

THE ROLE OF FKBP5 IN INFLUENZA VIRUS INFECTION

by

Pak Kei Chan

A thesis submitted in conformity with the requirements

for the degree of Master of Science

Graduate Department of Immunology

University of Toronto

© Copyright by Pak Kei Chan 2010

THE ROLE OF FKBP5 IN INFLUENZA VIRUS INFECTION

Degree of Master of Science, 2010

Pak Kei Chan

Graduate Department of Immunology

University of Toronto

Abstract

FK506 binding protein 5 (FKBP5) is a peptidyl propyl cis-trans isomerase that has been shown to interact with cellular immune pathways such as calcineurin and NF- κ B. During an influenza infection, FKBP5 is up-regulated at the lung in an *in vivo* ferret infection model, yet the effect of FKBP5 on influenza replication and immune response is not understood. An *in vitro* model of human alveolar epithelial cell line A549 was established to study the cause and the function of FKBP5 up-regulation during an influenza infection. In this *in vitro* model, FKBP5 was not up-regulated by influenza replication, but instead it was up-regulated when A549 cells were treated with glucocorticoid. FKBP5 up-regulation did not have any effect on rate of influenza replication. However, FKBP5 up-regulation mediated the suppressive effect of glucocorticoid on pro-inflammatory cytokine production, since FKBP5 knock-down by siRNA increased cytokine production in the presence of glucocorticoid. Overall, the results suggested that the up-regulation of FKBP5 is a physiological response of lung cells to the increase of glucocorticoid during influenza infections, which facilitates the suppressive effect of glucocorticoid on pro-inflammatory cytokine production.

Acknowledgements

First and foremost I would like to thank my supervisor Dr. David Kelvin for his guidance and support during the course of my project, and for providing me with an environment and a unique project that allowed me to learn the essence of being a researcher.

I would also like to thank my committee members, Dr. Michele Anderson and Dr. Rupert Kaul, for providing excellent feedback and helpful comments during the course of this project.

All the members of the Kelvin lab have provided great support for the project. I would like to thank Dr. Mark Cameron, Dr. Cheryl Cameron and Dr. Longsi Ran for all useful discussion. I would also like to thank Dr. Luoling Xu for her teaching and help in the laboratory. I would like to acknowledge Marina Sequeira for the administrative work. A special thank also go to Thomas Rowe, Ali Danesh and Abubaker Sidamesh for their teaching on influenza, CBA and siRNA techniques. Without them, the project cannot go smoothly. I also thank David Banner and Dr. Atsuo Ochi for technical support, and Stephen Huang and Yuan Fang for all their helps and friendships during the course of the project.

I would like to thank Dr. Alyson Kelvin, Stephen Job, Ryan Janzen and Stephanie Wilson for their inputs and comments on my thesis.

Last, but not least, I would like to thank my parents and my brother for their constant support in many aspects of my life.

TABLE OF CONTENTS

Abstract	ii
Acknowledgements	iii
Table of Contents	iv
List of Tables	vii
List of Figures	vii
List of Abbreviations	ix
1. INTRODUCTION	1
1.1 Influenza virology	2
1.2 Immune response to influenza infection	5
1.3 Differentiated H5N1 influenza immune response	7
1.4 Up-regulation of FKBP5 during influenza infection	11
1.5 FK506 binding protein 5 (FKBP5)	12
1.6 FKBP5 interaction with immune response pathway	13
1.7 FKBP5 and influenza infection	14
1.8 FKBP5 and the action of glucocorticoid	15
1.9 Rational, hypothesis and objectives	16
2. MATERIALS AND METHODS	18
2.1 Cells and cell culture conditions	19
2.2 Hydrocortisone and TNF α treatments	19
2.3 Cytometric bead array	20

2.4	Transfection of siRNA	20
2.5	Trizol RNA extraction	22
2.6	Reverse transcriptase polymerase chain reaction (RT-PCR)	22
2.7	Quantitative real time PCR (qPCR)	23
2.8	Influenza production	23
2.9	Influenza infection	24
2.10	Plaque assay	25
2.11	Haemagglutinin assay	25
2.12	MTS Assay	26
2.13	Protein extraction	26
2.14	Western blot	27
 3. RESULTS		 29
3.1	A/WS/33 H1N1 influenza was viable in A549 cells and produced viable Virus	30
3.2	A/WS/33 H1N1 influenza decreased the viability of A549 cells	34
3.3	A/WS/33 H1N1 influenza infection did not increase FKBP5 expression at mRNA and protein levels in A549 cells	37
3.4	FKBP5 expression was enhanced by hydrocortisone but not by the influenza strains A/NWS/33 H1N1 and A/PR/8/34 H1N1	40
3.5	FKBP5 expression was not enhanced by influenza infection in MDCK cells	44
3.6	Effect of hydrocortisone on growth of A549 cells	47

3.7	FKBP5 expression did not affect A/WS/33 H1N1 influenza replication	50
3.8	TNF α stimulation of A549 cells enhanced IL-6 and IL-8 production	53
3.9	A549 cells did not produce IL-1, IL-10 and IL-12 in response to TNF α	59
3.10	Glucocorticoid stimulation inhibited IL-6 and IL-8 production in A549 cells	62
3.11	siRNA transfection of A549 cells down-regulated FKBP5 expression	65
3.12	FKBP5 knock down decreased the suppressive effect of hydrocortisone on cytokine production	69
4. DISCUSSION		77
5. REFERENCES		87

List of Tables

Table 1.	The Influenza A virus genome and gene products	4
----------	--	---

List of Figures

Figure 1.	A/WS/33 H1N1 influenza replicated and produced infectious virus in A549 cells	32
Figure 2.	A/WS/33 H1N1 influenza decreased A549 cells viability	35
Figure 3.	A/WS/33 H1N1 influenza infection did not enhance FKBP5 mRNA and protein level in A549 cells	38
Figure 4.	FKBP5 expression was increased by hydrocortisone stimulation	42
Figure 5.	A/WS/33 H1N1 infection did not up-regulate FKBP5 mRNA and protein level in MDCK cells	45
Figure 6.	Hydrocortisone has minimal effect on the growth of A549 cells.	48
Figure 7.	Hydrocortisone stimulated FKBP5 up-regulation did not affect A/WS/33 H1N1 influenza replication	51
Figure 8.	Cytometric bead array standard curves for IL-6 and IL-8	55
Figure 9.	A549 cells produced IL-6 and IL-8 in response to TNF α stimulation	57
Figure 10.	A549 cells did not produce IL-1, IL-10 and IL-12 in response to TNF α stimulation	60
Figure 11.	Hydrocortisone suppressed IL-6 and IL-8 production in A549 cells	63

Figure 12	FKBP5 siRNA transfection of A549 cells decreased FKBP5 expression	67
Figure 13.	FKBP5 knockdown minimized the effect of hydrocortisone and increased IL-6 level.	71
Figure 14.	FKBP5 knockdown minimizes the effect of hydrocortisone and increased IL-8 level.	73
Figure 15.	FKBP5 expression knockdown by FKBP5 siRNA transfection	75

List of Abbreviations

ACTH	Adrenocorticotrophic hormone
ATCC	American Type Culture Collection
APC	Antigen Presenting Cell
ARDS	Acute Respiratory Distress Syndrome
BSA	Bovine Serum Albumin
CBA	Cytometric Bead Array
CCL	Chemokine (C-C motif) ligand
cDNA	Complementary Deoxyribonucleic Acid
CRH	Corticotrophin-Releasing Hormone
DC	Dendritic cell
DIGE	Difference gel electrophoresis
DMEM	Dulbecco's Modified Eagle Medium
dNTP	Deoxynucleo Triphosphate
dsRNA	Double Strand Ribonucleic Acid
ECL	Enhanced chemiluminescence
F12-K	Kaighn's Modification of Ham's F-12 medium
FBS	Fetal Bovine Serum
FKBP5	FK506 Binding Protein 5
GC	Glucocorticoid
GRE	Glucocorticoid Response Element
HA	Haemagglutinin Assay
HCV	Hepatitis C Virus
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HIV	Human Immunodeficiency Virus
HPA	Hypothalamic-Pituitary-Adrenal
HRE	Hormone Response Elements
IFN	Interferons
I κ B α	Inhibitor of kappa B alpha
I κ K α	Inhibitor of kappa B kinase alpha
IP-10	IFN-inducible protein 10
IPS-1	Interferon beta promoter stimulator-1
LPO	Lactoperoxidase
MAPK	p38 mitogen-activated protein kinase
MDCK	Madine Darby Canine Kidney
MFI	Median Fluorescence Intensity
MOI	Multiplicity of infection
MTS	tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt]
MuLV	Murine Leukemia Virus
Myd88	Myeloid differentiation primary response gene 88
NA	Neuraminidase
NFAT	Nuclear factor of activated T cells
NF- κ B	Nuclear factor kappa-light-chain-enhancer of activated B cells
OAS	2'-5' oligoadenylate synthetase
PBMCs	Peripheral Blood Mononuclear Cells
PBS	Phosphate-Buffered Saline
PCR	Polymerase Chain Reaction

PE	Phycoerythrin
Pen/strep	Penicillin and Streptomycin
Pfu	Plaque forming units
PPIase	Peptidylproline cis-trans isomerase
PVDF	Poly-vinyl difluoride
RIG-1	RNA helicase retinoic acid inducible gene 1
RNaseL	Ribonuclease L
Rpm	Rotations per minute
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SDS	Sodium Dodecyl Sulfate
SPC	Specific Pathogens Free
TCR	T cell receptor
TLR3	Toll like receptor 3
TNF	Tumor Necrosis Factor
TPR	Tetratricopeptide repeat
TRIF	TIR domain-containing adapter protein inducing interferon-beta

CHAPTER 1 - INTRODUCTION

Since the beginning of the current H5N1 influenza outbreak in November 2003, 6629 confirmed outbreaks in poultry have been reported in 50 countries (1). H5N1 influenza killed the majority of the birds infected in each outbreak, and many of the humans who have caught avian flu have displayed acute symptoms and died. To date, there have been 445 human cases of avian influenza reported to the World Health Organization, of which 263 cases were fatal, presenting a mortality rate in excess of 50% (2). Recently, the emergence of a swine-origin H1N1 influenza virus in 2009 has resulted in 246571 confirmed cases of human infection and over 9596 deaths worldwide (3). Unfortunately, it is unclear how exactly influenza infection leads to lethality in birds and humans, and no effective treatments currently exist. An understanding of the influenza pathology, and the host immune response, will be essential to establish an effective treatment for patients with severe avian influenza infection.

1.1 Influenza virology

Influenza viruses belong to the *Orthomyxoviridae* family of RNA viruses, and there are three specific types of influenza. Influenza A infects multiple animal species, and causes pandemics such as seasonal flu in humans as well as lethal avian influenza in poultry and waterfowl (4). Influenza B is less lethal and comprises a minority of human influenza cases. Influenza C causes only mild respiratory illness, and is less studied (5). Influenza A viruses are subtyped based on the antigenicity of their surface glycoproteins, haemagglutinin and neuraminidase (NA) (6). All 16 HA subtypes and all 9 NA subtypes of influenza A are found in waterfowl, whereas subtypes H1N1, H2N2, and H3N2

constitute the majority of all human infections (5). However, when humans are infected by H5N1 or H9N2 avian influenza viruses, the ensuing illness is usually deadly.

The Influenza virus is about 100nm in size and is either spherical or filamentous in shape (7). The influenza genome consists of eight individual RNA gene segments; the gene products of each segment, and their functions, are summarized in Table.1 (6). Influenza is an envelope virus and the HA, NA, and M2 proteins reside at the surface membrane envelope. The M1 protein is a major interior structural component of the virus, forming a shell around the eight vRNP gene segments. The remaining proteins, PA, PB1, PB2, NP, and NS2, bind with the vRNP segments. The NS1 protein is not part of the influenza virion, but is produced during the early phase of an influenza infection (5).

During infection, HA influenza surface proteins bind to the sialic acid moiety of the host cell's glycoprotein. Through endocytosis, the influenza virus enters the host cell's endosomes. The low pH of endosomes triggers a conformational change in the viral HA protein, which allows the fusion of the viral membrane with the host endosome's membrane, in turn releasing interior viral components including vRNPs (8, 9). The trafficking of vRNP into the host cell nucleus is facilitated by the nuclear localization signals of viral NP proteins (10). Inside the nucleus, the viral RNA dependent-RNA polymerase produces viral mRNA for viral protein production, and also produces positive sense complementary RNA, which then acts as a template for making more negative sense viral genomic RNA (11). Viral products HA, NA, and M2 are processed in the Golgi apparatus and exported to the cytoplasmic membrane (6). On the other hand, RNA polymerase components (PA, PB1, PB2), NP, M1, and NS2 are imported into the nucleus (5). The RNA polymerases and NP proteins form vRNP by binding to the newly

synthesized genomic RNA, while M1 and NS2 proteins facilitate the export of vRNP from the nucleus to the cytoplasm (12, 13). Finally, at the lipid raft of the cytoplasm, M1 proteins bring the vRNP and the associated polymerases and NS2 proteins close to the cytoplasm, which contains HA, NA, and M2 proteins (5, 14). The influenza virus escapes, with all of its structural proteins. Since the HA of a newly synthesised influenza can bind to the sialic acid of the producing host cell, the NA cleaves the sialic acid in order to release the virus (15).

Table 1. Influenza A genome and gene products

Genome Segment	Encoded Protein	Description and Function of Protein
1	PB2	RNA transcriptase complex component Binds to host cell capped mRNA
2	PB1	RNA transcriptase complex component RNA-dependent RNA polymerase
3	PA	RNA transcriptase complex component Replicates and transcribes RNA
4	HA Haemagglutinin	Virus surface protein Binds to sialic acid of host cell for entry
5	NP Nucleoprotein	Viral RNP component Binds to RNA genomic segments and RNA polymerase to form viral ribonucleoprotein (vRNP) Contains nuclear localization signal
6	NA Neuraminidase	Viral surface protein Cleaves sialic acid for virus release
7	M1 Matrix protein	Major viral internal component Transports viral RNP out of nucleus
	M2	Ion channel at virus surface

	Ion channel	Lowers the pH on the interior of the virus during endocytosis, in order to dissociate M1 and release vRNP to pass the nucleus pore
8	NS1 Non structural protein 1 NS2 Non structural protein 2	NS1 : multi functional protein Antagonizes interferon, and inhibits host cell mRNA transport from nucleus NS2: Transport vRNP out of nucleus

1.2 Immune response to infection by influenza

In humans, influenza primarily targets the epithelial cells of the respiratory tract. The immune system is activated in order to contain the infection and eliminate the virus. During an influenza infection, influenza double stranded RNA (dsRNA) accumulates in the infected cells. However since dsRNA is not normally present in cells, it is immediately recognized as a pathogen-associated molecular pattern by cellular receptors including the toll like receptor 3 (TLR3) and the RNA helicase retinoic acid inducible gene 1 (RIG-1), which initiate an anti-viral immune response (16, 17). Upon recognition of viral dsRNA in endosomes, TLR3 recruits myeloid differentiation primary response gene 88 (Myd88), and TIR domain-containing adapter proteins, inducing interferon-beta (TRIF) to activate NF- κ B and the Type I interferon response (16). RIG-1 recognizes cytoplasmic non-cap viral RNA (18) and activates NF- κ B and the Type I interferon response through interferon beta promoter stimulator-1 (IPS-1) (19, 20). RIG-1 also activates AP-1 through p38 mitogen-activated protein kinases (MAPK) (16, 21). The resulting IFN α/β secretion activates STAT (22) to sensitize the cells to IFN α/β , providing

a positive feedback mechanism (23) that sustains the cellular anti-viral state and cytokine production for influenza clearance.

The production of interferons (IFN) by influenza infected cells is essential in initiating a cellular antiviral state and an innate and adaptive immunity. Upon IFN stimulation, the influenza infected cells produce 2'-5' oligoadenylate synthetase (OAS) (23), which add 2'-5'-linked oligomers of adenosine to the viral RNA (24), so that the RNA can be targeted for digestion by ribonuclease L (RNaseL) (25). IFN also stimulates the expression of MxA proteins, inhibiting virus replication (26). In terms of innate immune response, IFN α/β stimulates macrophages to produce chemokine (C-C motif) ligand 2 (CCL2), chemokine (C-C motif) ligand 7 (CCL7) and IFN-inducible protein 10 (IP-10) (27), which further recruit monocytes/macrophages and Th1 cells (28) to contain the infection. For the generation of adaptive immunity, IFN α/β stimulates the maturation of dendritic cells (DCs) (29) and increases the major histocompatibility complex (MHC) expression on DC (30). IFN α/β is essential for the proliferation and survival of helper T cells (31), and controls the activity of helper T cells. This is done by inducing the expression of IL-12 (32) and IL-18 (33) receptors, and the enhancement of IFN γ production (34). The activation of antigen presenting cells (APCs) and helper T cells leads to the development of an influenza-specific Th1 response.

The activation of NF- κ B and AP-1 leads to the production of pro-inflammatory cytokines such as IL-1, IL-6, and TNF α (35) and chemokines such as CCL5 (21) that are important for innate immunity. In response to CCL5, NK cells are recruited to the site of infection (36). NK cells recognize the up-regulation of haemagglutinin on the infected cell through the NKp46 receptor (37) and kill the infected cell before the influenza virus

can be replicated. The innate antiviral mechanism restricts the replication of influenza virus at the earliest stages of infection, prior to the adaptive immune response.

Concurrently, DCs are recruited to the site of infection by chemokines such as CCL-2, initiating adaptive immunity (38). The DCs internalize the influenza virus through endocytosis. Viral particles are digested into peptides in the lysosome and are presented on the DC surface by MHC class II (39). The DCs undergo maturation and increases the expression of CCR7 so that it can migrate to the lymph node (40). The DCs then present the viral antigen to naïve CD4 (41) and CD8 T cells (42).

After antigen presentation, naïve CD8 T cells expressing an appropriate T cell receptor (TCR) mature into CD8 effector T cells and move to the site of infection (43), where they recognize infected epithelial cells and induce the apoptosis of infected cells either through the release of perforin or through Fas-FasL interactions (44). CD4 T cells mature into CD4 effector T cells and activate antigen presenting B cells to proliferate and produce influenza-virus specific antibodies (45). The antibodies bind to the surface of influenza virus, preventing the virus from infecting and spreading. The virus is eliminated within 7 to 10 days of infection (46), and effector B cells and T cells become memory B cells (47) and T cells (46), producing immunity against future infection by the same strain of influenza.

1.3 Differentiated H5N1 influenza immune response

Regular human influenza symptoms include fever and coughing, which are not life-threatening; individuals usually recover within two weeks of infection (48). Immuno-compromised individuals, such as transplantation patients taking immunosuppressive

drugs (49), young children, and the elderly (50), may develop more severe symptoms such as bronchitis and viral pneumonia (48), and may die due to complications such as secondary bacterial infections (51). However, some influenza strains are very lethal, such as H5N1, which has a mortality rate exceeding 50% (2). H5N1 patients frequently suffer severe viral pneumonia, which progresses to acute respiratory distress syndrome (ARDS) (52). Systemic infection can also occur in other organs, including the brain and liver, leading to multi-organ failure (53). Patients usually die within 9 days of the onset of disease (54).

One of the hallmarks of H5N1 influenza infection is an excess infiltration of monocytes, neutrophils, and activated lymphocytes in the lung (55). The inflammatory response caused by the macrophages and neutrophils leads to a number of severe lung injuries, including apoptosis of alveolar cells (55), hemorrhaging (56), and ARDS caused by pulmonary congestion (56). Avian influenza infections are also characterized by high serum cytokine levels, or hypercytokinemia (57). For instance, IL-6, IL-8, and MIG are respectively 200, 16, and 13 times higher in H5N1 patients than patients with regular human influenza (57). IL-6 is a pro-inflammatory cytokine (58), whereas IL-8 and MIG are chemokines which respectively recruit neutrophils (59) and T cells (60). Therefore, it has been postulated that hypercytokinemia is the cause of severe inflammation and excess leukocyte infiltration. Lymphopenia is another characteristic of severe avian influenza infections, and is often associated with the development of ARDS, as well as death (61-63). Lymphocyte levels in blood are three times lower in ARDS patients than in patients without ARDS (61), which may explain the inability of the immune system to clear some influenza viruses.

Although the exact mechanisms of hypercytokinemia, lung inflammation and systemic infection are not understood, research on influenza pathology has provided some insight. For example, avian influenza replicates more effectively than regular flu. For instance, the viral load of avian influenza is 10 times higher than regular influenza in the nose, and 160 times higher in the throat (57). Avian influenza has special proteins that counteract the immune system. For instance, the NS1 protein antagonizes the anti-viral IFN response by binding to dsRNA and RIG-1 (64). The PB2 polymerase, with a mutation at Glu627Lys, is also more effective in replication (65). The overall result is that avian influenza viruses replicate more effectively, causing a larger number of epithelial cells to be infected. The recruitment of monocytes and macrophages causes a higher production of pro-inflammatory cytokines, leading to severe lung inflammations. Clinically, the plasma level of cytokines and chemokines correlates positively with the pharyngeal viral load, suggesting that these responses are driven by high viral replication (57). In addition to a high viral load, avian influenza has a unique molecular mechanism that leads to a higher cytokine production. The NS1 protein of the H5N1 influenza virus induces pro-inflammatory cytokines, such as TNF- α in macrophages, to much higher levels than seen in H1N1 and H3N2 influenza (66). A recombinant H1N1 influenza, with the NS1 genes from the H5N1 influenza, induced high level of pulmonary pro-inflammatory cytokines in pigs, as well as prolonged viral production (67).

Infections are more systemic when caused by avian influenza, as compared to regular influenza, due to differing amino acid sequences in the HA protein's proteolytic cleavage site. Regular influenza has a single basic amino acid arginine and is specifically cleaved by trypsin-like extracellular serine protease Clara (68), which is secreted from

Clara cells in the epithelial lining of the respiratory tract (5). When the virus is released, the HA is cleaved by the extracellular serine protease to form functional HA1 and HA2 subunits for a second round of infection. Since the extracellular serine protease has a limited tissue distribution in the respiratory lining, regular influenza infections are limited in the upper respiratory tract (5). However, for avian influenza, the HA proteolytic cleavage site has poly basic residues, and can be cleaved by the furin protease, which is ubiquitously expressed in the Golgi apparatus of many cell types (69). Therefore, the HA is already cleaved and functional when the avian influenza virus is shed, and can initiate a second round of infection on any cell type, leading to systemic infection in organs such as the brain and kidney (53).

Since lung inflammation and the subsequent ARDS are the cause of mortality, reducing lung inflammation may save patients' lives. Since a high influenza viral load is associated with hypercytokinemia and a fatal outcome (57), research is underway on controlling virus replication through vaccination and anti-viral drugs. Yet, due to the high mutation rate of influenza viruses, different strains emerge and new vaccines have to be developed constantly (70). Influenza viruses can also develop resistance to anti-viral drugs such as oseltamivir (71), making influenza a moving target. However, drugs that target the aberrant host immune response could have the advantage of alleviating lethal pathophysiology such as lung inflammation, regardless of the influenza strain. Therefore, studying host immune responses is essential, and microarray is a useful technology to do so.

1.4 Up-regulation of FKBP5 during influenza infection

Several microarray studies on the cell line and animal models of influenza infection have been conducted, and have yielded useful information on virus-host interaction. For instance, a microarray analysis has found that numerous genes, which were related to antiviral and interferon responses, were down-regulated by the NS1 protein of influenza viruses when A549 cells were infected (72). This result is consistent with the finding that NS1 attenuates the interferon response (73) and the 2'5'-OAS/RNaseL antiviral response (74) through the NS1 double stranded RNA-binding domain, confirming the validity of using microarray analysis when studying virus-host interaction.

Our laboratory has performed a microarray analysis on a ferret model to study the host immune response during influenza infection (75). Ferrets are chosen because ferrets can be infected with human influenza viruses, and because ferret-to-ferret transmission is possible (76). Moreover, the clinical symptoms of influenza infected ferrets are similar to the symptoms of humans (77). Of the numerous genes characterized by the microarray analysis, FK506 binding protein 5 (FKBP5) is up-regulated at the site of influenza infection, particularly for H5N1 avian influenza infections. FKBP5, as a peptidylproline cis-trans isomerase (78), can assist protein folding, which may affect influenza replication. FKBP5 is known to interact with immune pathways such as NF- κ B (79) and calcineurin (80), which may affect the host immune response. The goal of this thesis is to characterize the cause of FKBP5 up-regulation, and to determine the function of FKBP5 in influenza infection.

1.5 FK506 binding protein 5 (FKBP5)

FKBP5 belongs to the FK506 binding protein family of peptidylproline cis-trans isomerases (PPIases). Its main function is to assist protein folding by converting the peptide bond preceding proline (propyl bond) into a correct cis-trans confirmation (81). FKBP5 was first discovered in T cells (78) and the preadipocyte cell line (82) in mice, and is expressed in a variety of mouse (82) and human tissues (83). FKBP5 is a monomeric cytoplasmic protein with 457 amino acids, and has a size of 51 kDa (83). The FKBP5 polypeptide is divided into 3 functional domains: the FK1 FKBP domain (33-138 aa), the FK2 domain (147-251), and the TPR (tetratricopeptide repeat) domain (261-400); there is also a linker sequence between the FK2 and TPR domains (84). The FK1 domain is the source of the PPIase activity of FKBP5, and Phe-67 and Asp-68 are the two highly conserved codons in the domain. A double point mutation with Phe-67 changed to Asp and Asp-68 changed to Val decreases the FKBP5 enzymatic activity by more than 90% (85). The FK2 domain is generally structurally similar to the FK1 domain. However, FK2 has only 26% sequence conservation compared to the FK1 domain, and has three amino acid insertions (D195, H196 and D197) in the enzymatic binding pocket. FK2 lacks the PPIase activity (84), but the distinct orientation of the FK2 domain in FKBP5 allows protein-protein interaction such as with progesterone receptor (84). The TPR domain is essential in Hsp90 binding for the interaction with steroid receptors (86). Rapamycin, an antagonist of FKBP5, can bind to the FK1 domain, thereby inhibiting PPIase activity; Rapamycin can also bind to the FK2 domain, thereby inhibiting the protein interaction activity of FKBP5 (84).

The human FKBP5 gene is about 115 kb in size, is situated in chromosome 6, and

is composed of 11 exons and 10 introns (87). The promoter of FKBP5 is 3.4 kb upstream of the gene (88). There are also two hormone response elements (HRE) in the intron E of the gene that respond to glucocorticoid and progestin (89). For the splicing pattern, alternative FKBP5 isoforms have not been described. However, techniques in proteomics such as 2D fluorescence difference gel electrophoresis (DIGE) and 2D immunoblotting reveal the possible existence of several FKBP5 isoforms (90). FKBP5 is known to interact with different proteins, including the glucocorticoid receptor, the Hsp90 protein, and the NF- κ B components (79, 86). With PPIase activity, FKBP5 can induce a conformational change in its interaction partner, and may act as a switch in controlling signal transduction along a cellular pathway.

1.6 FKBP5 interaction with immune response pathways

FKBP5 has been shown to interact with various immune response pathways. One of the important pathways is calcineurin, which is a serine/threonine protein phosphatase that controls cellular pathways through the phosphorylation of transcription factors, such as the nuclear factor of activated T cells (NFAT) (91). In the presence of the immunosuppressive drug FK506, the FKBP5/FK506 complex binds to calcineurin and inhibits the phosphatase activity of calcineurin (92). Therefore, the dephosphorylation of transcription factors such as NFAT is prevented and transcription factors cannot translocate to the nucleus to initiate the cellular response through gene expression (92). The end result is that FKBP5, in combination with FK506, can suppress the calcineurin pathway. Since FKBP5 can bind to calcineurin even in the absence of FK506 (93), it is also possible that FKBP5 interacts with other proteins and synergistically controls the

calcineurin pathway using different yet uncharacterized mechanisms.

Another important pathway that FKBP5 interacts with is NF- κ B. A mass spectrometry study of HeLa cells has shown that FKBP5 interacts with the inhibitor of kappa B kinase alpha (IKK α) during TNF α stimulation, and that the siRNA knock-down of FKBP5 decreases the NF- κ B activation measured by a luciferase reporter assay (94). In another study on primary vascular smooth muscle cells, rapamycin, an inhibitor of FKBP5, down-regulates NF- κ B activation as the degradation of the inhibitor of kappa B alpha (I κ B α) protein is not observed. FKBP5 siRNA also down-regulates NF- κ B activation as the translocation of the NF- κ B protein to the nucleus is inhibited (95). These findings suggest that FKBP5 plays an important role in the function of NF- κ B and may provide an entry point in controlling NF- κ B pathway through the interaction with other protein partners. Of note, FKBP5 is up-regulated by anti-inflammatory agents such as glucocorticoid (96), aspirin (97) and lactoperoxidase (LPO) (98). It is possible that FKBP5 act synergistically with other proteins that are also up-regulated by anti-inflammatory agents to inhibit cytokine production.

1.7 FKBP5 and influenza replication

To date, no study has described the relationship between influenza and FKBP5. However, being a PPIase, FKBP5 may play a role in viral replication. This is true for cyclophilin A and cyclophilin B, two PPIases that are required for human immunodeficiency virus (HIV) and hepatitis C virus (HCV) replication respectively (99, 100). Cyclophilin A binds to the HIV Gag protein, and the disruption of cyclophilin A-Gag interaction by cyclosporine A leads to a reduction in HIV replication (99).

Cyclophilin B is a cofactor of the RNA-dependent RNA polymerase NS5B of HCV (100), and the replication of HCV is inhibited by cyclosporine A (101). Since FKBP5 is up-regulated in the influenza-infected cells, it is worthwhile to study whether FKBP5 has any effect on influenza replication.

1.8 FKBP5 and the suppression of inflammation by glucocorticoid

Another important aspect of FKBP5 in influenza pathology is the suppression of inflammation through glucocorticoid. During influenza infection, pro-inflammatory cytokines such as IL-1, TNF α and IFN are secreted by infected cells and immune cells to sustain an inflammatory response (35). However, an uncontrolled inflammation can have detrimental consequences, as exemplified by septic shock syndrome (102). To suppress an excessive inflammation, the hypothalamic-pituitary-adrenal (HPA) axis is activated to increase adrenal glucocorticoid production (103). In response to the increase in the blood level of pro-inflammatory cytokines such as IL-1 and IL-6, the hypothalamus secretes corticotrophin-releasing hormone (CRH) (104). CRH stimulates the pituitary gland to secrete adrenocorticotrophic hormone (ACTH) (103). As the blood level of ACTH increases, the adrenal gland secretes glucocorticoid in response (103).

Glucocorticoid (GC) acts on different cell types, such as macrophages and T cells, to decrease pro-inflammatory cytokine production (105). GC first passes through the cell membrane and binds to the GC receptor (106), which translocates into the nucleus (106) and binds to the negative glucocorticoid response element (GRE), suppressing the transcription of inflammatory genes such as IL-1 and IL-2 (107). The GC receptor can also interact with transcription factors such as NF- κ B and AP-1 to prevent them from

binding to the DNA sequences of pro-inflammatory cytokines such as TNF α (108). The GC receptor can also bind to the positive GRE and up-regulate the expression of anti-inflammatory proteins such as I κ B (107).

During influenza infection, pro-inflammatory cytokines activate the HPA axis, leading to an increase in GC production. It is possible that the FKBP5 up-regulation observed in influenza infected cells is caused by GC. Since FKBP5 is shown to interact with different immune response pathways, it is further possible that FKBP5 alone, or in conjunction with other proteins, facilitates the suppressive effect of GC on cytokine production.

1.9 Rationale, hypothesis and objectives

Rationale

Up-regulation of FK506 binding protein (FKBP5) gene expression is observed at the site of influenza infection in a ferret model, yet the functions of FKBP5 in mediating the inflammation and virus production are not understood. Immunosuppressive agents such as glucocorticoid, aspirin, and lactoperoxidase up-regulate FKBP5 and suppress pro-inflammatory cytokine production. FKBP5 also interacts with various immune signalling pathways which control cytokine production, such as NF- κ B and calcineurin. It is possible that immunosuppressive action is facilitated by FKBP5. During an influenza infection, the level of glucocorticoid in the blood is up-regulated to decrease cytokine production. The up-regulation of FKBP5 at the site of infection may facilitate the function of glucocorticoid.

Hypothesis

FKBP5 plays a positive role in mediating the suppressive effect of glucocorticoid on pro-inflammatory cytokine production.

Objectives

The specific objectives for this study were:

1. To compare the suppressive effect of glucocorticoid on pro-inflammatory cytokine production in A549 lung cells, in the presence and absence of FKBP5 siRNA;
2. To examine whether influenza infection up-regulates FKBP5 expression in A549 cells;
3. To determine if glucocorticoid treatment and the subsequent FKBP5 up-regulation have an effect on influenza production in A549 cells.

CHAPTER 2 – MATERIALS AND METHODS

2.1 Cells and cell culture conditions

The human alveolar epithelial cell line A549 and the Madin-Darby Canine Kidney (MDCK) cell line were purchased from the American Type Culture Collection (ATCC). A549 cells were maintained in Kaighn's Modification of Ham's F-12 (F-12K) medium (ATCC) supplemented with 10% Fetal Bovine Serum (FBS) (GIBCO) and 1% penicillin and streptomycin (pen/strep) (Invitrogen). MDCK cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco) supplemented with 10% FBS, 1% pen/strep, 25mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (Gibco) and 0.2% bovine serum albumin fraction V (BSA) (Gibco). Cells were grown in T-75 tissue culture flasks (Corning or Sarstedt) at 37°C with 5% CO₂. Cells with passage number between 3 and 10 were used for experiments.

2.2 Hydrocortisone and TNF α treatments

Hydrocortisone (Sigma-Aldrich) was dissolved in DMEM to make a working stock at a concentration of 50 μ g/ml or 137.9 μ M. For experiments in a 6-well plate, 235 μ l of hydrocortisone stock was added to 3ml of culture medium to give a final concentration of 10 μ M. For experiments in a 96-well plate, hydrocortisone stock was serially half-log diluted in culture medium. 50 μ l each of diluted hydrocortisone was added to 50 μ l of culture medium in each well to give a final concentration of 32 μ M, 10 μ M, 3.2 μ M and serially down to 0.001 μ M. Human tumour necrosis factor alpha (TNF α) (R&D Systems) at a concentration of 1 μ g/ μ l was 10-fold diluted in F-12K medium to make a 100ng/ μ l working stock. 3 μ l of diluted TNF α was added to 3ml of culture medium in a 6-well plate to give a final concentration of 100ng/ml for the experiments.

2.3 Cytometric bead array

Cytometric bead array (CBA) human inflammation kit (Becton Dickenson) was used according to the manufacturer's instruction manual. In brief, 50 μ l of culture medium from cell samples was collected and frozen at -80°C, and was thawed prior to CBA measurement. Human inflammation standard, containing 5000pg/ml IL-1 β , IL-6, IL-8, IL-10 and IL-12p70, was serially 2-fold diluted. Human inflammation capture beads were prepared by mixing 10 μ l of each capture beads for the five cytokines to give a total volume of 50 μ l. The beads were then added to 50 μ l of diluted standard or 50 μ l of samples, and 50 μ l of PE detection reagent was added to provide a fluorescence signal. After 3 hours of incubation at room temperature, the beads were washed once with the wash buffer provided in the kit and resuspended in 300 μ l wash buffer. Three cytometer setup beads were used to calibrate the FACSCalibur flow cytometer (Becton Dickenson). The calibrated flow cytometer was then used to measure the fluorescence signal of the sample beads, and the results were analyzed by the BD CBA software (Becton Dickenson) and Microsoft Excel. The amount of cytokine in the culture medium sample was calculated from the standard curve generated by the human inflammation standard.

2.4 Transfection of siRNA

Lyophilized FKBP5 specific siRNA duplexes (Sigma-Aldrich and Ambion) and scrambled sequence siRNA duplex (Ambion) were dissolved in RNase free water (Ambion) to generate a storage stock at 50 μ M and a working stock at 10 μ M. The sequences of siRNAs were the following:

FKBP5 siRNA set 2 (Sigma-Aldrich):

sense: 5'-rGrArGrCUUrAUrAUrAUrGrArArGUrGrATT-3'

anti-sense: 5'-UrCrArCUUrCrAUrAUrAUrArArGrCUrCTT-3'

FKBP5 siRNA set 3 (Ambion):

sense: 5'- rGrArGrArArArGrGrCUUrGUrAUrArGrGrATT -3'

anti-sense: 5'- UrCrCUrAUrArCrArArGrCrCUUUrCUrCrAT-3'

Scrambled sequence siRNA (Ambion):

sense: 5'-rGrAUrCrAUrArCrGUrGrCrGrAUrCrArGrATT-3'

anti-sense: 5'-UrCUrGrAUrCrGrCrArCrGUrAUrGrAUrCTT-3'

The siPORT NeoFx transfection agent (Ambion) was used, and the siRNA transfection was performed according to the procedures in the manufacturer's manual. 5µl of NeoFx transfection agent was mixed in 100µl in Opti-MEM I medium (Invitrogen). A calculated amount of siRNA was dissolved in Opti-MEM I medium and added to the NeoFx agent, so that the final concentration of siRNA would be 30, 15 and 5nM in the 6-well plate. The RNA/NeoFx transfection agent complex was incubated for 10 minutes at room temperature. After incubation, the transfection complex was added to the 6-well plate and 3×10^5 A549 cells in 3ml regular growth medium were seeded on the transfection complex. The siRNA and transfection agent was left on the A549 cells and incubated at 37°C. At specific time points after transfection, mRNA was extracted from the cells and the efficiency of gene silencing was determined by quantitative real time polymerase chain reaction (qPCR).

2.5 Trizol RNA extraction

RNA was extracted from A549 and MDCK cells by Trizol reagent (Invitrogen) according to product manual instructions. Culture medium was removed from the 6-well plate and replaced with 1ml Trizol for 5 minutes. Trizol, with dissolved cellular materials, was homogenized by pipetting, transferred to a 1.5ml Eppendorf tube and frozen at -80°C. The Trizol sample was thawed prior to RNA extraction, and 200µl of chloroform was added to Trizol. After vigorous shaking, the Trizol sample was centrifuged at 12000 x g for 15 minutes at 4°C. The aqueous phase containing RNA was separated to another Eppendorf tube. RNA was precipitated by adding 500µl of isopropanol and centrifuging at 12000 x g for 10 minutes at 4°C. Finally, the precipitated RNA was washed once with 1ml of 75% ethanol and dissolved in 20µl of RNase free water (Ambion). The concentration and purity of the dissolved RNA was determined by ultra violet (uv) absorbance measurement at 260nm and 280nm using BioPhotometer 6131 (Eppendorf).

2.6 Reverse transcriptase polymerase chain reaction (RT-PCR)

Reverse transcriptase polymerase chain reaction (RT-PCR) was employed to make complementary DNA (cDNA) from RNA. The reaction was performed according to the manufacturer's instructions for Murine Leukemia Virus (MuLV) reverse transcriptase (Applied Biosystems). 2µg of RNA was mixed with: RNase free water (Ambion), 2µl of 10X PCR Buffer II (Applied Biosystems), 4µl of 25mM MgCl₂ (Applied Biosystems), 2µl of 10mM dNTP (Promega), 1µl of 20 unit RNase inhibitor, 1µl of 25µM random Hexamer or oligo d(T)₁₆ primers (Applied Biosystems), and 1µl of 50 units MuLV reverse transcriptase to give a total volume of 20µl. The reaction mix was

incubated for 10 minutes at room temperature, 40 minutes at 42°C and heat-inactivated for 5 minutes at 65°C. The cDNA produced was used for quantitative real time PCR.

2.7 Quantitative real time PCR (qPCR)

Quantitative real time PCR (qPCR) was used to measure the expression of FKBP5 relative to a house keeping gene β -actin. Following primers (Invitrogen) were used to amplify a 150bp fragment from gene FKBP5 and β -actin in cDNA.

FKBP5:	sense:	5'-ACCAAAGCTGTTGAATGCTG-3'
	anti-sense:	5'-GGCTGACTCAAACCTCGTTCA-3'
β -actin	sense:	5'-TGACCGGATGCAGAAGGA-3'
	anti-sense:	5'-CCGATCCACACCGAGTACTT-3'

0.5 μ l of cDNA, 3.5 μ l of RNase free water (Ambion), 1 μ l of 2.5 μ M primers and 5 μ l Power CYBR Green PCR Master Mix (Applied Biosystems) were mixed and applied to a 384-well plate (Applied Biosystems). RT-PCR was performed in the 7900HT Fast Real-Time PCR System (Applied Biosystems), and the result was analyzed using SDS software version 2.2 (Applied Biosystems) and Microsoft Excel.

2.8 Influenza production

Influenza Strains A/WS/33 H1N1, A/NWS/33 H1N1, and A/PR/8/34 H1N1 were purchased from ATCC. Following the procedures of World Health Organization manual on animal influenza diagnosis and surveillance (109), 100 μ l of 1000-fold diluted influenza virus with 100U/ml penicillin, 100 μ g/ml streptomycin and 50 μ g/ml gentamicin (Gibco) was inoculated into the amniotic cavity of specific pathogen free (SPC) 10-day

old embryonated hens' eggs (Health Canada). After 48 hours of incubation at 33.5°C with 5% CO₂, the eggs were chilled either for 24 hours at 4°C or 30 minutes at -20°C. 3-5ml amniotic fluid was collected and centrifuged at 600 x g for 10 minutes. The influenza virus in amniotic fluid was distributed in 1ml aliquot and stored at -80°C. The influenza titre was quantified by plaque assay and haemagglutination assay.

2.9 Influenza infection

Influenza infection experiments were carried out in 6-well plates. A549 or MDCK cells were seeded at a density of 3×10^5 per well in normal growth medium supplemented with FBS and pen/strep. After 24 hours of incubation at 37°C, cells were washed three times with 1ml pure growth medium stripped of any supplements. One well of cells were trypsinized and the number of cells was counted for calculating the multiplicity of infection (MOI). MOI=1 and MOI=5 were used for influenza infection. Based on the MOI, the correct amount of influenza virus was dissolved in 100µl of pure growth medium. The washing medium in the well was removed and 100µl virus medium was overlaid on the cells. The cells and virus were incubated for 1 hour at 37°C with 5% CO₂ to allow virus binding on the cells. After 1 hour of incubation, the 100µl virus medium was removed and the cells were washed three times with 1ml pure growth medium to remove any unbinding virus. The infected cells were then maintained in growth medium supplemented with 0.2% BSA, 25mM HEPES Buffer, 1% pen/strep and TPCK-trypsin (Sigma-Aldrich). 0.125 µg/ml TPCK-trypsin in F-12K medium was used for A549 cells while MDCK cells required 2 µg/ml TPCK-trypsin in DMEM.

2.10 Plaque assay

Plaque assay was performed according to the infection procedures described above and the procedures reported by Matrosovich *et al* (110). In brief, 3×10^5 MDCK cells were seeded in each well of a 6-well plate. Following 24 hours of 37°C incubation, the cells were washed three times with 1ml MDCK medium. The influenza virus was serially 10-fold diluted in MDCK medium. The wash medium was removed and 100µl each of 10^2 to 10^7 fold diluted influenza was added to each well. After 1 hour of 37°C incubation, the virus was removed and the cells were washed three times with 1ml MDCK medium. Finally, 3ml of 0.9% overlay agar supplemented with 1X DMEM medium, 0.2% BSA, 25mM HEPES Buffer, 1% pen/strep and 2 µg/ml TPCK-trypsin was put on top of the infected cells. After 3 days of incubation at 37°C with 5% CO₂, the agar was removed and the cells were fixed in 10% neutral buffered formalin for 30 minutes at 4°C. After fixing, the cells were stained with crystal violet. The number of plaques was counted in each well, and the titre of the influenza samples was reported in plaque forming units per ml (pfu/ml).

2.11 Haemagglutinin assay

The procedures for haemagglutination assay are documented in the World Health Organization manual on animal influenza diagnosis and surveillance (109). 50µl of pH=7.2, 0.01M phosphate-buffered saline (PBS) were added to each well in a 96-wells plate. 50µl of influenza virus was added to the first well and serially two-fold diluted by transferring 50µl from the first well to the next well down the row. 50µl of PBS washed 0.5% Alsever's turkey red blood cells were added to the diluted influenza in each well.

After 30 minutes of incubation at room temperature, haemagglutination patterns were recorded. The HA titre was reported as the reciprocal of the highest dilution of virus that caused complete haemagglutination.

2.12 MTS assay

CellTiter 96® Aqueous MTS Reagent (Promega) was used according to the manufacturer's instruction. The tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] MTS solution was stored at -20°C and thawed prior to the assay. A549 cells were cultivated in 96-well plate at a density of 20000 cells per well in 50µl of F12K growth medium. A/WS/33 H1N1 influenza stock was serially ten-fold diluted with F12K virus medium and hydrocortisone was serially half-log diluted in regular F12K growth medium. 50µl each of diluted virus or hydrocortisone were added to the wells. After 72 hours of influenza infection or 24, 48 and 72 hours of hydrocortisone treatment, 50µl of MTS reagents were added. After 1-1.5 hour incubation at 37°C, 100µl of 10% SDS were added to each well to stop the reaction. The light absorbance at 490nm was measured by a 96-well plate reader.

2.13 Protein extraction

Protein extraction followed the manufacturer's instruction for Trizol reagent. After removing the aqueous phase for mRNA extraction as described above, 300µl of 100% ethanol was added to the organic phase per 1ml of Trizol reagent used. The samples were mixed and centrifuged at 2000 x g for 5 minutes at 4°C. After

centrifugation, the phenol-ethanol supernatant was separated into a 15ml falcon tubes (Sarstedt). 1.5ml of isopropanol was added to precipitate the protein. The sample was incubated for 10 minutes at room temperature followed by centrifugation at 12000 x g for 10 minutes at 4°C. Finally, the protein precipitate was washed 3 times with 2ml of 0.3M guanidine hydrochloride in 95% ethanol, vacuum dried and dissolved in 300µl of 1% SDS. The protein product was then quantified using Bradford Protein Assay reagent (Bio-Rad) following the manufacturer's instruction. Alternatively, for extracting protein samples from cells, where mRNA extraction was not required, the cells were directly dissolved in 300µl of Triton X-100 lysis buffer (1% Triton X-100, 150mM NaCl, 50mM Tris-HCl, pH 7.5 and 1X Complete Protease Inhibitor Cocktail (Roche)) in a 6-well plate. The protein dissolved in the Triton X-100 buffer was quantified by Bradford Protein Assay reagent.

2.14 Western blot

Protein samples were mixed with 2x protein loading buffer (62.5 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 2% β-mercaptoethanol, 0.0005% bromophenol blue) and boiled at 100°C for 5 minutes. Samples were loaded to a 10% SDS-polyarylamid gel and were run at 120V for 30-40 minutes. After electrophoresis, the protein was transferred to nitrocellulose membranes (Millipore) by semi dry transfer method at 210mA for 3 hours. Membranes were blocked by 5% non-fat dry milk in Tris-buffered saline (TBS) with 0.05% Tween-20 (Sigma-Aldrich) for 1 hour at room temperature. Membranes were then incubated with 1:5000 diluted mouse anti-β-actin monoclonal antibody (Becton Dickenson) or rabbit anti-FKBP5 polyclonal antibody (Santa Cruz) in

Tween TBS (TTBS) milk solution for 1 hour at room temperature. The membranes were washed 3 times, 5 minutes each, in TTBS, and incubated with 1:10000 diluted goat anti-mouse IgG-HPR (Santa Cruz) or goat anti-rabbit IgG-HPR (Santa Cruz) secondary antibodies in TTBS milk solution for 1 hour at room temperature. Finally, the membranes were washed again 3 times, 5 minutes each, in TTBS and visualized by Amersham Enhanced Chemiluminescence (ECL) Plus Western Blotting Detection Reagents (General Electric Healthcare).

CHAPTER 3 - RESULTS

3.1 A/WS/33 H1N1 influenza was viable in A549 cells and produced viable virus

Here, I investigated FKBP5 up-regulation during influenza infection and its function in disease development. Our laboratory has previously shown that FKBP5, a peptidylproline cis-trans isomerase (PPIase), is up-regulated at the site of influenza infection such as lung and nasal turbinate in a ferret infection model (75). PPIases, such as cyclophilin A and cyclophilin B, are required for human immunodeficiency virus (HIV) and hepatitis C virus (HCV) replication respectively (99, 100). However, the function of FKBP5 in influenza replication is not well understood. I established an *in vitro* system to test the hypothesis that influenza infection increased FKBP5 expression which affected influenza replication. Since epithelial cells are the primary cellular targets for influenza infection in lung, A549 alveolar epithelium cell culture model was chosen to determine if A/WS/33 H1N1 influenza could be cultured.

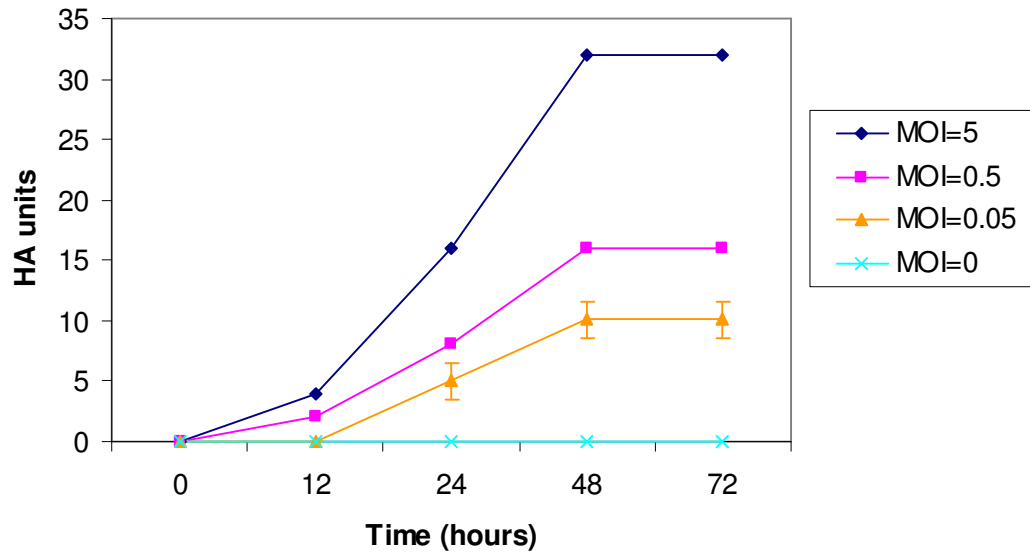
A549 cells were infected with A/WS/33 H1N1 influenza at the multiplicity of infection (MOI) of 5, 0.5, 0.05 and MOI=0 as an uninfected control. Culture medium was collected at 0, 12, 24, 48 and 72 hours and influenza replication was assessed by haemagglutination assay (HA) and plaque assay. The viral protein haemagglutinin level increased throughout the course of the experiment from 0 to 72 hours (Figure 1A). Influenza replication increased at a dose-dependent manner, since the higher initial inoculation of MOI=5 led to higher haemagglutinin production than the lower doses inoculations MOI=0.5 and MOI=0.05 (Figure 1A). At MOI=0 (no infection control), no haemagglutinin was detected. These results demonstrated that A/WS/33 H1N1 influenza was able to replicate in A549 cells.

To confirm that the influenza virus produced by A549 cells was viable and infectious, plaque assays were performed on samples from the MOI=5 infection. Consistent to the HA results, influenza was replicated in A549 cells as the number of infectious particle increased to over 80000 pfu/ml after 12 hours of infection (Figure 1B). The number of infectious particles decreased gradually after 24 hours continuing to 72 hours as the influenza virus destabilized (Figure 1B). This decrease was not observed in HA (Figure 1A), as HA was measuring viral protein levels from both the viable and non-viable viruses. Taken together, the results of the HA and plaque assays suggested that A/WS/33 H1N1 influenza replicated and produced infectious viral particles in A549 cells.

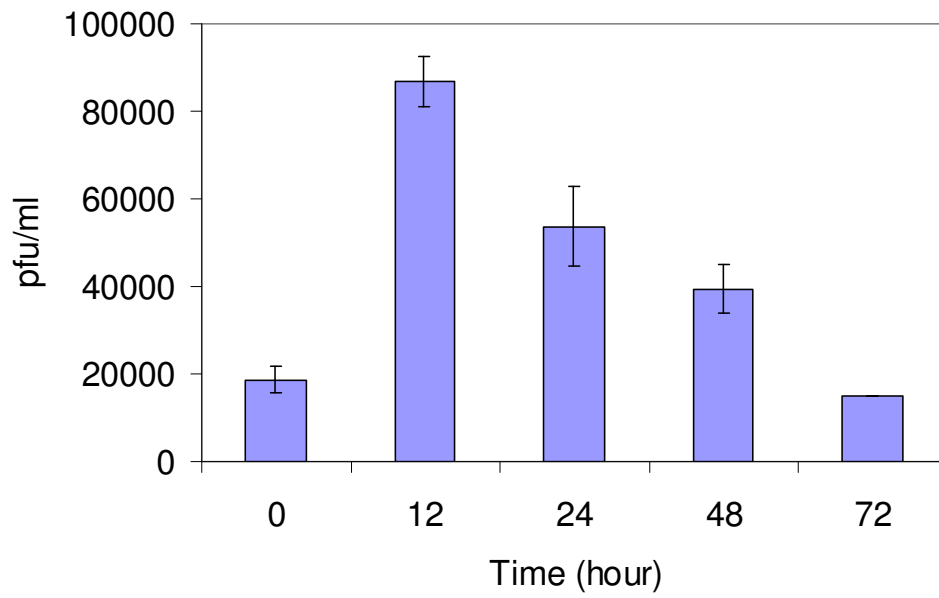
Figure 1. A/WS/33 H1N1 influenza replicated and produced infectious virus in A549 cells.

A549 cells were infected by A/WS/33 H1N1 at MOI=5, 0.5 and 0.05 with MOI=0 as an uninfected control. Culture medium was collected at 0, 12, 24, 48 and 72 hours post infection, and influenza replication was monitored by (A) haemagglutination assay and (B) plaque assay. (A) Haemagglutinin viral protein level increased from 0 to 72 hours. Higher haemagglutinin level was observed in samples from MOI=5 than MOI=0.5 and MOI=0.05. No haemagglutinin was detected in the uninfected negative control. (B) Plaque assay on samples from MOI=5 confirmed that influenza virus produced in A549 cells were viable. The number of viable viruses peaked at 12 hours and gradually decreased from 24 to 72 hours.

A



B



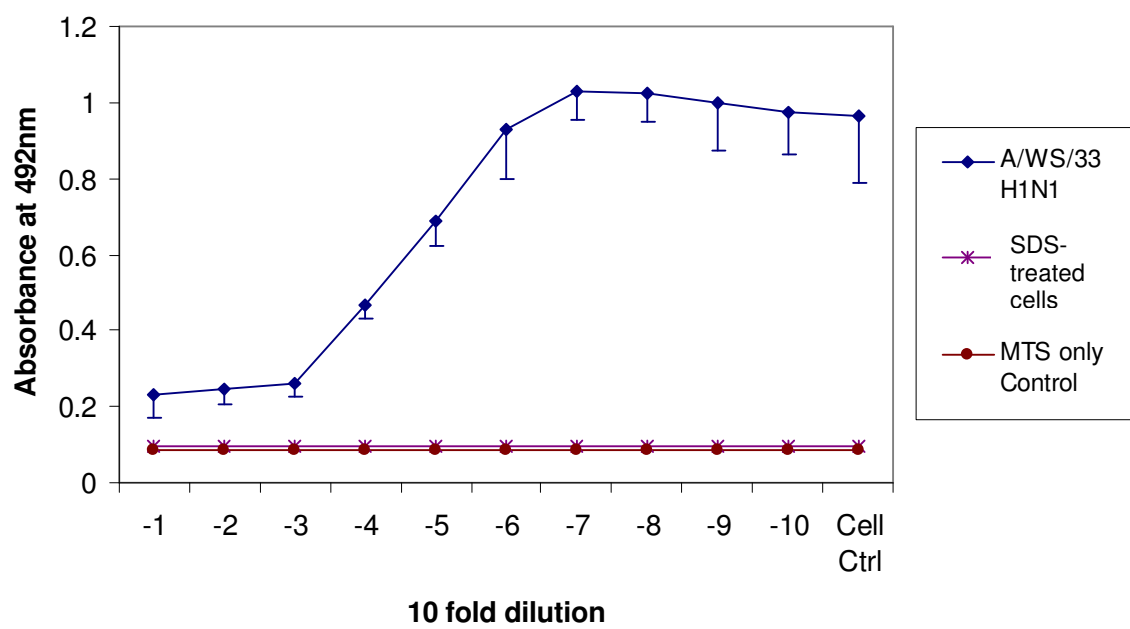
3.2 A/WS/33 H1N1 influenza decreased the viability of A549 cells

After demonstrating that A/WS/33 H1N1 influenza can replicate in A549 cells, I next examined the effect of influenza replication on the metabolism and viability of A549 cells. A549 cells were cultivated and infected with ten-fold serial dilutions of A/WS/33 H1N1 influenza. 72 hours after infection, the viability of the A549 cells was examined by [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] MTS assay.

When A549 cells were infected with A/WS/33 H1N1 influenza, the cells showed low absorbance of 0.2 for 1/10, 1/100 and 1/1000 fold diluted influenza samples (Figure 2) indicating low viability. When the virus was diluted from 10^4 to 10^6 fold, the absorbance gradually increased (Figure 2). As expected, for 10^7 to 10^{10} fold highly diluted influenza and no infection control, the absorbance remained high at 1.0 (Figure 8). The absorbance value of 0.2 for influenza samples was comparable to the absorbance value of 0.1 for dead A549 cells killed by SDS and MTS alone control, suggesting A/WS/33 H1N1 decreased the viability of A549 cells (Figure 2). The results demonstrated that A/WS/33 H1N1 infection of A549 cells affected cellular metabolism and viability and suggested that A549 cells as the cell model for investigating influenza replication.

Figure 2. A/WS/33 H1N1 influenza decreased A549 cells viability.

A549 cells were infected by serially diluted A/WS/33 H1N1 influenza for 72 hours. Cell viability after infection was measured by MTS assay. SDS treated cells and MTS reagents alone without cells were used as control. At high influenza titre (10^1 to 10^3 fold dilution), the viability of A549 cells was low with A_{492} at around 0.2, which was comparable to A_{492} value of 0.1 for killed cells and MTS alone controls. As the influenza titre decreased (10^4 to 10^7 fold dilution), the A_{492} values increased. For highly diluted influenza samples (10^4 to 10^7 fold dilution), the A_{492} of 1.0 was comparable to uninfected cells control.



3.3 A/WS/33 H1N1 influenza infection did not increase FKBP5 expression at mRNA and protein levels in A549 cells

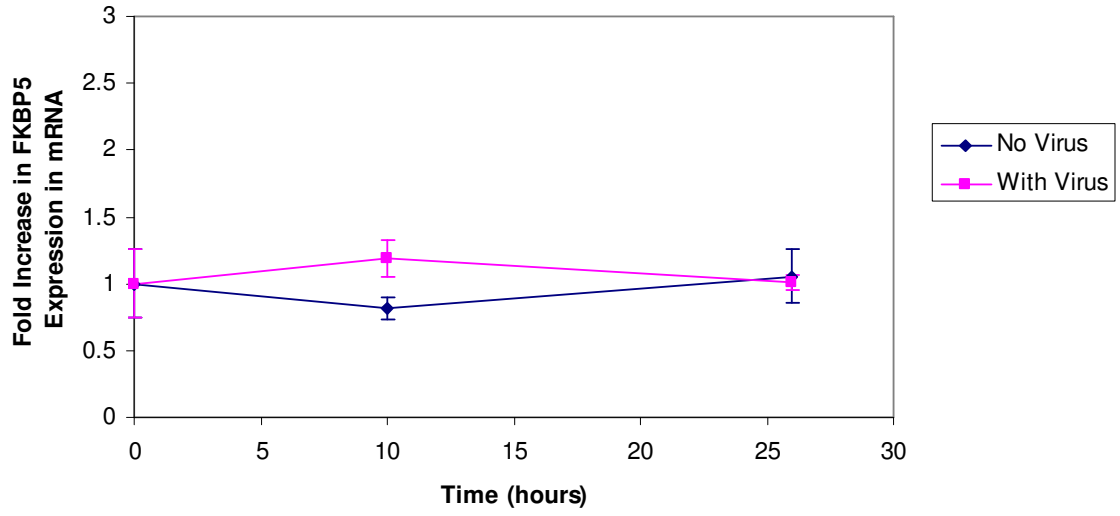
I next tested the hypothesis that influenza infection could up-regulate FKBP5 expression in the host cells. A549 cells were infected by A/WS/33 H1N1 influenza at MOI=1. At 0, 10 and 26 hours post-infection, mRNA and protein were extracted from A549 cells. FKBP5 expression in mRNA was determined by RT-PCR and FKBP5 protein level was measured by Western blot with the use of densitometry quantification.

FKBP5 mRNA level remained constant throughout the course of the experiments for both infected and uninfected A549 cells (Figure 3A). Furthermore, FKBP5 protein levels also did not change in both infected and uninfected A549 cells (Figure 3B). The results of the RT-PCR and Western blot analysis showed that A/WS/33 H1N1 influenza infection did not increase the FKBP5 expression level.

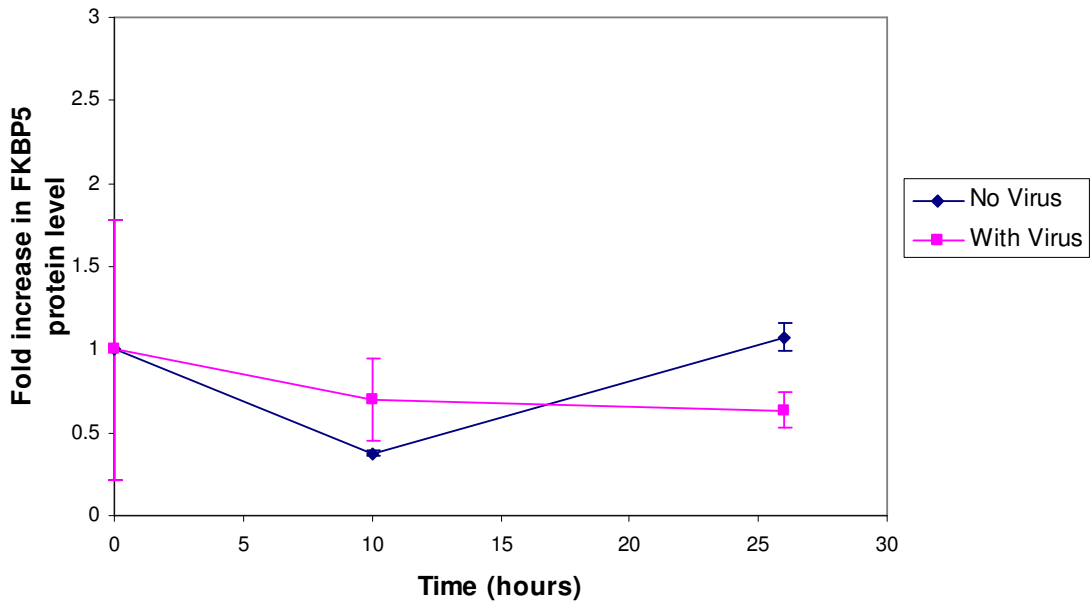
Figure 3. A/WS/33 H1N1 influenza infection did not enhance FKBP5 mRNA and protein level in A549 cells.

A549 cells were infected by A/WS/33 H1N1 at MOI=1. FKBP5 mRNA and protein level were measured by (A) RT-PCR and (B and C) western blot at 0, 10 and 26 hours post infection. (A) FKBP5 mRNA level remained constant for both infected and uninfected A549 cells. (B) FKBP5 protein levels for infected and uninfected A549 cells were similar. No increase of FKBP5 protein level was observed. (C) A section of western blot for 10 hours protein sample is shown to demonstrate densitometry quantification.

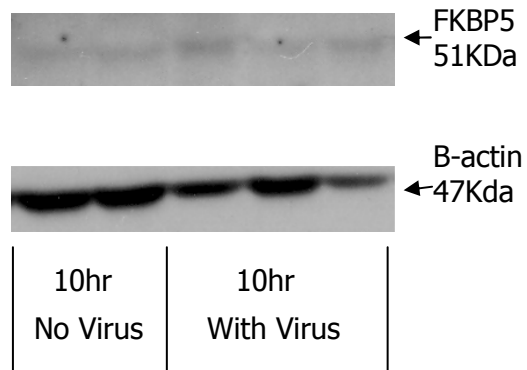
A



B



C



3.4 FKBP5 expression was enhanced by hydrocortisone but not by the influenza strains A/NWS/33 H1N1 and A/PR/8/34 H1N1

Influenza infection activates the anti-viral immune responses and the production of pro-inflammatory cytokines such as TNF α and IL-6 by cells such as neutrophils and macrophages (111). These pro-inflammatory cytokines in turn activate the Hypothalamic-Pituitary-Adrenal (HPA) axis and to increase adrenal glucocorticoid levels (112). It has previously been shown that glucocorticoid stimulation can increase FKBP5 expression in human peripheral blood mononuclear cells (PBMCs) (113). Furthermore, an *in vivo* study investigating NewCastle disease virus infection in chickens reported that viral infection led to the up-regulation of glucocorticoid expression to induce FKBP5 expression in the lung (114). From these previously published studies, I investigated if increased levels of glucocorticoid levels at the site of influenza infection could induce FKBP5 expression.

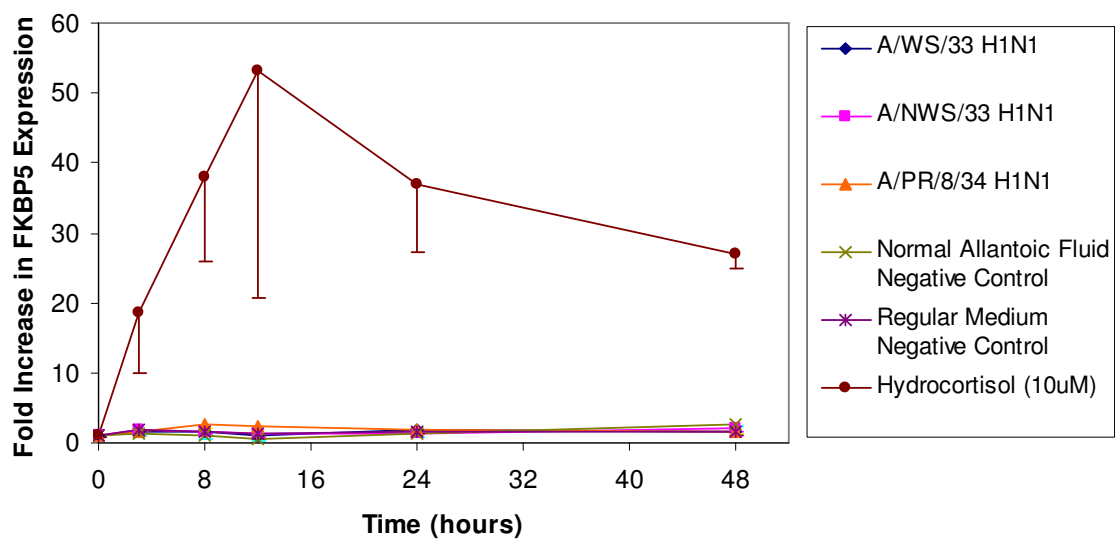
To test this hypothesis, hydrocortisone, the native form of human adrenal glucocorticoid, was added to A549 cells and FKBP5 expression was measured by RT-PCR. Moreover, infection with the influenza strains A/NWS/33 H1N1 and A/PR/8/34 H1N1 were also tested for the ability to induce FKBP5. A549 cells were cultivated and infected with A/WS/33 H1N1, A/NWS/33 H1N1 and A/PR/8/34 H1N1 at MOI=1 or stimulated with 10 μ M hydrocortisone. Samples were taken at 0, 4, 8, 12, 24 and 48 hours post infection/stimulation. Following RNA extraction, FKBP5 expression was measured by RT-PCR. Normal allantoic fluid and regular F12K medium were used as negative controls.

Consistent with the previous results, A/WS/33 H1N1, A/NWS/33 H1N1 and A/PR/8/34 H1N1 did not increase FKBP5 expression at early or late infection time points

(Figure 4). Furthermore, the FKBP5 expression levels were similar to normal allantoic fluid and regular medium negative controls (Figure 4), confirming that influenza replication did not increase FKBP5. In contrast, 10 μ M hydrocortisone enhanced FKBP5 expression by 19 to 53 fold throughout the time course. The up-regulation of FKBP5 peaked at 12 hours and gradually reduced at 24 and 48 hours following hydrocortisone stimulation (Figure 4).

Figure 4. FKBP5 expression was increased by hydrocortisone stimulation

A549 cells were infected with A/WS/33 H1N1, A/NWS/33 H1N1 or A/PR/8/34 H1N1 influenza at MOI=1 or stimulated by 10 μ M hydrocortisone. Normal allantoic fluid and regular F12K medium were used as negative controls. FKBP5 expression was measured by RT-PCR at 0, 4, 8, 12, 24 and 48 hours. All three influenza strains did not increase FKBP5 expression throughout the experiment. Stimulation with 10 μ M hydrocortisone increased FKBP5 expression by 19 to 53 fold.



3.5 FKBP5 expression was not enhanced by influenza infection in MDCK cells

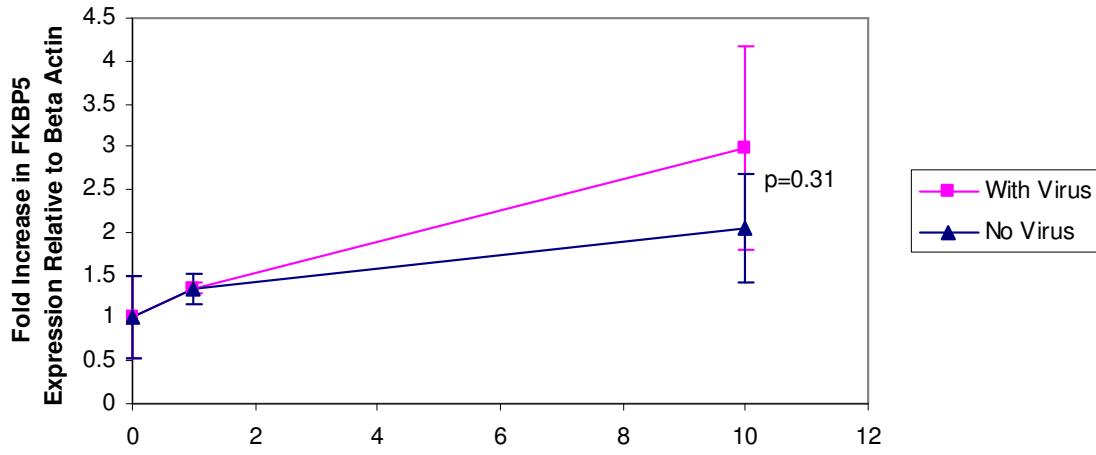
Since influenza infection did not induce FKBP5 expression in A549 cells, Madine Darby Canine Kidney (MDCK) cells were also investigated for influenza stimulated FKBP5 expression. MDCK cells are standard cells for influenza isolation and support the replication of different influenza strains. Therefore, MDCK cells made a suitable cell line to investigate influenza stimulated up-regulation of FKBP5.

MDCK cells were infected by A/WS/33 H1N1 influenza at MOI=1. FKBP5 expression in MDCK cells was examined by RT-PCR at of RNA extracted from samples at 0, 1 and 10 hours and by Western blot with densitometry quantification at 10 hours. At 0 and 1 hours, FKBP5 mRNA expression in infected and uninfected MDCK cells was similar (Figure 5A). At 10 hours, infected MDCK cells showed a slight increase in FKBP5 expression but the increase was not significant with $p=0.31$ (Figure 5A). Furthermore, Western blot analysis demonstrated that at 10 hours, there was no significant difference in FKBP5 protein levels between infected and uninfected MDCK cells (Figure 5B).

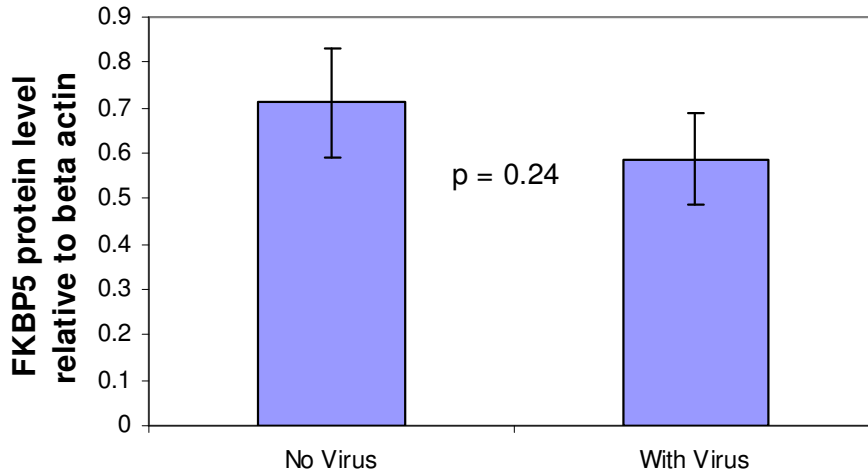
Figure 5. A/WS/33 H1N1 infection did not up-regulate FKBP5 mRNA and protein level in MDCK cells.

MDCK cells were infected by A/WS/33 H1N1 at MOI=1. FKBP5 mRNA and protein level were measured by (A) RT-PCR and (B and C) western blot at 0, 1 and 10 hours post infection. (A) FKBP5 mRNA levels for infected and uninfected MDCK cells were similar at 0 and 1 hours. At 10 hours, there was a slight increase in FKBP5 expression but the increase was not statistically significant ($p=0.31$). (B) At 10 hours, FKBP5 protein levels for infected and uninfected A549 cells were comparable with $p=0.24$. (C) A section of western blot at 10 hours protein sample was analysed by densitometry quantification.

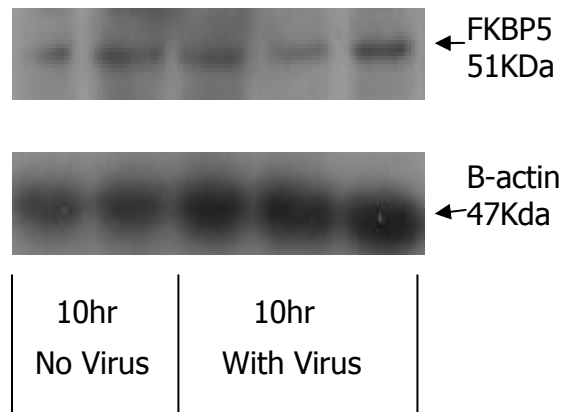
A



B



C



3.6 Effect of hydrocortisone on growth of A549 cells

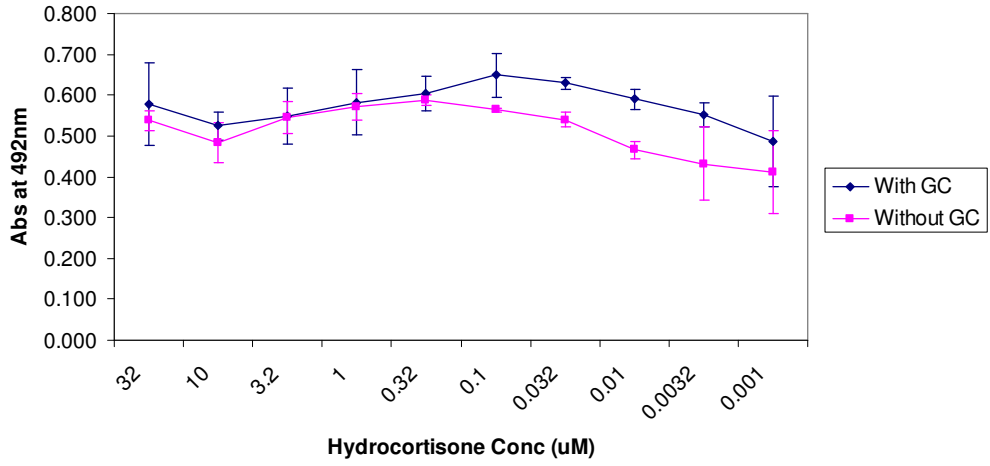
It has previously been shown that FKBP5 is up-regulated at the site of influenza infection such as lung and nasal turbinate in a ferret infection model (75). I have shown that FKBP5 was not increased by influenza infection but was enhanced by hydrocortisone in A549 cells, possibly as a physiological response to suppress inflammation. However, it is not known whether FKBP5 up-regulation has an effect on viral replication. I went on to investigate the effect of hydrocortisone stimulated up-regulation of FKBP5 on the influenza replication.

Prior to infection studies, I first sought to determine the effect of hydrocortisone alone on the growth of A549 cells. Cells were seeded and stimulated with hydrocortisone. After 24, 48 and 72 hours, the growth of A549 cells was measured by MTS assay. The growth of the A549 cells in the presence and absence of hydrocortisone was similar at 24, 48 and 72 hours (Figure 6A-C). These results showed that stimulation with 10 μ M of hydrocortisone, which was used in previous experiments, did not affect the growth of A549 cells and indicated that 10 μ M hydrocortisone was suitable to be used in testing FKBP5 affected influenza infection.

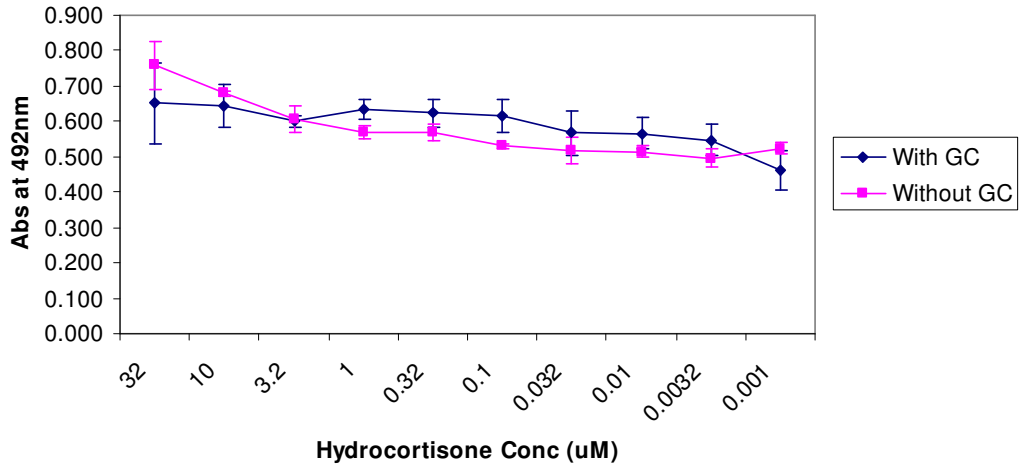
Figure 6. Hydrocortisone had minimal effect on the growth of A549 cells.

A549 cells were treated with serially diluted hydrocortisone in F12K medium. At 24, 48 and 72 hours, cell growth was measured by MTS assay. Hydrocortisone treated A549 cells and untreated cells show similar A_{492} value at (A) 24 hours, (B) 48 hours and (C) 72 hours. GC: glucocorticoid

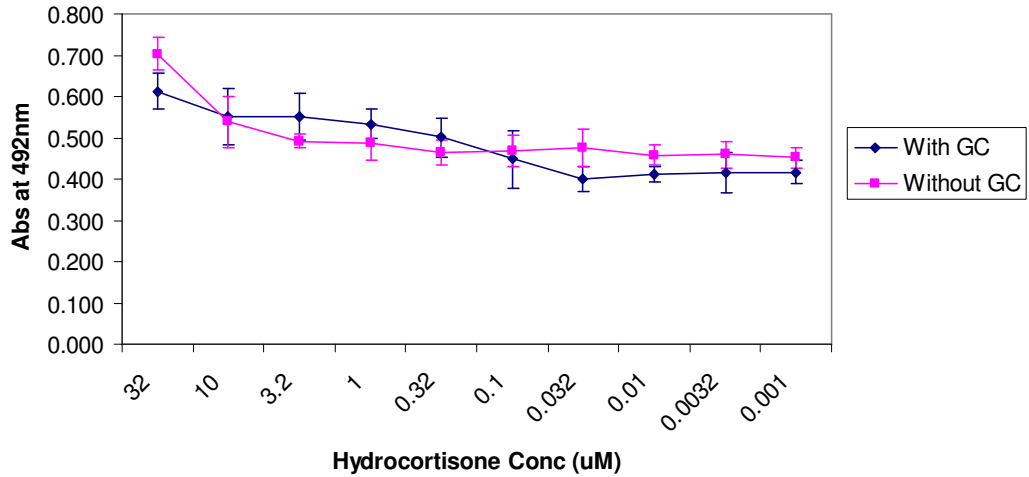
A



B



C



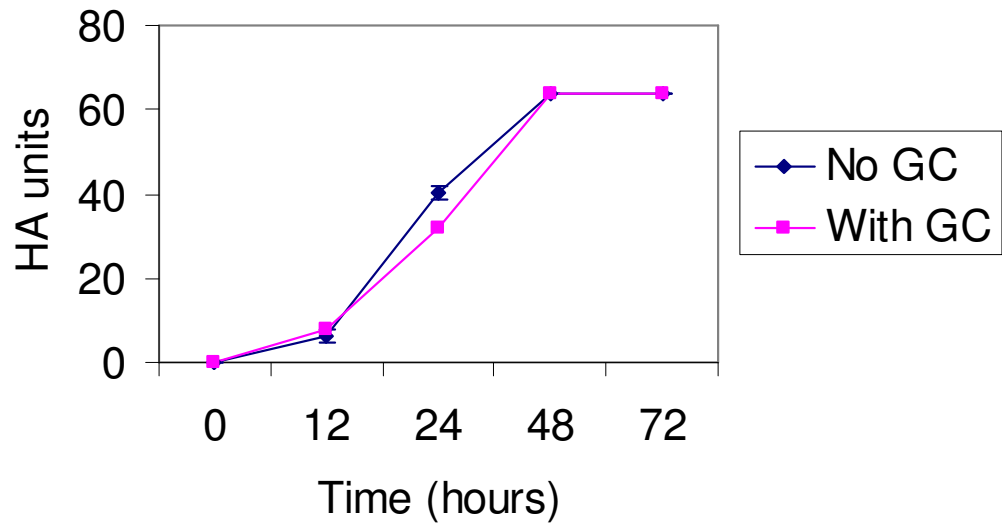
3.7 FKBP5 expression did not affect A/WS/33 H1N1 influenza replication

Next, I went on to investigate the effect of hydrocortisone stimulated FKBP5 up-regulation on influenza replication. A549 cells were stimulated with hydrocortisone to up-regulate FKBP5 and subsequently infected with A/WS/33 H1N1 influenza at MOI=5. Culture medium was collected at 0, 12, 24, 48 and 72 hours and influenza was titred by HA and plaque assay. Interestingly, influenza replicated well in both hydrocortisone-treated A549 cells and no treatment control (Figure 7A). As well, viral protein haemagglutinin levels were similar in both hydrocortisone-treated and untreated samples, suggesting that FKBP5 up-regulation had no effect on influenza replication (Figure 7A). Infectious influenza particles in 0 and 24 hours samples were measured by plaque assay. Consistent with the results from HA assays, the increase in viral particles were similar in hydrocortisone-treated and untreated samples with $p=0.74$ (Figure 7B). Together, these results indicated that hydrocortisone stimulated FKBP5 up-regulation does not affect influenza replication.

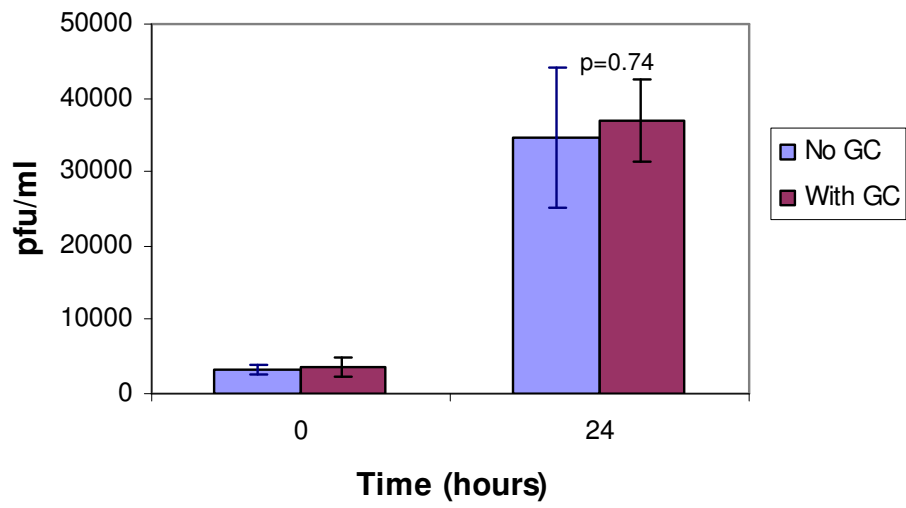
Figure 7. Hydrocortisone stimulated FKBP5 up-regulation did not affect A/WS/33 H1N1 influenza replication.

A549 cells were treated with 10 μ M hydrocortisone 12 hours after seeding to increase FKBP5 expression. 12 hours after hydrocortisone addition, A549 cells were infected by A/WS/33 H1N1 influenza at MOI=5 and MOI=0 for uninfected control. At 0, 12, 24, 48 and 72 hours post infection, culture medium was collected and influenza replication was measured by (A) haemagglutination assay and (B) plaque assay. (A) Haemagglutinin viral protein level increased from 0 to 72 hours. Haemagglutinin levels were similar in both hydrocortisone treated and untreated samples. (B) Viable influenza particles at 24 hours were similar for hydrocortisone treated and untreated samples with $p=0.74$. GC:glucocorticoid

A



B



3.8 TNF α stimulation of A549 cells enhanced IL-6 and IL-8 production

The above experimental results suggested that up-regulation of FKBP5 during influenza infection was not caused by influenza replication. Rather, it appeared to be a physiological response to glucocorticoid in viral infection. Although my results showed that FKBP5 up-regulation did not affect influenza replication, the role of FKBP5 in other aspects of influenza disease remains poorly understood.

During influenza infection in the lung, infected epithelial cells of the lung secrete IL-8 and CCL2 to recruit neutrophils and monocytes (115). Neutrophils and monocytes/macrophages then produce TNF α , IL-1 and IL-6 to initiate inflammation process (111). These pro-inflammatory cytokines activate the HPA axis and as a negative feedback mechanism adrenal glucocorticoid is secreted to suppress pro-inflammatory cytokine production (112) to control inflammation. Since glucocorticoid increased FKBP5 expression and FKBP5 has been shown to interact with immune response pathways such as NF- κ B (94), I went on to investigate whether FKBP5 up-regulation facilitates the suppressive effect of glucocorticoid on cytokine production in lung cells.

Prior to investigation of this hypothesis, I first sought to determine if A549 cells responded to TNF α by producing pro-inflammatory cytokines such as IL-6 and IL-8. Cytometric bead array (CBA) was the tool used to measure cytokines secreted from the cells to the medium. CBA employs five populations of beads which specifically capture human pro-inflammatory cytokines IL-1 β , IL-6, IL-8, IL-10 and IL-12p70. Due to the distinct fluorescence intensity of each bead, the five bead populations can be resolved in the FL3 channel of a flow cytometer, allowing the simultaneous measurement of five cytokines within one sample. The amount of cytokine captured on the bead is then

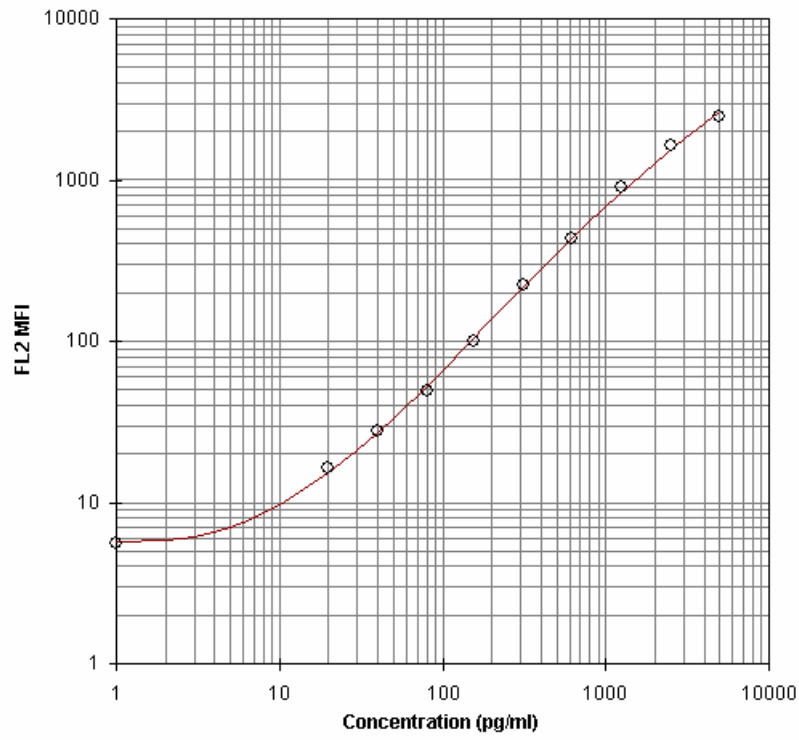
quantified by phycoerythrin(PE)-conjugated detection antibodies and measured in the FL2 channel of a flow cytometer. Prior to sample measurement, a standard curve correlating the FL2 fluorescence intensity with the amount of cytokine was made by using serially 2-fold diluted human inflammation standard. Standard curves for IL-6 and IL-8 were shown here as an example (Figure 8).

A549 cells were stimulated with 100ng/ml TNF α and the culture medium was collected at 0, 12, 24 and 48 hours after TNF α stimulation. Pro-inflammatory cytokines were quantified by cytometric bead array (CBA). In response to TNF α , IL-6 increased 13, 31 and 81 fold at 12, 24 and 48 hours, respectively (Figure 9A). Similarly, IL-8 production increased from 303 pg/ml to over 5000 pg/ml, the detection limit of CBA after TNF α stimulation (Figure 9B). This increase was not observed in the no treatment control. These data suggest that A549 cells are capable of producing pro-inflammatory cytokine following TNF α stimulation.

Figure 8. Cytometric bead array standard curves for IL-6 and IL-8

Human inflammation standards were serially 2-fold diluted and incubated with the cytometric beads. The median fluorescence intensity of the beads in FL2 channel was plotted against the concentration of the cytokine to generate a standard curve for IL-6 (A) and IL-8 (B). MFI: median fluorescence intensity

A



B

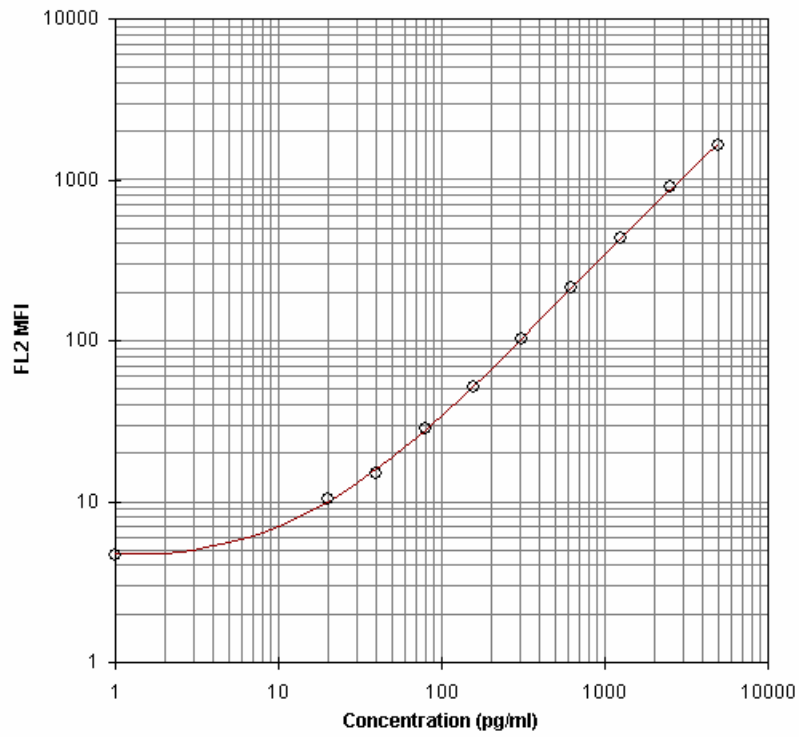
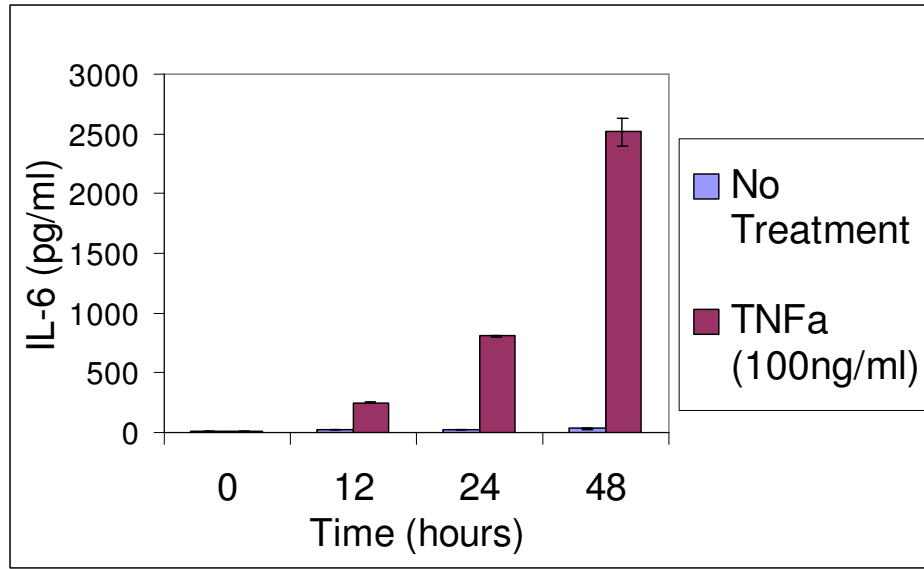


Figure 9. A549 cells produced IL-6 and IL-8 in response to TNF α stimulation.

A549 cells were stimulated with 100ng/ml TNF α and supernatant IL-6 (A) and IL-8 (B) cytokine levels were measured by CBA at 0, 12, 24 and 48 hours. (n=3, mean \pm SD)

A



B

	IL-8 level (pg/ml)			
Time (hours)	0	12	24	48
No Treatment	333 ± 13	486 ± 18	597 ± 34	803 ± 8
TNFα (100ng/ml)	303 ± 2	>5000	>5000	>5000

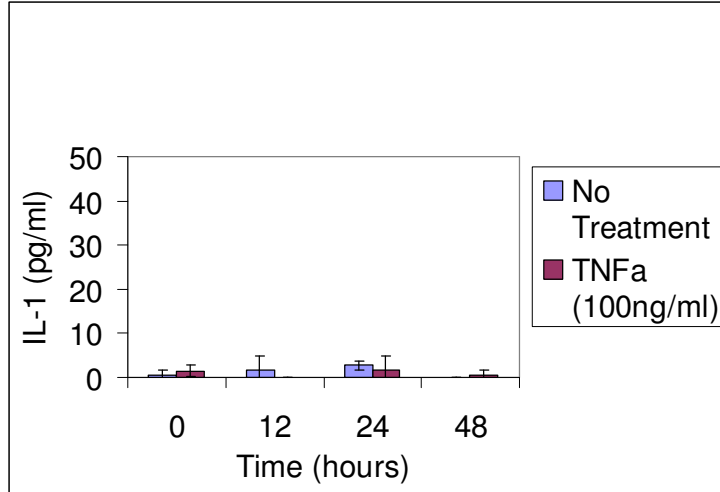
3.9 A549 cells did not produce IL-1, IL-10 and IL-12 in response to TNF α

IL-1 and IL-12 are important pro-inflammatory cytokines produced by neutrophils and macrophages to sustain the pro-inflammatory state during influenza infection (111). Conversely, IL-10 is an anti-inflammatory cytokine produced by effector T cell to regulate the inflammation process (116). I next sought to investigate the production of IL-1, IL-10 and IL-12 by A549 cells following TNF α stimulation. Furthermore, I wanted to determine if glucocorticoid stimulation could suppress the production of these cytokines. A549 cells were stimulated with 100ng/ml TNF α and cytokine production was measured at 0, 12, 24 and 48 hours by CBA. In contrast to IL-6 and IL-8, A549 cells did not produce IL-1, IL-10 and IL-12 in response to TNF α (Figure 10A-C). These data are consistent with the literature that IL-10 and IL-12 are primarily produced by white blood cells and not by epithelial cells (6).

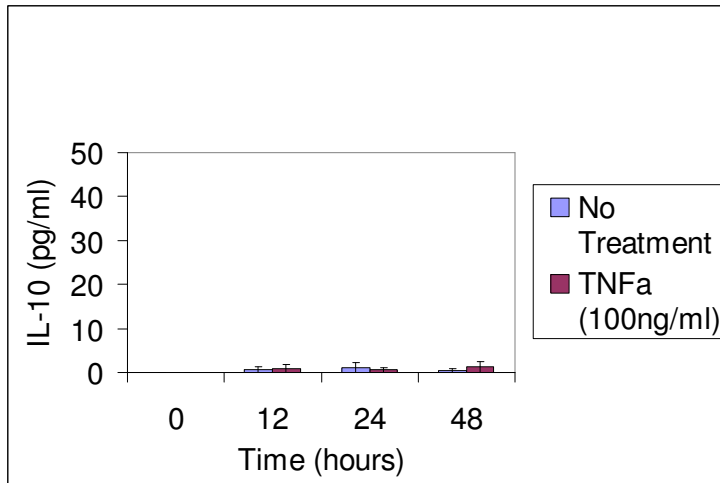
Figure 10. A549 cells did not produce IL-1, IL-10 and IL-12 in response to TNF α stimulation.

A549 cells were stimulated with 100ng/ml TNF α and cytokine production in the supernatant was measured by CBA at 0, 12, 24 and 48 hours. (A) IL-6 (B) IL-10 (C) IL-12 were not produced in response to TNF α stimulation.

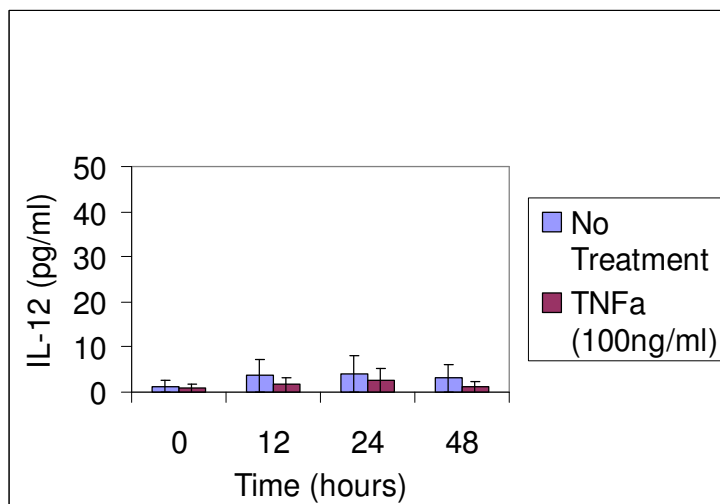
A



B



C

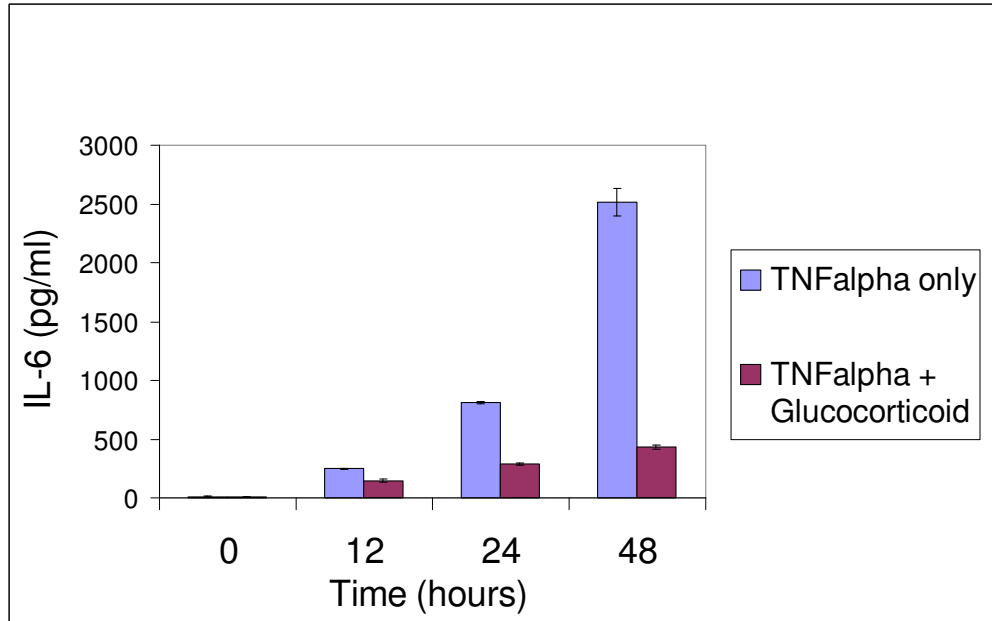


3.10 Glucocorticoid stimulation inhibited IL-6 and IL-8 production in A549 cells

After demonstrating that A549 cells produced IL-6 and IL-8 in response to TNF α , I next sought to investigate the effects of glucocorticoid on cytokine production. Cultured A549 cells were stimulated with hydrocortisone, at a final concentration of 10 μ M. Following the addition of hydrocortisone, A549 cells were stimulated with 100ng/ml TNF α and cytokine production was measured by CBA at 0, 12, 24 and 48 hours. IL-6 production increased in the cells stimulated with TNF α without hydrocortisone treatment (Figure 11A). Interestingly, in the presence of hydrocortisone, the production of IL-6 was suppressed with 1.7, 2.8 and 5.8 fold at 12, 24 and 48 hours respectively (Figure 11A). Furthermore, hydrocortisone reduced IL-8 production from over 5000 pg/ml to 3456 pg/ml at 12 hours (Figure 11B), suggesting that hydrocortisone can suppress pro-inflammatory cytokine production in A549 cells. Taken together, these results demonstrated that A549 cells produced pro-inflammatory cytokines in response to TNF α and that hydrocortisone stimulation suppressed cytokine production. Furthermore, this suggested that A549 cells are a good model to study the molecular events responsibly involved in the suppression of cytokine production and particularly the role of FKBP5.

Figure 11. Hydrocortisone suppressed IL-6 and IL-8 production in A549 cells.

A549 cells were cultivated in F12K medium and subsequently treated with 10 μ M of hydrocortisone. The cells were then stimulated with 100ng/ml TNF α , and the cytokine production was measured by CBA at 0, 12, 24 and 48 hours. (A) IL-6 production increased after TNF α stimulation and was inhibited in the hydrocortisone treated samples. (B) Hydrocortisone inhibited TNF α stimulated IL-8 increase. (n=3, mean \pm SD)

A**B**

Time (hours)	IL-8 level (pg/ml)			
	0	12	24	48
TNF α only	303 \pm 2	>5000	>5000	>5000
TNF α + Glucocorticoid	148 \pm 5	3456 \pm 116	>5000	>5000

3.11 siRNA transfection of A549 cells down-regulated FKBP5 expression

Above I have shown that FKBP5 expression is inducible upon hydrocortisone treatment. I next wanted to further investigate the suppressive effects of FKBP5 expression on cytokine production. To investigate effects of FKBP5 expression, I developed FKBP5 siRNA to knock down FKBP5. As a quality control step, I tested the siRNA transfection reagent and double strand RNA itself to ensure there were not non-specific effects leading to inhibition of FKBP5 expression. Other factors such as time of transfection and concentration of siRNA were also optimized. Cultured A549 cells were transfected with siRNA in F12K medium using Ambion Neo f(x) transfection reagent. Following transfection 10 μ M hydrocortisone was added to A549 cells to induce FKBP5 expression. The cells were then harvested and mRNA was extracted and FKBP5 expression was measured by RT-PCR.

Consistent to previous experimental results, when A549 cells were treated with hydrocortisone, there was a 22 fold increase in FKBP5 expression (Figure 12A). Comparing A549 cells treated with GC in the presence and absence of Neo f(x) transfection reagent, there is 17% decrease in FKBP5 expression when transfection reagent was present. However, the difference was not statistically significant as demonstrated by a Student's T-test ($p=0.21$) (Figure 12A). These results indicated that the transfection conditions had only minimal effects on FKBP5 expression.

I next tested whether FKBP5 specific siRNA could knock down FKBP5. FKBP5 siRNA inhibited expression by 53%, 45% and 45% with $p=0.03$, $p=0.06$, $p=0.06$ for siRNA concentration of 30, 15 and 5nM respectively (Figure 12A). The scrambled sequence siRNA that did not target any human gene had an 8%, 16% and 20% decrease

in FKBP5 expression at siRNA concentration of 30, 15 and 5nM respectively. However, these decreases were not statistically significant with $p=0.22$, $p=0.26$ and $p=0.29$. In summary, FKBP5 siRNA significantly decreased FKBP5 expression in A549 cells by 40%, while mock transfection and non-specific siRNA did not have any statistically significant effect on FKBP5 expression.

To further confirm the effects of FKBP5 siRNA and to optimize the parameters for efficient gene silencing, the siRNA experiment was repeated using two sets of FKBP5 siRNA changing the time of hydrocortisone addition to 12 hours instead of 36 hours as used before. Similar to the previous experiment, FKBP5 siRNA set 2 decreased FKBP5 expression by 43%, 22% and 42% at concentrations of 30, 15 and 5nM (Figure 12B). FKBP5 siRNA set 3 also inhibited FKBP5 expression by 39% at 5nM concentration (Figure 12B).

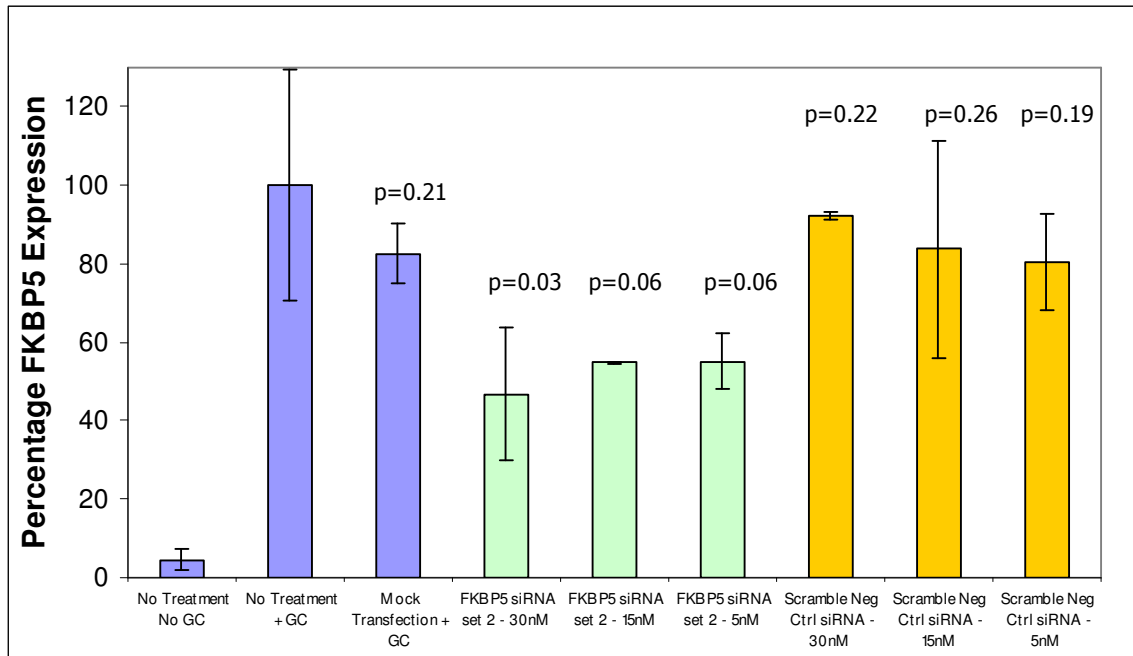
The results from these two experiments demonstrated that two sets of FKBP5 siRNA were able to knock down FKBP5 expression as early as 12 hours post-transfection and continue to be effective at 36 hours post-transfection. Furthermore, the 5nM siRNA concentration was as effective as 30nM for siRNA set 2 and 5nM was most effective for set 3. Therefore, for subsequent experiments in testing the effect of FKBP5 knock down on the ability of hydrocortisone to suppress cytokine production, 5nM of siRNA was chosen and 10 μ M hydrocortisone was added 24 hours post-transfection.

Figure 12. FKBP5 siRNA transfection of A549 cells decreased FKBP5 expression.

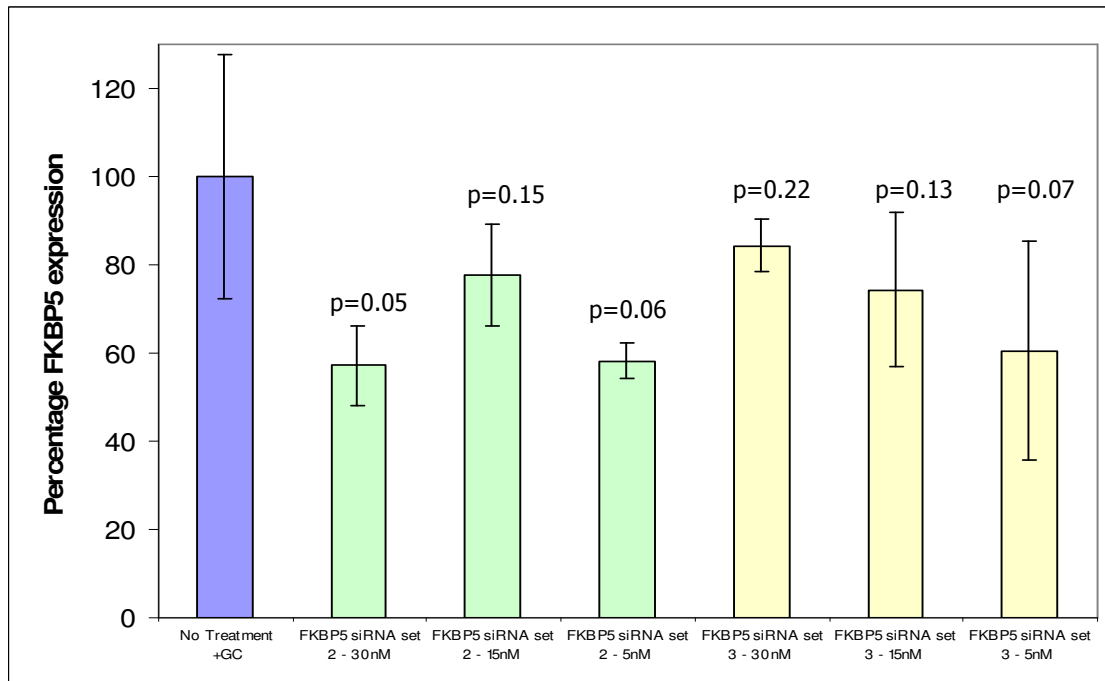
(A) A549 cells were transfected with FKBP5 siRNAs and control siRNAs. After 36 hours, 10 μ M hydrocortisone was added. 12 hours after hydrocortisone addition, mRNA was extracted and FKBP5 expression was analysed by RT-PCR. In response to hydrocortisone, FKBP5 expression was increased by 22 fold (1st and 2nd bars). Transfection reagent (3rd bar) and non-specific scrambled sequence siRNAs (yellow bars) did not have a statistically significant effect on FKBP5 expression. In contrast, FKBP5 specific siRNA set 2 (light green bars) significantly decreased FKBP5 by 45-53%. (n=3, error bars represent standard deviation, results analyzed by Student's T-test compared to no treatment + GC value)

(B) A549 cells were transfected with FKBP5 specific siRNA. After 12 hours, 10 μ M hydrocortisone was added. mRNA was extracted 12 hours later and FKBP5 expression was analyzed by RT-PCR. FKBP5 specific siRNA set 2 (light green bars) significantly decreased FKBP5 expression by 42% at 30nM and 5nM concentration. FKBP5 siRNA set 3 (orange bars) also decreased FKBP5 expression by 39% at 5nM concentration. (n=3, error bars represent standard deviation, results analyzed by Student T-test compared with the no treatment + GC sample). GC: glucocorticoid

A



B



3.12 FKBP5 knockdown decreased the suppressive effect of hydrocortisone on cytokine production

Since A549 cells responded to TNF α and hydrocortisone and FKBP5 siRNA was effective in knocking down FKBP5 expression in A549 cells, I examined the effect of FKBP5 knockdown on the suppressive action of hydrocortisone by measuring IL-6 and IL-8 production. A549 cells were seeded and transfected with 5nM FKBP5 and control siRNA. 24 hours following transfection, A549 cells were treated with 10 μ M hydrocortisone. Subsequently, the cells were stimulated with 100ng/ml of TNF α and cytokine production in culture medium was quantified by cytometric bead array.

Consistent with previous results, TNF α increased IL-6 production and hydrocortisone suppressed IL-6 production (Figure 5A). When FKBP5 was knocked down by FKBP5 siRNA set 2 and set 3, both increased IL-6 production at 24 and 48 hours compared to the negative control scrambled sequence siRNA (Figure 13A). The FKBP5 siRNA set two showed a 31% increase and a 22% increase at 24 and 48 hours with $p=0.03$ and $p=0.06$ compared to the scrambled sequence siRNA control (Figure 13B). For FKBP5 siRNA set 3, there was also a 21% increase and a 17% increase in IL-6 production at 24 and 48 hours with $p=0.09$ and $p=0.05$ compared to scrambled sequence siRNA control (Figure 13B).

The results of IL-8 were similar to the IL-6 results: A549 cells increased IL-8 production following TNF α stimulation and IL-8 production was suppressed by hydrocortisone treatment (Figure 14A). When FKBP5 was knocked down by siRNA set 2 and 3, IL-8 production increased at 24 hours compared to scrambled sequence siRNA (Figure 14A). There was a 19% and 21% increase, $p=0.11$ and $p=0.08$, for set 2 and set 3

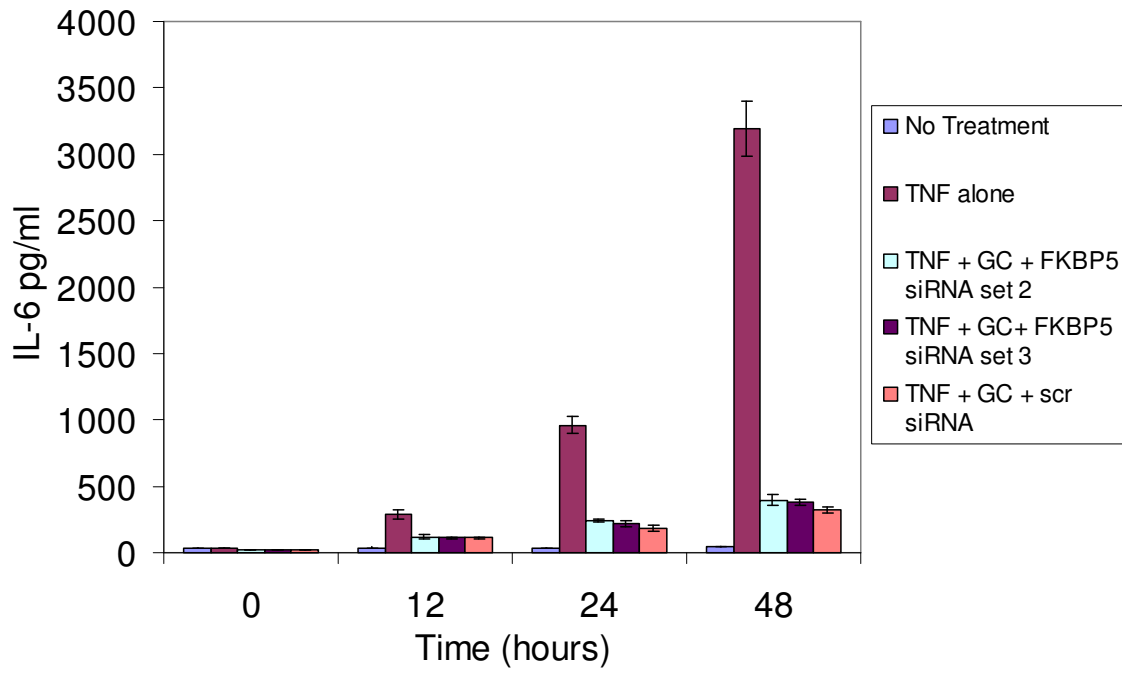
respectively (Figure 14B). At 48 hours, the IL-8 level in the culture medium exceeded the detection limit of CBA at 10000µg/ml.

In conclusion, when FKBP5 was knocked down, the suppressive effect of hydrocortisone was reduced and IL-6 and IL-8 production tended to be increased. Furthermore, RT-PCR was performed to ensure that siRNA was knocking down FKBP5 expression. Similar to the previous siRNA experiments, both set 2 and set 3 were effective in minimizing FKBP5 expression by 40% (Figure 15).

Figure 13. FKBP5 knockdown minimized the effect of hydrocortisone and increased IL-6 level.

A549 cells were seeded and transfected with FKBP5 siRNA or scrambled siRNA. After 24 hours, 10 μ M hydrocortisone was added followed by the treatment with 100ng/ml of TNF α . IL-6 production was measured by CBA at 0, 12, 24 and 48 hours. (A) IL-6 production increased with TNF α and was suppressed by hydrocortisone (1st, 2nd and 5th bars). When FKBP5 was knocked down, a trend of increasing of IL-6 production at 24 and 48 hours compared to scrambled sequence siRNA (3rd, 4th and 5th bar) was seen. (B) IL-6 production increased by 30.7% and 20.5% in FKBP5 siRNA set 2 and set 3 samples (2nd and 3rd bars) compared to scrambled sequence siRNA (4th bars) with p=0.03 and p=0.09, respectively. At 48 hours, there were also a 21.8% increase and a 16.8% increase in IL-6 production in FKBP5 siRNA set 2 and set 3 samples (2nd and 3rd bars) compared to scrambled siRNA (4th bars) with p=0.06 and p=0.05 respectively. (n=3, mean \pm SD) GC: glucocorticoid

A



B

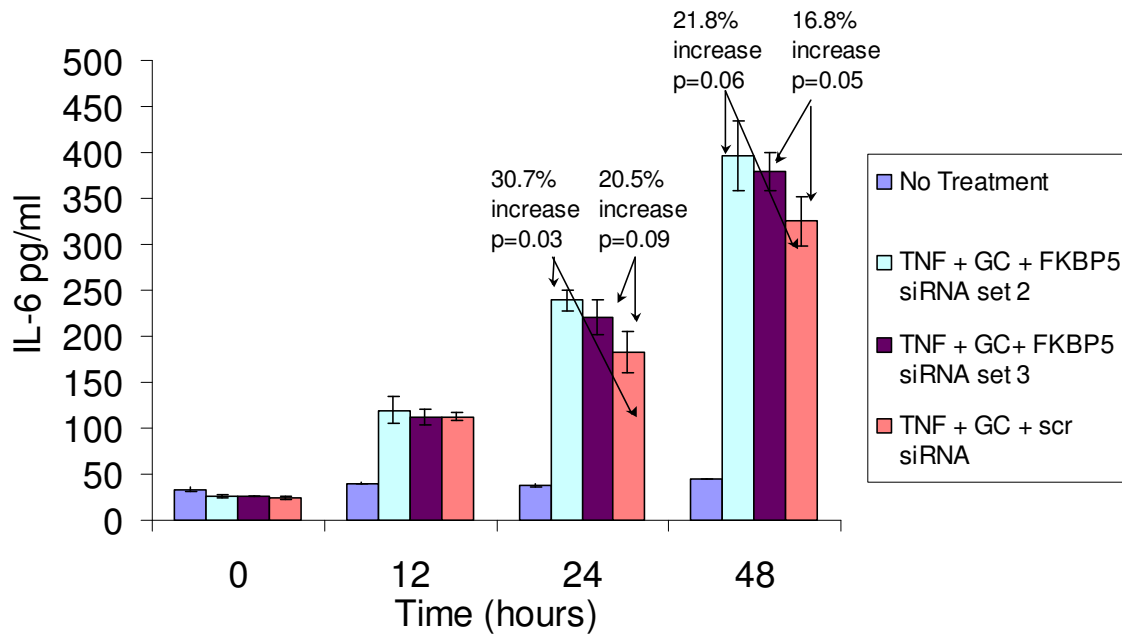
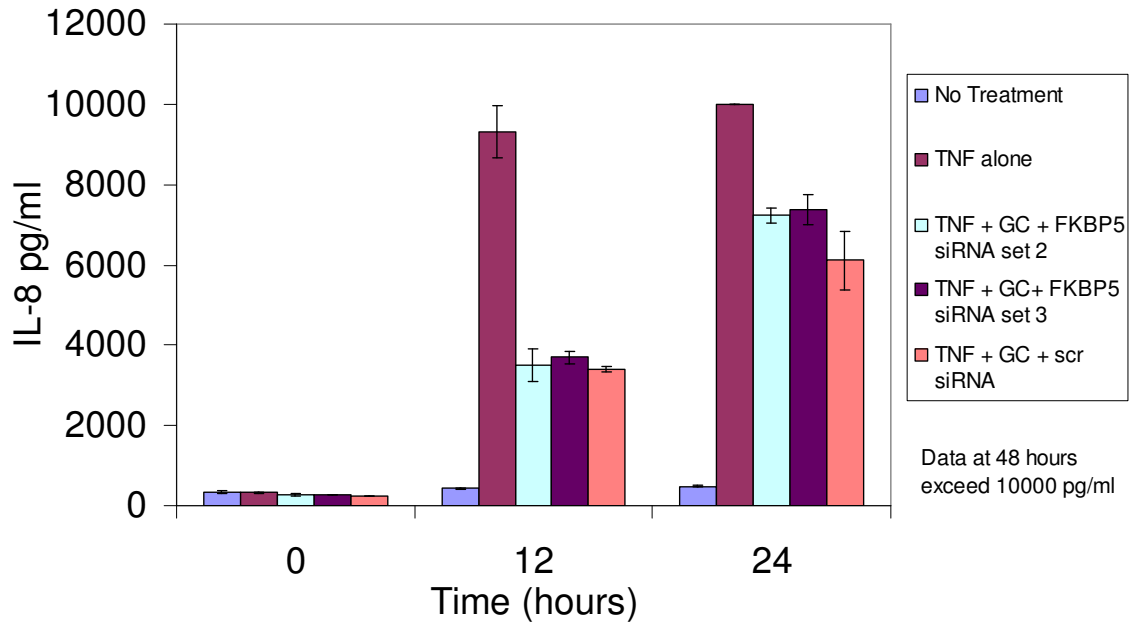


Figure 14. FKBP5 knockdown minimized the effect of hydrocortisone and increased IL-8 level.

IL-8 production was measured by CBA under the same conditions as the assessment of IL-6. (A) Similar to IL-6, IL-8 production was increased by TNF α and suppressed by hydrocortisone (1st, 2nd and 5th bars). When FKBP5 was knocked down IL-8 production increased at 24 hours (3rd and 4th bars) compared to the scrambled siRNA (5th bar). At 48 hours, the IL-8 production exceeded the detection limit of CBA at 10000 pg/ml. (B) IL-8 production at 24 hours increased 18.5% and 20.8% in FKBP5 siRNA set 2 and set 3 samples (2nd and 3rd bars) compared to the scrambled siRNA (4rd bars) with p=0.11 and p=0.08 respectively. (n=3, mean \pm SD) GC: glucocorticoid

A



B

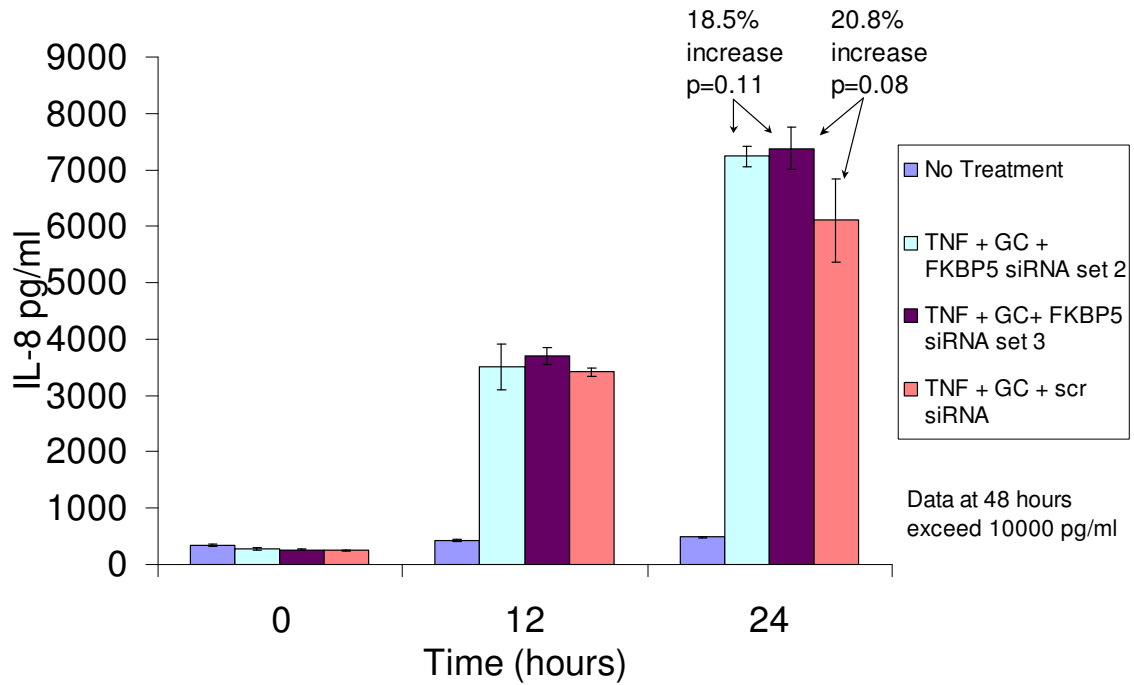
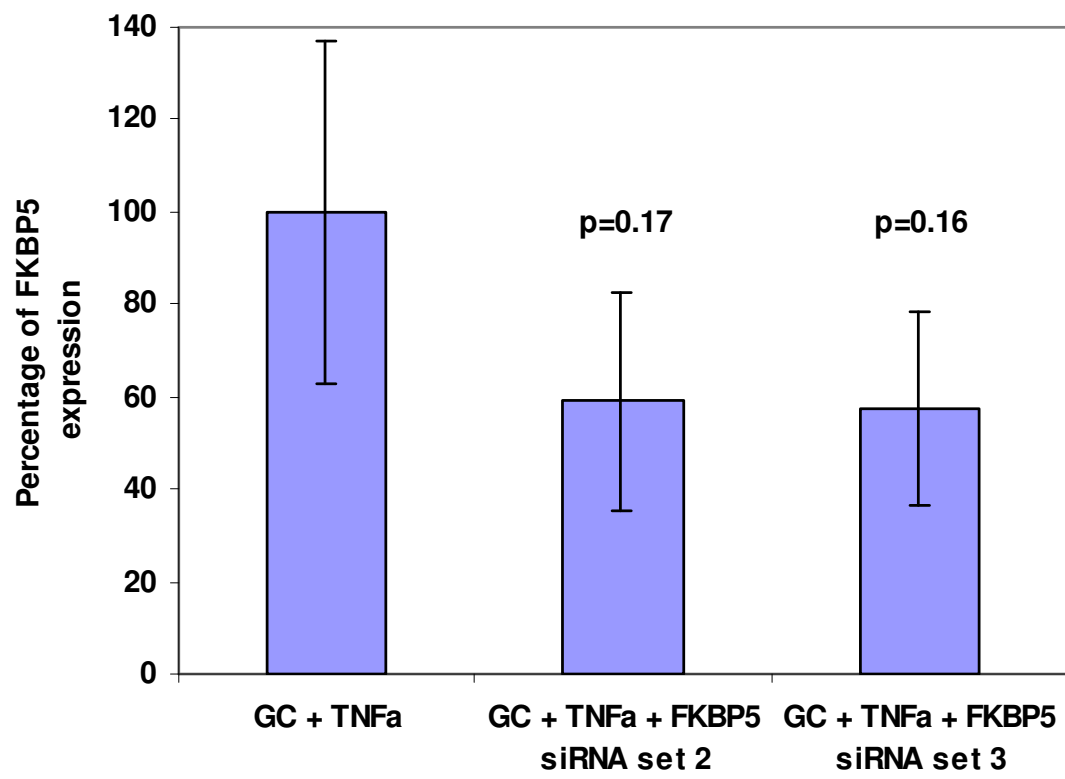


Figure 15. FKBP5 expression knockdown by FKBP5 siRNA transfection.

RT-PCR was performed on mRNA from A549 cells at TNF α stimulation 0 hours. Consistent to the results of two previous siRNA experiments, FKBP5 siRNA set 2 and set 3 at 5nM concentration knocked down FKBP5 expression by 40% with $p=0.17$ and $p=0.16$ respectively. GC: glucocorticoid



CHAPTER 4 – DISCUSSION

Microarray technology has been used to study host gene expression of various viral infections such as HIV (117) and Herpes Simplex Virus (118). Importantly, this technique has yielded valuable information of host immune responses and disease progression events. This thesis is based on the finding that FKBP5 is up-regulated in the lung during influenza ferret infection (75). Furthermore, FKBP5 has been shown to interact with cellular immune pathways such as calcineurin and NF- κ B (80, 94), and to be up-regulated by immunosuppressive agents (97, 98, 113). Here, an A549 lung cell line model was used to investigate the cause of FKBP5 up-regulation and the function of FKBP5 up-regulation during influenza infection.

I first determined whether FKBP5 up-regulation at the site of infection was directly caused by influenza infection and replication. Infections by different strains of influenza did not up-regulate FKBP5 in A549 cells in either early (4-12 hour) or late (24-48 hour) infections (Figures 3 and 4), suggesting that influenza replication was not the direct cause of FKBP5 up-regulation. This result is consistent with findings from the literature which have not shown FKBP5 to be up-regulated by influenza infection *in vitro*. A microarray study of influenza infection in A549 cells revealed that many important immune response genes such as IFN- β and NF- κ B are directly up-regulated by influenza replication (72), but FKBP5 was not included in the list of up-regulated genes. The result was further confirmed here where MDCK cells, which are susceptible to influenza infection, were also unable to up-regulate FKBP5 following influenza infection (Figure 5). This is consistent with another microarray study which showed that influenza infections did not up-regulate FKBP5 in other cell lines such as the human cervical epithelial HeLa cell line (119).

As mentioned above, although influenza replication did not directly up-regulate FKBP5 at the cellular level, FKBP5 up-regulation was observed in a ferret *in vivo* study (75). It is possible that FKBP5 up-regulation was indirectly caused by the host immune response to influenza, and it is proposed here that glucocorticoid is the cause of the FKBP5 increase. During an influenza infection, infected lung cells and infiltrating leukocytes secrete pro-inflammatory cytokines (111). In response to inflammation, the adrenal gland secretes glucocorticoid to suppress inflammation and acts as a negative feedback mechanism (112). Since glucocorticoid was shown to increase FKBP5 levels in different cells such as human PBMC (113) and the Pre-B human leukemia 697 cell (120), glucocorticoid may be the stimulus of FKBP5 up-regulation in the ferret lung. Here I showed that glucocorticoid treatment caused a substantial increase of FKBP5 expression in A549 lung cells (Figure 4). This finding is in agreement with a study of New Castle Disease Virus in chickens, which found that glucocorticoid up-regulation during a viral infection led to a systemic increase of FKBP5 in twelve organs of the infected animal, including the lung (114).

Following the result that glucocorticoid up-regulated FKBP5 in lung cells, two aspects of the function during influenza infection were examined: the effect of FKBP5 regulation on influenza replication, and the role of FKBP5 in mediating the suppressive action of glucocorticoid on pro-inflammatory cytokine production. Influenza virus often replicates by exploiting host immune response pathways. For instance, the virus uses Tumor Necrosis Factor-related Apoptosis-inducing Ligand (TRAIL) and Cluster of Differentiation 95 Ligand (CD95L), both of which are up-regulated by the anti-viral NF- κ B pathway, to enhance virus replication (121, 122). However, FKBP5 up-regulation

induced by glucocorticoid did not have any effect on A/WS/33 H1N1 replication in A549 cells at the viral particle or the viral protein levels (Figure 7), suggesting that FKBP5 up-regulation provided no advantage to influenza replication. Even though there has been no single study in the literature that directly investigated the relationship between FKBP5 and influenza replication, other evidence is consistent with this result. For example, lactoperoxidase (LPO) has been shown to up-regulate FKBP5 in intestinal epithelial cells (98); and, in an *in vivo* study, mice which had been fed with lactoperoxidase (LPO) were infected with influenza and subsequently exhibited reduced lung inflammation and an improved recovery (123). In that study, however, there was no difference in influenza titre in the lungs of the mice after LPO treatment, suggesting that up-regulation of FKBP5 did not affect the influenza replication. Humans with a severe avian influenza infection usually exhibit lung inflammation, which is sometimes suppressed by the administration of glucocorticoid (124, 125). Safety of this medical practice is supported by my findings that influenza replication is not promoted by glucocorticoid stimulation and the accompanying up-regulation of FKBP5.

I further examined the function of FKBP5 in mediating the suppressive effect of glucocorticoid on cytokine production. Since TNF α is the pro-inflammatory cytokine secreted by infiltrating macrophages and neutrophils, as well as by lung cells infected with influenza (111, 115), inflammation events were mimicked by stimulating A549 lung cells with TNF α . A549 cells responded to TNF α , producing high amounts of IL-6 and IL-8 (Figure 9), a result which is consistent with findings in the literature that IL-6 and IL-8 are produced in the lung during an influenza infection and at a particularly high rate in the case of severe inflammations (126, 127). Furthermore, the production of IL-6 and

IL-8 was reduced when glucocorticoid was introduced (Figure 11). Finally, when FKBP5 was down-regulated by siRNA in the presence of glucocorticoid, there was a trend of increase in the IL-6 and IL-8 production compared to the glucocorticoid-only control (Figure 13 and 14), suggesting that the suppressive effect of glucocorticoid was at least partially mediated by FKBP5.

The concentration of TNF α used in this study (100ng/ml) was actually higher than the concentration required for inducing IL-8 expression in A549 cells, which is 2ng/ml-20ng/ml (128). It is possible that if a lower concentration of TNF α is used to induce a medium expression of IL-6 and IL-8, a lower amount of glucocorticoid is required and the effect of FKBP5 knock-down by siRNA can be uncovered.

The finding that FKBP5 played a positive role in suppressing cytokine production concurs with previous findings in the literature. When mice were fed with LPO, which can up-regulate FKBP5 (98), the mice exhibited a significant decrease in IL-6 levels in their lungs, as well had reduced lung inflammation during influenza infection (123). This is consistent with the idea that FKBP5 plays a positive role in suppressing IL-6 production. Aspirin is an anti-inflammatory drug that can up-regulate FKBP5, as shown in a cellular study and an *in vivo* study in mice (97). Since I have shown that FKBP5 can suppress inflammatory cytokine production, it is possible that the anti-inflammatory effect of aspirin is mediated by FKBP5 up-regulation.

In summary, the up-regulation of FKBP5 in the lung during an influenza infection is not caused by the replication of the influenza. Rather it is likely a physiological response of lung cells to the increased circulating levels of glucocorticoid during virus infections. This physiological increase in FKBP5 had a positive role in facilitating the

suppressive effect of glucocorticoid on pro-inflammatory cytokine production, yet glucocorticoid treatments and the subsequent increase of FKBP5 do not have any effect on influenza replication.

The finding that FKBP5 mediated the down-regulation of pro-inflammatory cytokine production has several implications, especially pertaining to the therapeutic use of glucocorticoid in treating severe cases of lung inflammation caused by avian influenza. Glucocorticoid is a double-edged sword: although it suppresses inflammation, it also decreases the ability of immune cells such as macrophage, neutrophils, cytotoxic T cells and natural killer cells to clear the influenza virus (112, 129). Furthermore, glucocorticoid treatments have been associated with side effects such as osteonecrosis, which affected many patients who were treated with glucocorticoid during severe acute respiratory syndrome (SARS) viral infection (130). Moreover, there is considerable variability in the patient response to the use of glucocorticoid treatment of avian influenza. Some patients responded well with high doses of glucocorticoid, while others responded to a lower dose or did not respond at all (125). It is possible that a lower dose of glucocorticoid can minimize the suppressive effect on the function of crucial immune cells, such as T cell activation, while still causing the up-regulation of FKBP5 (129). The finding that FKBP5 facilitated the suppressive effect of glucocorticoid on cytokine production suggested that FKBP5 stimulators such as LPO and aspirin can be used instead of glucocorticoid in treating lung inflammations, thereby minimizing the side effects of glucocorticoid. My finding that FKBP5 up-regulation does not affect influenza replication also suggested that FKBP5 stimulators are safe drugs for controlling inflammation caused by influenza.

Another implication of my findings is that there are multiple pathways of glucocorticoid action. The anti-inflammatory property of glucocorticoid has been well studied, and mechanisms of action of glucocorticoid are mainly classified into two categories: genomic mechanism, which affects gene expression, and non-genomic mechanism, which acts through protein-protein interaction and is less characterized (107). The finding that FKBP5 mediated the suppressive effects of glucocorticoid may reveal a novel mechanism in the non-genomic category, especially since FKBP5 interacts with various immune response pathways (80, 94). Study of this novel mechanism may also reveal some therapeutic targets for suppressing pro-inflammatory cytokine production.

The findings here contribute to the understanding of FKBP5's role in influenza disease progression, and also have implications for the use of FKBP5 targeting influenza therapeutics. However, these findings are limited by the fact that my experiments are based on *in vitro* A549 cell line model. To confirm these results, future *in vivo* work is suggested, as detailed below.

My *in vitro* work in A549 cells has determined that FKBP5 up-regulation was not caused by influenza, but by lung cells responding to the increase of glucocorticoid which occurs during infection. To confirm that glucocorticoid is the cause of FKBP5 up-regulation during an *in vivo* influenza infection, it is proposed that glucocorticoid antagonist RU-486 be used to treat infected mice or ferrets. It is expected that FKBP5 up-regulation will be reduced when the animals are treated with RU-486 and infected with influenza, since the effects of glucocorticoid are disrupted by RU-486 treatment. For control animals infected with influenza in the absence of RU-486, a normal level of FKBP5 up-regulation should be observed. Thus, the animal model would support my

findings that glucocorticoid caused FKBP5 up-regulation during influenza infection.

In order to confirm the finding that FKBP5 mediated the suppressive effect of glucocorticoid, it is proposed that FKBP5 knockout mice be used, since FKBP5 knockout mice have previously been used to study the functions of FKBP5 (131). To induce pro-inflammatory cytokine production in the lung, mice would be treated with TNF α . Then, the administration of glucocorticoid would suppress cytokine production, shown by IL-6 and IL-8 measurements by CBA and RT-PCR. For FKBP5 knockout mice, the IL-6 and IL-8 expression in lung would be expected to be higher, as FKBP5 would not be there to mediate glucocorticoid's suppressive effect. Glucocorticoid treatment is expected to suppress IL-6 and IL-8 cytokine production in wild type mice, confirming the mediating role of FKBP5 in glucocorticoid action.

Following confirmation that FKBP5 mediated the suppressive effect of glucocorticoid at both cellular and animal levels, the exact mechanism of FKBP5 should be investigated. It is uncertain whether FKBP5 up-regulation alone directly suppressed cytokine production, or if another protein acted synergistically with FKBP5. To determine FKBP5's mechanism for mediating glucocorticoid, the *in vitro* A549 cell model established in this thesis be used. A549 cells could be transfected with a plasmid to constitutively express FKBP5 protein. Subsequently, A549 cells should be treated with TNF α to stimulate IL-6 and IL-8 production. If A549 cells expressing the FKBP5 plasmid yielded a lower cytokine production than untransfected A549 cells, I will be able to conclude that FKBP5 up-regulation alone suppresses cytokine production. However, if cytokine production in the A549 cells with the FKBP5 expression plasmid was similar to

that of untransfected A549 cells, then I will be able to conclude that another protein acted synergistically with FKBP5 to suppress cytokine production.

It is also proposed that an investigation to determine which interacting protein partners facilitated the function of FKBP5. This can be done by treating A549 cells with TNF α and glucocorticoid and extracting the cellular protein for immuno-precipitation to identify FKBP5 and its interacting partners by immunoblotting. Mass spectrometry could then also be used to identify the interacting protein partners. Subsequently, the cellular pathways on which FKBP5 has an effect should be studied, in order to determine the mechanism of FKBP5 action.

The findings in this thesis also set the groundwork for future investigations into FKBP5 as a therapeutic target in the treatment of influenza. To establish the protective function of FKBP5 in controlling inflammation, it is proposed that FKBP5 knockout mice be infected with a mouse specific influenza, such as the A/PR/8/34 strain. It is expected that in the absence of FKBP5, the physiological increase in glucocorticoid during the infection will not effectively suppresses lung inflammation, causing the FKBP5 knockout mice to die early. This will demonstrate that FKBP5 up-regulation is an essential therapeutic target. It is then proposed that FKBP5 knockout mice be treated with drugs known to up-regulate FKBP5 that also have a positive effect in controlling influenza and inflammation, such as aspirin and LPO. The expected result is that the protective effects of the drugs will be reduced in FKBP5 knockout mice, confirming that FKBP5 mediates the therapeutic function of the drug in controlling inflammation. Based on the new understanding of the FKBP5 action mechanism, new drugs which up-regulate FKBP5 can be developed and used to mitigate severe lung inflammation in avian

influenza patients, potentially reducing morbidity from the current rate of greater than 50%.

CHAPTER 5 – REFERENCES

1. World Organization for Animal Health. 2009. Outbreaks of avian influenza (subtype H5N1) in poultry.
2. World Health Organization. 2009. Cumulative number of confirmed human cases of avian influenza A/(H5N1) reported to WHO.
3. World Health Organization. 2009. Pandemic (H1N1) 2009 - update 78.
4. Neumann, G. and Y. Kawaoka. 2006. Influenza Epidemics. *Encyclopedia of Life Sciences*
5. Whittaker, G. R. 2001. Intracellular trafficking of influenza virus: clinical implications for molecular medicine. *Expert Rev. Mol. Med.* 2001: 1-13.
6. Neumann, G. and Y. Kawaoka. 2007. Influenza viruses. *Encyclopedia of Life Sciences*
7. Mosley, V. M. and R. W. G. Wyckoff. 1946. Electron micrography of the virus of influenza. *Nature* 157: 263.
8. Carr, C. M. and P. S. Kim. 1994. Flu virus invasion: halfway there. *Science* 266: 234-236.
9. Hernandez, L. D., L. R. Hoffman, T. G. Wolfsberg, and J. M. White. 1996. Virus-cell and cell-cell fusion. *Annu. Rev. Cell Dev. Biol.* 12: 627-661.
10. O'Neill, R. E., R. Jaskunas, G. Blobel, P. Palese, and J. Moroiianu. 1995. Nuclear import of influenza virus RNA can be mediated by viral nucleoprotein and transport factors required for protein import. *J. Biol. Chem.* 270: 22701-22704.
11. Krug, R. M., F. V. Alonso-Caplen, I. Julkunen, and M. G. Katze. 1989. Expression and replication of the influenza virus genome. In *The influenza viruses*, R. M. Krug ed. Plenum Press, New York. 89.
12. Martin, K. and A. Helenius. 1991. Nuclear transport of influenza virus ribonucleoproteins: the viral matrix protein (M1) promotes export and inhibits import. *Cell* 67: 117-130.
13. Neumann, G., M. T. Hughes, and Y. Kawaoka. 2000. Influenza A virus NS2 protein mediates vRNP nuclear export through NES-independent interaction with hCRM1. *EMBO J.* 19: 6751-6758.
14. Scheiffele, P., A. Rietveld, T. Wilk, and K. Simons. 1999. Influenza viruses select ordered lipid domains during budding from the plasma membrane. *J. Biol. Chem.* 274: 2038-2044.

15. Colman, P. M. 1989. Neuraminidase: enzyme and antigen. In *The influenza viruses*, R. M. Krug ed. Plenum Press, New York. 175.
16. Kawai, T. and S. Akira. 2008. Toll-like receptor and RIG-I-like receptor signaling. *Ann. N. Y. Acad. Sci.* 1143: 1-20.
17. Yoneyama, M. and T. Fujita. 2007. Function of RIG-I-like receptors in antiviral innate immunity. *J. Biol. Chem.* 282: 15315-15318.
18. Pichlmair, A., O. Schulz, C. P. Tan, T. I. Naslund, P. Liljestrom, F. Weber, and C. Reis e Sousa. 2006. RIG-I-mediated antiviral responses to single-stranded RNA bearing 5'-phosphates. *Science* 314: 997-1001.
19. Kawai, T., K. Takahashi, S. Sato, C. Coban, H. Kumar, H. Kato, K. J. Ishii, O. Takeuchi, and S. Akira. 2005. IPS-1, an adaptor triggering RIG-I- and Mda5-mediated type I interferon induction. *Nat. Immunol.* 6: 981-988.
20. Seth, R. B., L. Sun, C. K. Ea, and Z. J. Chen. 2005. Identification and characterization of MAVS, a mitochondrial antiviral signaling protein that activates NF-kappaB and IRF 3. *Cell* 122: 669-682.
21. Kujime, K., S. Hashimoto, Y. Gon, K. Shimizu, and T. Horie. 2000. p38 mitogen-activated protein kinase and c-jun-NH2-terminal kinase regulate RANTES production by influenza virus-infected human bronchial epithelial cells. *J. Immunol.* 164: 3222-3228.
22. Lehtonen, A., S. Matikainen, and I. Julkunen. 1997. Interferons up-regulate STAT1, STAT2, and IRF family transcription factor gene expression in human peripheral blood mononuclear cells and macrophages. *J. Immunol.* 159: 794-803.
23. Julkunen, I., T. Sareneva, J. Pirhonen, T. Ronni, K. Melen, and S. Matikainen. 2001. Molecular pathogenesis of influenza A virus infection and virus-induced regulation of cytokine gene expression. *Cytokine Growth Factor Rev.* 12: 171-180.
24. Hovnanian, A., D. Rebouillat, M. G. Mattei, E. R. Levy, I. Marie, A. P. Monaco, and A. G. Hovanessian. 1998. The human 2',5'-oligoadenylate synthetase locus is composed of three distinct genes clustered on chromosome 12q24.2 encoding the 100-, 69-, and 40-kDa forms. *Genomics* 52: 267-277.
25. Bisbal, C., C. Martinand, M. Silhol, B. Lebleu, and T. Salehzada. 1995. Cloning and characterization of a RNase L inhibitor. A new component of the interferon-regulated 2-5A pathway. *J. Biol. Chem.* 270: 13308-13317.
26. Haller, O., P. Staeheli, and G. Kochs. 2007. Interferon-induced Mx proteins in antiviral host defense. *Biochimie* 89: 812-818.

27. Matikainen, S., J. Pirhonen, M. Miettinen, A. Lehtonen, C. Govenius-Vintola, T. Sareneva, and I. Julkunen. 2000. Influenza A and sendai viruses induce differential chemokine gene expression and transcription factor activation in human macrophages. *Virology* 276: 138-147.
28. Zlotnik, A. and O. Yoshie. 2000. Chemokines: a new classification system and their role in immunity. *Immunity* 12: 121-127.
29. Cella, M., M. Salio, Y. Sakakibara, H. Langen, I. Julkunen, and A. Lanzavecchia. 1999. Maturation, activation, and protection of dendritic cells induced by double-stranded RNA. *J. Exp. Med.* 189: 821-829.
30. Taylor, P., T. Tamura, and K. Ozato. 2006. IRF family proteins and type I interferon induction in dendritic cells. *Cell Res.* 16: 134-140.
31. Matikainen, S., T. Sareneva, T. Ronni, A. Lehtonen, P. J. Koskinen, and I. Julkunen. 1999. Interferon-alpha activates multiple STAT proteins and upregulates proliferation-associated IL-2Ralpha, c-myc, and pim-1 genes in human T cells. *Blood* 93: 1980-1991.
32. Rogge, L., L. Barberis-Maino, M. Biffi, N. Passini, D. H. Presky, U. Gubler, and F. Sinigaglia. 1997. Selective expression of an interleukin-12 receptor component by human T helper 1 cells. *J. Exp. Med.* 185: 825-831.
33. Sareneva, T., I. Julkunen, and S. Matikainen. 2000. IFN-alpha and IL-12 induce IL-18 receptor gene expression in human NK and T cells. *J. Immunol.* 165: 1933-1938.
34. Sareneva, T., S. Matikainen, M. Kurimoto, and I. Julkunen. 1998. Influenza A virus-induced IFN-alpha/beta and IL-18 synergistically enhance IFN-gamma gene expression in human T cells. *J. Immunol.* 160: 6032-6038.
35. Ghosh, S., M. J. May, and E. B. Kopp. 1998. NF-kappa B and Rel proteins: evolutionarily conserved mediators of immune responses. *Annu. Rev. Immunol.* 16: 225-260.
36. Robertson, M. J. 2002. Role of chemokines in the biology of natural killer cells. *J. Leukoc. Biol.* 71: 173-183.
37. Mandelboim, O., N. Lieberman, M. Lev, L. Paul, T. I. Arnon, Y. Bushkin, D. M. Davis, J. L. Strominger, J. W. Yewdell, and A. Porgador. 2001. Recognition of haemagglutinins on virus-infected cells by NKp46 activates lysis by human NK cells. *Nature* 409: 1055-1060.
38. Xu, L. L., M. K. Warren, W. L. Rose, W. Gong, and J. M. Wang. 1996. Human recombinant monocyte chemotactic protein and other C-C chemokines bind and induce directional migration of dendritic cells in vitro. *J. Leukoc. Biol.* 60: 365-371.

39. Grayson, M. H. and M. J. Holtzman. 2007. Emerging role of dendritic cells in respiratory viral infection. *J. Mol. Med.* 85: 1057-1068.
40. Luster, A. D. 2002. The role of chemokines in linking innate and adaptive immunity. *Curr. Opin. Immunol.* 14: 129-135.
41. Stoll, S., J. Delon, T. M. Brotz, and R. N. Germain. 2002. Dynamic imaging of T cell-dendritic cell interactions in lymph nodes. *Science* 296: 1873-1876.
42. Lawrence, C. W. and T. J. Braciale. 2004. Activation, differentiation, and migration of naive virus-specific CD8+ T cells during pulmonary influenza virus infection. *J. Immunol.* 173: 1209-1218.
43. Flynn, K. J., G. T. Belz, J. D. Altman, R. Ahmed, D. L. Woodland, and P. C. Doherty. 1998. Virus-specific CD8+ T cells in primary and secondary influenza pneumonia. *Immunity* 8: 683-691.
44. Topham, D. J., R. A. Tripp, and P. C. Doherty. 1997. CD8+ T cells clear influenza virus by perforin or Fas-dependent processes. *J. Immunol.* 159: 5197-5200.
45. Bishop, G. A. and B. S. Hostager. 2001. B lymphocyte activation by contact-mediated interactions with T lymphocytes. *Curr. Opin. Immunol.* 13: 278-285.
46. Eichelberger, M., W. Allan, M. Zijlstra, R. Jaenisch, and P. C. Doherty. 1991. Clearance of influenza virus respiratory infection in mice lacking class I major histocompatibility complex-restricted CD8+ T cells. *J. Exp. Med.* 174: 875-880.
47. Joo, H. M., Y. He, and M. Y. Sangster. 2008. Broad dispersion and lung localization of virus-specific memory B cells induced by influenza pneumonia. *Proc. Natl. Acad. Sci. U. S. A.* 105: 3485-3490.
48. Roxas, M. and J. Jurenka. 2007. Colds and influenza: a review of diagnosis and conventional, botanical, and nutritional considerations. *Altern. Med. Rev.* 12: 25-48.
49. Harris, K., J. Baggs, R. L. Davis, S. Black, L. A. Jackson, J. P. Mullooly, and L. E. Chapman. 2009. Influenza vaccination coverage among adult solid organ transplant recipients at three health maintenance organizations, 1995-2005. *Vaccine* 27: 2335-2341.
50. Centers for Disease Control and Prevention. 2008. Prevention and control of influenza: recommendations of the advisory committee on immunization practices (ACIP), 2008. *Morbidity and Mortality Weekly Report* 57:
51. Thompson, W. W., D. K. Shay, E. Weintraub, L. Brammer, N. Cox, L. J. Anderson, and K. Fukuda. 2003. Mortality associated with influenza and respiratory syncytial virus in the United States. *JAMA* 289: 179-186.

52. Writing Committee of the Second World Health Organization Consultation on Clinical Aspects of Human Infection with Avian Influenza A (H5N1) Virus, A. N. Abdel-Ghafar, T. Chotpitayasunondh, Z. Gao, F. G. Hayden, D. H. Nguyen, M. D. de Jong, A. Naghdaliyev, J. S. Peiris, N. Shindo, S. Soeroso, and T. M. Uyeki. 2008. Update on avian influenza A (H5N1) virus infection in humans. *N. Engl. J. Med.* 358: 261-273.
53. de Jong, M. D. and T. T. Hien. 2006. Avian influenza A (H5N1). *J. Clin. Virol.* 35: 2-13.
54. World Health Organization. 2007. Weekly epidemiological record. 82, 6: 41.
55. Uprasertkul, M., R. Kitphati, P. Puthavathana, R. Kriwong, A. Kongchanagul, K. Ungchusak, S. Angkasekwinai, K. Chokephaibulkit, K. Srisook, N. Vanprapar, and P. Auewarakul. 2007. Apoptosis and pathogenesis of avian influenza A (H5N1) virus in humans. *Emerg. Infect. Dis.* 13: 708-712.
56. Ng, W. F., K. F. To, W. W. Lam, T. K. Ng, and K. C. Lee. 2006. The comparative pathology of severe acute respiratory syndrome and avian influenza A subtype H5N1--a review. *Hum. Pathol.* 37: 381-390.
57. de Jong, M. D., C. P. Simmons, T. T. Thanh, V. M. Hien, G. J. Smith, T. N. Chau, D. M. Hoang, N. V. Chau, T. H. Khanh, V. C. Dong, P. T. Qui, B. V. Cam, Q. Ha do, Y. Guan, J. S. Peiris, N. T. Chinh, T. T. Hien, and J. Farrar. 2006. Fatal outcome of human influenza A (H5N1) is associated with high viral load and hypercytokinemia. *Nat. Med.* 12: 1203-1207.
58. Heinrich, P. C., I. Behrmann, S. Haan, H. M. Hermanns, G. Muller-Newen, and F. Schaper. 2003. Principles of interleukin (IL)-6-type cytokine signalling and its regulation. *Biochem. J.* 374: 1-20.
59. Baggiolini, M. and I. Clark-Lewis. 1992. Interleukin-8, a chemotactic and inflammatory cytokine. *FEBS Lett.* 307: 97-101.
60. Liao, F., R. L. Rabin, J. R. Yannelli, L. G. Koniaris, P. Vanguri, and J. M. Farber. 1995. Human Mig chemokine: biochemical and functional characterization. *J. Exp. Med.* 182: 1301-1314.
61. Chotpitayasunondh, T., K. Ungchusak, W. Hanshaoworakul, S. Chunsuthiwat, P. Sawanpanyalert, R. Kijphati, S. Lochindarat, P. Srisan, P. Suwan, Y. Osotthanakorn, T. Anantasetagoon, S. Kanjanawasri, S. Tanupattarachai, J. Weerakul, R. Chaiwirattana, M. Maneerattanaporn, R. Poolsavathitikoool, K. Chokephaibulkit, A. Apisarntharak, and S. F. Dowell. 2005. Human disease from influenza A (H5N1), Thailand, 2004. *Emerg. Infect. Dis.* 11: 201-209.
62. Tran, T. H., T. L. Nguyen, T. D. Nguyen, T. S. Luong, P. M. Pham, V. C. Nguyen, T. S. Pham, C. D. Vo, T. Q. Le, T. T. Ngo, B. K. Dao, P. P. Le, T. T. Nguyen, T. L. Hoang,

- V. T. Cao, T. G. Le, D. T. Nguyen, H. N. Le, K. T. Nguyen, H. S. Le, V. T. Le, D. Christiane, T. T. Tran, J. Menno de, C. Schultsz, P. Cheng, W. Lim, P. Horby, J. Farrar, and World Health Organization International Avian Influenza Investigative Team. 2004. Avian influenza A (H5N1) in 10 patients in Vietnam. *N. Engl. J. Med.* 350: 1179-1188.
63. Yuen, K. Y., P. K. Chan, M. Peiris, D. N. Tsang, T. L. Que, K. F. Shortridge, P. T. Cheung, W. K. To, E. T. Ho, R. Sung, and A. F. Cheng. 1998. Clinical features and rapid viral diagnosis of human disease associated with avian influenza A H5N1 virus. *Lancet* 351: 467-471.
64. Mibayashi, M., L. Martinez-Sobrido, Y. M. Loo, W. B. Cardenas, M. Gale Jr, and A. Garcia-Sastre. 2007. Inhibition of retinoic acid-inducible gene I-mediated induction of beta interferon by the NS1 protein of influenza A virus. *J. Virol.* 81: 514-524.
65. Subbarao, E. K., W. London, and B. R. Murphy. 1993. A single amino acid in the PB2 gene of influenza A virus is a determinant of host range. *J. Virol.* 67: 1761-1764.
66. Cheung, C. Y., L. L. Poon, A. S. Lau, W. Luk, Y. L. Lau, K. F. Shortridge, S. Gordon, Y. Guan, and J. S. Peiris. 2002. Induction of proinflammatory cytokines in human macrophages by influenza A (H5N1) viruses: a mechanism for the unusual severity of human disease? *Lancet* 360: 1831-1837.
67. Seo, S. H., E. Hoffmann, and R. G. Webster. 2002. Lethal H5N1 influenza viruses escape host anti-viral cytokine responses. *Nat. Med.* 8: 950-954.
68. Kido, H., K. Sakai, Y. Kishino, and M. Tashiro. 1993. Pulmonary surfactant is a potential endogenous inhibitor of proteolytic activation of Sendai virus and influenza A virus. *FEBS Lett.* 322: 115-119.
69. Steinhauer, D. A. 1999. Role of hemagglutinin cleavage for the pathogenicity of influenza virus. *Virology* 258: 1-20.
70. Carrat, F. and A. Flahault. 2007. Influenza vaccine: the challenge of antigenic drift. *Vaccine* 25: 6852-6862.
71. Gubareva, L. V., R. G. Webster, and F. G. Hayden. 2002. Detection of influenza virus resistance to neuraminidase inhibitors by an enzyme inhibition assay. *Antiviral Res.* 53: 47-61.
72. Geiss, G. K., M. Salvatore, T. M. Tumpey, V. S. Carter, X. Wang, C. F. Basler, J. K. Taubenberger, R. E. Bumgarner, P. Palese, M. G. Katze, and A. Garcia-Sastre. 2002. Cellular transcriptional profiling in influenza A virus-infected lung epithelial cells: the role of the nonstructural NS1 protein in the evasion of the host innate defense and its potential contribution to pandemic influenza. *Proc. Natl. Acad. Sci. U. S. A.* 99: 10736-10741.

73. Garcia-Sastre, A., A. Egorov, D. Matassov, S. Brandt, D. E. Levy, J. E. Durbin, P. Palese, and T. Muster. 1998. Influenza A virus lacking the NS1 gene replicates in interferon-deficient systems. *Virology* 252: 324-330.
74. Min, J. Y. and R. M. Krug. 2006. The primary function of RNA binding by the influenza A virus NS1 protein in infected cells: Inhibiting the 2'-5' oligo (A) synthetase/RNase L pathway. *Proc. Natl. Acad. Sci. U. S. A.* 103: 7100-7105.
75. Cameron, C. M., M. J. Cameron, J. F. Bermejo-Martin, L. Ran, L. Xu, P. V. Turner, R. Ran, A. Danesh, Y. Fang, P. K. Chan, N. Mytle, T. J. Sullivan, T. L. Collins, M. G. Johnson, J. C. Medina, T. Rowe, and D. J. Kelvin. 2008. Gene expression analysis of host innate immune responses during Lethal H5N1 infection in ferrets. *J. Virol.* 82: 11308-11317.
76. Fornek, J. L., M. J. Korth, and M. G. Katze. 2007. Use of functional genomics to understand influenza-host interactions. *Adv. Virus Res.* 70: 81-100.
77. Govorkova, E. A., J. E. Rehg, S. Krauss, H. L. Yen, Y. Guan, M. Peiris, T. D. Nguyen, T. H. Hanh, P. Puthavathana, H. T. Long, C. Buranathai, W. Lim, R. G. Webster, and E. Hoffmann. 2005. Lethality to ferrets of H5N1 influenza viruses isolated from humans and poultry in 2004. *J. Virol.* 79: 2191-2198.
78. Baughman, G., G. J. Wiederrecht, N. F. Campbell, M. M. Martin, and S. Bourgeois. 1995. FKBP51, a novel T-cell-specific immunophilin capable of calcineurin inhibition. *Mol. Cell. Biol.* 15: 4395-4402.
79. Hinz, M., M. Broemer, S. C. Arslan, A. Otto, E. C. Mueller, R. Dettmer, and C. Scheidereit. 2007. Signal responsiveness of IkappaB kinases is determined by Cdc37-assisted transient interaction with Hsp90. *J. Biol. Chem.* 282: 32311-32319.
80. Li, T. K., S. Baksh, A. D. Cristillo, and B. E. Bierer. 2002. Calcium- and FK506-independent interaction between the immunophilin FKBP51 and calcineurin. *J. Cell. Biochem.* 84: 460-471.
81. Fischer, G. and F. Schmid. 2001. Peptidylproline cis-trans-Isomerases. *Encyclopedia of Life Sciences*
82. Yeh, W. C., T. K. Li, B. E. Bierer, and S. L. McKnight. 1995. Identification and characterization of an immunophilin expressed during the clonal expansion phase of adipocyte differentiation. *Proc. Natl. Acad. Sci. U. S. A.* 92: 11081-11085.
83. Nair, S. C., R. A. Rimerman, E. J. Toran, S. Chen, V. Prapapanich, R. N. Butts, and D. F. Smith. 1997. Molecular cloning of human FKBP51 and comparisons of immunophilin interactions with Hsp90 and progesterone receptor. *Mol. Cell. Biol.* 17: 594-603.

84. Sinars, C. R., J. Cheung-Flynn, R. A. Rimerman, J. G. Scammell, D. F. Smith, and J. Clardy. 2003. Structure of the large FK506-binding protein FKBP51, an Hsp90-binding protein and a component of steroid receptor complexes. *Proc. Natl. Acad. Sci. U. S. A.* 100: 868-873.
85. Barent, R. L., S. C. Nair, D. C. Carr, Y. Ruan, R. A. Rimerman, J. Fulton, Y. Zhang, and D. F. Smith. 1998. Analysis of FKBP51/FKBP52 chimeras and mutants for Hsp90 binding and association with progesterone receptor complexes. *Mol. Endocrinol.* 12: 342-354.
86. Davies, T. H., Y. M. Ning, and E. R. Sanchez. 2005. Differential control of glucocorticoid receptor hormone-binding function by tetratricopeptide repeat (TPR) proteins and the immunosuppressive ligand FK506. *Biochemistry* 44: 2030-2038.
87. Scammell, J. G., W. B. Denny, D. L. Valentine, and D. F. Smith. 2001. Overexpression of the FK506-binding immunophilin FKBP51 is the common cause of glucocorticoid resistance in three New World primates. *Gen. Comp. Endocrinol.* 124: 152-165.
88. Hubler, T. R., W. B. Denny, D. L. Valentine, J. Cheung-Flynn, D. F. Smith, and J. G. Scammell. 2003. The FK506-binding immunophilin FKBP51 is transcriptionally regulated by progestin and attenuates progestin responsiveness. *Endocrinology* 144: 2380-2387.
89. Hubler, T. R. and J. G. Scammell. 2004. Intronic hormone response elements mediate regulation of FKBP5 by progestins and glucocorticoids. *Cell Stress Chaperones* 9: 243-252.
90. Billing, A. M., F. Fack, J. Renaut, C. M. Olinger, A. B. Schote, J. D. Turner, and C. P. Muller. 2007. Proteomic analysis of the cortisol-mediated stress response in THP-1 monocytes using DIGE technology. *J. Mass Spectrom.* 42: 1433-1444.
91. Rusnak, F. and P. Mertz. 2000. Calcineurin: form and function. *Physiol. Rev.* 80: 1483-1521.
92. Weiwad, M., F. Edlich, S. Kilka, F. Erdmann, F. Jarczowski, M. Dorn, M. C. Moutty, and G. Fischer. 2006. Comparative analysis of calcineurin inhibition by complexes of immunosuppressive drugs with human FK506 binding proteins. *Biochemistry* 45: 15776-15784.
93. Li, T. K., S. Baksh, A. D. Cristillo, and B. E. Bierer. 2002. Calcium- and FK506-independent interaction between the immunophilin FKBP51 and calcineurin. *J. Cell. Biochem.* 84: 460-471.
94. Bouwmeester, T., A. Bauch, H. Ruffner, P. O. Angrand, G. Bergamini, K. Croughton, C. Cruciat, D. Eberhard, J. Gagneur, S. Ghidelli, C. Hopf, B. Huhse, R. Mangano, A. M.

Michon, M. Schirle, J. Schlegl, M. Schwab, M. A. Stein, A. Bauer, G. Casari, G. Drewes, A. C. Gavin, D. B. Jackson, G. Joberty, G. Neubauer, J. Rick, B. Kuster, and G. Superti-Furga. 2004. A physical and functional map of the human TNF-alpha/NF-kappa B signal transduction pathway. *Nat. Cell Biol.* 6: 97-105.

95. Giordano, A., R. Avellino, P. Ferraro, S. Romano, N. Corcione, and M. F. Romano. 2006. Rapamycin antagonizes NF-kappaB nuclear translocation activated by TNF-alpha in primary vascular smooth muscle cells and enhances apoptosis. *Am. J. Physiol. Heart Circ. Physiol.* 290: H2459-65.

96. Vermeer, H., B. I. Hendriks-Stegeman, B. van der Burg, S. C. van Buul-Offers, and M. Jansen. 2003. Glucocorticoid-induced increase in lymphocytic FKBP51 messenger ribonucleic acid expression: a potential marker for glucocorticoid sensitivity, potency, and bioavailability. *J. Clin. Endocrinol. Metab.* 88: 277-284.

97. Silva, A. M. and L. F. Reis. 2000. Sodium salicylate induces the expression of the immunophilin FKBP51 and biglycan genes and inhibits p34cdc2 mRNA both in vitro and in vivo. *J. Biol. Chem.* 275: 36388-36393.

98. Wakabayashi, H., H. Miyauchi, K. Shin, K. Yamauchi, I. Matsumoto, K. Abe, and M. Takase. 2007. Orally administered lactoperoxidase increases expression of the FK506 binding protein 5 gene in epithelial cells of the small intestine of mice: a DNA microarray study. *Biosci. Biotechnol. Biochem.* 71: 2274-2282.

99. Franke, E. K. and J. Luban. 1996. Inhibition of HIV-1 replication by cyclosporine A or related compounds correlates with the ability to disrupt the Gag-cyclophilin A interaction. *Virology* 222: 279-282.

100. Watashi, K., N. Ishii, M. Hijikata, D. Inoue, T. Murata, Y. Miyanari, and K. Shimotohno. 2005. Cyclophilin B is a functional regulator of hepatitis C virus RNA polymerase. *Mol. Cell* 19: 111-122.

101. Nakagawa, M., N. Sakamoto, N. Enomoto, Y. Tanabe, N. Kanazawa, T. Koyama, M. Kurosaki, S. Maekawa, T. Yamashiro, C. H. Chen, Y. Itsui, S. Kakinuma, and M. Watanabe. 2004. Specific inhibition of hepatitis C virus replication by cyclosporin A. *Biochem. Biophys. Res. Commun.* 313: 42-47.

102. McEwen, B. S., C. A. Biron, K. W. Brunson, K. Bulloch, W. H. Chambers, F. S. Dhabhar, R. H. Goldfarb, R. P. Kitson, A. H. Miller, R. L. Spencer, and J. M. Weiss. 1997. The role of adrenocorticoids as modulators of immune function in health and disease: neural, endocrine and immune interactions. *Brain Res. Brain Res. Rev.* 23: 79-133.

103. Turnbull, A. V. and C. L. Rivier. 1999. Regulation of the hypothalamic-pituitary-adrenal axis by cytokines: actions and mechanisms of action. *Physiol. Rev.* 79: 1-71.

104. Navarra, P., S. Tsagarakis, M. S. Faria, L. H. Rees, G. M. Besser, and A. B. Grossman. 1991. Interleukins-1 and -6 stimulate the release of corticotropin-releasing hormone-41 from rat hypothalamus in vitro via the eicosanoid cyclooxygenase pathway. *Endocrinology* 128: 37-44.
105. Buttgereit, F., K. G. Saag, M. Cutolo, J. A. da Silva, and J. W. Bijlsma. 2005. The molecular basis for the effectiveness, toxicity, and resistance to glucocorticoids: focus on the treatment of rheumatoid arthritis. *Scand. J. Rheumatol.* 34: 14-21.
106. Almawi, W. Y. and O. K. Melemedjian. 2002. Molecular mechanisms of glucocorticoid antiproliferative effects: antagonism of transcription factor activity by glucocorticoid receptor. *J. Leukoc. Biol.* 71: 9-15.
107. Stahn, C., M. Lowenberg, D. W. Hommes, and F. Buttgereit. 2007. Molecular mechanisms of glucocorticoid action and selective glucocorticoid receptor agonists. *Mol. Cell. Endocrinol.* 275: 71-78.
108. Reily, M. M., C. Pantoja, X. Hu, Y. Chinenov, and I. Rogatsky. 2006. The GRIP1:IRF3 interaction as a target for glucocorticoid receptor-mediated immunosuppression. *EMBO J.* 25: 108-117.
109. World Health Organization. 2002. World Health Organization manual on animal influenza diagnosis and surveillance.
110. Matrosovich, M., T. Matrosovich, W. Garten, and H. D. Klenk. 2006. New low-viscosity overlay medium for viral plaque assays. *Virol. J.* 3: 63.
111. Brydon, E. W., S. J. Morris, and C. Sweet. 2005. Role of apoptosis and cytokines in influenza virus morbidity. *FEMS Microbiol. Rev.* 29: 837-850.
112. Silverman, M. N., B. D. Pearce, C. A. Biron, and A. H. Miller. 2005. Immune modulation of the hypothalamic-pituitary-adrenal (HPA) axis during viral infection. *Viral Immunol.* 18: 41-78.
113. Vermeer, H., B. I. Hendriks-Stegeman, D. van Suylekom, G. T. Rijkers, S. C. van Buul-Offers, and M. Jansen. 2004. An in vitro bioassay to determine individual sensitivity to glucocorticoids: induction of FKBP51 mRNA in peripheral blood mononuclear cells. *Mol. Cell. Endocrinol.* 218: 49-55.
114. Park, J., M. Kim, G. Na, I. Jeon, Y. K. Kwon, J. H. Kim, H. Youn, and Y. Koo. 2007. Glucocorticoids modulate NF-kappaB-dependent gene expression by up-regulating FKBP51 expression in Newcastle disease virus-infected chickens. *Mol. Cell. Endocrinol.* 278: 7-17.
115. Julkunen, I., K. Melen, M. Nyqvist, J. Pirhonen, T. Sareneva, and S. Matikainen. 2000. Inflammatory responses in influenza A virus infection. *Vaccine* 19 Suppl 1: S32-7.

116. Sun, J., R. Madan, C. L. Karp, and T. J. Braciale. 2009. Effector T cells control lung inflammation during acute influenza virus infection by producing IL-10. *Nat. Med.* 15: 277-284.
117. Imbeault, M., M. Ouellet, and M. J. Tremblay. 2009. Microarray study reveals that HIV-1 induces rapid type-I interferon-dependent p53 mRNA up-regulation in human primary CD4+ T cells. *Retrovirology* 6: 5.
118. Kamakura, M., A. Nawa, Y. Ushijima, F. Goshima, Y. Kawaguchi, F. Kikkawa, and Y. Nishiyama. 2008. Microarray analysis of transcriptional responses to infection by herpes simplex virus types 1 and 2 and their US3-deficient mutants. *Microbes Infect.* 10: 405-413.
119. Geiss, G. K., M. C. An, R. E. Bumgarner, E. Hammersmark, D. Cunningham, and M. G. Katze. 2001. Global impact of influenza virus on cellular pathways is mediated by both replication-dependent and -independent events. *J. Virol.* 75: 4321-4331.
120. U, M., L. Shen, T. Oshida, J. Miyauchi, M. Yamada, and T. Miyashita. 2004. Identification of novel direct transcriptional targets of glucocorticoid receptor. *Leukemia* 18: 1850-1856.
121. Wurzer, W. J., C. Ehrhardt, S. Pleschka, F. Berberich-Siebelt, T. Wolff, H. Walczak, O. Planz, and S. Ludwig. 2004. NF-kappaB-dependent induction of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and Fas/FasL is crucial for efficient influenza virus propagation. *J. Biol. Chem.* 279: 30931-30937.
122. Mazur, I., W. J. Wurzer, C. Ehrhardt, S. Pleschka, P. Puthavathana, T. Silberzahn, T. Wolff, O. Planz, and S. Ludwig. 2007. Acetylsalicylic acid (ASA) blocks influenza virus propagation via its NF-kappaB-inhibiting activity. *Cell. Microbiol.* 9: 1683-1694.
123. Shin, K., H. Wakabayashi, K. Yamauchi, S. Teraguchi, Y. Tamura, M. Kurokawa, and K. Shiraki. 2005. Effects of orally administered bovine lactoferrin and lactoperoxidase on influenza virus infection in mice. *J. Med. Microbiol.* 54: 717-723.
124. Hien, N. D., N. H. Ha, N. T. Van, N. T. Ha, T. T. Lien, N. Q. Thai, V. D. Trang, T. Shimbo, Y. Takahashi, Y. Kato, A. Kawana, S. Akita, and K. Kudo. 2009. Human infection with highly pathogenic avian influenza virus (H5N1) in northern Vietnam, 2004-2005. *Emerg. Infect. Dis.* 15: 19-23.
125. Carter, M. J. 2007. A rationale for using steroids in the treatment of severe cases of H5N1 avian influenza. *J. Med. Microbiol.* 56: 875-883.
126. Peiris, J. S., W. C. Yu, C. W. Leung, C. Y. Cheung, W. F. Ng, J. M. Nicholls, T. K. Ng, K. H. Chan, S. T. Lai, W. L. Lim, K. Y. Yuen, and Y. Guan. 2004. Re-emergence of fatal human influenza A subtype H5N1 disease. *Lancet* 363: 617-619.

127. Chan, M. C., C. Y. Cheung, W. H. Chui, S. W. Tsao, J. M. Nicholls, Y. O. Chan, R. W. Chan, H. T. Long, L. L. Poon, Y. Guan, and J. S. Peiris. 2005. Proinflammatory cytokine responses induced by influenza A (H5N1) viruses in primary human alveolar and bronchial epithelial cells. *Respir. Res.* 6: 135.
128. Standiford, T. J., S. L. Kunkel, M. A. Basha, S. W. Chensue, J. P. Lynch 3rd, G. B. Toews, J. Westwick, and R. M. Strieter. 1990. Interleukin-8 gene expression by a pulmonary epithelial cell line. A model for cytokine networks in the lung. *J. Clin. Invest.* 86: 1945-1953.
129. Ashwell, J. D., F. W. Lu, and M. S. Vacchio. 2000. Glucocorticoids in T cell development and function. *Annu. Rev. Immunol.* 18: 309-345.
130. Chan, M. H., P. K. Chan, J. F. Griffith, I. H. Chan, L. C. Lit, C. K. Wong, G. E. Antonio, E. Y. Liu, D. S. Hui, M. W. Suen, A. T. Ahuja, J. J. Sung, and C. W. Lam. 2006. Steroid-induced osteonecrosis in severe acute respiratory syndrome: a retrospective analysis of biochemical markers of bone metabolism and corticosteroid therapy. *Pathology* 38: 229-235.
131. Yong, W., Z. Yang, S. Periyasamy, H. Chen, S. Yucel, W. Li, L. Y. Lin, I. M. Wolf, M. J. Cohn, L. S. Baskin, E. R. Sanchez, and W. Shou. 2007. Essential role for Co-chaperone Fkbp52 but not Fkbp51 in androgen receptor-mediated signaling and physiology. *J. Biol. Chem.* 282: 5026-5036.