture

HEK293T-TLR9 cells are maintained in high glucose DMEM medium. The media is supplement with 10% heat-inactivated fetal bovine serum, MEM Non-essential Amino Acid, 1mM Sodium Pyruvate and 55uM 2-Merceptoethanol. In addition, 10uM puromysin and 5uM of Blasto. antibodies are added to the medium.

b) Reverse siRNA Transfection

First, cells are detached by using Trypsin in addition to complete medium(pre-made cell culture medium) without antibodies. Aliquot 15000 cells in 100ul medium per well, flat-bottom 96 micro-plate is used. 0.25ul RNAiMAX pre-mixed with 10ul OptiMEM per well are added then incubated for 5 minutes. In the separate tube, 10ul of siRNA with 10ul of pre-mixed RNAiMAX are mixed then incubated for 20 minutes. In the final step, add RNAiMAX mixture into cells then incubated for 24 hours. After 24 hours of incubation, following stimulation is completed with CpG ODN ligands. The ligand is directly added to 96 micro-plate well and stimulated for 48hours.

c) Luciferase Reporter Assay System

First, remove medium from cultured cells then rinse cultured cells with the 1X PBS reagent. The stimulated cells are lysed with 1X Passive Lysis Buffer, then transfer sample into 96-plate well which is specifically designed to use in Luciferase Assay experiment. Use Luciferase Assay Substrate in Luciferase Assay Buffer II and Stop & Glo reagent provided from Promega to measure the Dual-Luciferase reporter activity.

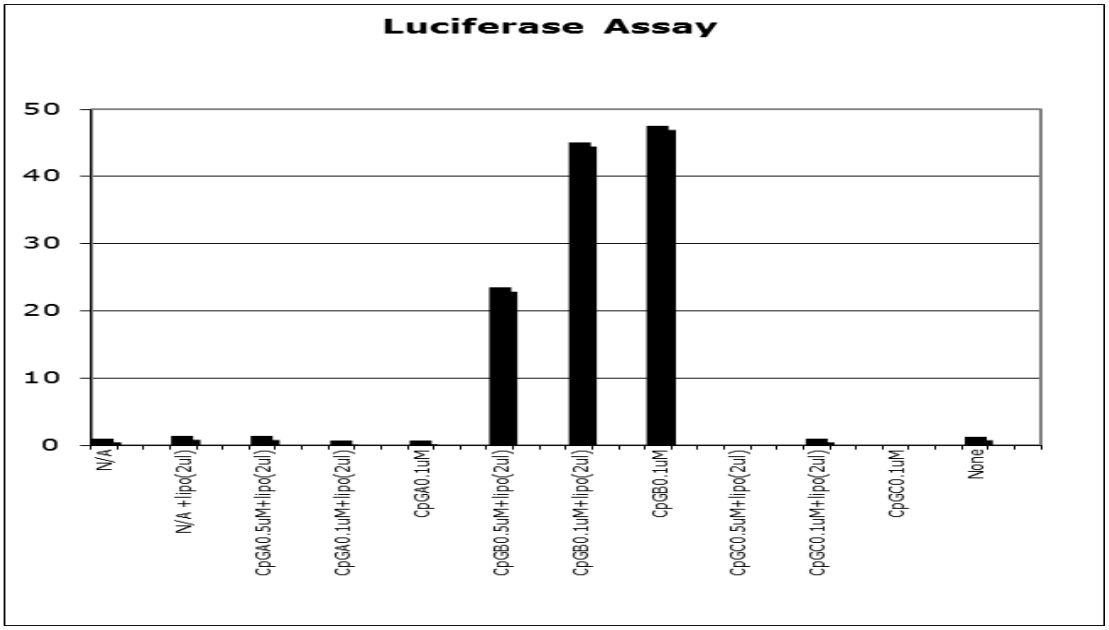
Data

Experiment #1.TLR9 Dosage Response

The focus of the study is to determine the early innate immune response cellular network to HIV-1 infection. We hypothesized that several of the candidate innate response factors identified from preliminary studies are components of the host antiviral sensing machinery and/or pathways downstream of this sensing machinery. Restriction factors may include known and unknown stimulators of the antiviral sensing machinery induce IFN and/or antiviral responses. Our study primarily focus is on extracytoplasmic receptors known as the Toll-like receptors(TLR). Since little is known about how HIV interact with TLR sensing pathway induces an antiviral response such as the induction of IFN and/or IFN-stimulated genes(ISGs) with anti-HIV activity, we study the interaction of HIV and HIV restriction factors with TLRs. We hypothesize that the attenuated responsiveness of HIV-infected cells towards TLR is a requirement for HIV replication, and consequently enhancers or restorers of the TLR responses will act as viral restriction factors. Furthermore, attenuation of TLR signals is likely to create a window for the opportunistic infections frequently observed in HIV-positive individuals. Thus, the question is whether the identified restriction factors act via the extra-cytoplasmic sensors TLRs and their downstream signaling components, and whether TLRs are involved in the IFN induction during the HIV infection. There are numbers of different TLR families some are engaged in negative effect of TLR engagement by HIV-derived PAMP on virus replication, yet TLR2 and TLR9 by constituents of bacteria can lead to an increase in HIV-1 gene expression, and viral production.(2-2”75/231). We wanted to identify 1) whether the identified restriction factors are induced or repressed by TLR activation and 2) whether stimulation of specific TLR alters the ability of

HIV to infect host cells, replicate and egress. My project is primarily focus on TLR9 response, before we start identified the role of restriction factors, we wanted to identify the right dosage and type of ligands that stimulate TLR9. Since TLR9 is known to be activated by unmethlyated CpG-DNA or its analog, synthetic oligonucleotides that contains CpG motif called CpG-ODN were used to identify the ligand. Thus, in the first experiment we used three different type of CpG-ODN such as A, B, and C. Moreover, we also tried using different concentration of each ligand, then measure the activation of TLR9 with luciferase assay.

Table 2.Luciferase Assay-TLR 9 with different CpG ligands. CpG A, CpG B, CpG C is used to stimulate TLR 9. Three different concentrations are used 0.5uM, 0.1uM. Also to confirm that lipofectamine do not interfere with the TLR9 activity we conducted experiment with and without the lipofectamine.



First, we want to identify the type of ligand that stimulates TLR9. Since Three types of CpG ligand (CpG A, CpG B, CpG C) is commercially made and available, we used them to stimulate TLR9. In addition, we used different concentration from 0.5uM, 1.0uM with and

without lipofectamine. For the control, we unstimulated one sample. To show that lipofectamine does not interfere with CpG ligand activity we also used lipofectamine alone. As result indicated in figure1, CpG B ligand is only ligand stimulated TLR9 and resulted noticeable luciferase assay around 45. However, we did not see the great difference between using lipofectamine along with CpG ligands. Expect for CpG B, other synthetic ligands did not result activation upon addition of ligand.

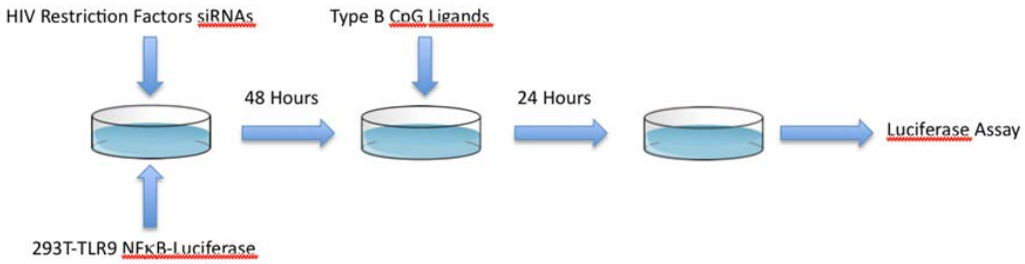


Figure2. Functions of Identified HIV Restriction Factors in Pattern Recognition Receptors (PRRs) Signaling.This shows the overall process of how we deduce luciferase assay. We first add HIV Restriction factors eliminated from preliminary studies by reverse transfection to 294T-TLR9 NF-kB-luciferase cell. After 48 hours we stimulated with Type B CpG ligand based on our first experiment. After 24 hours of stimulation we measure the activation of NF-kB with luciferase assay.

Luciferase Assay Result

Table 3.Inhibition of TLR9 Signaling by Knocking-down(KD) of Identified HIV RF>=90%. This includes 6 restriction factors that had greater and equal to 90 percent inhibition NF-kB activity on luciferase assay. The table also includes gene ID, NCBI gene symbol, gene description, average normalized NF-kB activity percent and standardized normalized NF-kB activity.

Row	Column	Entrez Gene ID	NCBI gene symbol	Gene Description	AVG. Normalized NFκB Acitivity	STDEV. Normalized NFκB Acitivity
C	10	8454	CUL1	cullin 1	3.1%	1.0%
F	6	255631	COL24A1	collagen, type XXIV, alpha 1	6.8%	0.3%
D	11	55072	RNF31	ring finger protein 31	6.9%	2.1%
C	7	84570	COL25A1	collagen, type XXV, alpha 1	8.4%	0.4%
C	10	127829	ARL8A	ADP-ribosylation factor-like 8A	9.8%	3.8%
B	5	4171	MCM2	minichromosome maintenance complex component 2	9.9%	6.0%

Table 4.Inhibition of TLR9 Signaling by KD of Identified HIV RF 75%-90% (I). This includes 49 restriction factors that had 75 to 90 percent inhibition of NF-kB activity on luciferase assay. The table also includes gene ID, NCBI gene symbol, gene description, average normalized NF-kB activity percent and standardized normalized NF-kB activity.

Row	Column	Entrez Gene ID	NCBI gene symbol	Gene Description	AVG. Normalized NFkB Acitivity	STDEV. Normalized NFkB Acitivity
B	12	6223	RPS19	ribosomal protein S19	12.5%	7.0%
E	3	57157	PHTF2	putative homeodomain transcription factor 2	13.6%	6.7%
B	7	5111	PCNA	proliferating cell nuclear antigen	13.8%	7.7%
B	6	4792	NFKBIA	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	14.1%	2.8%
H	4	5154	PDGFA	platelet-derived growth factor alpha polypeptide	14.2%	2.2%
A	10	10206	TRIM13	tripartite motif-containing 13	14.8%	1.3%
F	12	375	ARF1	ADP-ribosylation factor 1	15.6%	10.2%
G	2	705	BYSL	bystin-like	15.9%	6.4%
D	8	23205	ACSBG1	acyl-CoA synthetase bubblegum family member 1	16.1%	8.3%
B	9	55763	EXOC1	exocyst complex component 1	16.1%	2.2%
B	10	56097	PCDHGC5	protocadherin gamma subfamily C, 5	16.6%	2.1%
C	5	7484	WNT9B	wingless-type MMTV integration site family, member 9B	16.6%	0.7%
F	8	100271374	RPS27AP5	ribosomal protein S27a pseudogene 5	17.1%	2.8%
E	12	93973	ACTR8	ARP8 actin-related protein 8 homolog (yeast)	17.3%	13.4%
A	4	375	ARF1	ADP-ribosylation factor 1	17.6%	4.9%
G	3	983	CDK1	cyclin-dependent kinase 1	17.7%	0.6%
F	9	1	A1BG	alpha-1-B glycoprotein	17.9%	2.8%
C	2	80198	MUS81	MUS81 endonuclease homolog (S. cerevisiae)	18.0%	0.9%
E	6	80198	MUS81	MUS81 endonuclease homolog (S. cerevisiae)	18.0%	1.2%
C	2	7133	TNFRSF1B	tumor necrosis factor receptor superfamily, member 1B	18.3%	10.5%
A	12	2294	FOXF1	forkhead box F1	18.4%	4.9%
H	11	7187	TRAF3	TNF receptor-associated factor 3	18.5%	5.5%
B	11	6117	RPA1	replication protein A1, 70kDa	18.5%	5.5%
A	1	1	A1BG	alpha-1-B glycoprotein	18.5%	1.7%
C	11	9616	RNF7	ring finger protein 7	18.6%	3.0%

Table 5. Inhibition of TLR9 Signaling by KD of Identified HIV RF 75%-90% (II). This includes 49 restriction factors that had 75 to 90 percent inhibition of NF-kB activity on luciferase assay. The table also includes gene ID, NCBI gene symbol, gene description, average normalized NF-kB activity percent and standardized normalized NF-kB activity.

Row	Column	Entrez Gene ID	NCBI gene symbol	Gene Description	AVG. Normalized NFkB Acitivity	STDEV. Normalized NFkB Acitivity
D	12	1757	SARDH	sarcosine dehydrogenase	18.8%	1.0%
B	1	2534	FYN	FYN oncogene related to SRC, FGR, YES	18.9%	0.6%
B	2	3006	HIST1H1C	histone cluster 1, H1c	19.0%	5.2%
A	1	7484	WNT9B	wingless-type MMTV integration site family, member 9B	19.1%	4.0%
E	2	5422	POLA1	polymerase (DNA directed), alpha 1, catalytic subunit	19.2%	0.3%
G	9	2534	FYN	FYN oncogene related to SRC, FGR, YES	19.2%	4.1%
B	11	57157	PHTF2	putative homeodomain transcription factor 2	19.4%	2.0%
B	8	5154	PDGFA	platelet-derived growth factor alpha polypeptide	19.5%	0.0%
B	3	3077	HFE	hemochromatosis	19.6%	7.5%
D	9	29801	ZDHHC8	zinc finger, DHHC-type containing 8	19.7%	2.9%
A	3	333	APLP1	amyloid beta (A4) precursor-like protein 1	19.8%	3.2%
H	2	4792	NFKBIA	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	19.8%	5.5%
A	7	983	CDK1	cyclin-dependent kinase 1	19.9%	3.4%
E	2	56097	PCDHGC5	protocadherin gamma subfamily C, 5	20.5%	5.8%
E	10	84465	MEGF11	multiple EGF-like-domains 11	21.0%	15.3%
B	3	22820	COPG	coatomer protein complex, subunit gamma	21.7%	0.3%
B	7	55072	RNF31	ring finger protein 31	21.8%	6.0%
F	3	131616	TMEM42	transmembrane protein 42	22.6%	8.6%
E	5	64854	USP46	ubiquitin specific peptidase 46	23.1%	3.3%
D	3	10399	GNB2L1	guanine nucleotide binding protein (G protein), beta polypeptide 2-like 1	23.5%	5.9%
E	11	84570	COL25A1	collagen, type XXV, alpha 1	23.5%	8.6%
G	5	1267	CNP	2',3'-cyclic nucleotide 3' phosphodiesterase	23.8%	0.0%
D	11	94030	LRRC4B	leucine rich repeat containing 4B	24.1%	9.9%
F	7	339799	EIF3FP3	eukaryotic translation initiation factor 3, subunit F pseudogene 3	24.6%	12.5%

Table 6. Inhibition of TLR9 Signaling by KD of Identified HIV RF 50%-75% (I). This includes 49 restriction factors that had 75 to 90 percent inhibition of NF-kB activity on luciferase assay. The table also includes gene ID, NCBI gene symbol, gene description, average normalized NF-kB activity percent and standardized normalized NF-kB activity.

Row	Column	Entrez Gene ID	NCBI gene symbol	Gene Description	AVG. Normalized NFkB Acitivity	STDEV. Normalized NFkB Acitivity
D	9	8971	H1FX	H1 histone family, member X	25.8%	4.6%
H	1	4171	MCM2	minichromosome maintenance complex component 2	26.0%	8.8%
A	9	1267	CNP	2',3'-cyclic nucleotide 3' phosphodiesterase	26.2%	4.5%
D	10	64094	SMOC2	SPARC related modular calcium binding 2	26.3%	3.9%
B	10	5597	MAPK6	mitogen-activated protein kinase 6	27.1%	8.1%
G	8	2294	FOXF1	forkhead box F1	27.2%	0.4%
G	12	3619	INCENP	inner centromere protein antigens 135/155kDa	27.2%	7.8%
F	4	162517	FBXO39	F-box protein 39	27.2%	9.9%
D	12	55196	C12orf35	chromosome 12 open reading frame 35	27.3%	17.8%
B	4	3619	INCENP	inner centromere protein antigens 135/155kDa	27.8%	1.1%
B	4	23205	ACSBG1	acyl-CoA synthetase bubblegum family member 1	28.1%	2.1%
C	9	7918	BAT4	HLA-B associated transcript 4	28.1%	1.7%
B	2	11334	TUSC2	tumor suppressor candidate 2	28.4%	13.0%
F	2	127829	ARL8A	ADP-ribosylation factor-like 8A	28.9%	2.2%
G	4	1153	CIRBP	cold inducible RNA binding protein	29.5%	1.1%
D	10	54472	TOLLIP	toll interacting protein	30.3%	23.5%
G	11	3077	HFE	hemochromatosis	30.3%	6.9%
C	12	9619	ABCG1	ATP-binding cassette, sub-family G (WHITE), member 1	30.6%	24.9%
A	5	7918	BAT4	HLA-B associated transcript 4	31.6%	6.4%
H	3	5111	PCNA	proliferating cell nuclear antigen	32.2%	17.8%
E	4	57822	GRHL3	grainyhead-like 3 (Drosophila)	33.2%	4.6%
D	6	11334	TUSC2	tumor suppressor candidate 2	33.7%	0.0%
D	4	100271374	RPS27AP5	ribosomal protein S27a pseudogene 5	33.8%	16.4%
G	1	596	BCL2	B-cell CLL/lymphoma 2	35.2%	12.6%
E	1	55763	EXOC1	exocyst complex component 1	35.3%	14.2%
F	11	333	APLP1	amyloid beta (A4) precursor-like protein 1	35.5%	1.1%

Table 7. Inhibition of TLR9 Signaling by KD of Identified HIV RF 50%-75% (II). This includes 49 restriction factors that had 75 to 90 percent inhibition of NF-kB activity on luciferase assay. The table also includes gene ID, NCBI gene symbol, gene description, average normalized NF-kB activity percent and standardized normalized NF-kB activity.

Row	Column	Entrez Gene ID	NCBI gene symbol	Gene Description	AVG. Normalized NFκB Acitivity	STDEV. Normalized NFκB Acitivity
G	6	1291	COL6A1	collagen, type VI, alpha 1	35.6%	12.0%
F	10	86	ACTL6A	actin-like 6A	36.0%	23.0%
D	3	339799	EIF3FP3	eukaryotic translation initiation factor 3, subunit F pseudogene 3	36.4%	7.4%
H	7	6117	RPA1	replication protein A1, 70kDa	36.4%	10.0%
A	10	1291	COL6A1	collagen, type VI, alpha 1	37.7%	5.4%
A	2	86	ACTL6A	actin-like 6A	37.8%	6.2%
A	12	10563	CXCL13	chemokine (C-X-C motif) ligand 13	38.0%	14.4%
A	6	8454	CUL1	cullin 1	39.3%	0.3%
D	5	10941	UGT2A1	UDP glucuronosyltransferase 2 family, polypeptide A1	39.3%	38.1%
C	1	64854	USP46	ubiquitin specific peptidase 46	40.5%	3.4%
C	6	84465	MEGF11	multiple EGF-like-domains 11	40.9%	10.3%
D	2	255631	COL24A1	collagen, type XXIV, alpha 1	42.1%	7.9%
B	9	5515	PPP2CA	protein phosphatase 2, catalytic subunit, alpha isozyme	42.2%	7.9%
F	1	124245	ZC3H18	zinc finger CCCH-type containing 18	42.7%	0.7%
H	12	7447	VSNL1	visinin-like 1	43.7%	21.2%
C	5	84293	C10orf58	chromosome 10 open reading frame 58	43.9%	6.1%
E	6	94030	LRRC4B	leucine rich repeat containing 4B	44.8%	3.7%
D	7	22820	COPG	coatomer protein complex, subunit gamma	45.1%	10.1%
A	6	705	BYSL	bystin-like	45.2%	13.9%
B	12	57822	GRHL3	grainyhead-like 3 (Drosophila)	45.7%	19.4%
A	7	9616	RNF7	ring finger protein 7	46.0%	10.8%
E	7	81620	CDT1	chromatin licensing and DNA replication factor 1	46.1%	4.0%
H	10	7133	TNFRSF1B	tumor necrosis factor receptor superfamily, member 1B	46.3%	31.0%
D	2	10206	TRIM13	tripartite motif-containing 13	47.7%	15.0%
C	8	93973	ACTR8	ARP8 actin-related protein 8 homolog (yeast)	48.0%	6.5%
A	5	596	BCL2	B-cell CLL/lymphoma 2	48.3%	37.8%
C	4	7447	VSNL1	visinin-like 1	49.4%	8.5%

Table 9. Inhibition of TLR9 Signaling by KD of Identified HIV RF 25%-50%. This includes 49 restriction factors that had 25 to 50 percent inhibition of NF-kB activity on luciferase assay. The table also includes gene ID, NCBI gene symbol, gene description; average normalized NF-kB activity percent and standardized normalized NF-kB activity.

Row	Column	Entrez Gene ID	NCBI gene symbol	Gene Description	AVG. Normalized NFκB Acitivity	STDEV. Normalized NFκB Acitivity
B	8	55196	C12orf35	chromosome 12 open reading frame 35	50.6%	16.3%
C	11	131616	TMEM42	transmembrane protein 42	50.7%	13.2%
D	4	10563	CXCL13	chemokine (C-X-C motif) ligand 13	51.2%	10.0%
E	1	3184	HNRNPD	heterogeneous nuclear ribonucleoprotein D (AU-rich element RNA binding protein 1, 37kDa)	52.4%	1.6%
C	8	7803	PTP4A1	protein tyrosine phosphatase type IVA, member 1	52.4%	1.5%
C	3	7187	TRAF3	TNF receptor-associated factor 3	55.2%	21.9%
F	5	170685	NUDT10	nudix (nucleoside diphosphate linked moiety X)-type motif 10	55.4%	27.4%
C	4	83860	TAF3	TAF3 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 140kDa	55.6%	11.2%
G	10	3006	HIST1H1C	histone cluster 1, H1c	56.0%	33.7%
D	6	3192	HNRNPU	heterogeneous nuclear ribonucleoprotein U (scaffold attachment factor A)	57.0%	8.0%
D	1	170685	NUDT10	nudix (nucleoside diphosphate linked moiety X)-type motif 10	57.5%	10.9%
E	8	83860	TAF3	TAF3 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 140kDa	57.9%	13.2%
A	8	1153	CIRBP	cold inducible RNA binding protein	58.0%	1.9%
A	11	10399	GNB2L1	guanine nucleotide binding protein (G protein), beta polypeptide 2-like 1	60.7%	21.7%
H	8	6223	RPS19	ribosomal protein S19	64.1%	26.1%
C	3	81620	CDT1	chromatin licensing and DNA replication factor 1	65.0%	1.3%
C	9	124245	ZC3H18	zinc finger CCCH-type containing 18	65.6%	4.6%
E	4	8971	H1FX	H1 histone family, member X	66.3%	27.8%
H	6	5597	MAPK6	mitogen-activated protein kinase 6	66.8%	26.3%
C	6	7514	XPO1	exportin 1 (CRM1 homolog, yeast)	68.3%	1.6%
A	4	7803	PTP4A1	protein tyrosine phosphatase type IVA, member 1	71.5%	4.8%
H	5	5515	PPP2CA	protein phosphatase 2, catalytic subunit, alpha isozyme	72.7%	21.8%
D	1	9994	CASP8AP2	caspase 8 associated protein 2	74.5%	25.6%

Table 10. Inhibition of TLR9 Signaling by KD of Identified HIV RF 0-25%. Table10. includes 49 restriction factors that had 0 to 25 percent inhibition of NF-kB activity on luciferase assay. The table also includes gene ID, NCBI gene symbol, gene description; average normalized NF-kB activity percent and standardized normalized NF-kB activity.

Row	Column	Entrez Gene ID	NCBI gene symbol	Gene Description	AVG. Normalized NFkB Acitivity	STDEV. Normalized NFkB Acitivity
B	5	29801	ZDHHC8	zinc finger, DHHC-type containing 8	75.1%	33.4%
C	12	162517	FBXO39	F-box protein 39	75.7%	15.7%
E	5	64094	SMOC2	SPARC related modular calcium binding 2	77.1%	14.1%
A	8	9619	ABCG1	ATP-binding cassette, sub-family G (WHITE), member 1	79.7%	19.8%
B	1	10941	UGT2A1	UDP glucuronosyltransferase 2 family, polypeptide A1	82.4%	37.5%
H	9	6241	RRM2	ribonucleotide reductase M2	85.6%	22.6%
B	6	54472	TOLLIP	toll interacting protein	90.0%	30.1%
A	2	7514	XPO1	exportin 1 (CRM1 homolog, yeast)	90.0%	10.4%
D	8	7319	UBE2A	ubiquitin-conjugating enzyme E2A (RAD6 homolog)	92.6%	16.6%
G	7	1540	CYLD	cylindromatosis (turban tumor syndrome)	97.1%	33.7%
A	11	1540	CYLD	cylindromatosis (turban tumor syndrome)	103.5%	13.0%
E	9	84293	C10orf58	chromosome 10 open reading frame 58	106.4%	21.4%
C	7	7701	ZNF142	zinc finger protein 142	112.1%	0.9%
D	5	1757	SARDH	sarcosine dehydrogenase	114.6%	36.7%
C	1	6241	RRM2	ribonucleotide reductase M2	132.1%	49.9%
A	3	7701	ZNF142	zinc finger protein 142	141.3%	16.6%
E	3	7319	UBE2A	ubiquitin-conjugating enzyme E2A (RAD6 homolog)	141.9%	26.3%
D	7	5422	POLA1	polymerase (DNA directed), alpha 1, catalytic subunit	161.1%	77.1%
A	9	9994	CASP8AP2	caspase 8 associated protein 2	171.8%	62.1%

Result

The preliminary set up for the siRNA screening experiment is to identify the type of PAMP in an effective dosage to stimulate down-stream activity of TLR9 receptor. The purpose of the preliminary experiment is to identify ideal condition to run the future experiment such as determining cell concentration, ligand functions most ideally, and it's most effective dosage. After the preliminary set up experiment, we discovered that about 15000cells per 100ul well with CpG B ODN ligand stimulation induce best stimulation of TLR9 receptor. In addition, 1.0uM of CpG B ODN works best than other dosages. We also obtained that using lipofectamine does not interfere with function of CpG ligands since the luciferase assay data was very similar between CpG ligand alone and an addition of lipofectamine.

Next part of the experiment is to identify the restriction factors that contain effect in TLR9 stimulated innate immune reponse. HEK293T cell were reverse-transfected with various siRNAs (received from lab, and selected based on preliminary study) and incubated for 48 hours.

After the luciferase assay, we categorized the samples based on their NF-kB inhibition percentage. 6 restriction factors had 90 percent or even higher inhibition of NF-kBactivity. 49 restriction factors had 75-90 percent NF-kB inhibition rate, 51 restriction factors had 50-75 percent NF-kB inhibition rate. 23 restriction factors had 25-50 percent inhibition rate, and 19 restriction factors had 0-25 inhibition NF-kB activity.

Due to large number of siRNA samples, we concentrated on positive restriction factors with ninety percent or higher inhibition of NF-kB activity.

RNF31

NF-kB is tightly regulated by the ubiquitin pathway, a significant transcription factor in inflammatory response, anti-apoptotic and immune processes pathway. It is widely known that the ubiquitin system is important in the various cellular functions such as cell cycle progression, DNA repair, signal transduction and membrane protein transport[26]. Interestingly, the importance of NF-kB has been found in many diseases such as cancer and neurodegenerative disorder[26]. Ubiquitination is carried out by a cascade of reactions catalyzed by three classes of enzymes as follow: a ubiquitin activating enzyme(E1), a ubiquitin conjugation enzyme(E2) and a ubiquitin ligases(E3). First, E1 activate c-terminus of ubiquitin by using a large energy generated by hydrolysis reaction of ATP. Then E1 is now conjugated to the active site of cysteine residue by a reactive thioester bond. The ubiquitin then transfer to an active site of E2 cysteine. Finally, E3 selectively choose substrates and E2, and catalyzed ubiquitin transfer from E3 to mostly the NH2 group of Lys in target proteins. E3 plays significant roles in the spatiotemporal-specific recognition of the target proteins for ubiquitination. According to studies, ubiquitins are linked via isopeptide bonds between an internal Lys and the C-terminal Gly. In addition, the K48-linked polyubiquitin serves as a signal for proteasomal degradatio[28], whereas the K63-linked polyubiquitin chain functions in signal transduction and DNA repair without functioning as a degradation[29].

We have found siRNA factors cause more than ninety or higher reduction on NF-kB activity involves with the ubiquitin pathway. RING finger ubiquitin ligase is reported with another familiar name called HOIL-1 for heme-oxidized IRP2. HOIL-1 belongs to the RING-IBR(in between ring)-RING(RBR) protein family[31]. The ubiquitin associated (UBA) domain

of HOIP binds straight to the ubiquitin like (UBL) domain of HOIL-1L, but not to ubiquitin, and the interaction is critical for formation of the stable complex. The linear ubiquitin chain assembly complex(LUBAC) is composed of two RNF which also known as HOIL-1L and HOIP, conjugates head-to-tail-linked linear polyubiquitin chain to substrates.

Cul1

CUL1, the first member of the Cullin family[23], was first identified in 1996[24], which plays essential role in the protein degradation and the protein ubiquitination[25]. Cul1 is known to form the SKP1-CUL1-F-box protein, SCF E3 ubiquitin ligase complex, which mediates the ubiquitination of proteins involved in various biological processes such as cell proliferation, differentiation, and cell death[25]., The SCF E3 ligases among all E3 ubiquitin ligases are the largest family and are responsible for regulating turnover of many significant regulatory proteins[23]. Cullin 1 is known to construct a large part of SCF E3, and the SCF E3 promotes the degradation of many key regulatory proteins in cell-context, temporally, controlling precisely numerous important cellular processes. Therefore, by knocking-down CUL1, which used to function as molecular regulator and in the post-translational modification of cellular proteins involving ubiquitin, resulted in decreased of NF-kB activity more than ninety percent. Thus, we can conclude that activity of Cul1 is important to immune system and can consider as a positive regulator.

Col24A1

Collagen XXIV is a newly discovered, therefore poorly characterized member of the fibril-forming family of collagen molecules. Col24A1 contains an interesting structural feature of invertebrate fibrillar collagens and is predominantly existed in bone and retina but

to a less extent in cornea, skin, and tendon[34]. Col24A1 is involved in regulation of osteoblastic cells. Interestingly, the activation of Col24A occurs sometime before onset of the late osteoblast differentiation[32]. The structural part of Col24A1 composes of a long triple helical domain flanked by typical propeptide-like sequences. A short imperfection in the triple helix distinguishes Col24A1 unique from others vertebrate fibrillar collagen family, and study proposed that collagen XXIV is an ancient molecule that may contribute to the regulation of type I collagen fibrillogenesis at specific anatomical locations during fetal development[33].

Col25A1

Collagen alpha-1(XXV) chain is a protein encoded by *COL25A1* gene, a brain-specific membrane-bound collagen and a member of collagenous transmembrane proteins[37]. Proteolytic processing releases collagenous Alzheimer amyloid plaque component (CLAC), a soluble form of COL25A1 containing the extracellular collagen domains that associates with senile plaques in Alzheimer disease (AD)[35]. AD is a common elderly onset neurodegenerative disease results in dementia due to massive accumulation of amyloid deposits in senile plaques[35]. According to recent studies on COL25A1, they have identified a novel collagenous protein as a component of senile plaque amyloid, specifically expressed in neurons. However, the correlation between a large inhibition of NF-kB activity with COL25A1 cannot be determined.

ARL8A

ADP-robosylation factors (ARLs) are known as GTP-binding proteins, and regulates the membrane trafficking and structure[39]. The six mammalian Arf proteins are expressed

ubiquitously and so it is anticipated that each will have a distinct localization and function within the cell[39]. They depart from other small GTP-binding proteins by a unique structural device, Arf proteins rotates between inactive GDP-bound and active GTP-bound forms that bind effectors, which allows front-back communication from the N-terminus to the nucleotide binding[40]. Moreover, similar to other GTP-binding proteins, a complex of Arf-GTP with an effector protein mediates Arf functions. The studies determined that Arf interacts with at least three different types of effectors. First, it interacts with structural protein, the vesicle coat protein. The second type of effector is lipid-metabolizing enzymes, and the third comprises those proteins that bind to Arf-GTP, however biological functions are not yet understood[41].

Along with other restriction factors with multiple names also ARL is named Triparite motif protein 23(TRIM23). It functions as an E3 ligase for NF-kB essential modulator (NEMO). The recent study found that the role of NEMO is significant for virus-induced antiviral response. Ubiquitin E3 ligases are involved in the regulation of innate immune signaling. TRIM 23 up-regulates the NF-kB-driven reporter genes in cells expressing NEMO by TRIM23-mediated ubiquitin conjugation. TRIM23 slightly activated NF-kB in cells expressing large amount of NEMO and significantly activated NF-kB in cells expressing NEMO. NEMO serves as a critical component to coupling upstream receptor signaling to the canonical NF-kB pathway.

MCM2

Mini-Chromosome maintenance (MCM) is initiates the eukaryotic genome replication. Hexameric protein complex is known as a key player in the regulation of DNA replication, and it is highly regulated by the MCM complex. MCM complex involves in the

pre-replication complex and allow the formation of replication forks during the transcription, and help recruit proteins for the DNA replication. MCMs identified initially for their role in plasmid maintenance and cell cycle progression, and cell proliferation[48]. Therefore from the therapeutic view, *MCM* genes are ideal candidates for targeted cell proliferation related disease such as cancer therapy[48].

Discussion

In this study, we first identified best PAMP among CpG ODNs to that activated TLR9 because it is important part of preliminary experiment. In the early innate immunity, receptors bind with specific PAMPs, directly or together with cellular cofactors. PAMPs initiate the induction of several intracellular signaling events such as activation of the NF-kB and IRF pathway, and the inflammasome via TLR receptors. Based on our experiment, CpGB ODN interacts best with TLR9 receptor. This is an important discovery because potential PAMP present in HIV include single-stranded RNA and a short double-stranded DNA region. During the life cycle of HIV-1, cytoplasmic viral double stranded DNA and DNA/RNA intermediates are involved. The presence of these viral nucleic acid structures strongly suggests that nucleic sensing TLR9 and CpGB sequence may be important components of the innate immune surveillance machinery that detects HIV infection.

According to pre-existing evidences indicate that many TLR family signaling pathways regulate HIV infection such as TLR8 and TLR8 which can be activated by HIV-1 ssRNA pathway. Induction of TLR4 signaling leads to secretion of type 1 IFNs that act to decrease HIV-1 infection of DCs and decrease transmission of HIV-1 from DCs to $CD4^+$ T cells. In contrast to these negative effects on virus replication, induction of TLR2 and TLR9 by constituents of bacteria, leads to an increase in HIV-1 gene expression and viral production. Thus, taken together, these finding indicate that HIV-1 infection may be recognized and regulated by TLR9 and most if not all major branches of the host innate immune system. We now can take other TLR families to test whether those are significant components of the innate immune surveillance machinery to detect HIV infection.

The next part of the experiment is to determine effector or regulator among numerous restriction factors that activated via TLR9 pathway. We hypothesized that some of the identified restriction factors act to trigger and amplify TLR-mediated innate immune responses. These restriction factors are found by using three genome-wide genetic lost-of-function screens of cellular sensors and effectors involved in the innate signaling pathways that respond to HIV-1 infection. We incorporated these restriction factors by siRNA prior to exposure of the cells to specific TLR ligands and tested NFkB activity. Among various candidates, we found six those highly inactivate production of NF-kB activity. Since siRNA incorporated with the genome by reverse transfection, we know that higher the NF-kB inhibition percentage resulting restriction factors acts as positive regulator for downstream innate signaling process. We narrowed our focus on six restriction factors, which resulted in significantly high(more than 90 percent and up) reduction of NF-kB activity via TLR9 receptor. Among six restriction factors, three of them are components of human ISG which known to restrict HIV-1 infection at early or late stage such as TRIM, APOBEC3G. Not surprisingly, all three of restriction factors: CUL1, RNF31, ARL8A are involved with ubiquitination function via TLR9 receptor mediated innate signaling pathway. ARL8L (also known as TRIM23) functioned as an E3 ligase for NEMO. The recent study found that the role of NEMO is significant for virus-induced antiviral response. RNF31 is component of LUBAC regulates immune and inflammatory signaling pathways. CUL1 is involved in APOBEC which are counteracted by the HIV-1 accessory protein Vif. HIV-1 infection also lead to IRF3 degradation through either Vpr- or Vif-dependent proteasomal degradation, and the HIv-1 Vpr protein can block type 1 IFN production by plasmacytoid DC (Additional details for each restriction factors are written below).

In contrast to recent study about TLR9, the induction of TLR9 by constituents of bacteria can lead to an increase in HIV-1 gene expression and viral production, thus interplay between HIV and opportunistic bacterial pathogens may not be true. Instead in our study, increased activation of NF-kB occur with stimulation of TLR9 receptor with CpGB ligand was observed and evidence was supported by identification of restriction factors, which is known human ISGs identified to restrict HIV-1 infection at early or late steps. These restriction factors we identified plays essential role in ubiquitination that occurs through an enzymatic cascade involving three enzymes: E1, E2, E3 and lead proteasomal degradation.

Detail Explanation of Each Restriction Factors Act Via TLR9 Medicated Pathway

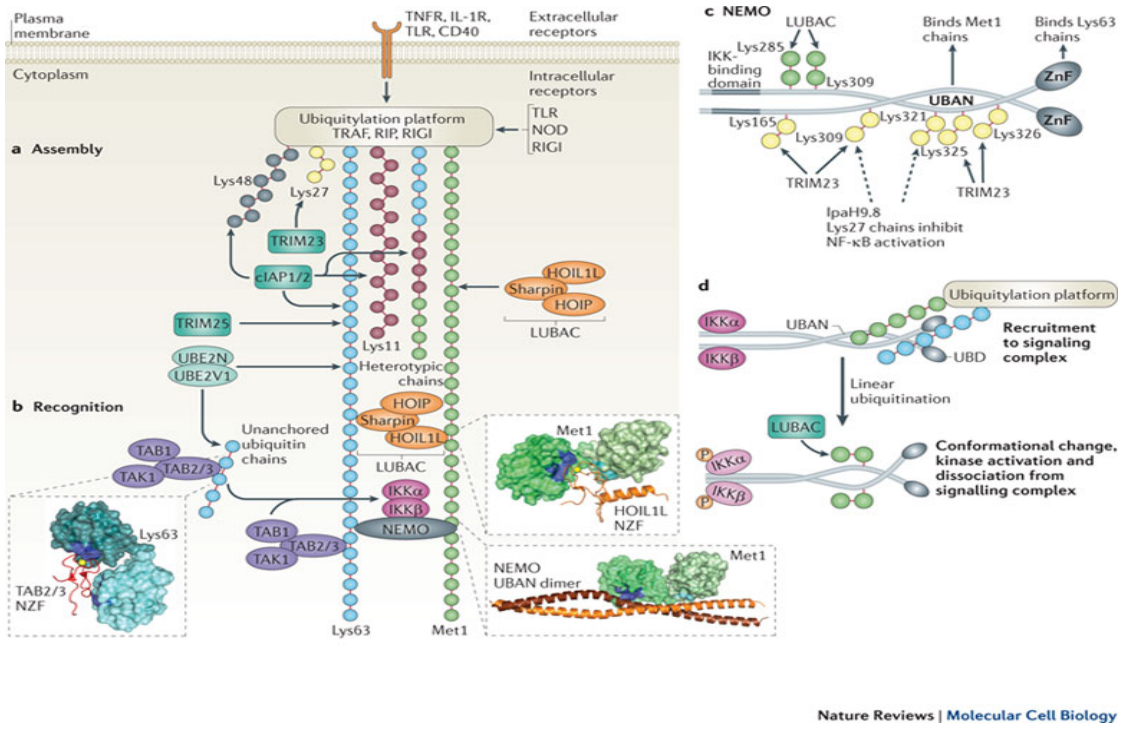


Figure3. NF-κB signalling regulated by atypical Ubiquitylation**.** Assembly of signalling complexes including both extracellular and intracellular receptors to trigger nuclear factor-κB (NF-κB) signalling.

Since there are many siRNA samples we tested, we would like to focus on sample with ninety or greater NF-kB inhibition rate after reverse transfected with siRNA. Six samples are as following:

Table 4: Inhibition of TLR9 Signaling by KD of Identified HIV RF>=90%. This includes 6 restriction factors that had greater and equal to 90 percent inhibition NF-kB activity on luciferase assay. The table also includes gene ID, NCBI gene symbol, gene description, average normalized NF-kB activity percent and standardized normalized NF-kB activity.

Row	Column	Entrez Gene ID	NCBI gene symbol	Gene Description	AVG. Normalized NFκB Acitivity	STDEV. Normalized NFκB Acitivity
C	10	8454	CUL1	cullin 1	3.1%	1.0%
F	6	255631	COL24A1	collagen, type XXIV, alpha 1	6.8%	0.3%
D	11	55072	RNF31	ring finger protein 31	6.9%	2.1%
C	7	84570	COL25A1	collagen, type XXV, alpha 1	8.4%	0.4%
C	10	127829	ARL8A	ADP-ribosylation factor-like 8A	9.8%	3.8%
B	5	4171	MCM2	minichromosome maintenance complex component 2	9.9%	6.0%

Among many samples, we identified six restriction factors those most inhibited NF-kB activity more than ninety percents are listed in Table1. Since we incorporated siRNA by reverse transfection, these are considered possible positive regulators of NF-kB activation. Although the specific role of both collagen (COL24A1 and COL24A1) and MCM have not yet clearly understand(regards to innate immunity), however, we were able to identify the roles of rest of restriction factors are strongly related to activation of NF-kB. CUL1, RNF31, and ARL8A are involved with upstream reaction, thus, in order to activate NF-kB, it is essential to have three restriction factors.

Role of ADP-ribosylation factor-like 8A (ARL8A)

ADP-robosylation factors (ARLs) are known as GTP-binding proteins, and regulates the membrane trafficking and structure[39]. The six mammalian Arf proteins are expressed ubiquitously and so it is anticipated that each will have a distinct localization and function within the cell[39]. They depart from other small GTP-binding proteins by a unique structural device, Arf proteins rotates between inactive GDP-bound and active GTP-bound forms that bind effectors, which allows front-back communication from the N-terminus to the nucleotide binding[40]. Moreover, similar to other GTP-binding proteins, a complex of Arf-GTP with an effector protein mediates Arf functions. The studies determined that Arf interacts with at least three different types of effectors. First, it interacts with structural protein, the vesicle coat protein. The second type of effector is lipid-metabolizing enzymes, and the third comprises those proteins that bind to Arf-GTP, however biological functions are not yet understood[41].

Role of RNF31

First, linear ubiquitin ligase complex (LUBAC) is a RING E3 ligase regulates immune and inflammatory signaling pathways. However, unlike classic RING E3 ligase, LUBAC determines the type of ubiquitin chain being formed, an activity normally associated with the E2 enzyme via a thioester intermediate. LUBAC is found to composed of HOIL-1L (heme-oxidized IRP ligase1), HOIP(HOIL-1L-interacting protein, also known as RNF31, ZIBRA, and PAUL), and SHARPIN(SHANK-associated RH domain interacting protein). HOIP contains three zinc fingers, of which two are NZW type as well as a ubiquitin-associated (UBA) domain and an RBR domain. The E3 activity of HOIP to generate linear

polyubiquitin is required for the efficient activation of canonical IKK, and the subsequent phosphorylation of IKK targets NF-kB activation pathway.

Ubiquitination occurs through an enzymatic cascade involving three enzymes: E1, E2, E3. E1, the activating enzyme forms a reactive thioester with the C-terminal glycine of ubiquitin in an ATP-dependent fashion then this thioester passed on to an E2-conjugating enzyme and finally transferred to E3 enzyme.

Role of the Skp, Cullin, F-box containing (SCF) complex

The ubiquitination of protein lead proteasomal degradation by using multi-protein E3 ubiquitin ligase complex called SCF complex. The role of E3 in the ubiquitination of proteins, which involves in the cell cycle and other cellular protein for destruction is known. SCF complex is formed with three component as following: 1)F-box protein serves in formation of proteins independently of the complex and let proteins to attach the Skp1 component. 2)Skp1is bridges protein between F-box and cullin. 3)Cullin(CUL1), which also known as the major part of the SCF complex, allows the connection between the Skp1 and RBX1. RBX1 contains a zinc-binding domain call RING Ringer (RNF) to E2 allowing the transferral of the ubiquitin to a lysine residue of the target protein. The regulation of cell cycle in eukaryotes completed through synthesis and degradation or phosphorylation and dephosphorylation of cell-cycle-regulating proteins by controlling the transitions between G1/S and G2/M phases.

Role of CUL1 related to HIV

Interestingly, we were able to find the correlation between CUL family and HIV. The Vif is found to play critical role in generating initiation of infection. Recent study identified

that CEM/APOBEC3G is only contained in non-permissive cells, and function as mediator of anti-HIV-1 activity, which is suppressed by Vif. In order for Vif to suppress APOBEC3G which results in antiviral activity is specifically depend on Cul5-SCF function. HIV-1 Vif function along with Cul5, Elngin B, Elonging C, and Rbx1 that create a complex similar to SCF-like complex, and more specifically named VCB-like complex. VCB-like complex belongs to ubiquitin protein ligases, ubiquitinating a broad type of proteins play role in cell cycle regulation, signal transduction, transcription, and other cellular processes. APOBEC3G is defeated by the Vif which acts as an adaptor between it and VCB-like complex. This reaction causes the polyubiquitination and subsequent proteasomal destruction of APOBEC3G and thus its clearance from virus producer cells.

Target specificity toward NEMO is determined by LUBAC

Not surprisingly, the interaction between LUBAC and NEMO determines a target's specificity. The activation of NF-kB is important in many post-translational modifications such as phosphorylation and ubiquitination. The formation of Lys-63-linked and linear ubiquitin chains is found to be crucial step to activate of the canonical NF-kB pathway. This formation of ubiquitin chain is done by an activation of NEMO with linear ubiquitin chains, and IKKbeta, which leads to the phosphorylation and degradation of the inhibitors of NF-kB.

The ubiquitination of NEMO with linear ubiquitin chains is performed by the LUBAC. LUBAC contains three components known as HOIP, HOIL, and SHARPIN. HOIP serve as a catalytic unit of the overall complex which determine the specificity of LUBAC. HOIP transfers the ubiquitin from its active site on Cys-885 RING2 to the N-terminus of the target ubiquitin located on the unique C-terminal linear ubiquitin chain determining domain.

In conclusion, in our study we categorized potential restriction factors, which activated by stimulation of TLR9 receptor, based the inhibition of NF-kB activity. Later in the study, among 150 restriction factors, we concentrated on restriction factors those stimulated through TLR9 receptors response to CpG B ligands, and were able to identify many potential positive NF-kB regulators. Once we narrowed down our focus to six restriction factors with ninety or more inactivation of NFkB activity compare to their normal NF-kB activity function, we went through the previous study conducted with those restriction factors. Then we found out that among six potential NF-kB regulators, three of them play crucial roles in upstream of NFkB activation. Interestingly, there is new study published in 2013 correlation between CUL and HIV. The role of three restriction factors is interconnected to each other by formatting the ubiquitin chain. The ubiquitination of NEMO(ARL8) with linear ubiquitin chains in depended on LUBAC(RNF31). And the formation of ubiquitin chains is mediated by a cascade of E1-E2(SCF complex:CUL1 containing complex)-E3(LUBAC) enzyme. Therefore, it is clearly understood why inactivation of NF-kB resulted and distinguished as positive regulator of NF-kB through TLR9 receptors. Further study can be perform with different TLR families, and see whether these CUL1, RNF31, and ARL8A restriction factors results in high NF-kB inactivation percentage by using same experiment strategy. If we can identify other TLR receptors that produce similar activity, then we can possibly design novel anti-HIV therapies to increase specific TLR receptors stimulation with ligands.

As part of global immune project efforts were divided into multiple parts as early innate immune response to HIV infection, genome-wide siRNAs screens which revealed numerous HIV restriction factors act via TLR signaling as being responsible for HIV

infection. After study of TLR9, we identified there is strong correlation between HIV infection and TLR innate immune signaling pathway. Our laboratory continued the study on putative HIV restriction factors with another TLR family member, TLR3. Unexpectedly, a significant number of the putative HIV restriction factors acted as strong negative regulators of TLR3 signaling, suggesting that activation of TLR3 signaling enhances HIV infection rather than antagonizing the virus.

Stable expression of exogenous TLR3 in HEK293 cells significantly increased infection with HIV-1 virus as determined by enhanced transcription of proviral DNA. Interestingly, the enhancement was dependent of the TRIF expression, which is known as the adaptor protein for TLR3 and partial TLR4 signaling pathway. Moreover, TLR3/TRIF relies on the activation of TAK1 and IRF7. Activation IRF7 appears to facilitate HIV-1 transcription by recruiting the histone acetyltransferase E1A Binding Protein p300 to initiate chromatin changes within the integrated viral DNA. Manipulation of TLR3 signaling and consequent modulation of the chromatin structure within the viral promoter region may therefore not only be a novel means to change HIV-1 infection, but might also prove useful in the re-activation of latent HIV-1 infection.

Our laboratory's continuous study on TLR families support more evidence that HIV-1 and TLR families-mediated innate immune responses to increase or decrease viral replication.

References

1 Sepkowitz KA (June 2001). "AIDS—the first 20 years". N. Engl. J. Med. 344 (23): 1764–72

2 AIDS epidemic update". World Health Organization. Retrieved 2011-07-29.

3 Del Rio C, Curran JW. Epidemiology and prevention of acquired immunodeficiency syndrome and human immunodeficiency virus infection. In: Mandell GL, Bennett JE, Dolin R, eds. Principles and Practice of Infectious Diseases . 7th ed. Philadelphia, Pa: Elsevier Churchill Livingstone; 2009:chap 118.

4 Piot P. Human immunodeficiency virus infection and acquired immunodeficiency syndrome: A global overview. In: Goldman L, Ausiello D, eds. Cecil Medicine . 23rd ed. Philadlelphia, Pa: Saunders Elsevier; 2007:chap 407.

5 Sterling TR, Chaisson RE. General clinical manifestations of human immunodeficiency virus infection (including the acute retroviral syndrome and oral, cutaneous, renal, ocular, metabolic, and cardiac diseases). In: Mandell GL, Bennett JE, Dolin R, eds. Principles and Practice of Infectious Diseases . 7th ed. Philadelphia, Pa: Elsevier Churchill Livingstone; 2009:chap 121.

6 Cunningham, A.; Donaghy, H.; Harman, A.; Kim, M.; Turville, S. (2010). "Manipulation of dendritic cell function by viruses". Current opinion in microbiology 13 (4): 524–529.

7 Gilbert, PB; McKeague, IW; Eisen, G; Mullins, C; Guéye-Ndiaye, A; Mboup, S; Kanki, PJ (28 February 2003). "Comparison of HIV-1 and HIV-2 infectivity from a prospective cohort study in Senegal". Statistics in Medicine 22 (4): 573–593

8 Lawn SD (2004). "AIDS in Africa: the impact of coinfections on the pathogenesis of HIV-1 infection". J. Infect. Dis. 48 (1): 1–12

9 International Committee on Taxonomy of Viruses(2002). "61. Retroviridae". National Institutes of Health. Retrieved 2006-02-28

10 Cunningham, A.; Donaghy, H.; Harman, A.; Kim, M.; Turville, S. (2010). "Manipulation of dendritic cell function by viruses". Current opinion in microbiology 13 (4): 524–529.

11 Rolls A, Shechter R, London A (September 2007). Toll-like receptors modulate adult hippocampal neurogenesis. 9. pp. 1081–8

12 Ausubel (2005). "Are innate immune signaling pathways in plants and animals conserved?". Nature Immunology 6 (10): 973–9.

13 Du X, Poltorak A, Wei Y, Beutler B (September 2000). "Three novel mammalian toll-like receptors: gene structure, expression, and evolution". Eur. Cytokine Netw. 11 (3): 362–71.

14 Medzhitov R (October 2007). "Recognition of microorganisms and activation of the immune response". Nature 449 (7164): 819–26.

15 Shigeoka AA, Holscher TD, King AJ (May 2007). "TLR2 is constitutively expressed within the kidney and participates in ischemic renal injury through both MyD88-dependent and -independent pathways". J. Immunol. 178 (10): 6252–8.

16 Hamilton A, Baulcombe D (1999). "A species of small antisense RNA in posttranscriptional gene silencing in plants". Science 286 (5441): 950–2.

17 Wyatt R, Sodroski J (1998). "The HIV-1 envelope glycoproteins: fusogens, antigens, and immunogens". Science 280 (5371): 1884–8.

18 Hiscott J., Pitha P, Genin P, Nguyen H. Triggering the interferon response: the role of IRF-3 transcription factor. J Interferon Cytokine Res, 1999. 19(1): p. 1-13

19 Lemaitre B, Nicolas E, Michaut L, Reichhart JM, Hoffmann JA (September 1996). "The dorsoventral regulatory gene cassette spätzle/Toll/cactus controls the potent antifungal response in Drosophila adults". Cell 86 (6): 973–83

20 Du X, Poltorak A, Wei Y, Beutler B (September 2000). "Three novel mammalian toll-like receptors: gene structure, expression, and evolution". Eur. Cytokine Netw. 11 (3): 362–71.

20 H. Hemmi, O. Takeuchi, T. Kawai, T. Kaisho, S. Sato, H. Sanjo, M. Matsumoto, K. Hoshino, H. Wagner, K. Takeda, S. Akira A Toll-like receptor recognizes bacterial DNA. Nature, 408 (2000), pp. 740–745

21 Weiner, GJ; Liu, HM; Wooldridge, JE; Dahle, CE; Krieg, AM (1997). "Immunostimulatory oligodeoxynucleotides containing the CpG motif are effective as immune adjuvants in tumor antigen immunization". Proceedings of the National Academy of Sciences of the United States of America 94 (20).

22 Dalpke, AH; Zimmermann, S; Albrecht, I; Heeg, K (2002). "Phosphodiester CpG oligonucleotides as adjuvants: polyguanosine runs enhance cellular uptake and improve immunostimulative activity of phosphodiester CpG oligonucleotides in vitro and in vivo". Immunology 106 (1): 102–12

23 Sarikas A, Hartmann T, Pan ZQ. The cullin protein family. Genome Biol. 2011;12:22.

24 Kipreos ET, Lander LE, Wing JP, He WW, Hedgecock EM. Cul-1 is required for cell cycle exit in C. elegans and identifies a novel gene family. Cell. 1996;85:829–839.

25 Kipreos ET, Lander LE, Wing JP. (1996). "cul-1 is required for cell cycle exit in C. elegans and identifies a novel gene family.". Cell 85 (6): 829–39.

26 Hershko A, Ciechanover A (1998) The ubiquitin system. Annu Rev Biochem 67: 425–479.

27 Hicke L, Dunn R (2003) Regulation of membrane protein transport by ubiquitin and ubiquitin-binding proteins. Annu Rev Cell Dev Biol 19: 141–172.

28 Chau V, Tobias JW, Bachmair A, Marriott D, Ecker DJ, Gonda DK, Varshavsky A (1989) A multiubiquitin chain is confined to specific lysine in a targeted short-lived protein. Science 243: 1576–1583

29 Hofmann RM, Pickart CM (1999) Noncanonical MMS2-encoded ubiquitin-conjugating enzyme functions in assembly of novel polyubiquitin chains for DNA repair. Cell 96: 645–653.

30 Tokunaga C, Kuroda S, Tatematsu K, Nakagawa N, Ono Y, Kikkawa U (1998) Molecular cloning and characterization of a novel protein kinase C-interacting protein with structural motifs related to RBCC family proteins. Biochem Biophys Res Commun 244: 353–359.

31 Marin I, Lucas JI, Gradilla AC, Ferrus A (2004) Parkin and relatives: the RBR family of ubiquitin ligases. Physiol Genomics 17: 253–263.

32 Matsuo N, Tanaka S, Gordon MK, Koch M, Yoshioka H, Ramirez F. J Biol Chem. 2006 Mar 3;281(9):5445-52. Epub 2005 Dec 22.

33 Matsuo N, Tanaka S, Gordon MK, Koch M, Yoshioka H, Ramirez F. J Biol Chem. 2006 Mar 3;281(9):5445-52. Epub 2005 Dec 22.

34 Koch M, Laub F, Zhou P, Hahn RA, Tanaka S, Burgeson RE, Gerecke DR, Ramirez F, Gordon MK. J Biol Chem. 2003 Oct 31;278(44):43236-44. Epub 2003 Jul 21

35 Hashimoto T, Wakabayashi T, Watanabe A, Kowa H, Hosoda R, Nakamura A, Kanazawa I, Arai T, Takio K, Mann DM, Iwatsubo T (Apr 2002). ClAC: a novel Alzheimer amyloid plaque component derived from a transmembrane precursor, CLAC-P/collagen type XXV *EMBO J* **21** (7): 1524–34

36 Franzke, C.-W., Bruckner, P., and Bruckner-Tuderman, L. (2005) J. Biol. Chem. 280, 4005-4008

37 Kowa, H., Sakakura, T., Matsuura, Y., Wakabayashi, T., Mann, D. M. A., Duff, K., Tsuji, S., Hashimoto, T., and Iwatsubo, T. (2004) Am. J. Pathol. 165, 273-281

38 Chavrier P. and Goud B. (1999) The role of ARF and rab GTPases in membrane transport. Curr. Opin. Cell Biol., 11, 466–475.

40 Cherfils J. and Chardin P. (1999) GEFs: structural basis for their activation of small GTP-binding proteins. Trends Biochem. Sci., 24, 306–311.

41 "Arf and its many interactors". Current opinion in cell biology 15 (4): 396–404. 2003.

42 "An N-terminally acetylated Arf-like GTPase is localised to lysosomes and affects their motility." Hofmann I., Munro S. J. Cell Sci. 119:1494-1503(2006)

43 Todorov IT, Lavigne J, Sakr F, Kaneva R, Foisy S, Bibor-Hardy V (Jul 1991). "Nuclear matrix protein mitotin messenger RNA is expressed at constant levels during the cell cycle". *Biochem Biophys Res Commun* 177 (1): 395–400.

44 Maine, G. T., Sinha, P., and Tye, B. K. (1984) Genetics 106, 365–385