EFFICIENCY OF RECOMBINANT VACCINIA VIRUS CONTAINING INFLUENZA H5 *HA* GENE IN THE INDUCTION OF IMMUNE RESPONSE IN MICE

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ABSTRACT

This research studied both the cell mediated immune response (CMIR) and humoral immune response (HIR) in BALB/c mice immunized with replicating recombinant vaccinia virus harboring H5 *HA* (rVaccinia H5 HA) gene insert.

CMIR was studied by intraperitoneally immunizing mice with rVaccinia H5 HA , wild type vaccinia virus containing pSC11 plasmid backbone (rVaccinia pSC11), or PBS (control) using poly I:C as the adjuvant. The mice were boosted once without poly I:C after two weeks of priming. Splenocytes of the immunized mice were used as the effector cells in the *in vitro* cytotoxic activity assay, and P815 cells ($H2^d$) infected with rVaccinia H5 HA were used as target cells. NK cell cytotoxicity assay employing mouse splenocytes as the effector cells and Yac-1 cells as the target cells was also performed. In addition, immune sera from the immunized mice were investigated by antibody-dependent cell cytotoxicity (ADCC) assay using splenocytes as the effector cells and recombinant vaccinia virus infected P815 cells as the target cells. The results from four independent experiments of cytotoxic activity assay showed an average cytotoxic activity of 20-25 % at effector:target ratios of 50:1. No cytotoxic activity was observed in the control mice. In addition, immune sera demonstrated 30% cytotoxicity in ADCC at the effector:target ratio of 50:1 and NK cell cytotoxicity yielded 12% cytotoxicity at both the effector:target ratio of 12.5:1 and 50:1.

HIR was studied by immunizing mice intraperitoneally with rVaccinia H5 HA or rVaccinia pSC11 using poly I:C as the adjuvant. Unimmunized mice were used as the control. The immunized mice were boosted without poly I:C, either once at three weeks after priming or twice at three weeks intervals. Seroconversion to H5 HA antigen was detected in sera collected from immunized BALB/c mice by hemagglutination inhibition assay (HI) and micro-neutralization (micro-NT) assay. Geometric mean HI antibody titer in immune mouse sera after first boost and second boost were 113 and 139, respectively. Similarly, the geometric mean neutralizing antibody titers after first boost and second boost were 226 and 320, respectively. The second boost did not significantly increase the level of antibody titers as compared to those obtained after the first boost (paired *t*-test; $p > 0.005$).

The results suggest that the H5 HA protein exhibited both functional B and T antigenic determinants that can be presented in $H2^d$ molecules of BALB/c mice and stimulate CMIR and HIR to a certain level.

KEY WORDS: CMIR/ HIR/ HIGHLY PATHOGENIC AVIAN INFLUENZA H5N1 VIRUS/ HEMAGGLUTININ/ RECOMBINANT VACCINIA VIRUS

87 pages

ความสามารถของ RECOMBINANT VACCINIA VIRUS ที่มียีนฮีแมกกลูตินินของไวรัสไข้หวัดนก H5N1 ในการกระตุ้นระบบภูมิคุ้มกันของหนู

EFFICIENCY OF RECOMBINANT VACCINIA VIRUS CONTAINING INFLUENZA H5 *HA* GENE IN THE INDUCTION OF IMMUNE RESPONSE IN MICE

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บทคัดย่อ

งานวิจัยนี้ศึกษาการตอบสนองของระบบภูมิคุ้มกันแบบอาศัยเซลล์ และสารน้ำ ในหนู BALB/c ซึ่งถูกกระตุ้นด้วยการฉีด recombinant vaccinia virus ที่มียีนฮีแมกกลูตินินของไวรัสไข้หวัดนก H5N1 (rVaccinia H5 HA)

การศึกษาการตอบสนองของระบบภูมิคุ้มกันแบบอาศัยเซลล์ทำโดยการฉีดเชื้อ rVaccinia H5N1 HA หรือเชื้อ recombinant vaccinia virus ที่มี plasmid pSC11 (rVaccinia pSC11) หรือฉีด PBS เข้าทางช่องท้องของหนูโดยใช้ poly I:C เป็นตัวแอดจูแวนท์ หนูจะถูกฉีดกระตุ้นซ้ำหนึ่งครั้งหลังจากการกระตุ้นครั้งแรกนานสองสัปดาห์โดยไม่ใช้ poly I:C จากนั้นนำเซลล์ม้ามของหนูมาใช้ในการทดสอบความเป็นพิษต่อเซลล์ P815 ($H2^d$) ที่ติดเชื้อ rVaccinia H5 HA ซึ่งเป็นเซลล์เป้าหมาย (cytotoxic T lymphocyte assay) และทดสอบความสามารถของ NK cells ของหนูในการทำลาย Yac-1 cells (NK cell cytotoxicity assay) นอกจากนี้ยังได้นำซีรั่มของหนูที่ถูกกระตุ้นภูมิคุ้มกันมาใช้ในทดสอบความเป็นพิษต่อเซลล์ชนิดพึ่งแอนติบอดี (antibody-dependent cell cytotoxicity, ADCC) ผลจากการทดลองที่ไม่มีความเกี่ยวเนื่องกัน 4 ครั้ง แสดงค่าเฉลี่ยความเป็นพิษต่อเซลล์ที่ระดับ 20-25% โดยใช้อัตราส่วนเซลล์ effector ต่อเซลล์เป้าหมายเท่ากับ 50:1 ความเป็นพิษนี้ไม่พบในเซลล์จากหนูกลุ่มควบคุม และยังพบว่าความเป็นพิษต่อเซลล์ที่เกิดจาก NK cell มีค่าต่ำประมาณ 12% ที่อัตราเซลล์ effector ต่อเซลล์เป้าหมายเท่ากับ 12.5:1 และ 50:1 นอกจากนี้การทดสอบความเป็นพิษต่อเซลล์ชนิดพึ่งแอนติบอดีพบว่ามีความเป็นพิษต่อเซลล์เป้าหมายมากขึ้นเป็น 30% ที่อัตราส่วนเซลล์ effector ต่อเซลล์เป้าหมายเท่ากับ 50:1

การศึกษาการตอบสนองของระบบภูมิคุ้มกันแบบสารน้ำ ทำโดยการฉีดเชื้อ rVaccinia H5 HA หรือเชื้อ rVaccinia pSC11 โดยใช้ poly I:C เป็นตัวแอดจูแวนท์ และ หนูที่ไม่ได้รับการฉีดสารใดๆเป็นกลุ่มควบคุม หนูในการทดลองนี้จะได้รับการฉีดกระตุ้นภูมิคุ้มกันซ้ำโดยไม่มี poly I:C หนึ่งครั้งหลังการกระตุ้นภูมิคุ้มกันครั้งแรก 3 สัปดาห์ หรือกระตุ้นสองครั้งโดยมีช่วงห่าง 3 สัปดาห์ และทดสอบซีรั่มหนูเพื่อหาแอนตีบอดีที่จำเพาะต่อแอนติเจน H5N1 HA โดยวิธี Hemagglutination inhibition (HI) assay และ micro-neutralization (micro-NT) assay ผลการทดลองแสดงว่าหนูที่ได้รับการฉีด rVaccinia H5 HA มีการสร้างแอนติบอดีเพิ่มสูงขึ้นอย่างชัดเจน (seroconversion) โดยค่า geometric mean HI antibody titer (GMT) ของหนูที่ฉีดกระตุ้นซ้ำหนึ่งครั้งและสองครั้ง มีค่าเท่ากับ 113 และ 139 ตามลำดับ ในขณะเดียวกัน ค่า geometric mean NT antibody titer ของหนูที่ฉีดกระตุ้นซ้ำหนึ่งครั้งและสองครั้งเท่ากับ 226 และ 320 ตามลำดับ การกระตุ้นภูมิคุ้มกันครั้งที่สองไม่ได้เพิ่มปริมาณแอนติบอดีให้สูงขึ้นอีกเมื่อเปรียบเทียบกับกลุ่มที่กระตุ้นซ้ำครั้งเดียวอย่างมีนัยสำคัญ (paired *t*-test; $p>0.05$)

ผลจากการศึกษาแสดงให้เห็นว่าโปรตีนฮีแมกกลูตินินของไวรัสไข้หวัดนก H5N1 มีความเป็นแอนติเจนที่จะถูกนำเสนออยู่บน โมเลกุล $H2^d$ของหนู BALB/c และสามารถกระตุ้นทั้ง T และ B cells ให้สร้างภูมิคุ้มกันตอบสนองได้ทั้งแบบอาศัยเซลล์ และสารน้ำ

87 หน้า

CONTENTS

CONTENTS (cont.)

CONTENTS (cont.)

CONTENTS (cont.)

CONTENTS (cont.)

CONTENTS (cont.)

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

Abbreviation	Term
ADCC	Antibody dependent cell cytotoxicity
APC	Antigen presenting cell
^{o}C	Celsius
CMIR	Cell mediated cell immune response
CPE	Cytopathic effect
CTL	Cytotoxic T lymphocytes
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic acid
DW	Distilled water
EDTA	Ethylenediaminetetraacetic acid
EMEM	Earl's minimal essentials medium
FBS	Fetal bovine serum
HA	Hemagglutinin/ Hemagglutination assay
HI	Hemagglutination inhibition assay
HIR	Humoral immune response
Hr.	Hour(s)
MDCK	Mardin Darby canine kidney cells
MHC	Major histocompatibility complex
min	Minute(s)
ml	Millilitre(s)
NSS	Normal saline solution
PCR	Polymerase chain reaction
PBS	Phosphate buffer saline
pfu	Plaque forming unit
RBC	Red blood cell

LIST OF ABBREVIATIONS (cont.)

Abbreviation	Term
RDE	Receptor destroying enzyme
rpm	Round per minute
rVaccinia pSC11	Recombinant vaccinia containing plasmid pSC11
rVaccinia H5 HA	Recombinant vaccinia containing H5 *HA* gene insert
$TCID_{50}$	Tissue culture infectious dose
TPCK	Tosylsulfonyl Phenylalanyl Chloromethyl Ketone
VGM	Virus growth medium

CHAPTER I
INTRODUCTION

Immune response is an important mechanism of the body to get rid of foreign substances and pathogens, and maintain homeostasis. In response to virus infection, cytotoxic T lymphocytes (CTL) and antibodies are the key players for virus removal. Virus infected cells present viral antigenic proteins on major histocompatibility molecules and stimulate T cells [1]. CTL specifically kills the infected cells by releasing cytotoxic granules [2- 4]. Roles of antibodies in the defense against virus infection is either to neutralize the virus and blocking cell entry or functions as a specific antibody in antibody –dependent cell cytotoxicity (ADCC). After the virus is removed from the body, T and B lymphocytes become memory cells, which on next encounter, will rapidly and effectively combat the virus by memory cytotoxic T cell or produce high level and potent antibodies respectively [5, 6].

H5N1 avian influenza is one of the emerging diseases with over 50% mortality rate. Almost all human cases got H5N1 virus infection from the infected poultry. Human to human transmission of H5N1 virus is rare due to restriction of receptor binding preference. Avian H5N1 virus has higher propensity to galactose α 2,3 linked- sialic acid (SA α 2,3 gal); while human influenza viruses prefer target cells with SA α 2,6 gal [7] H5N1 avian influenza virus caused mainly severe pneumonia which may progress to acute respiratory distress syndrome, renal dysfunction, multiorgan failure and death [7- 9]. Hypercytokine production might play a key role in pathogenesis [10, 11].

HA is a membrane glycoprotein located on the virus surface and is synthesized as a precursor known as HA0. In order to gain infectivity, HA0 is cleaved in to HA1 and HA2 domains. HA1 binds to the host cellular receptor; and according to its high variability in the amino acid sequence, the antibody to HA1 is specific to strain or subtype. HA2 functions during the uncoating step of the virus life cycle as the fusion mediator of viral envelope and endosomal membrane. This domain is highly

conserved between different influenza virus [12]. HA is highly immunogenic and induces protective immunity. Previous investigators reported that HA epitopes induce both cellular immune response and humoral immune response [13, 14]. There are several methods for determining cellular immunity such as cytotoxic T lymphocyte (CTL) assay, antibody dependent cell cytotoxicity (ADCC) and NK cell cytotoxicity. On the other hand, microneutralization assay (micro-NT) measures neutralizing antibody to HA; while hemagglutination-inhibition (HI) assay is employed for an assessment of vaccine immunogenicity.

Several recombinant systems are used to express a desired protein. The systems previously reported included the recombinant vaccinia virus system, baculovirus insect cell system and the recombinant yeast cells [15]. Also, recombinant vaccinia virus carrying H1 HA and H5 HA gene has been used to study the effect of HA gene to the immune response by other researches [16]. This study has used the availability of recombinant system in the production of active H5 HA protein and has shown its immunogenicity in humoral immune response (HIR) over cell mediated immune response (CMIR) in BALB/c ($H2^d$) mice. Moreover, the HIR results have indicated that the production of the immunogenic H5 HA by using rVaccinia H5 HA can be applied in the study of immune response, disease diagnosis and vaccine development of viral influenza A infection.

CHAPTER II
OBJECTIVES

The objective of this study is to investigate the adaptive immune response in both cell mediated immune response (CMIR) and humoral immune response (HIR) in BALB/c mice ($H2^d$) to HA protein of influenza H5N1 by intraperitoneally immunization with recombinant vaccinia virus encoding H5 *HA* gene (rVaccinia H5 HA).

Part 1

CMIR to H5 HA influenza gene of BALB/c ($H2^d$) mice were studied *in vitro*. Mice were immunized with rVaccinia H5 HA with one boost. Wild type vaccinia with pSC11 plasmid (rVaccinia pSC11), and PBS immunized mice were used as control. The investigation is composed of cytotoxic T lymphocytes (CTL) activity, antibody-dependent cell-mediated cell cytotoxic (ADCC) activity and natural killer cell (NK) activity. P815 cells ($H2^d$) expressing H5 HA protein are target cells. Three effector:target ratio were used in CTL, ADCC and NK assay: 12.5:1, 25:1, and 50:1. The cytotoxicity is detected by non-radioactive assay kit.

Part 2

HIR in BALB/c mice to H5 *HA* influenza gene were studied *in vitro*. Mice were immunized with rVaccinia H5 HA with one or two boost. Mice immunized with rVaccinia pSC11 or unimmunized mice were used as control. Specific antibodies to H5 HA protein are investigated by measuring effective hemagglutination inhibition (HI) and neutralization (NT) activity of immunized mice sera.

CHAPTER III
LITERATURE REVIEW

3.1 Immune response

Cells and molecules of the immune system coordinately react against pathogens. It also has the ability to distinguish foreign from self particles which is known as self-recognition. The system is found in a wide range of organisms including plants [6]. The goal of immune system is to remove pathogens, such as, bacteria, fungi, virus, and worms. Other than pathogens, the immune system acts against other foreign substances and tissues such as allergens, and tissue transplant. Then, the body will return to homeostasis state.

When a person is wounded, the phagocytic leukocytes, such as macrophages, arrive at the scene. They engulf and destroy microbes while releasing signaling proteins to activate other immune cells. These signaling proteins are known as cytokines. Chemokines are the cytokines the draw immune cells the wounded location. The immune cells and substances that can act immediately are part of innate immune response. The phagocytes activate adaptive immune response which consists of T and B lymphocytes. When the pathogens or foreign substances are removed from the body, T and B lymphocytes will become memory cells [5, 6].

Immune response can be classified into two types based on its readiness to response and specificity to foreign substances or pathogens.

3.1.1 Innate immune response

3.1.1.1 Innate immune cells

Innate immune response can act immediately when they encounter foreign particle but have less specificity than adaptive immune response. The innate immune cells are neutrophil, basophil, eosinophil, macrophages, dendritic cells, monocytes, and NK cells. The first cells to arrive at the site of infection are Neutrophils. The chemokines IL-1, IL-8 released from activated macrophages and

activated T cells draw them out of the vascular area into the tissue. Neutrophils have phagocytic activity like macrophages but they have granules that generate antimicrobial substances. Eosinophils have phagocytic properties and are drawn to tissue areas. Their major role is to defense against parasitic organisms, such as worms. They release basic proteins, sulfotransferase and sulfatases from eosinophilic granules to damage the parasite. Basophils are non phagocytic cells that release mediators from their granules. Macrophages and dendritic cells are known as professional antigen presenting cells (APC), they engulf the cells or pathogen by phagocytosis then present antigen peptide on major histocompatibility complex (MHC). NK cells can control infections and can kill tumor cells. The cells that are susceptible to NK cells are cells that MHC class I are not exported to the cell surface which are found in some viral infections and some tumor cells. Its killing is also known as "The kiss of death". Specific antibodies to target pathogens, such as helminthes or infected cells, can aid NK cytotoxicity which is known as antibody dependent cell cytotoxicity (ADCC). [5,6]

3.1.1.2 Inflammation

When there is an injury or an infection, inflammation occurs. The inflammatory signs are swelling, increase in temperature, redness, and pain. The series of events begins with vasodilation which the capillaries increase in diameter. This event leads to tissue redness and increase in local temperature. Then the capillary permeability increases and immune cells such as neutrophil and macrophages enter the area which results in the swelling of the area. These cells release chemokines that attract phagocytes to the site of infection. The phagocytes will engulf these cells and process to present on MHC molecules [2].

3.1.1.3 Recognition of foreign substances

Innate immune response can recognize pathogens associated molecular pattern (PAMPs) or danger-associated molecular patterns (DAMPs) by interaction with pattern recognition receptor (PRRs) and responses to pathogen in a non specific way. PAMPs are conserved motif expressed by microbial pathogens such as bacterial Lipopolysaccharide (LPS), peptidoglycans, flagellins (bacteria), and double stranded RNA. DAMPs are molecules from necrotic or dying cells such as heat shock protein or protein fragments. Some of the PAMPs are used as an adjuvant

to induce the immune response. There are varieties of PRRs locating on the cell surface on in the cytosol. Toll like receptor (TLR) molecules is one of the important PRRs. TLR can recognize a wide range of pathogens including bacteria and virus Other PRRs are RIG-1 like receptors (RLRs) and nod-like receptors (NLRs). The PRRs located on cell surface recognize extracellular pathogens proteins and lipids such as TLR2, TLR4 and TLR5 which recognize lipoteichoic acid (gram positive bacteria), lipopolysaccharide (gram negative bacteria) and flagellin respectively. TLR3, TLR7 and TLR9 recognise PAMPs from virus and are located in the cytosol. TLR3 recognize double stranded RNA, TLR7 and TLR8 recognize single stranded RNA from viruses and TLR 9 recognize unmethylated CpG motifs [18, 19]. TLR receptor interaction leads to MyD88 dependent signaling or MyD88 independent signaling. These two types of signaling lead to different cytokine production. MyD88 dependent pathway leads to proinflammatory cytokines and MyD88 independent stimulate IFNβ and dendritic cell maturation. My-D88 dependent pathway activates NFκB via IL-1 receptor associated kinases and TRAF-6, resulting in inflammatory cytokines. MyD88 independent, which are found in TLR3, TLR7, TLR8, TLR9 signaling pathway, uses TRIF dependent pathway. TRIF activates the transcription factor, interferon regulatory factor 3 (IRF3). IRF3 activation results in production of type one interferons (IFNα, or IFNβ) which is involved in antiviral responses [6].

3.1.2 Bridging between innate and adaptive immune response

In order to activate adaptive immune response, antigen presentation by cell- cell interaction between antigen presenting cells (APCs) and adaptive immune cell (T lymphocytes) is needed. Cytokines and chemokines are also required to stimulate effective adaptive immune response.

3.1.2.1 Antigen presentation to T lymphocytes

Antigen presentation to T lymphocytes is classified to MHC class I and MHC class II restriction which interacts with $CD8^+$ T cells and $CD4^+$ T cells respectively. In virus infection, the virus was engulf by professional APCs (macrophages, dendritic cells) or infected to the target cells. In the infected cells, the viral genome is translated to viral protein and protein is processed by proteasome. The peptides bind to MHC class I in the endoplasmic reticulum (ER) domain and were

transported to Golgi apparatus, vesicle and cell surface. The antigenic peptide on the MHC interacts with T cell receptor (TCR) and $CD8^+$ molecule of $CD8^+$ T cells which results in activating cytotoxic T cell. On the other hand, pathogens or infected cells presenting pathogenic peptide are engulfed into endosome by professional APC. The endosome fuse with lysosome and the pathogens are processed. The MHC class II are synthesized and transported from ER to Golgi apparatus and exocytic vesicle. The processed antigen in endosome-lysosome binds to the MHC class II groove and is transported to cell surface. MHC class II will interact with TCR and CD4 molecule of $CD4^+$ T cells [20].

3.1.2.2 Antigen presentation to B lymphocytes

B cells recognize proteins, polysaccharides, lipids and small chemical that are expressed either on microbial surface or as soluble form. B cells can recognize microbe and are activated or requires T helper ($CD4^+$) cell interaction [20].

3.1.3 Adaptive immune response

Adaptive (acquire) immunity requires activation and stimulation to function. Not only it has higher specificity than innate immunity, it also has memory. Memory of adaptive immunity is important in eliciting fast and effective immune response when re-encounter the same infectious agents. This type of immunity is classified into cell mediated immunity and humoral immunity.

3.1.3.1 Major histocompatibility molecules (MHC)

In order for APC to present antigenic proteins, the pathogens must be processed and present peptides on MHC molecule. The MHC molecules are classified according to their biochemical structute mainly MHC class I and class II.

3.1.3.1.1 MHC class I

All cell types are able to present antigen on MHC class I molecule. If the protein were engulfed into endosome-phagosome it will exit the phagosome into the cytosol. In the infected cells, the virus will translated to viral proteins. The protein will be processed into peptides by proteasome. Peptides enter the ER via transporter associated with antigen processing (TAP) and associate with MHC class I molecule. The MHC class I complex is transported to golgi apparatus, exocytic

vesicle and present on cell surface. The interaction is specific to $CD8^+$ T lymphocytes [20].

3.1.3.1.2 MHC class II

Only professional antigen presenting cells present MHC class II. The first step is engulfing extracellular pathogens (bacteria, fungal), proteins or infected cells by APC. The protein antigen enters the vesicular endosome and lysosome. At the same time MHC class II with class II invariant chain peptide (CLIP) is synthesize in ER. The complex is transported to Golgi apparatus and endosomal vesicle. Inside the vesicle contains a protein known as DM which will remove CLIP and make the MHC cleft accessible to peptides. The processed peptide binds to the binding cleft of MHC class II molecule. MHC-antigenic peptide complex is presented on cell surface. MHC class II is specific to $CD4^+$ T cells [20].

3.1.3.2 Memory of adaptive immune response

The adaptive immune response requires activation time which makes it unable to react immediately. In turn, it is more specific and has memory. When the body encounters the pathogens again, the memory cells are ready to response. After T cell and B cell activation and clonal expansion, effector cells and memory cells are produced. When the pathogen is removed from the body, effector cells die by apoptosis. However, memory T cells and antibodies will reside in blood circulation and secondary lymphoid organs for a certain period [20].

3.1.3.2.1 Vaccination

The purpose of vaccination is to induce high level of specific adaptive immune response and provide long term or life-long protection against the same infection.

3.1.3.2.1.1 Vaccines

Vaccines are classified based on the nature of immunogen; for example, live attenuated, killed inactivate, subunit, and toxoid.

Live attenuated vaccine is from the pathogen that has replicated and has mutated until it has low virulence. This is a very good approach for RNA virus vaccine because it has high mutation rate. This method has an advantage of mimicking the real pathogen. However, the vaccine may revert to have high virulence and cannot be used in immunosuppressed persons. The vaccine is

enters the target cell and present on MHC class I molecule to CTL. CTL will kill the infected cell and will become memory cells. If immature phagocytose vaccine, it will leads to neutralizing IgG antibodies and memory B cells.

Killed/ inactivated vaccine: The term killed is used for bacteria vaccines, while inactivated is used for virus vaccines. An example of this vaccine is the Polio or Hepatitis vaccine. The adaptive immune reponse to the immunogen has a wider range than the toxoid vaccine. After injection, the whole organism is phagocytosed by immature dendritic cells. Then they are presented on different MHC class II. They are recognized by BCR and TCR. The method is safer, unactively multiplying, stable and long lasting. However, there are disadvantages to this method. The vaccine requires adjuvant and several doses. Secondly, there may be sone acute local inflammatory reaction.

Toxoid vaccines use toxoid molecules from pathogens, such as tetranus, which are taken up by immature dendritic cells. They are processed through the endosomal pathway and presented on MHC class II. The TCR of T_h2 recognized the antigen presented on MHC class II and start T cell proliferation. B cell can recognize the antigen directly and produce IgM and IgG. Toxoid vaccine is not highly immunogenic. Therefore, high dose and adjuvant is required for this vaccine. The usage of high dose may lead to tolerance. The advantages and disadvantages are similar to that of killed/inactivated vaccine.

Subunit vaccines use a particular antigen or combination of antigens to prevent infection. Therefore the key is to identify that particular immunogen. The adaptive immune response to this vaccine depends on whether it is protein based or polysaccharide based. The protein based is T dependent while polysaccharide based is T independent. The advantage is the same with toxoid vaccine with the addition of ability to distinguish vaccinated from infected persons. This vaccine also needs adjuvant and local reaction at injection site [21].

3.1.3.2.1.2 Adjuvants

The adjuvants functions in aiding the vaccine in generation of strong immune response *in vivo*. Using adjuvants in vaccine will increase antibodies titer, decrease the dose number or used antigen, enhance the speed and duration of vaccine, and induce cell mediated immunity, mucosal immunity and

broader immune response. Adjuvants can be divided to delivery system and immunopotentiators. Virosome, liposome, virus-like particles or viral vector are the antigen delivery adjuvants. The immunopotentiators are sense by the TLR family. The immunopotentiators are alternate PAMPs, double stranded RNA and CPG. Most of the traditional vaccine with aluminium containing compounds stimulate T-helper 2 cells which leads to the production of IgG, IgM and various cytokines. However, recent vaccine developments stimulate T_h1 immunity to prevent infectious diseases, cancer, and autoimmune diseases. Nowadays, alum is the only adjuvant allowed to use in human. Even though there are other potent adjuvants, they are highly toxic [22].

In order to have an effective vaccination three main factors are required; immunogen, host immune response, vaccine handling and environment. The booster, antigenic properties and adjuvant are part of the vaccine that leads to the high level of immunity. Due to each animal or person has different immunogenic background, their response to vaccine may vary. For example, the maternal antibody and the function of immune response has been shown to effect to vaccine potential. The handling and administration of vaccine is also crucial. It was shown that different administration sites lead to different level of immune response. Also if it was injected different from the route which was developed it may cause harm. The vaccination programs were designed to protect against normal condition of exposure but cannot be used in severe condition of challenge [23].

3.1.3.3 Cell-mediated immune response (CMIR)

T lymphocytes (T cells) are produced in bone marrow and mature in thymus. Maturation of T lymphocytes requires antigen presentation on MHC of APC. T lymphocytes maturation is divided into two phases: early phase and late phase. Early phase covers the developmental stages prior to $CD4^+CD8^+$ T lymphocytes. Late phase maturation is depending on cell surface expression of a functional T cell receptor. Development of T lymphocytes is divided into 4 stages: double negative, double positive, single negative-single positive and triple negatives. The immature T lymphocytes lack TCR-CD3 expression and are in $CD4^-CD8^-$ population which are the triple negatives. Then the next stage is double positive which $CD4^+$and $CD8^+$ T lymphocytes were generated. During this phase pre-T cell receptor are present. Double positive went through generation of cloning cells with a single T

cell receptor (TCR) β. T lymphocytes that express αβ TCR CD3 complex chose two single positive pathway to develop: $CD4^+$ T lymphocytes (10%) and $CD8^+$ T lymphocytes (5%). T lymphocytes went through positive and negative selection. In the thymic cortex, T lymphocytes interact with MHC class I or II of their epithelial cells with. RAG-1, RAG-2 and TdT which facilitate rearrangement of αβ TCR proteins will be expressed during positive selection. The T lymphocytes that fail to express αβ T lymphocytes will die by apoptosis. T lymphocytes that went through positive selection will show low affinity and high affinity. Interaction with macrophages in thymus medulla or bone marrow derived dendritic cells will lead to cell apoptosis of T lymphocytes with high affinity. Interleukin-2 (IL-2) and costimulation is required for completing the activation of proliferation. The first co-stimulation signal is from an interaction of antigenic peptide on TCR with CD3. The second co-stimulation signal is from CD28 of T lymphocytes and B7 on antigen presenting cells.

CMIR was shown to get rid of the intracellular pathogens. The APC can be either the infected cell or the phagocytes that ingest pathogens or infected cells. T lymphocytes can be categorized into 2 types based on the presentation of costimulatory molecules: $CD4^+$Tcells and $CD8^+$ Tcells.

3.1.3.3.1 $CD4^+$ T lymphocytes

$CD4^+$ T cells recognize antigen by interaction of TCR, MHC class II and costimulatory molecules. The cells play role in secreting cytokines and chemokines that help with activation of other immune cells. Because of the ability to secrete cytokine to help other immune cell, it is known as T helper cells. The cell contact through CD40 and CD40 ligand leads to secretion of cytokines such as IFNγ, and TNF. These cytokines have the ability to aid phagocytes in killing pathogens. T helper cells are characterized into T_h1 and T_h2 based on the cytokine production. T_h1 cells secrete IL-2, IFN-γ and TNFβ which aids cytotoxic T cells and are involved in delayed type hypersensitivity. On the other hand, T_h2 cells secrete IL-4, IL-5 IL-6 and IL-10 which help B cell activation and play role in worm infection.

Based on many researches, $CD4^+$ T cells also have cytotoxic activity [24-26]. Absence of CD28, presence of NK receptors such as killer immunoglobulin like receptor (NKG2D) and CD57 expression, and lack of CD27 were shown to be the phenotypic properties of these cells. Cytotoxic $CD4^+$ T cells are

found circulating in low frequencies. The $CD4^+CD28^-$ does not express CCR7, and CD62L but able to migrate to lymph nodes by CCR5, CxCr3 and CX3CR1. However the cells do not express CD38, CD69, HLA-DK and ki67 which are signs of activation. These cells are found be able to kill infected cell that present peptide on MHC class II. The first function is to get rid of pathogens that avoid or inhibits presentation of MHC class I complex such as viruses from Herpesviridae family. The killing of infected cells can be done by granules exocytosis or by binding of Fas (also known as CD95) to FasL. The cytotoxicity of these cells can be inhibited with concanamycin A which suggests that the killing is granule dependent. The presence of $CD4^+$ T lymphocytes are found in many infections such as Cytomegalovirus, Epstein-barr virus, Vaccinia virus, Human immunodeficiency virus, mycobacteria and *C.neoformans*. Cytomegalovirus infected cells are killed by peptide recognition of MHC class II which leads to granule release. In Human immunodeficiency virus infection with coinfection of Cytomegalovirus, high level of cytotoxic $CD4^+$ T cell presence is shown. However, their research does not show that whether it is caused by human immunodeficiency virus or Cytomegalovirus. The lack of CD27 and CD28 are overcome by using NK receptors such as NKG2D ligand. There are two suggested function of cytotoxic $CD4^+$ T cells. The another possible function is to provide negative feedback killing of antigen presenting cells that travels to the lymph node. Interestingly, cytotoxic $CD4^+$ T cells are present in autoimmune disease, vascular disease, and inflame bowel disease. The developments of $CD4^+$ T helper have been studied but not for $CD4^+$ cytotoxic T cells. Therefore, the development of $CD4^+$ cytotoxic T cells has become interesting [24]. Deborah M. Brown and her colleagues studied on the factors, such as cytokines and antigen presentation, involving in $CD4^+$ cytotoxic T cell development [25]. Their studies use TCR Tg mice and cytotoxicity experiments were done by using different methods such as 3H-thymidine cytotoxic assay, and flow cytometry. They found that IL-2 has an important effect on the cytotoxicity of $CD4^+$ T cells especially at antigen presentation with lower peptide concentrations whereas the exogeneous IL-2 is required to drive the perforin mediated cytotoxicity. $CD4^+$ cytotoxic T cell roles in viral infection have also been studied. Kathleen A. Stuller and her colleagues study on the *in vivo* functions of cytotoxic $CD4^+$ T cells in gamma herpesvirus68 (γHV68) infection [26]. The experiments

include depletion of $CD8^+$ T cells, by anti-CD8 antibodies, in γHV68 infected mice and measuring cytotoxic activity. The results show that $CD4^+$ T cells can kill γHV68 infected cell by direct cytotoxicity which leads to control of γHV68 infection.

A recently found subset of $CD4^+$ T cells is T_h17. Th_{17} secretes IL-17 and IL-22 which is different from T_h1 and T_h2. The differentiation from naïve $CD4^+$ T cells requires stimulation with antigen with the presence of TGF-β, IL-6 and IL-1. It was suggested that this type of T lymphocytes plays role in extracellular bacterial and fungal infection [6].

Another subtype of $CD4^+$ T lymphocytes is T regulatory cell (Treg). These cells regulate the functions of other T lymphocytes by releasing suppressing cytokines (IL-10). Treg cells also plays role in autoimmune diseases. Treg express $CD4^+$, $CD25^+$ and $FoxP_3$ costimulatory molecules [27].

3.1.3.3.2 $CD8^+$ T lymphocytes

These T lymphocytes have cytotoxic activity against abnormal cells or infected cells are known as Cytotoxic T cells (CTL) [6]. CTL express TCR and $CD8^+$ on their surface which interacts specifically to MHC class I complex. Recognition of antigen leads to T cell activation and leads to target cell death. T lymphocytes bind to the target cells using TCR receptor, coreceptors, and adhesion molecules. This leads to immunological synapse which activates the T cell. Activated T cells will kill the infected cell by two major mechanisms; binding of Fas to Fas ligand and granules exocytosis. The granules of CTL cells are granzyme, perforin, and granulosin. Perforin form holes at the surface of the infected cells. Then granzymes enter the cell causing cell death. The activated T cells also release cytokines and chemokines. Interferon (IFN) is one of the important cytokine that was proposed to have an antiviral role. Also chemokines will recruit more T cells into the area. The cytotoxicity can be evaluated by many cytotoxic assays.

3.1.3.3.2.1 Radioactive cytotoxic assays

a) Conventional cytotoxic assay (^{51}Cr)

This assay has been used for many years in several studies [14]. In brief, target cells of this assay are labeled with ^{51}Cr. The effector cytotoxic cells are incubated at 37^oC with labeled target cells at different ratios. Then,

supernatant are collected and level of ^{51}Cr release was measured. The calculation of specific cell lysis is as followed:

$$\% \text{ specific lysis} = \frac{E - S}{T - S} \times 100$$

E = experimental release in the presence of killers (in cpm),

S = spontaneous release without killers, and

T = total incorporated label

This method is expensive, and dangerous because the use of Chromium which is a radioactive substance in the assay. However, they give high cytotoxicity sensitivity.

b) JAM assay [28]

Due to the limitations of using ^{51}Cr to detect cell death, this method is developed. [3H] thymidine is used in this assay. Even though it is radioactive, it is less dangerous and can be used to detect cytotoxicity at a lower cost. This method is different to the conventional assay in the labeling target cells, harvesting step, and detection of cytotoxicity. While the conventional method harvests the supernatant, this assay requires cell harvest onto glass fiber filters. Also the conventional method detects cell death, while JAM assay detects living cells. Therefore the calculation for cell death is different.

When substitute into the standard equation used by conventional assay

$$\% \text{ specific DNA loss} = \frac{(T-E) \times (T-S)}{T-(T-S)} \times 100$$

Which becomes

$$\% \text{ specific killing} = \frac{S - E}{S} \times 100$$

E = experimentally retained DNA in the presence of killers (in cpm),

S = retained DNA in the absence of killers (spontaneous),

and T= total incorporated label

3.1.3.3.2.2 Non-radioactive cytotoxic assay

Radioactive assays have to be approved by Office of Atom for Peace organization. Moreover, the person that use radioactive methods must went through special training. Therefore, non radioactive assays have been developed which does not require radioactive labeling of target cells

a) Lactate dehydrogenase (LDH) based assay

Each cell produced LDH inside the nucleus. When cells die, the cell membrane will erupt causing lesion of LDH. This assay uses that principle. In order to measure the amount of cells killed by CTL, the LDH level in the supernatant was measured. According to the protocol effector cells were cultured with target cells for 4 hrs in different E:T ratios. The supernatant were then collected and LDH were measured. [29,30]

Calculation of cytotoxicity is as followed

$$\% \text{ cytotoxicity} = \frac{\text{Exp-Eff-Tar}}{\text{Max-Tar}} \times 100$$

Exp= Experiment – media

Eff= Effector spontaneous – media

Tar= Target spontaneous – media

Max= Maximum Target Release – Volume correction

b) Flow cytometry

CFSE can be labeled on target cells. When the cells proliferate, the level of CFSE will be divided between daughter cells. Proliferation level can be used to represent cytotoxicity level. This assay label target cells with CFSE then incubate at 37^{o}C for 1-3 days which is longer than conventional assay (4hr). Then, they are incubated together with effector cells and later on with specific antibodies. Finally cells are analyzed by using flow cytometry [31].

$$\% \text{ survival} = \frac{[\text{absolute no. viable CFSE+ target cells } (t = x)]}{[\text{absolute no. viable CFSE+ target cells } (t = 0)]} \times 100$$

3.1.3.4 Humoral immune response (HIR)

The humoral immunity can recognize not only proteins but also lipid and carbohydrates antigens as well. [20] The effector function of antibodies created from B lymphocytes are able to neutralize the virus, aids in complement

activation and functions in antibody dependent cell cytotoxicity (ADCC). The memory antibodies can be used to prevent future encounter to pathogens which is a goal of vaccination. In terms of diagnoses, the subjects/patients antibodies can tell which pathogen they have encountered or vaccinated.

B cells are generated and mature in the bone marrow. B cell development starts with lymphoid stem cells differentiation into progenitor B-cell and precursor B cells. IL-7, VLA-4 (progenitor B cells) and VCAM-1(precursor B cells) helps in cell differentiation. The next step in B cell maturation is Ig gene rearrangement. RAG-1, RAG-2 and terminal deoxynucleotidyl transferase (TdT) play important role in heavy chain, light chain rearrangement. Immature B cell that can react to self antigens will be killed.

B cells activation is either T helper cell dependent or T helper cell independent. The antigens that induce T independent activation are divided into two types. Type 1 antigens are bacterial cell wall components including Lipopolysaccharides (LPS). Type 2 antigens are bacterial cell wall with repetitive units conjugated to polymeric proteins. In cases where the antigen cannot produce competent signal, signals from T helper cell is required. The signals include T and B cells conjugate signal, CD40/CD40L, and T helper cytokines. Then B cells become plasma cells that produce IgM, and IgG respectively. IgG has higher specificity and affinity than IgM.

Functions of the antibodies are mediated by the heavy chain of immunoglobulin molecules. IgM, IgG, IgA, IgD and IgG are heavy chains that can be switched by recognition of pathogens, with or without CD40L interaction, and cytokines. IgM is the first type of immunoglobulin secreted. It requires no signal from T helper cells in transcription and translation to IgM. Other immunoglobulin requires signal from T helper cell in RNA splicing and translation. IgG requires signal from T helper cell and IFN-γ in class switching. The Iγ-Sγ-Cγ locus of γ mRNA is transcribed in to γ protein. IgG functions in Fc receptor-dependent phagocytes response, complement activation and neonatal immunity. IL-4 induces class switching into IgE. IgE functions against helminthes and mast cell degranulation. IgA is involved in mucosal immunity, and requires several cytokines, such as APRIL, BAFF and TGF-β.

The RNA splicing, transcription and translation of IgA and IgE is similar to that of IgG except that the locus that was transcribed is Iα-Sα-Cα and Iε-Sε-Cε respectively.

The effector functions of antibodies are neutralization of microbes and microbial toxins, antibody-mediated opsonization and phagocytosis, and complement activation. Antibodies can bind to microbes and prevent infection by stearic hindrance or induce conformal changes in surface molecules. Toxin-specific antibodies bind and change the interactions with host cells and prevent tissue injury and diseases. The binding requires the specific antigen binding region. Most of circulating neutralization antibody is of IgG type, and in mucosal tract the neutralization antibody is IgA. Antibodies can also promote phagocytosis by binding to Fc receptor on phagocytes, which is known as opsonization. Opzonization leads to Fc receptor signaling and phagocyte activation. An example of Fc receptor is FcγRI which is found on macrophages, neutrophils and epsinophils. FcγRI binds to IgG at the Fc unit. Antibody can aids killing of infected cells by binding to Fc receptor of leukocytes. This function of antibody is known as antibody-dependent cell-mediated cytotoxicity (ADCC). The antibody binds to the antigen expressed on infected cell or phagogytes. Then the Fc receptor of lymphocytes (FcγRIII) such as macrophages or NK cells binds to the Fc unit of antibody. The antibody activates the macrophages or NK cells and leads to the killing of infected cell [6].

During the infection of cytopathic virus such as vaccinia (viral loading red line), specific $CD4^+$ and $CD8^+$ T cells are activated and expanded. Polyreactive antiviral IgM antibodies are likely induced as a first defense in antiviral responses. Long-lived plasma cells are induced, providing neutralizing antibodies. The half-life of long-lived plasma cells has been reported to be longer than that of $CD4^+$ or $CD8^+$ T cells. It has been argued that there is no intrinsic half-life of long-lived plasma cells, but rather that their survival is conditional on being in a survival niche. The vertical dotted line indicates the assumed lower level of detection. [32]

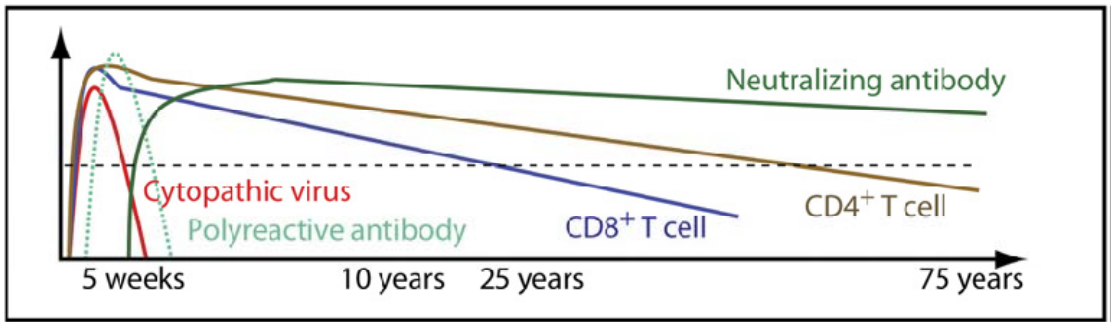

Figure 3.1: Immune cells and antibodies reponse to cytopathic virus [32]

3.2 Influenza virus

Viruses are non cellular organisms that have nucleic acid (DNA or RNA) as their genome. Because the lack of their own metabolisms, they use the host's machineries for themselves. Virus properties such as genomic structure, type of the capsid, or existence of envelope including host range, and pathogenesis can be used for viral classification. [33] After entering the cell, virus transcribes to RNA and translates to protein. The viral protein form together as a complete virion and leave the cell leading to infection of other cells. [33, 34]

3.2.1 Nature of influenza virus.

Influenza virus is a member of *Orthomyxoviridae* family. It is divided into type A, B, C based on its nucleoprotein and matrix protein (NP and M). Influenza A and B virus causes diseases in human and in animals.. Influenza virus can be divided in to subtypes by Hemagglutinin (HA) and Neuraminidase (NA). HA is divided into 16 subtypes and NA is divided into 9 subtypes. Generally, influenza that cause diseases in human belong to subtype H1 and H3 (H2 was extinct). All subtypes of influenza can be infected to avian. Of these avian influenza some of them are able to infect human such as H9N2, H7N7, H7N1 H7N3 and H5N1. Most of the avian influenza virus show low pathogenic (LPAI) (including influenza B). However H5N1 influenza is highly pathogenic and causes severe symptoms in human. Therefore H5N1 influenza is classified as HPAI.

Influenza virus is an enveloped virus with eight (seven in type C) segmented negative stranded RNA (HA, NA, NP, M, NS, PB1, PB2, and PA). The diagram of virus is shown in figure 3. The eight genes of influenza virus are encoded

into ten proteins which are PB1, PB2, PA, HA, NA, NP, M1, M2, NS1 and NS2. PB1, PB2 and PA are RNA transcriptase complex. The viral genome with nucleoprotein is also known as ribonucleoprotein (RNP). M1 covers the NP. On the surface of the virus are HA, NA and M2. Nucleoprotein (NP) is component for nucleoprotein or viral capsid. Hemagglutinin (HA) aids cell entry. Neuraminidase (NA) is important for viral release from cell and spreading. M1 is a component under the envelope while M2 is an ion channel. Non-specific protein 1 (NS1) was found to inhibit the innate immune function and non-specific protein 2 (NS2) transport RNP to cytoplasm. As HA and NA are presented on the surface of virus, it is known that they are potent antigens for influenza virus. [35, 36]

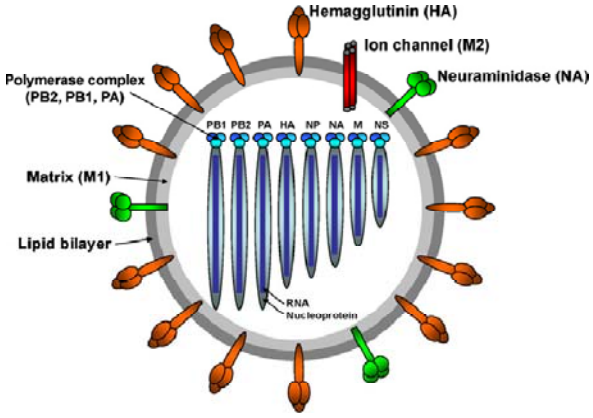

Figure 3.2: Influenza virus structure [37]

The genetic of influenza virus have high mutational rate which leads to changes in antigen. This is known as antigenic variation and is classified into antigenic drift and antigenic shift. [38]

a) Antigenic drift

The new antigen is caused by point mutation in HA and NA genes. The difference between the new and the original antigen does not lead to a new subtype of influenza virus.

b) Antigenic shift

The new antigen is very different from the original. This is caused by genetic reassorment of two influenza virus which is caused by two different subtypes of viruses infect the same cell and during the self assembly process, trading of genome might happen. This leads to a hybrid virus with a new HA or NA

3.2.1.1 Hemagglutinin (HA)

HA is encoded by RNA segment no.4 and contains more than 1,000 bp. The preunit hemagglutinin (HA0) is located in ER of infected cells. HA chain composed of ectodomian of 512 residues, 27 transmembrane residues and 20 cytoplasmic tail residues. When HA0 is cleaved it become two sulfide linked chain; HA1 and HA2. This is an important part of pathogenicity and spread of the infection. The N-terminus of HA2 (fusion peptide) is conserved between influenza virus and is essential in HA fusion activity.

Three dimensional structure of both cleaved and uncleaved HA was determined. Crystallization and from x-ray diffraction data of trimeric ectodomain of HA were treated with bromelain. The HA trimer is composed of two domains a long fibrous stem from HA2 residues and the globular head from HA1 residues. Uncleaved HA0, eight residues (including five hydrophobic domain of HA2) that surround the clevage site project away from the molecules. The deep cavity is filled with the C-terminus of HA1 and the hydrophobic domain of HA2. When HA is cleaved, fusion peptides are buried in the stem of trimer. The rearrangement during cleavage that leads to ionizable residues in the cavity is implicated with the refolding during low pH event.

HA receptor binding site is a pocket located on each subunit at the distal end of the molecule. The binding site has different specificity between human trachea (α2,6), avian intestine (α2,3) and pig intestine (α2,3 and α2,6) [39].

Influenza is suggested to be transmitted from wild waterfowl to domestic, quail/pig chicken and human respectively. The host range of influenza expanded to other species such as felines, viverrids, stone martens and dogs. Swine is proposed as an intermediate host for interspecies spread and as a mixing vessel [40].

3.2.2 H5N1 Avian influenza

Influenza A virus subtype H5N1, is one of the known avian influenza virus (AIV) that can infect human and is highly pathogenic [37]. H5N1 are divided into two clades [41]. H5N1 clade two can be further divided into three subclasses. The vaccines and antiviral drug for each classes and subclasses are different. For example, all H5N1 clade I viruses are resistant to adamantanes (amantadine and rimantadine), but the

majority of H5N1 clade 2 are sensitive. Therefore rapid diagnostic of H5N1 treatment can lead to effective healing.

3.2.2.1 Pathogenesis

The two factors that are involved in pathogenesis are the viral factors and the immune response.

The first viral factor is the binding to SA 2,6 gal of H5N1 virus. This receptor is found in the lower respiratory tract and GI tract. This results in the location of symptoms. The second factor is the multiple basic amino acid at cleavage site domain on HA leads to highly pathogenicity

The severity of the avian influenza can be caused by the immune response. Cytokine storm which is proposed as one of the reasons that leads to high fever, inflammation in the location of infection. Some viral genes play an important role in inducing the immune response of the body, such as NS gene that will increase the level of proinflammatory cytokines and chemokines. However, in some studies on influenza virus infection, NS gene has also been shown to decrease IFNβ to evade immune response. This has been discussed as a possible way of causing severity in patients [39, 42].

3.2.2.2 Clinical symptoms

Symptoms of patients infected with H5N1 AIV are similar to that of other influenza. First, the patients will have high fever (above 38^{o}C) and will have abnormality in the function of lower respiratory tract which is the location of the H5N1 virus attachment. Later on, they will suffer from respiratory failure which is the main cause of death in H5N1 AIV patients [9]. According to the recent report on AIV by World Health Organization (WHO), 602 human cases has been confirmed to be caused by H5N1 influenza, out of these, 355 cases leads to mortality (12 April 2012) (http://www.who.int/csr/disease/avian_influenza/country/cases_table_2009_09_24/en/index.html), Therefore the fatality rate is higher than 50%

3.2.2.3 Immune response to H5N1 AIV

The innate immune induces by H5N1 HA leads to effective cells and cytokines. Inhibitory factors that reduce the infectivity of virus were found in mucous. These factors are similar to N-acetylneuraminic acid-containing receptors.

High level of cytokine secretion was found in the pulmonary such as IFNα and β. Macrophages were found to activate NK cells by releasing cytokines IL-1, IL-6, TNF-α and IL-12. The activated NK cells will lyse the infected cell. Complement pathway was also shown to play role in response to virus in mice system.

In order to have a complete protection against virus, adaptive immune response must be induced. Viral antigens from inactive viral particles and apoptotic infected cells were intake by antigen presenting cells (APC). The proteins are processed into peptides and presented on MHC class I or class II molecules. APC secretes IL-1β which activated the lymphocytes. Activation of T helper cells leads to secretion of IFNγ, IL-1 (T_h1), IL-4 and IL-5 (T_h2) which aids the antibody production. These antibodies neutralized the virus by binding to its antigen. Th1 enhances the $CD8^+$ T cell proliferation. The cytotoxic $CD8^+$ T cell recognize the antigenic peptide on MHC class I of the infected cell and kill the infected cells by releasing cytotoxic granules.

Humoral immune response counteracts against influenza virus by neutralizing antibodies. Neutralizing antibodies specific to HA will block cell entry. On the other hand neutralizing antibodies specific to NA will prevent viral release from infected cells. In influenza immune response, mucosal antibodies (IgA) is the second line of defense after innate immune response. During primary infection IgA, IgG and IgM is present. IgA and IgM is secreted locally and memory B cells can be found in the area. The serum antibody of patients after infection will have all three major Ig class present. During primary infection IgG and IgM are dominant, while during secondary infection IgA and IgG are more dominant. [5,6]

However, avian influenza virus has the abilities to avoid recognition by immune response. These are known as antigenic shift and antigenic drift. Antigenic drift is the mutation that causes slightly different surface glycoprotein. Antigenic shift uses gene reassortment between influenza virus and results in a new virus with new surface glycoprotein. An example of this is shown in 2009 where the new virus formed by gene reassortment between 1918 flu and swine flu. The spreading was globally and unlike normal animal flu, it has the ability to transmit from one person to another. It has reached the level of pandemic. Fortunately the severity of the virus was not high as avian flu.

3.2.2.4 Impact of H5N1 AIV to society and economy

The H5N1 epidemic has an impact on the society and economy of the country. Culling birds and vaccination were suggested to use on poultry to prevent spreading. Culling birds have the impact on all class of poultry owner and producers. This also leads to shortage of poultry exportation and importation of poultry. The population of the countries that have the epidemic or the countries that import poultry from the countries that have H5N1 epidemic has less intake of the poultry meat and eggs. Also, during the epidemic in Thailand there were reports of decrease in the tourism. As a whole, during the epidemics, Thailand loses money from import and from tourism. Another interesting fact that was found is in Vietnam where the virus was isolated from pigs, this shows that it is possible to cross the species barrier. One of the challenges that can be done to prevent spreading of the virus is to sterilize the poultry litter to be safe for handling and disposal [44].

3.2.3 H5 *HA* Gene Recombination

Gene recombination technology has shed light on molecular biology studies even on systems with complications such as eukaryotic cell. Enzyme restriction endonucleases have been used to cut DNA and clone into vectors such as plasmid or bacteriophage. Then the DNA sequence was check to see that there were no mistakes in cloning the gene into the vector. Then the gene was expressed and amplified by polymerase chain reaction (PCR) technique [45]. The goal of gene recombinant technology is to identify, isolate, conduct and re-express genes from a given host. [46]

Vaccinia recombination system is one of gene recombination techniques that use vaccinia virus to transport the gene into the cell. Then the inserted gene will be express inside the infected cell. The advantage of using vaccinia virus as a vector is that they can infect the cell similarly to viruses leading to a similar situation as of the real virus such the immune response.

Vaccinia virus is a virus in the family of *poxviridae*. It has a wide range of infection, infecting birds and mammals including humans. The approximate size of the virus in this family is 220-450 x 140-260 x 110-200 nm. It consists of an envelope, and double stranded DNA genome (approximately 200 kbp) [34]. The large genome

makes it able to carry genes. The virus has been used as a smallpox vaccine. After the disappearance of the disease, scientists use vaccinia to transport gene into tissues (gene therapy and genetic engineering) [37]. Nowadays terrorists use smallpox as biological weapon which increase the attention to vaccinia [38].

Studies have been done by using vaccinia recombination techniques to observe viral components effects on immune system have been done since 1980's [14].

CHAPTER IV
MATERIALS AND METHODS

4.1 Cells

4.1.1 P815 cells ($H2^d$)

P815, a mouse lymphoblast-like mastocytoma cell line is used as target cells to present H5 HA epitopes in CMIR experiments. The cells were grown at 37°C, 5% CO_2 in RPMI 1640 medium (Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco) (see appendix) and subpassaged at the splitting ratio 1:3 twice a week.

4.1.2 TK^- cells ($H2^k$)

TK^- cells are fibroblasts originated from subcutaneous connective tissue and are thymidine kinase negative. TK^- cells are used for propagation and plaque titration of recombinant vaccinia. The cells are maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS (see appendix) and incubated at 37°C in 5% CO_2. Cells were subpassage twice a week.

4.1.3 Madin-Darby Canine Kidney (MDCK) cells

MDCK is an epithelial cell line originated from an adult female cocker spaniel (*Canis familaris*) kidney. The cells are used for propagation and titration of the reverse genetic influenza virus and microNT assay. The cells were grown in Eagle's Minimal Essential Medium (EMEM, Gibco) supplemented with 10% FBS (see appendix) and maintained at 37°C in 5% CO_2. Cell subpassaging was done twice a week.

4.1.4 Yac-1 cells

Yac-1 cells are lymphoma cell line derived from A/Sn mouse that was induced with Moloney murine leukemia virus (Mo-MuLV). Yac-1 cells are target cells

of mouse NK-cells and are used to study the NK cytotoxic activity. The cells are maintained in RPMI 1640 medium (Gibco) supplemented with 10% FBS (Gibco) (see appendix). The cells were subpassaged at splitting ratio 1:3 twice a week and incubated at 37^{o}C in 5% CO_2.

4.2 Viruses

4.2.1 Recombinant vaccinia viruses

Recombinant vaccinia virus originated from vaccinia virus Lister strain harboring *HA* gene insert derived from A/Thailand/1(KAN-1)/2004 (H5N1) virus (rVaccinia H5 HA) and the recombinant vaccinia virus harboring pSC11 plasmid backbone (rVaccinia pSC11) were kindly provided by Prof. Pilaipan Puthavathana, Siriraj Cooperative Research Center, Department of Microbiology, Faculty of Medicine Siriraj Hospital, Mahidol University, Thailand.

4.2.1.1 Preparation of recombinant vaccinia virus stocks

TK^- cell monolayers were inoculated with the recombinant viruses at at multiplicity of infection (MOI) of 0.05 in DMEM supplemented with 2% FBS (see appendix). The inoculated cultures were incubated at 37^{o}C in 5% CO_2 for 1 hr and 30min with swirling at every 15 min. The excess inoculums were washed out and the inoculated cultures were further incubated at 37^{o}C, 5% CO_2 for approximately 48 hr or until the cell showed 4+ degree of cytopathic effect (CPE) before harvesting.

The infected cell monolayers were scraped into the maintenance media and centrifuged at 1500 rpm for 10 min at 4^{o}C. The infected cell pellet was resuspended with small volume of DMEM supplemented with 2% FBS and store at -80^{o}C overnight.

The frozen cell suspension underwent three freeze-thaw cycles. Each cycle comprised freezing at -80^{o}C for 30 min and thawing in water bath for 30 min. A 0.1 volume of 0.25% trypsin (GIBCO) was added into the cell suspension and incubated for 15 min in 37^{o}C water bath. The reaction was stop with 0.1 volume of FBS. The virus suspension was aliquot and store at -80^{o}C.

4.2.1.2 Plaque assay

The recombinant viruses were titrated by plaque assay in TK^- cells in triplicate.

TK^- cell monolayers in 6 well culture-plates were prepared 1-2 days before use. Stock of rVaccinia H5 HA or rVaccinia pSC11 virus were 10 fold-diluted in DMEM media supplemented with 2% FBS. Then, a 500 µl volume of each diluted virus suspension was inoculated onto each well of cell monolayer and incubated for 1 hr and 30 min with swirling at every 15 min. The excess inoculum was removed; and the infected monolayers were added with DMEM media supplemented with 2% FBS for 48 hr at 37^{o}C followed by staining with 1% crystal violet. Number of plaques in each well was counted. The counting number of approximately 50-100 plaques per well was further calculated for pfu/ml by using the following equation.

$$\text{Pfu/ml} = \frac{\text{number of plaques} \times 2 \times \text{dilution factor}}{3}$$

4.2.1.3 Detection of H5 *HA* gene insert in the recombinant vaccinia virus by polymerase chain reaction (PCR)

PCR technique was used to prove that H5 *HA* gene did exist in the recombinant virus, rVaccinia H5 HA. DNA was extracted from rVaccinia H5 HA virus and rVaccinia pSC11 virus as described in Qiagen extraction kit protocol and further used as the DNA template in PCR assay. A 50 µl of a reaction mixture was composed of 5 µl of 10X PCR buffer, 2 µl of $MgCl_2$, 2 µl of forward primer, 2 µl of reverse primer, 2 µl of dNTP mix, 0.5 µl of Hot star taq, 10 µl of recombinant vaccinia DNA template and 26.5 µl of distilled water. The H5 HA forward primer sequence is ACT CCA ATG GGG GCG ATA AA; and that of the reverse primer is CAA CGG CCT CAA ACT GAG TGT. The expected product is 350 bp in size.

The reaction tube was subjected to pre-denaturation step at 94^{o}C for 5 min.; and followed by 35 cycles of which each one was consisted of the denaturation step at 94 ^{o}C for 30 sec, annealing step at 55^{o}C for 30 sec, and extension step at 72 ^{o}C for 30 sec. with the final extension step at 72 ^{o}C for 10 min. PCR products were electrophoresed in 1.5% agarose gel before staining with ethidium bromide solution and visualized over UV illumination.

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4.2.2 Reverse genetics influenza virus

The reverse genetic influenza virus (rgPR8-H5 HA) was kindly provided by Prof. Pilaipan Puthavathana, Siriraj Influenza Cooperative Research Center, Department of Microbiology, Faculty of Medicine Siriraj Hospital, Mahidol University, Thailand. The virus obtained seven genes (PA, PB1, PB2, NP, NA, M and NS) from A/Puerto Rico/8/1934 (H1N1) backbone and *HA* gene with the elimination of multiple basic amino acids at cleavage site from A/Thailand/1/KAN-1/2004 virus (KAN-1 virus). This reverse genetic virus was used in both HI and micro-NT assays instead of using the wild type KAN-1 virus because it was safer to work with; and the results obtained from either virus were comparable.

4.3 Animals

In this research, female BALB/c ($H2^d$) mice from National Laboratory Animal Center were used. The mice immunization protocol (Ethical Clearance) was approved by the Faculty of Veterinary Science- Animal Care and Use Committee (FVS-ACUC) on October, 27 2009. The hardcopy of Ethical Clearance is attached with this thesis (See Appendix). Mice immunization is divided depending on their purposes.

BALB/c mice were housed at Faculty of Veterinary Science, Mahidol University, Thailand. They live in 7.5 x 11.5 x 5 plastic individual ventilated cage (IVC) in a hygiene environment at 25±2^{o}C with 55-100% humidity. Each cage contains 3-4 mice. The mice were routinely fed with standard food and autoclaved water. Mice abnormality was observed daily.

4.4 Mice immunization

4.4.1 Mice immunization for cell mediated immune response (CMIR) experiments

Female BALB/c mice ($H2^d$), 4-6 weeks old, were used in this experiment. Two mice were used for each condition. Four separate experiments were done. The

mice were immunized intraperitoneally with rVaccinia H5 HA or rVaccinia pSC11 virus or PBS at 10^7 pfu with 10 µg of poly I:C. poly I:C (Sigma) were kindly provided by Dr. Sathit Pichyangkul, U.S Army Medical Component, AFRIMS, Thailand.

Two weeks after first immunization, the booster containing 10^7 pfu of rVaccinia H5 HA, rVaccinia pSC11 or PBS will be injected into each mouse intraperitoneally. Seven days later, mice were euthanasia by intraperitoneal injection of pentobarbital (Nembutal) 0.05-0.1 ml. Blood was collected by cardiopuncture, and transfer to eppendorfs. Sera from clotted blood will be used in ADCC assay. Spleens were collected in 1X RPMI 1640 media in 15 ml tube. Spleens were crushed into single cell splenocytes and use as effector cells in cytotoxic assay.

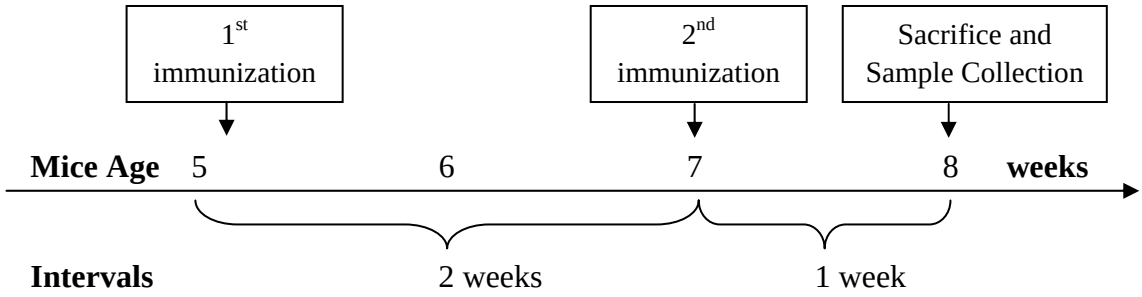


Figure 4.1: Diagram shows mice immunization protocol for CMIR experiments

4.4.2 Mice immunization for humoral immune response (HIR)

Female BALB/c mice ($H2^d$), 8-10 weeks old, were used in this experiment. In each experiment, total of at least six mice were intraperitoneally immunized with rVaccinia H5 HA or rVaccinia pSC11 at $5x10^6$ pfu with 10 µg of poly I:C (Sigma) and eight mice were unimmunized mice (control group). Three separate experiments were done.

Three weeks after the first immunization, the booster containing $5x10^6$ pfu of rVaccinia H5 HA, rVaccinia pSC11 or PBS was injected into each mouse. Three weeks later, three rVaccinia H5 HA immunized mice, three rVaccinia pSC11 immunized mice and four untreated mice will be sacrificed by intraperitoneal injection of pentobarbital (Nembutal) 0.05-0.1ml. Their blood and spleens were collected. On the same day, the remaining three mice of rVaccinia H5N1 HA immunized mice and

three mice of rVaccinia pSC11 virus immunized mice will be boosted with $5x10^6$ pfu of rVaccinia H5 HA or rVaccinia pSC11 respectively.

Three weeks later, three rVaccinia H5 HA immunized mice, three rVaccinia pSC11 virus immunized mice and four untreated mice will be sacrificed with intraperitoneal injection of pentobarbital (Nembutal) 0.05-0.1ml. Their blood and spleens were collected. Clotted blood from the heart was collected in eppendorfs and spleens were collected in 7 ml of 1X RPMI 1640 containing Pen-Strep. Clotted blood was spin down and serum was transferred individually to serum tubes and kept at -20°C until use in HI and micro-NT assay. Immunized mice spleen were separated into single cell splenocytes by overlay on with mouse phycoll (1.09 ± 0.001 g/ml), resuspend with 10% DMSO and kept at -80°C

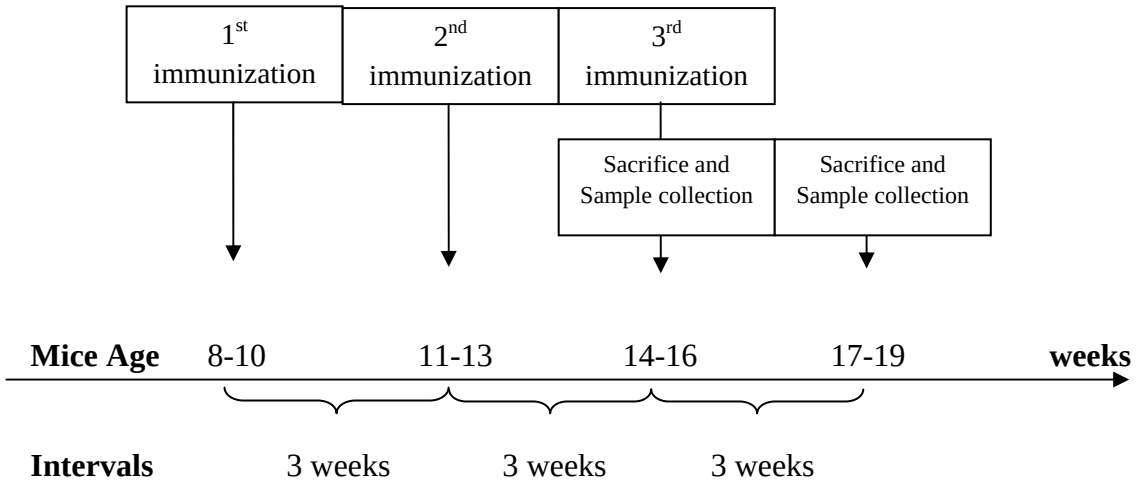


Figure 4.2: Diagram shows mice immunization protocol for HIR experiments

4.5 *In vitro* assay of cell mediated immune response (CMIR)

4.5.1 Cytotoxic T lymphocyte (CTL) assay procedure

4.5.1.1 Preparation of P815 infected target cells

As a result of H5 HA optimization on P815 target cells, P815 cells were infected with either rVaccinia H5 HA, rVaccinia pSC11 or PBS at MOI of 3 at 18 hr. P815 ($H2^d$) cells ($1x10^6$ cells) were inoculated with 0.5 ml inoculums of rVaccinia H5 HA or rVaccinia pSC11 at the MOI of 3 in RPMI 1640 media supplemented with 2% FBS. The infected cells were incubated at 37°C, 5% CO_2 for 1

hr and 30 min while swirling every 15 min. Further incubation at 37^{o}C, 5% CO_2 were done until 12 hr post infection. Then the infected cells were washed at 1500 rpm for 5 min at 20^{o}C. Cells were incubated until 18hr post infection. Target cells were overlayed on human lymphoprep (density 1.32 ± 0.001 g/ml), and resuspended in RPMI 1640 + 2% FBS. Target cells were count and cell viability was calculated. The cell viability percentage was above 99%. Target cells were adjusted to $1x10^6$ cells/ml by RPMI 1640 +2% FBS and 100µl ($5x10^3$ cells) of cell suspension were added to each well of 96 well culture plate (U-bottom).

4.5.1.1.1 Growth curve and cell viability

Uninfected P815 cells ($3x10^5$cells) were cultured and every 6 hr the cells were counted and cell viability was calculated.

4.5.1.1.2 Optimum H5 HA expression on P815 cells by varying MOI and time of infection

P815 cells ($1x10^6$ cells) were inoculated with 0.5 ml rVaccinia H5 HA inocculum with MOI of 3 or 4 at 12 hr (vary MOI), and MOI of 3 for 12 hr, 18 hr and 24 hr (vary time of infection) in RPMI 1640 supplemented with 2% FBS. Inoculated cells were incubated at 37^{o}C, 5% CO_2 while swirling every 15 min for 1 hr and 30min. Further incubation of inoculated cell is done at 37^{o}C, 5% CO_2 for 12 hr, 18 hr or 24 hr. The cells were spindown, resuspend with 1X PBS, spot on slide and fix with cold acetone.

4.5.1.1.3 Indirect immunofluorescent staining protocol

Immunofluorescent staining is used to observe the of H5 HA protein expression on target P815 cells which is crucial in studying the optimum MOI and time of infection.

Goat polyclonal-anti-H5 was used as primary antibody and Rabbit-anti-goat Ig was used as secondary antibody. Both were diluted with 1X PBS to the dilution of 1:20. Slide marker was used to draw a circle around the cell spot.

Primary antibody or normal mice serum (negative control), 30 µl, was added to each spot. Slides were incubated at 37^{o}C for 30 min in a

dark moist chamber. The slides were washed in 1X PBS pH 7.4 in a slide bucket for 10 min, and the slides were washed once with DW.

Secondary antibody, 30 µl, was added to each cell spot. Slides were incubated at 37 ^{o}C for 30 min in a dark moist chamber. The slides were washed with 1X PBS with Evan's blue in a slide bucket for 10 min and wash with DW. The slides were blown with a hair dryer, mount and cover with coverslip. The slides were observed under fluorescent microscope.

4.5.1.1.4 Preparation of P815 target cells

P815 cells ($1x10^6$ cells) were inoculated with 0.5 ml of rVaccinia H5 HA inocculum with MOI of 3 for 18 hr in RPMI 1640 supplemented with 2% FBS. Inoculated cells were incubated at 37^{o}C, 5% CO_2 while swirling every 15 min for 1 hr and 30 min. Further incubation at 37 ^{o}C, 5% CO_2 were done until 12 hr post infection. Then the infected cells were washed at 1500 rpm for 5 min at 20^{o}C and resuspend with RPMI 1640 supplemented with 2% FBS. Cells were incubated until 18hr post infection. Target cells were overlay on human lymphoprep with % viability above 99% and resuspended in RPMI 1640 + 2% FBS.

4.5.1.2 Preparation of effector splenocytes

The spleens were crush through sieve into 3 ml 1X RPMI 1640 media and overlay on 2 ml human lymphoprep (density 1.32 ± 0.001 g/ml) to isolate into single cells. The isolated single cell splenocytes were washed twice at 1500 rpm for 10 min and resuspend with RPMI 1640 media supplemented with 2% FBS. Cell number and cell viability were count and calculated. Effector splenocyte cells viability is above 99%. The effector cells and target cells were added into each well at three effector:target ratios; 12.5:1, 25:1 and 50:1 in a U bottom plate. The experiment was carried out at least in tripicate.

4.5.1.3 Cytotoxic activity assay

The cell plate was incubated at 37^{o}C for 4.5 hr. Supernatant from each well was collected for cytolysis assay. Forty-five minutes prior to transfer supernatant for cytolysis assay, the lysis buffer was added into Target Maximum and Volume Correction wells.

4.5.1.4 CytoTox 96® non-radioactive cytotoxicity assay

CytoTox 96® non-radioactive cytotoxicity assay (Promega) is a non-radioactive cytolysis assay that was used to determine cytolysis activity. The cytotoxic assay was done following the manufacture protocol. Briefly, 50 µl of cultured supernatant was transferred to flat bottom strips. After that, 50 µl of substrate mix was added to each well and incubate at room temperature in the dark for 30 min. After incubation, 50 µl of stop solution was added. The absorbance of each well was recorded at 490 nm.

4.5.1.5 Calculation of % cytotoxicity

The cytotoxicity percentage were calculated by using the following formula (obtained from manufacturing protocol)

$$\% \text{ cytotoxicity} = \frac{\text{Experimental - Effector spontaneous - Target spontaneous}}{\text{Target Maximum – Target spontaneous}} \times 100$$

4.5.2 Antibody-dependent cell cytoxicity (ADCC) assay

4.5.2.1 Preparation of P815 infected target cells

P815 target cells were prepared as mentioned in part 5.1.1.4 with an adjustment. After the target cells were count and calculated for cell viability, target cells were sensitized with heat inactivated pooled autologous mouse serum by adjusting target cells to $1x10^6$cells/ml by RPMI 1640 + 1% of heat-inactivated autologous immunized mice serum. Target cells suspension 100 µl ($5x10^3$ cells) were added to each well. Then they were incubated at 37^oC for 30 minutes.

4.5.2.2 Preparation of effector cells

Preparation of effector cells (immunized mice splenocytes) is the same as in CTL assay, which is mentioned in part 4.5.1.2

4.5.2.3 Preparation of immunized pooled mice serum

Clotted blood was spin down and pooled immunized serum was transferred to serum tube. Serum was heat inactivated at 56^oC for 30 min prior use in Antibody Dependent Cell Cytotoxicity (ADCC).

4.5.2.4 Cytotoxic assay and CytoTox 96® non-radioactive cytotoxicity assay

The ADCC cell plate was incubated for 4.5 hr. The non-radioactive cytotoxic LDH assay was done following manufacture's protocol, as mentioned in 4.5.1.4. Calculation of cytotoxicity percentage was done by using manufacture's equation as mentioned in 4.5.1.5.

4.5.3 Natural killer (NK) cell cytotoxicity assay (Positive control)

4.5.3.1 Preparation of Yac-1 cells

Yac-1 cells, a universally used cell line as a target for NK cells, is used as target cells. Target cells were overlay on human lymphoprep with % viability above 99% and resuspended in RPMI 1640 + 2% FBS. The cells were adjusted to $1x10^6$ cells/ml by 1X RPMI 1640 supplemented with 2% FBS and 100µl ($5x10^3$ cells) of cell suspension were added to each well of 96 U bottom well culture plate.

4.5.3.2 Preparation of effector cells

Preparation of effector cells (immunized mice splenocytes) is the same as in CTL assay which is mentioned in part 4.5.1.2.

4.5.3.3 Cytotoxic assay and CytoTox 96® non-radioactive cytotoxicity assay

The NK cytotoxicity cell plate was incubated for 4.5 hr. The non-radioactive cytotoxic LDH assay was done following manufacture's protocol, as mentioned in 4.5.1.4. Calculation of cytotoxicity percentage was done by using manufacture's equation as mentioned in 4.5.1.5.

4.5.4 Statistical analysis

Triplicate experiments were done and at least three mice were used for each experiment. Mean, SD, SE, and statistic analysis of variance were done by using GraphPad program

4.6 *In vitro* assay of humoral immune response (HIR)

4.6.1 Indirect immunofluorescence assay

Indirect immunofluorescence assay was used for detection of specific antibody in the mouse immune sera using rVaccinia H5 HA infected TK cell deposits as the test antigen. The assay protocol was as described in 4.5.1.1.3

4.6.2 Hemagglutination inhibition (HI) assay

4.6.2.1 Goose erythrocyte preparation

Goose erythrocytes from the National Laboratory Animal Center, Salaya were used in hemagglutination assay and HI assay. Goose erythrocytes were washed 3 times with 1X PBS pH 7.2 by spinning at 1500 rpm for 10 min at 4^{o}C. The 50% goose erythrocyte suspension was prepared for the absorption of non-specific agglutinators in the test sera and 0.5% goose erythrocyte suspension was used in hemagglutination assay and HI assay.

4.6.2.2 Serum treatment

Mouse serum was treated with receptor destroying enzyme (RDE, Japan) in order to eliminate non-specific agglutinator prior to running the HI assay. A 30 µl volume of a test serum was mixed with 90 µl of RDE for 16-18 hrs at 37^{o}C in water bath and followed by heat inactivation at 56^{o}C for 30 min. The RDE-treated serum was then absorbed with 30 µl of 50% goose erythrocytes for one hour at 4^{o}C, with shaking at every 15 min. A 150 µl volume of normal saline solution was added into the serum tube to obtain the serum dilution of 1:10. The treated sera were kept at 4^{o}C, and used within a week.

4.6.2.3 Detection for non specific agglutinators in the test sera

The test sera were checked for presence of non-specific agglutinators remaining after absorption by adding a 25 µl volume of the treated serum at dilution 1:10, 25µl of 1X PBS pH 7.2 and 25 µl of 0.5% goose erythrocyte suspension into a well of the V bottom 96-well plate. Then, the plate was mixed gently by tapping and incubated at 4 ^{o}C for 30 min. If the test serum was free of nonspecific agglutinators, the reaction well should not show hemagglutination.

4.6.2.4 Hemagglutination assay

Hemagglutination assay was performed in order to determine the titer of H5 HA antigen in the rgPR8-H5 HA virus. The stock virus was two-fold serially diluted with 1X PBS in 50µl/well in V-shaped bottom well plate. A 50 µl of the 0.5% goose erythrocyte suspension was added into each well. The plate was incubated at 4^{o}C for 30 min and the hemagglutination pattern was examined. The reciprocal of the highest virus dilution that shows complete hemagglutination is the virus titer; and one hemagglutination unit is defined as the highest virus dilution that completely hemagglutinates the test erythrocytes.

4.6.2.5 HI assay

HI assay was performed after that described in the WHO protocol [49]. rgPR8-H5 HA was used as the test antigen at the working concentration of 4HA units/25 µl; and 0.5% goose erythrocyte suspension was used as the indicator. Each serum was tested in duplicate. The treated serum at the initial dilution of 1:10 from 6.2.2 was two-fold diluted with 1X PBS pH7.2 in a 25 µl volume in V-shaped bottom well plate. The diluted serum was incubated with 25 µl of the test antigen for 30 min at room temperature. After that, 50 µl of erythrocyte suspension were added into each well; and the plate was incubated at 4^{o}C for 30 min. The HI antibody titer was determined by the highest serum dilution that completely inhibits hemagglutination. Antigen back titration was included in every test run. The antibody titer of <10 was assigned as 5 for GMT calculation.

	1	2	3	4	5	6	7	8	9	10	11	12
A	1:10										4 HA units	
B	1:20										2 HA units	
C	1:40										1 HA units	
D	1:80										0.5 HA unit	
E	1:160										0.25 HA unit	
F	1:320										Erythrocyte control	
G	1:640											
H	1:1280											

Figure 4.3: Plate plan for HI assay in duplicate

4.6.3 HA-based micro-neutralization assay (micro-NT assay)

4.6.3.1 Serum treatment

A 30 µl of the test serum was added with 150 µl of RDE followed by incubation for 16-18hr and heat inactivation at 56^{o}C for 30 min. Thereafter, a 180 µl of 1X EMEM was added to the treated serum to make the initial serum dilution of 1:10.

4.6.3.2 Virus titration

The rgPR8-H5 HA virus was titrated for tissue culture infective dose 50 ($TCID_{50}$) in a half-log dilution manner in MDCK cell monolayers. The protocol was followed that described in the WHO manual for avian influenza [49, 50]. The virus stock was diluted with viral growth media (VGM) supplemented with tosylsulfonyl phenylalanyl chloromethyl ketone treated trypsin (trypsin-TPCK) in quadruplicate in a 96-well plate. The plate was incubated for 2hr at 37 ^{o}C and a 100 µl volume of the diluted virus was transferred onto MDCK maintained in 100 µl of VGM. The plate of virus infected MDCK cell monolayers was incubated for 2 days or until the highest virus dilution showed cytopathic effect of 2+. For end point measurement, a 50 µl volume of culture supernatant from each well were transferred into the corresponding well in the second plate of V-shaped bottom and added with a 50 µl volume of 0.5% goose erythrocytes. Culture supernatant from the well with highest virus dilution that yielded 50% hemagglutination was considered positive for virus infection. $TCID_{50}$ was calculated by Reed-Muench method [49,51].

4.6.3.3 MDCK cell preparation

MDCK cell monolayer cell with 90-100% confluency was prepared. Cell monolayer was washed twice with 1X EMEM and 100 µl of VGM was added to each well

4.6.3.4 Micro-NT assay

The sera were two-fold serially diluted in EMEM in a U-shaped bottom 96 well plate, starting from the dilution of 1: 10 to 1: 1280 in a 60 µl volume. A 60 µl volume of the test virus at the amount of 200 $TCID_{50}$ in EMEM supplemented with trypsin TPCK was added into each well, and incubated at 37^{o}C for 2hr. Then, 100 µl of the serum-virus mixture were transferred onto the MDCK cell plate maintained in 100 µl of EMEM supplemented with trypsin TPCK. Positive

control serum of previously known antibody titer, antigen back titration and cell control were included in each test run. The reaction plate was incubated for two days or until the virus back titration well of 1 $TCID_{50}$ show CPE of 1+ - 2+. Fifty µl of culture supernatant from each well were transferred into the corresponding well of the second V-shaped bottom well plate, and added with 50 µl of 0.5% goose erythrocyte suspension. Culture supernatant from the well with highest serum dilution that showed 50% hemagglutination is considered as the antibody titer.

		1	2	3	4	5	6	7	8	9	10	11	12
	Dilution	**Serum1**		**Serum 2**		**Serum 3**		**Serum 4**		**Positive serum**		**Back Titration**	
A	**1:10**											100 $TCID_{50}$	
B	**1:20**											10 $TCID_{50}$	
C	**1:40**											1 $TCID_{50}$	
D	**1:80**											0.1 $TCID_{50}$	
E	**1:160**											Cell control	
F	**1:320**												
G	**1:640**												
H	**1:1280**												

Figure 4.4: Plate plan for micro-NT assay in duplicate

4.6.4 Statistical analysis

Three independent experiments were performed for HIR study with 3-5 mouse serum for each immunogen. Statistical analysis was done by using SPSS v.11.5 program. The antibody titers obtained after immunizing with each immunogen or after each boost were compared using either paired *t*-test or individual *t*-test with p-value of 0.05.

CHAPTER V
RESULTS

5.1 Preparation of recombinant vaccinia virus stocks

5.1.1 Recombinant vaccinia virus propagation

Recombinant vaccinia virus harboring H5 HA (rVaccinia H5 HA) or that harboring pSC11 plasmid backbone (rVaccinia pSC11) was propagated in TK^- cells. At two days post infection, the infected TK^- cell cultures showed cytopathic effect of 4+ with morphological changes such as roundness and detachment from the culture surfaces (Figures 5.1A and 5.1B).

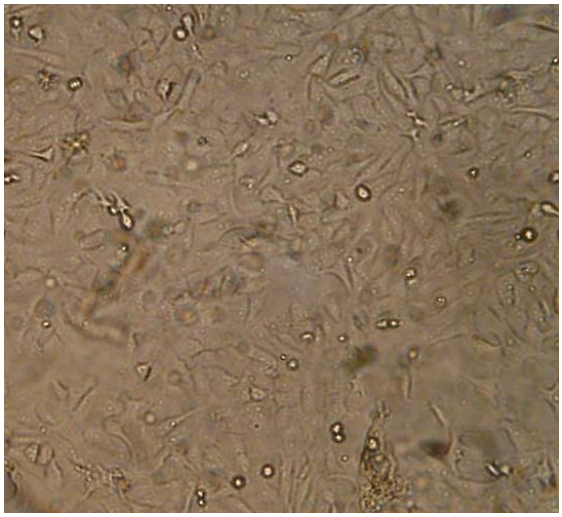
5.1.2 Virus titration by plaque assay

The virus stock was titrated in triplicate in TK^- cell monolayers. Plaques were counted after staining with 1% crystal violet as shown in figures 5.2A and 5.2B. Two lots of rVaccinia H51 HA virus and 6 lots of rVaccinia pSC11 viruses were prepared; and the titers obtained are as shown in Tables 5.1 and 5.2.

5.1.3 H5 *HA* PCR product

To assure that the rVaccinia H5 HA virus contained the H5 *HA* gene insert, DNA extracts from the recombinant viruses were amplified by H5 HA specific primer pairs available in our laboratory. As shown in Figure 5.3, the PCR product from amplification of the rVaccinia H5 HA virus was approximately 350 bp in size as expected. The rVaccinia pSC11 viral genome was not amplified by this primer pair. This suggested that the rVaccinia H5 HA virus contains H5 *HA* gene.

A



B

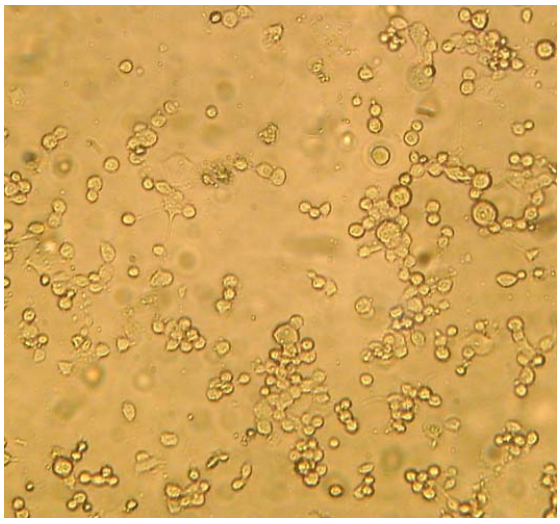
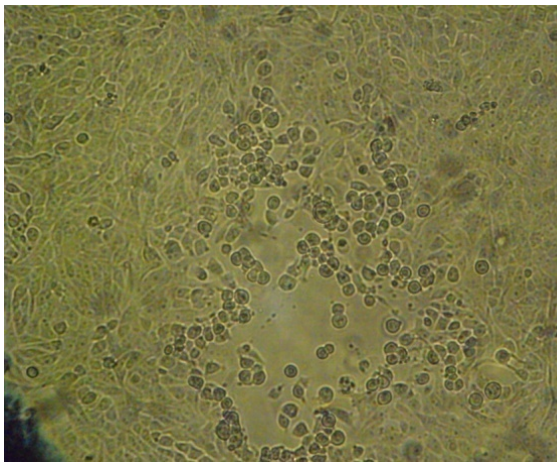


Figure 5.1: Microscopic views of TK^- cells. (A) Uninfected TK^- cells; (B) TK^- cells infected with recombinant vaccinia virus at 2 days p.i. with 4+ CPE

A



B

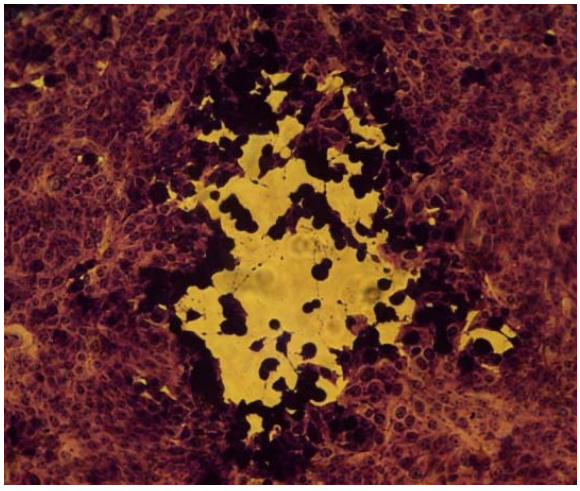


Figure 5.2: Microscopic view of plaques; (A) before staining; (B) after staining with 1% crystal violet.

Table 5.1: Titers of rVaccinia H5 HA virus

Virus lot	Titer ($x10^8$pfu/ml)
1	2.75
2	2.62

Table 5.2: Titers of rVaccinia pSC11 virus

Virus lot	Titer ($x10^8$pfu/ml)
1	1.95
2	2.21
3	2.6
4	2.16
5	1.9

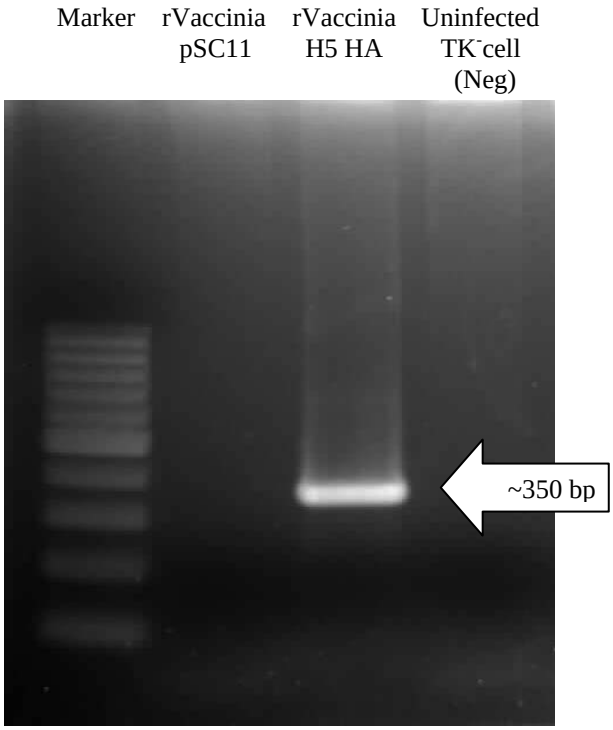


Figure 5.3: Gel electrophoresis of the amplified product at approximate size of 350 bp is shown for rVaccinia H5 *HA* gene amplification only

5.2 Cell mediated immune response (CMIR)

5.2.1 P815 target cell

5.2.1.1 Growth curve and cell viability of normal P815 cells

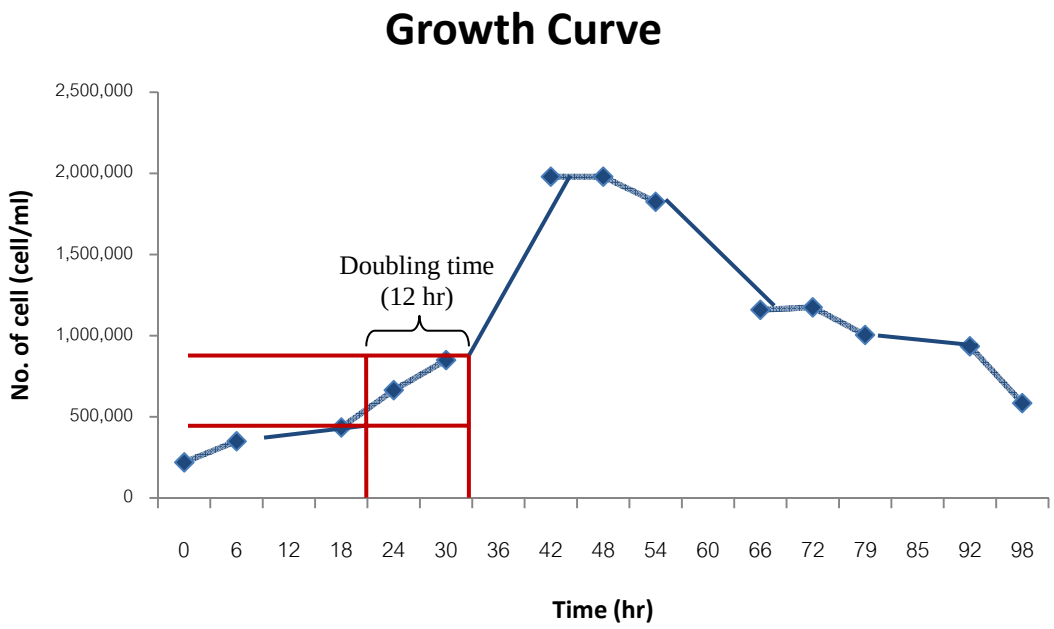
The growth curve and % cell viability of uninfected P815 cells was shown in figure 5.4A and 5.4B respectively. The initial cell concentration that was added to the flask is $3x10^5$cells/ml. The results show that P815 cells reach log phase at 18 hr and went to stationary phase at 42 hr. The cell count number decreases after 54 hr which is the same time point that the % cell viability drop. The doubling time of P815 cells is 12 hr.

5.2.1.2 Immunofluorescent staining of P815 target cells

First, P815 cells infected with rVaccinia H5 HA at MOI of 3 or at 12 hr were counted, spot on slide, and indirect immunofluorescent stain. The % viability of the cell are shown in table 5.3, where there are not much differences between the MOI of 3 and 4 (94% and 95% respectively). Immunofluorescent infected cell spot staining was shown in figure 5.5. Percentage of H5 HA positive cells were shown in table 5.4. The % of positive H5 cells are 13% and 15% at MOI of 3 and 4, respectively. The viability and positive cells were not different from each other so MOI of 3 was selected to use in this experiment

Then, P815 cells was infected with rVaccinia H5 HA at MOI of 3 at 12,18 and 24 hr. Indirect immunofluorescent staining was performed and results were shown in figure 5.6. The cell viability of infected P815 cells at MOI 3 at 12, 18, and 24 hr are 96.67%, 95.67% and 93.67% respectively (Table 5.5). The % positive H5 HA expression in infected cells are 18.28%, 27.88%, 31.2% at 12, 18 and 24 hr, respectively (Table 5.6). From this information, target cells were prepared by infecting P815 cells at MOI 3 for 18 hr. to yield the highest positive cells with % viability more than 95%.

A



B

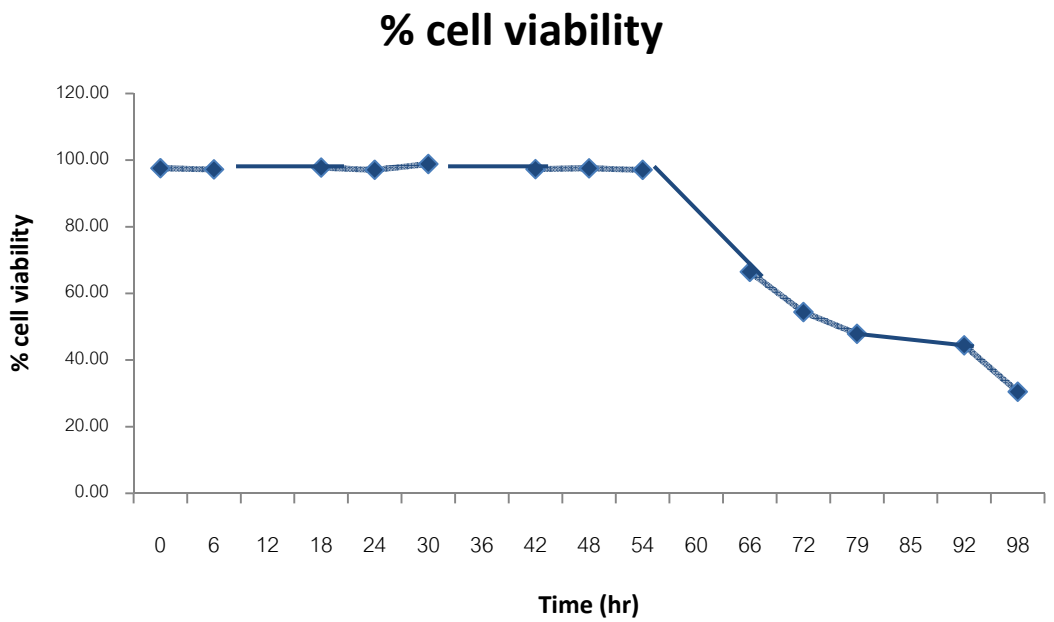


Figure 5.4: (A) Growth curve and (B) % cell viability of uninfected P815 cells

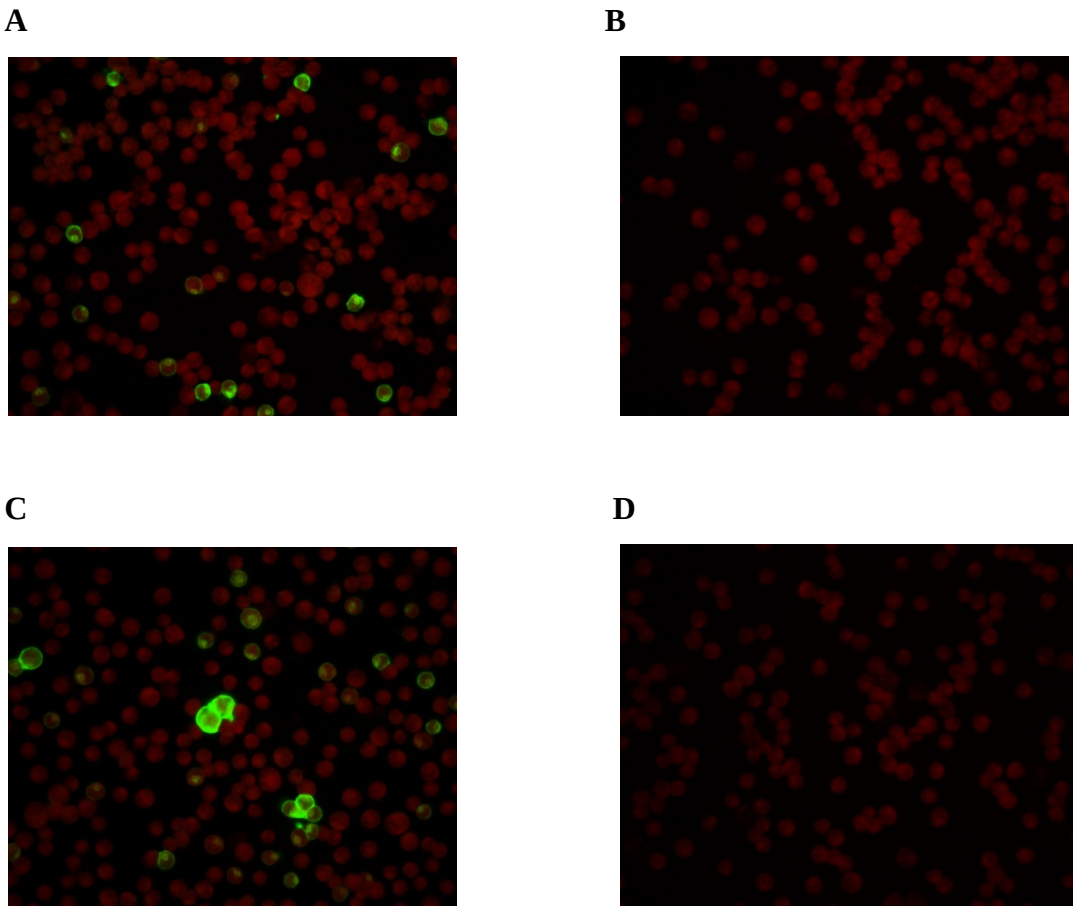


Figure 5.5: Immunofluorescence assay of P815 cells infect with rVaccinia H5 HA by using goat poly antibody to H5 *HA* gene compare to control (normal mouse antibody): (A) P815 cells infect with rVaccinia H5 HA at MOI =3 for 12 hrs with (B) control. (C) P815 cells infected with rVaccinia H5 HA at MOI =4 for 12 hrs with (D) control.

Table 5.3: % cell viability of rVaccinia H5 HA infected P815 cells

Infect virus	**rVacciniaH5 HA**	
MOI / Time (hr)	3	4
12	94%	95%

Table 5.4: % rVaccinia H5 HA infected P815 cells expressing H5 HA

rVaccinia H5 HA	% H5 HA positive cells
MOI 3 t=12hr	13%
MOI 4 t=12hr	15%

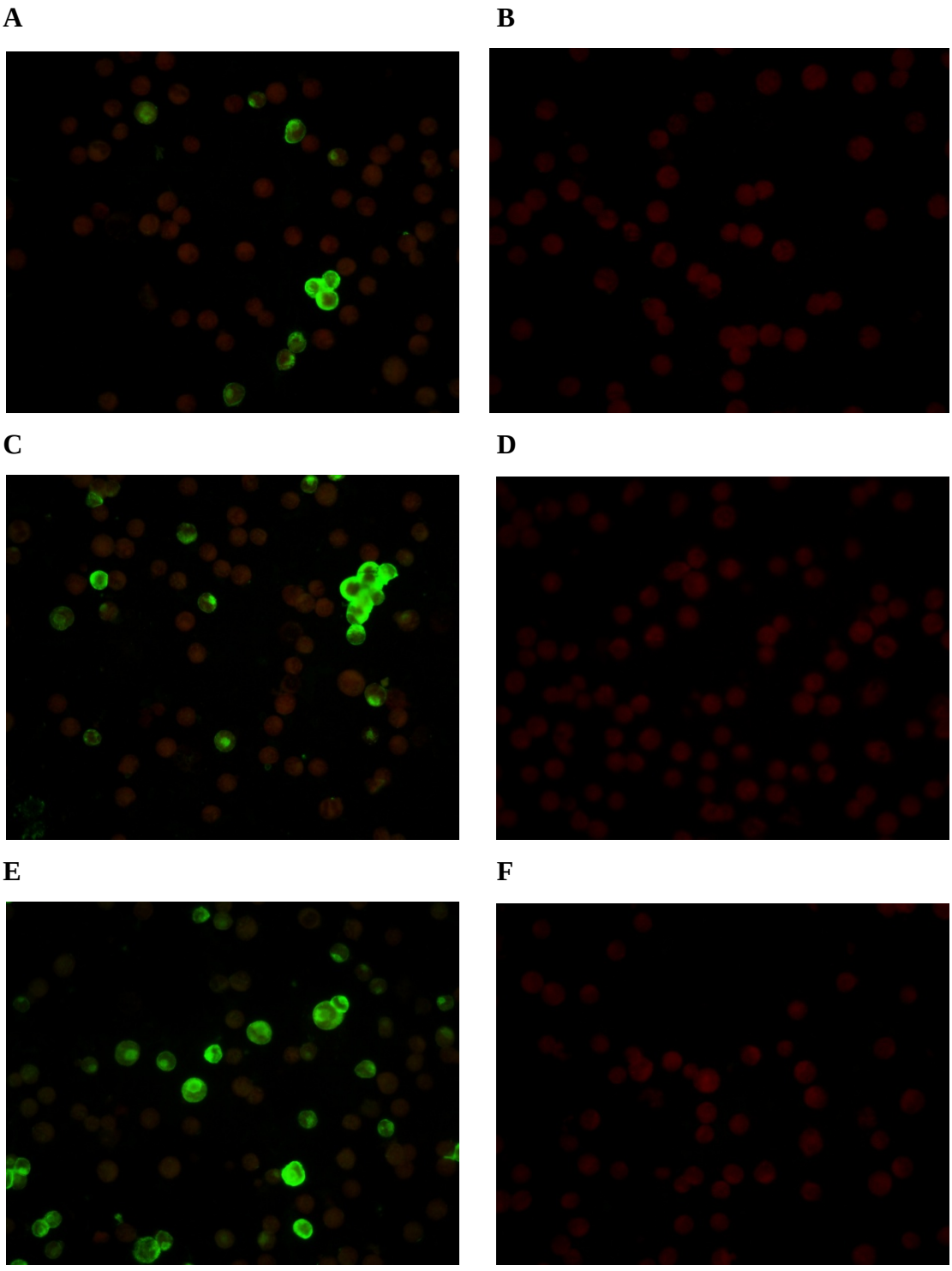


Figure 5.6: Immunofluorescence assay of P815 cells infect with rVaccinia H5 HA at (A,B)12 hr, (C,D)18hr and (E,F) 24 hr. The figures on the left (A, C, E) use antibody specific to H5 HA as primary antibody. While the figures on the right (B,D,F) use normal mice antibody as primary antibody (negative control).

Table 5.5: % cell viability of rVaccinia H5 HA infected P815 cells (MOI=3)

Time Post Infection	% viability
12	96.67%
18	95.67%
24	93.67%

Table 5.6: % rVaccinia H5 HA infected P815 cells expressing H5 HA (MOI=3)

Time Post Infection	% H5 HA positive cells
12	18.28
18	27.88
24	31.2

5.2.2 Cytotoxic T lymphocyte activity (CTL assay)

Three ratios of effector:target cells were done; 12.5:1, 25:1 and 50:1. Average % cytotoxic activity and standard error (SE) were shown in figure 5.7 A-C.

The % cytotoxicity of rVaccinia H5 HA immunized mice at ratio 12.5:1, 25:1, and 50:1 are 24.55 ± 0.33%, 9.579 ± 3.56%, and 22.79 ± 10.10% respectively (shown in figure 5.7A). The cytotoxicity of rVaccinia pSC11 immunized mice at ratio 12.5:1, 25:1, and 50:1 are 13.77 ± 12.36%, -21.86 ± 26.36%, and -13.68 ± 28.44% respectively (shown in figure 5.7B). The cytotoxicity of PBS immunized at ratio 12.5:1, 25:1, and 50:1 are 17.35 ± 8.56%, 3.48 ± 5.84%, and 2.56 ± 3.38% respectively (shown in figure 5.7C). Statistical analysis shows that cytotoxicity (20-25%) percentage at 50:1 effector:target ratio of rVaccinia H5 HA is statistically different from that of PBS at the same ratio (Mann-Whitney one tailed *t*-test; $p<0.05$).

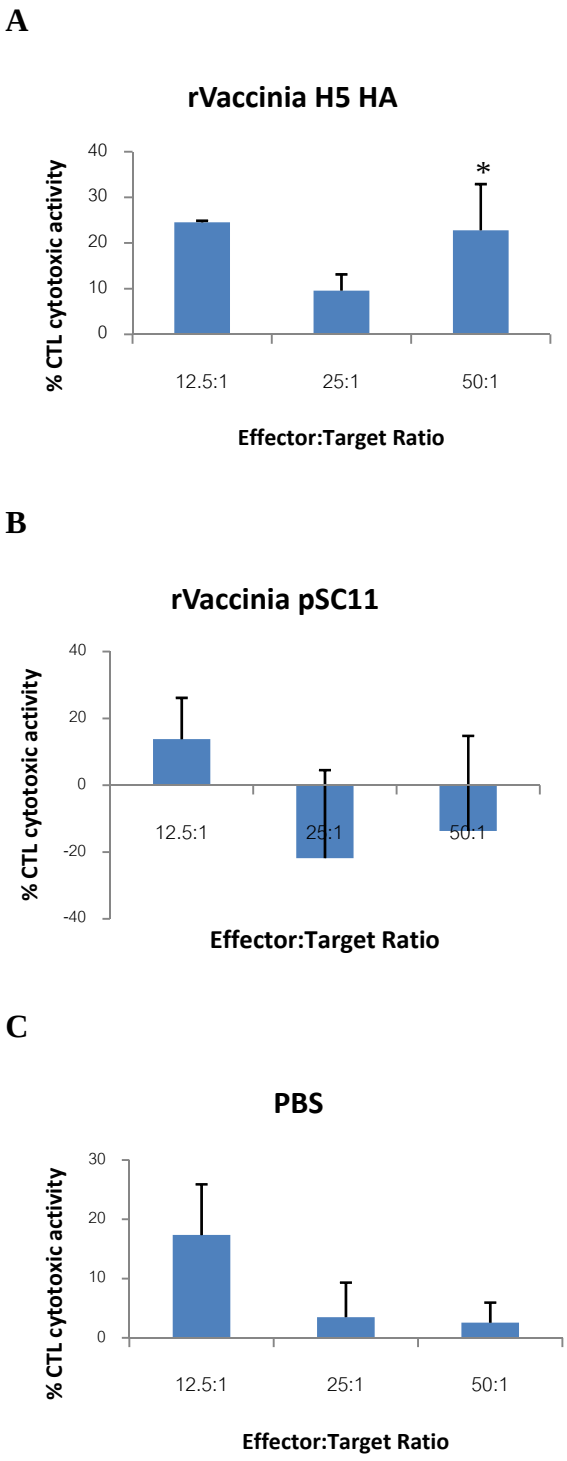


Figure 5.7: % CTL activity of mice splenocytes from mice immunized with either (A) rVaccinia H5 HA, (B) rVaccinia pSC11 or (C) PBS. The data are shown as mean ± SE of triplicate values which represent four independent experiments.

* = significant difference to PBS at CI of 95%

5.2.3 Antibody - dependent cell cytotoxicity (ADCC)

Infected P815 target cells, which were treated with 1% autologous heat inactivated mice serum, were added to mice splenocytes at effector:target ratio of 12.5:1, 25:1 and 50:1. The average cytotoxic activity from triplicate experiment and its SE was calculated and shown into figure 5.8 A-C.

The % ADCC of rVaccinia H5 HA immunized mice splenocytes at ratio 12.5:1, 25:1, and 50:1 are 24.88 ± 10.62%, 10.409 ± 6.81% and 30.51 ± 32.72% respectively (shown in figure 5.8A). The % ADCC of rVaccinia pSC11 immunized mice splenocytes at ratio 12.5:1, 25:1, and 50:1 are 4.94 ± 2.55%, 3.20 ± 14.41%, and -22.34 ± 10.92% respectively(shown in figure 5.8B). The % ADCC cytotoxicity of PBS immunized splenocytes at ratio 12.5:1, 25:1, and 50:1 are -11.296 ± 2.881%, 2.743 ± 0.406%, and -5.332 ± 0.398% (shown in figure 5.8C). In summary, statistical analysis show that ADCC activity of rVaccinia H5 HA at 12.5:1 ratio is statistically different to ADCC activity of rpSC11 vaccinia at the same ratio (Mann-Whitney one tailed *t*-test; $p<0.05$).

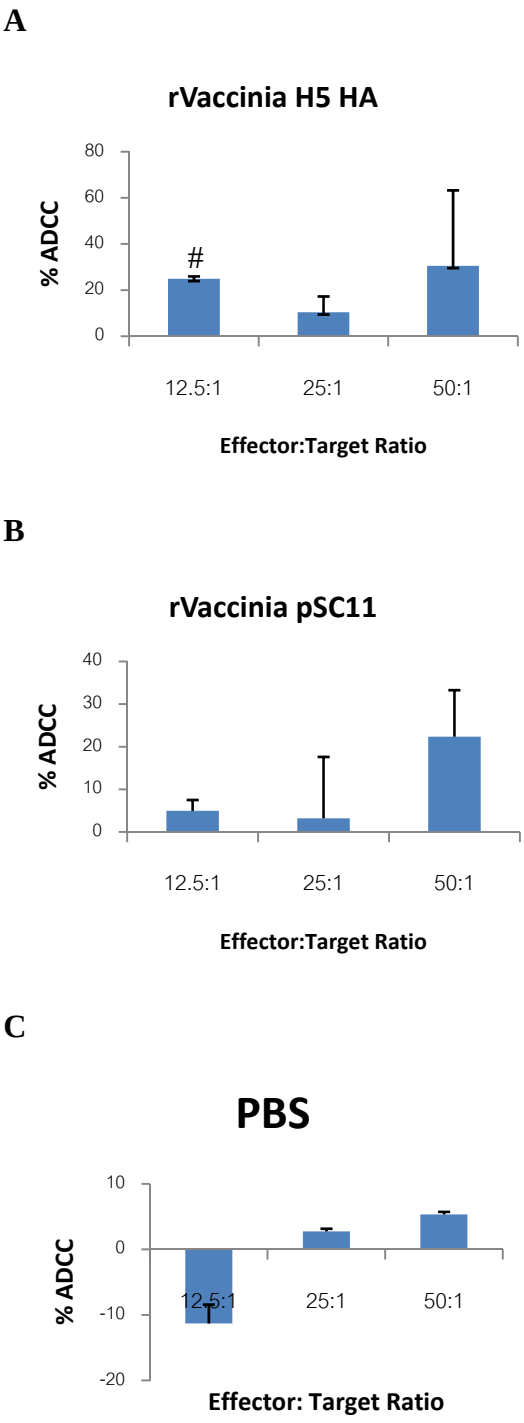


Figure 5.8: % ADCC of mice splenocytes using 1% pooled mice serum from mice immunized with either (A) rVaccinia H5 HA, (B) rVaccinia pSC11 or (C) PBS. The data are shown as mean ± SE of triplicate values which represent four independent experiments.

= significant different to rVaccinia pSC11 at CI of 95%

5.2.4 Natural killer cell cytotoxicity (NK assay)

NK cell cytotoxicity was done as a positive control for cytotoxic assay and splenocyte abilities. Immunized mice splenocytes were put together with target Yac-1 cells at ratio 12.5:1, 25:1, and 50:1.

% NK cytotoxic activity of rVaccinia H5 HA immunized mice splenocytes at 12.5:1, 25:1 and 50:1 ratios are 12.60 ± 3.88%, 3.11 ± 9.44%, and 11.63 ± 11.52% (shown in figure 5.9A). NK cytotoxic activity of rVaccinia pSC11 immunized mice splenocytes at 12.5:1, 25:1 and 50:1 ratios are 23.24 ± 18.25%, -32.32 ± 32.22%, and 11.88 ± 9.98% respectively (shown in figure 5.9B). NK cytotoxic activity of PBS immunized mice splenocytes at 12.5:1, 25:1 and 50:1 ratios are 8.60 ± 13.62%, 0.38 ± 2.47%, and 1.52 ± 14.32% respectively (shown in figure 5.9C).

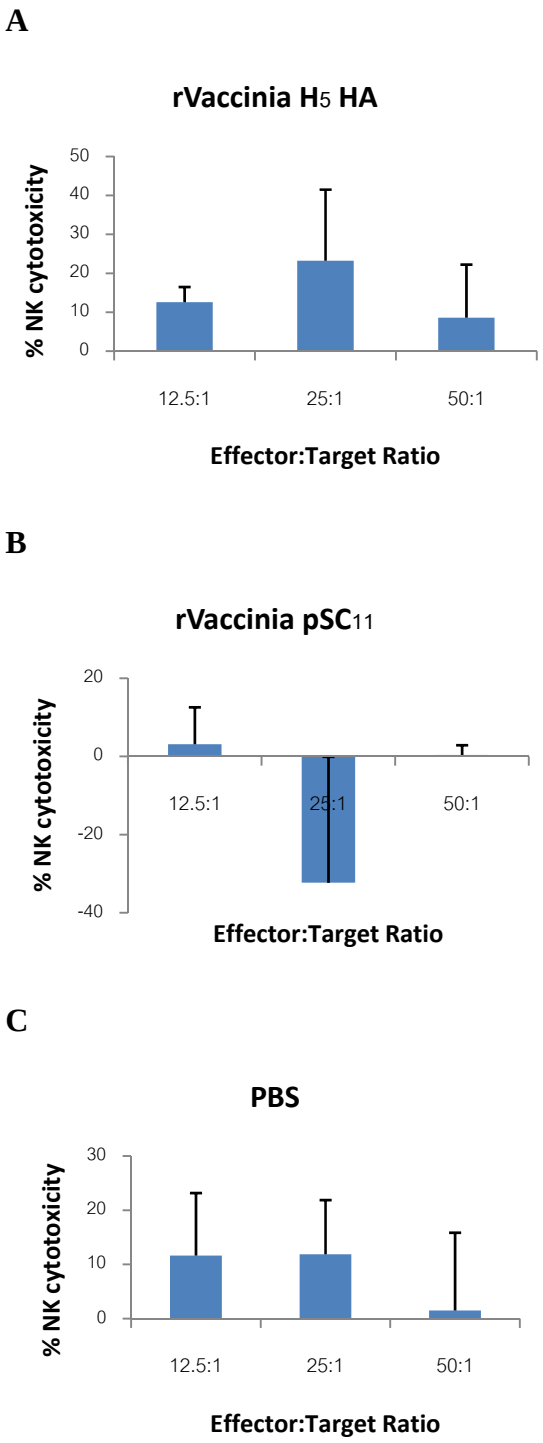


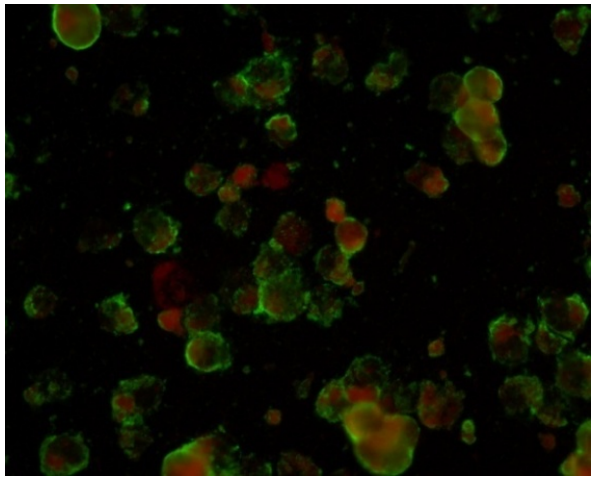
Figure 5.9: % NK cytotoxicity of mice splenocytes immunized with either (A) rVaccinia H5 HA, (B) rVaccinia pSC11 or (C) PBS. The data are shown as mean± SE of triplicate values which represent four independent experiments.

5.3 Humoral immune response (HIR)

5.3.1 Detection of specific antibody to H5 HA in mouse immune sera by indirect immunofluorescence staining

Pooled serum sample from immunized mice bled at one week after first boost (from the CMIR study) was assayed for presence of specific antibody to H5 HA by indirect immunofluorescence using TK^- cells infected with rVaccinia H5 HA as the test antigen. Pooled serum from the PBS immunized mice was used as negative control. The test sera were assayed at dilution 1:40. The results showed that the test antigen stained with pooled serum from rVaccinia H5 HA immunized mice showed green fluorescence color; while that from PBS immunized serum did not (Figures 5.10 A and 5.10B).

A



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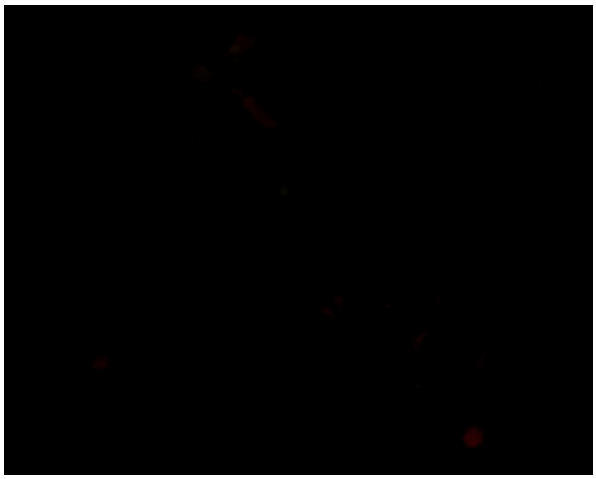

Figure 5.10: Immunofluoresence assay of rVaccinia H5 HA infected TK^- cells staining with pooled serum from: (A) rVaccinia H5 HA immunized mice; and (B) PBS immunized mice

5.3.2 Hemagglutination inhibition (HI) assay

Immune sera from 66 immunized mice from 3 independent experiments were determined for antibody to H5 HA by HI assay using reverse genetic viruses harboring H5 HA as the test antigen. The results catagorized by experiment number, immunogen, boosting and the geometric mean titer obtained are shown in Table 5.7. The antibody titers obtained after one boost and 2 boosts of the immunogen are compared as shown in Table 5.8. It was demonstrated that all of 20 mice immunized with rVaccinia H5 HA developed seroconversion to H5 HA antigen after one boost of immunization. Mice boosted once or twice after antigen priming were not different in level of antibody titer (Geometric mean titer; GMT 113 *vs.* 139) as compared by statistical analysis (parametric paired *t*-test; $p > 0.05$). No antibody response was detected in mice immunized with rVaccinia pSC11 or unimmunized mice.

Table 5.7: HI antibody titers to H5 HA in mouse immune sera (n=66)

Exp. No.	Immunizing protocol	Immunogen	Total mice	Number of mice with HI antibody titer of									GMT
				<10	10	20	40	80	160	320	640	>640	
1	1st boost	rVaccinia H5 HA	4	-	-	-	-	-	4	-	-	-	160
		rVaccinia pSC11	4	4	-	-	-	-	-	-	-	-	<10
		Unimmunized	5	5	-	-	-	-	-	-	-	-	<10
	2nd boost	rVaccinia H5 HA	4	-	-	-	-	-	4	-	-	-	160
		rVaccinia pSC11	4	4	-	-	-	-	-	-	-	-	<10
		Unimmunized	6	6	-	-	-	-	-	-	-	-	<10
2	1st boost	rVaccinia H5 HA	3	-	-	-	1	2	-	-	-	-	64
		rVaccinia pSC11	2	2	-	-	-	-	-	-	-	-	<10
		Unimmunized	4	4	-	-	-	-	-	-	-	-	<10
	2nd boost	rVaccinia H5 HA	3	-	-	-	-	2	1	-	-	-	101
		rVaccinia pSC11	3	3	-	-	-	-	-	-	-	-	<10
		Unimmunized	4	4	-	-	-	-	-	-	-	-	<10
3	1st boost	rVaccinia H5 HA	3	-	-	-	1	-	1	1	-	-	127
		rVaccinia pSC11	3	3	-	-	-	-	-	-	-	-	<10
		Unimmunized	4	4	-	-	-	-	-	-	-	-	<10
	2nd boost	rVaccinia H5 HA	3	-	-	-	-	1	1	1	-	-	160
		rVaccinia pSC11	3	3	-	-	-	-	-	-	-	-	<10
		Unimmunized	4	4	-	-	-	-	-	-	-	-	<10

Table 5.8: Comparison between HI antibody titers obtained after one boost or two boosts after antigen priming

Immunizing protocol	Immunogen	Total mice	Number of mice with NT antibody titer of									GMT
			<10	**10**	**20**	**40**	**80**	**160**	**320**	**640**	**>640**	
1st boost	rVaccinia H5 HA	10	-	-	-	2	2	5	1	-	-	113*
	rVaccinia pSC11	9	9	-	-	-	-	-	-	-	-	<10
2nd boost	rVaccinia H5 HA	10	-	-	-	-	3	6	1	-	-	139*
	rVaccinia pSC11	10	10	-	-	-	-	-	-	-	-	<10
Unimmunized	-	27	27	-	-	-	-	-	-	-	-	<10

* = significant different to rVaccinia pSC11 and unimmunized mice sera at CI of 95%

5.3.3 Hemagglutination based-micro neutralization assay

HA based-microNT assay was performed to determine neutralizing antibody in 61 immune mouse sera using reverse genetic viruses harboring H5 HA as the test virus. The results catagorized by experiment number, immunogen, boosting and the geometric mean titer obtained are shown in Table 5.9. The antibody titers obtained after one boost and 2 boosts of the immunogen are compared as shown in Table 5.10. Interestingly, our study found that sera obtained from mice immunized with rVaccinia pSC11 or unimmunized mice contained non-specific inhibitory factors that could block the virus infectivity despite treatment of the test sera by RDE followed by heat inactivation. Nevertheless, the cross neutralizing antibody titers in these sera did not exceed 40. The antibody titers from rVaccinia pSC11 immunized mice sera show GMT value are 18 and 14 for mice with first boost and second boost respectively, while the GMT of antibody titer of unimmunized mice sera are 19 and 18 for first and second boost; while the specific antibody titers obtained from all 20 serum samples from mice immunized with rVaccinia H5 HA exceeded 160. The GMT of one boost and two boost are 226 and 320 respectively implied that all immunized mice developed seroconversion as early as one boost after antigen priming. The antibody titers obtained after one boost or 2 boosts of rVaccinia H5 HA were significantly higher than those obtained from rVaccinia pSC11 or unimmunized mice (parametric independent t- test; $p < 0.05$ for all comparison between rVaccinia H5 HA and rVaccinia pSC11 or unimmunized mouse antibody NT titer). Nevertheless, there was no significant difference in antibody titers obtained after one boost or 2 boosts of the specific immunogen (parametric paired *t*-test; $p > 0.05$).

Table 5.9: HA-based micro NT antibody titers in mouse immune sera (n=61)

Exp. No.	Immunizing protocol	Immunogen	Total mice	Number of mice with NT antibody titer of									GMT
				<10	10	20	40	80	160	320	640	>640	
1	1st boost	rVaccinia H5 HA	4	-	-	-	-	-	2	2	-	-	226
		rVaccinia pSC11	2	-	-	2	-	-	-	-	-	-	20
		Unimmunized	2	-	-	2	-	-	-	-	-	-	20
	2nd boost	rVaccinia H5 HA	4	-	-	-	-	-	-	4	-	-	320
		rVaccinia pSC11	4	-	2	2	-	-	-	-	-	-	14
		Unimmunized	6	-	-	4	2	-	-	-	-	-	25
2	1st boost	rVaccinia H5 HA	3	-	-	-	-	1	-	2	-	-	202
		rVaccinia pSC11	2	-	1	1	-	-	-	-	-	-	14
		Unimmunized	4	-	1	3	-	-	-	-	-	-	16
	2nd boost	rVaccinia H5 HA	3	-	-	-	-	-	-	2	1	-	403
		rVaccinia pSC11	3	-	-	3	-	-	-	-	-	-	20
		Unimmunized	4	-	-	4	-	-	-	-	-	-	20
3	1st boost	rVaccinia H5 HA	3	-	-	-	-	-	1	2	-	-	254
		rVaccinia pSC11	3	-	-	3	-	-	-	-	-	-	20
		Unimmunized	4	-	1	2	1	-	-	-	-	-	20
	2nd boost	rVaccinia H5 HA	3	-	-	-	-	1	-	1	1	-	254
		rVaccinia pSC11	3	-	3	-	-	-	-	-	-	-	10
		Unimmunized	4	-	4	-	-	-	-	-	-	-	10

Table 5.10: Comparison between HA based micro-NT antibody titers obtained after one boost or two boosts after antigen priming

Immunizing protocol	Immunogen	Total mice	Number of mice with NT antibody titer of									GMT
			<10	10	20	40	80	160	320	640	>640	
1st boost	rVaccinia H5 HA	10	-	-	-	-	1	3	6	-	-	226*
	rpSC11	7	-	1	6	-	-	-	-	-	-	18
2nd boost	rVaccinia H5 HA	10	-	-	-	-	1	-	7	2	-	320*
	rpSC11	10	-	5	5	-	-	-	-	-	-	14
Unimmunized	-	24	-	6	15	3	-	-	-	-	-	18

* = significant different to rVaccinia pSC11 and unimmunized mice sera at CI of 95%

5.3.4 Correlation between HI and NT antibody titers in the immunized mice

Correlation between HI antibody titers and neutralizing antibody titers in 20 mice which were immunized and boost once or boost twice with rVaccinia H5 HA was determined, and poor correlation was obtained (r^2=0.183) as shown in figure 5.11.

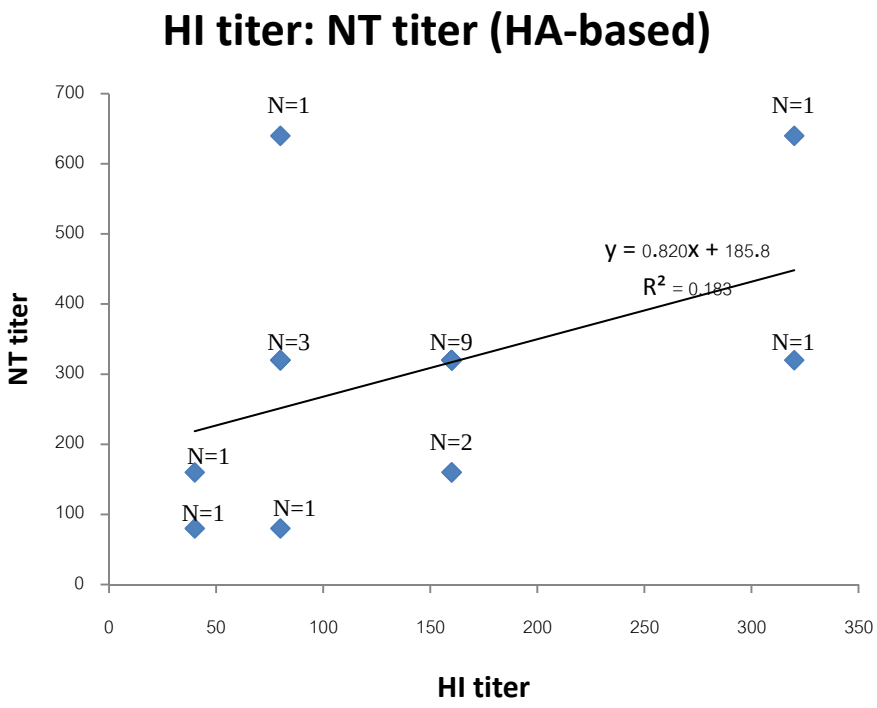

Figure 5.11: Correlation between HI antibody titer and neutralizing antibody titers of mice immunized with rVaccinia H5 HA (r^2 =0.183)

CHAPTER VI
DISCUSSION

By using animal model in assaying adaptive immune response, the immunization route and immunization protocol play the important roles. Mice immunization via intraperitoneal with rVaccinia H5 HA leads to some cytotoxic activity of CTL as done by *in vitro* experiment using H5 HA expressed target P815 cells as specific target cells. The specific antibodies with neutralizing antibody and agglutination inhibition to H5 HA were also performed. The discussion will be divided into two parts: Cell mediated immune response (CMIR) and humoral immune response (HIR).

Part 1: CMIR

Recombinant vaccinia virus containing influenza gene has been shown to induce CTL response in mouse system by other studies [14, 52, 53,54]. The influenza genes that were used in their studies are H1N1 HA, H7N2 nucleoprotein (NP) or both HA and NP of H1N1 influenza. The results from *in vitro* CTL cytotoxic assay from our CMIR experiment shows that splenocytes from mice immunized with rVaccinia H5N1 HA have less than 30% cytotoxic activity which correlates to other results. CTL of Meitin et al is 32% at 30:1 effector:target ratio [14]. Lawson et al shows the NP peptide (amino acid 147-155) induced 30-35% CTL activity at 25:1 effector:target ratio. The whole gene of NP expressed in recombinant vaccinia can induce more than 40% cytotoxicity when compared to their recombinant vaccinia containing H1N1 HA gene. These suggest the possibility of NP protein as a potent T epitope than HA protein [53]. Cytotoxicity to NA protein was also studied earlier by Wysoca et al. The cytotoxicity found toward this viral surface was low as similar to HA [55]. In general, percentage cytotoxic activity that will be considered to demonstrate strong cytotoxic activity should be higher than 50% [56]. The % cytotoxic activity in our study is less than 30%, may be explained by the following explanations.

a) The mice immunization protocol

1) Mice immunization protocol and the experiment time may not be a proper protocol to detect CTL activity since there is a report by Powell et al that shows the short persistence period of CTL post infection in patients [57]. The recombinant vaccinia encoded *HA* gene immunization dose is 10^6-10^7 pfu and were injected to each mouse via the intravenous, or intraperitoneal route. In our study, mice were immunized with 10^7 pfu of either rVaccinia H5 HA or rVaccinia pSC11 by intraperitoneal route. So the dose of rVaccinia H5 HA may not be the optimum concentration. Moreover, some researches boost mice via intranasal route with whole influenza virus at different interval time. [53, 58] In our experiment, recombinant vaccinia influenza virus is used to boost immune response instead of whole influenza virus.

2) The H5 HA protein is processed to present on MHC I molecule ($H2^d$) on BALB/c mice to stimulate CTL activities, however, it is not known which protein sequences will be presented as an effective T epitope on $H2^d$ molecule. Since other studies on T epitope of HA protein show that the HA sequence containing potent T epitope are 518-526 in H2K mice, 158-169 in H5N9 HA of BALB/c mice and 246-260 in chicken[13, 59, 60].

b) The *in vitro* cytotoxic assay system

1) In our experiment Lactate dehydrogenase (LDH) assay was used to determine the cytotoxicity. LDH assay detects LDH that leaks to the extracellular medium. This assay is suitable for measuring cell death by cell membrane damage which can be caused by cell membrane disruptive agents such as TritonX. [61] Effector splenocytes may cause target cell death without cell leakage. Therefore induced cell death may not be detected with this assay. Other non-radioactive assays are suggested [62, 63]. There are many non-radioactive assays are available and detect cell death at different aspects, for example, Aspartate amino transferase (ASP) and LDH are suitable to cell death with cell membrane rupture or necrosis. Caspase-3/7 activity was used to detect apoptosis cell death. Adenosine triphosphate (ATP)-based assay, MTT assay, and neutral red staining can be modified

to identify cell cytotoxicity. Glutamate dehydrogenase (GLDH) assay is another assay that can be used to identify mitochondria toxicity. Moreover, it is preferably that more than one cytotoxic assay should be used to determine the cytotoxicity. [61]

The effector spontaneous and target spontaneous values are high in LDH assay of our experiments. This leads to low % cytotoxicity of CTL, ADCC, and NK cytotoxic activities. Accurate cell titration into each experiment wells is one of the solutions suggested to improve results. Debra Burns thesis, Youngstown state University, which studied NK activity by measuring with LDH assay, also found the high effector spontaneous and target spontaneous values [29].

2) NK cell activity using Yac-1 cell, which is a susceptible target cell of mice NK cells, was done as the positive control to assess the *in vitro* cytolysis assay condition using LDH assay was tested, however, the % cytotoxic is low. Debra Burns research on NK cytotoxic activity in mice system by using LDH assay, showed low cytotoxic activity [29]. However, the percent cytotoxicity is high in other studies [56]

c) Antibody-dependent cell cytotoxicity (ADCC) assay

ADCC assay of this study demonstrates the % cytotoxicity higher than CTL assay. This suggests that there are specific antibodies to H5 HA in mice serum can function as antibody that lead to other cytotoxic cell apart from specific CTL. ADCC assay performed by other researchers has been shown to increase cytotoxicity percentage comparing to CTL assay which is similar to our results. [64, 65]. Bruckheimer, E.M. et al study the efficiency of antibody in killing cancer cell, by vary antibody concentration based on weight, but in this study, it was unable to do serum titration in ADCC assay [64]. Hashimoto, G et al studied ADCC activity to influenza by using cord plasma [65]. The results shows ADCC activity in human cord. In this study, high percentage cytotoxicity were found at 12.5 and 50:1 effector:target ratio while it is not shown in 25:1 effector:target ratio which may be indicated there was some error of pipetting.

Part 2: HIR

Immunization of mice with rVaccinia H5 HA shows the occurring of specific antibody that has the activities of both hemagglutination inhibition and neutralization. The presence of HA specific antibody is similar to whole influenza virus immunization, but rVaccinia H5 HA immunized mice produces a lower antibody level.[36] This may be explained by the following topics.

1) Mice Immunization protocol

The immunization protocol is different from CMIR experiment and is expected to be a better mice immunization protocol. Eight to ten weeks old mice are mature enough to elicit efficient humoral immune response with the ability to replenish new B cell [66]

2) Hemagglutination inhibition (HI) assay and micro-neutralization (micro-NT) assay

HI and micro-NT assay are the standard assay to determine antibody titer in individual mouse serum and in patients' blood. [67, 68]. HI assay shows geometric mean titer (GMT) of antibody at approximately 120 in rVaccinia H5 HA immunized mouse serum which was not found in rVaccinia pSC11 immunized mice and unimmunized mice serum. No non-specific background was found. Recombinant vaccinia with influenza HA was used in other studies in mice [14] and chicken [60]. The HI titer of mice experiment is 187 by immunizing mice with rVaccinia H1 HA via the intravenous route. The chicken were immunized by intramuscular or intraperitoneal with vaccinia H5 HA of Ty/Ire influenza virus then they were challenged with 10 EID_{50} of different virulent H5 influenza viruses by inoculation into the nasal cleft. The results after chicken challenge show titer of 60 and 230 in immunization route of intramuscular or intraperitoneal, respectively.

Micro-NT assay shows the seroconversion in rVaccinia H5 HA immunized mice serum since first boost. Non- specific background from mouse serum was detected even though the tested mouse sera were treated with receptor destroying enzyme (RDE). Micro-NT results show slightly higher titer when compared to HI titer which correlates with another [68].

Other researches show that HA immunized mice leads to neutralizing antibody which blocks the cell entry [69]. Interestingly the mouse serum from our experiment shows an unusual neutralization. When enzyme-link immunosorbent assay (ELISA) of micro-NT plate was done, H5 HA protein was detected in every serum dilution. The reason of this is unknown. HA based-micro NT assay was used as an end point determination to detect H5 HA protein. Antibody titer from HA-based micro-NT assay has GMT of is 226-320 in mice immunized with rVaccinia H5 HA. The GMT of rVaccinia pSC11 immunized and unimmunized mice sera are lower than 20. CPE based is a subjective method in observing the cytopathic changes in infected cells. It is important to note that infected cells might not show visible cytopathic effect during early stages of viral propagation.

The lack of booster effect in these HIR experiment may be due to the existing antibody in mice destroy the living recombinant vaccinia that was used to boost mice. For example the some canine or feline vaccine require booster but are not recommended [70]. Boosters of living vaccine are injected to dogs or cats after 1-3 years when necessary [71].

As it is known, that both $CD4^+$ and $CD8^+$ T cell are important in immune protection against microbial and virus infection including influenza virus infection [5,6]. $CD4^+$ T cell functions both in helping B cell or antibody response and $CD8^+$ (CTL) differentiation. Apart from $CD8^+$ CTL cytotoxic activity, some study also showed that cytotoxic activity was mediated by $CD4^+$ T cell is shown [24-26]. To initiate the effective immune protection to influenza virus, it is likely that both $CD4^+$ and $CD8^+$ T cell are involved. The immune prophylaxis of viral influenza infection by vacciniation is still under intensive research especially when there was avian influenza outbreak. To produce an effective vaccine, at least 3 main topics should be considered, the influenza viral immunogenic parts (epitope), the dose, route of immunization and the last is host genetic background. This study has applied rVaccinia H5 HA as an immunogen to immunize BALB/c mice and detect their CTL activity and HI and NT antibody activity. This may be able to suggest that there is a presentation of H5 HA protein in MHC molecule of BALB/c mice ($H2^d$). The preliminary CTL results of this study suggest that HA protein can be processed and presented with both MHC I and MHC II ($H2^d$) by certain type of antigen presenting cells that are not known. Other

studies demonstrate CTL activity of other influenza protein such as NP, M protein [53, 58]. However, those studies used whole virus particle and some influenza vaccine production studies use certain protein of known sequences to evaluate the suitable epitope.

For the antibody activity, both effective anti-influenza activity, HI and NT are observed in this study by immunization of BALB/c mice with rVaccinia H5 HA. This indicates the protective response elicited by HA protein itself. In most of the all studies of the antibody activities they detected antibodies when host are infected or immunized with whole influenza virus particle [67, 68]. According to the immune mechanism, it implies that HA proteins produced in BALB/c intraperitoneally immunized with rVaccinia H5 HA can be presented with $H2^d$ MHC molecule of the antigen presenting cells. What are the presenting cells and which part of the processed HA protein that are presented in association with $H2^d$ MHC molecule cannot be shown in this study. However, it is interesting to find out since it may provide useful information in vaccine research.

CHAPTER VII
CONCLUSION

Both cell mediated immune response and humoral immune response have important roles in killing influenza virus and influenza virus infected cells. We hypothesized that the cytolysis function of CMIR and antibody-dependent cell-mediated cytotoxicity (ADCC) may have an important role of getting rid of H5N1 influenza virus. In HIR experiment, immunized mice serum was expected to have neutralizing antibody as well. In order to study the immune response to H5 HA in BALB/c mice ($H2^d$), rVaccinia H5 HA is used in this study. Recombinant vaccinia with pSC11 plasmid (rVaccinia pSC11) and PBS (CMIR experiments) or unimmunized mice (HIR experiment) are used as control.

The results demonstrate activity of functioning CTL from rVaccinia H5 HA from immunized BALB/c mice ($H2^d$). Moreover, immunized mouse serum contains antibodies that help CTL and other immune cells in target cell cytolysis (ADCC). Immunization by rVaccinia H5 HA was also found to be able to produce a high level of specific antibodies to H5 HA with both neutralizing and hemagglutination inhibition function.

In conclusion, this study suggests that HA protein from rVaccinia H5 HA via intraperitoneal injection should have both B and T antigenic determinants. Moreover, the B epitope in HA protein has high immunogenicity property than T epitope as shown in BALB/c ($H2^d$) mouse in this study

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APPENDIX

Growth media for splenocytes and P815 cells

1. RPMI 1640 for washing splenocytes

1.1 Working solution (1X)

RPMI 1640 powder	1 bottle	
1M HEPES	2.86	g
$NaHCO_3$	2	g
L-glutamine	2.92	g
Penicillin &Streptomycin	1	ml

Add sterile distilled, deionized water to 1,000 ml and sterile through filtration with 0.22 µm millipore membrane and keep at 4^oC

2. RPMI 1640 for P815 cell maintenance and infection use commercial RPMI 1640 media from Gibco

2.1 Growth media (10% FBS) kept in 4^oC

RPMI 1640 (1Xworking solution)	90	ml
Fetal Bovine serum	10	ml
L-glutamine	1	ml

2.2 Virus infection media (2% FBS) kept in 4^oC

RPMI 1640 (1Xworking solution)	98	ml
Fetal Bovine serum	2	ml
L-glutamine	1	ml

3. 1X PBS for washing cells

NaCl	8	g
KCl	0.2	g
KH_2PO_4	0.2	g
Na_2HPO_4	9.1	g
Deionized distilled water to	1,000	ml

Sterilize by autoclaving at 121^oC under pressure for 15 min, then store at room temperature

4. 0.25% trypsin used is a commercialized product from Gibco

Growth media for TK cells

1. Dulbecco's modified eagle medium (DMEM)

1.1 Stock solution 5X

DMEM powder	134	g

Add deionized water to final volume of 2,000 ml. Sterile by membrane filtration (0.22µm membrane filter). Store at -20^{o}C

1.2 Working solution 1X (100 ml)

Sterile deionized water	80	ml
DMEM 5X	20	ml
5% $NaHCO_3$	5	ml
1M HEPES	1	ml
Penicillin (40,000 U/ml)	0.5	ml
Gentamicin (4,000 µg/ml)	0.5	ml

Store at 4^{o}C

1.3 Growth media (10% FBS in EMEM) kept at 4 ^{o}C

DMEM (Working solution 1X)	90	ml
Fetal bovine serum	10	ml

1.4 Mainternance media for recombinant vaccinia virus infection

DMEM (Working solution 1X)	98	ml
Fetal bovine serum	2	ml

Growth media for MDCK cells

1. Earle's minimal essential medium (EMEM)

1.1 Stock solution 10X

EMEM powder	95.3	g

Add sterile distilled, deionized water to 1,000 ml and sterile through filtration with 0.45 µm millipore membrane. Aliquot the stock solution into 100ml/tube and kept frozen at -20^{o}C

1.2 Working solution 1X

10X EMEM	10	ml
1M HEPES	1	ml

Penicillin 40,000 U/ml	0.5	ml
Gentamycin 4 mg/ml	0.5	ml
Fungizone 1 mg/ml	0.1	ml
5% $NaHCO_3$	4	ml

Add sterile distilled, deionized water to 100 ml and kept at 4^{o}C

1.3 Growth media (10% FBS in EMEM) kept at 4^{o}C

EMEM (Working solution 1X)	90	ml
Fetal bovine serum	10	ml

1.4 Mainternance media for influenza virus infection/Virus growth media (VGM)

EMEM (Working solution 1X)	100	ml
Trypsiin-TPCK 500µg/ml	0.4	ml

2. 0.125% Trypsin-EDTA

0.25% Trypsin-EDTA	50	ml
(Gibco, Carlsbad, California, Cat No. LTG-25200-056)		
PBS	50	ml

3. Phosphate buffer saline (PBS) without Ca^{2+} and Mg^{2+}, pH7.2

The stock solution (10X) is used for preparing trypsin solution and the working solution is used for cell washing in the trypsinization process.

3.1 PBS stock solution (10X)

NaCl	80.0	g
KCl	2.0	g
KH_2PO_4 (anhydrous)	1.2	g
Na_2HPO_4 (anhydrous)	9.1	g
Deionized distilled water to	1,000	ml

Adjust pH to 7.2 by 1N NaOH. Sterilize by autoclaving at 121^{o}C under pressure of 15lb/ square inch for 15minutes, then store at room temperature

3.2 Working solution (1X)

PBS stock solution (10X)	10	ml

Deionized distilled water	90	ml

4. Trypsin – TPCK 500 µg/ml

TPCK-trypsin	10	mg
(Sigma-Alidrich Co., St. Louis, USA, Cat No. P-3688)		
MEM 1X	20	ml

Sterile by filtration with 0.45 µm millipore membrane. Aliquot 200µl/tube and kept frozen at -20^{o}C

5. 5% $NaHCO_3$

$NaHCO_3$	25	g

Add sterile distilled deionized water to 1,000 ml. Sterile through filtration with 0.45 µm millipore membrane. Aliquot 50 ml/tube and kept at 4^{o}C

6. 1M HEPES

HEPES	23.83	g

Add sterile distilled deionized water to 100 ml. Sterile through filtration with 0.45 µm millipore membrane and kept at 4^{o}C

7. Antibiotics

7.1 Penicillin 40,000 U/ml (10X)

Penicillin	1,000,000 U/bottle

Add sterile distilled deionized water to 25 ml. Aliquot 5 ml and kept at 4^{o}C

7.2 Gentamycin 4 µg/ml (10X)

Gentamycin	80	mg

Add sterile distilled deionized water to 20 ml. Aliquot 5 ml and kept at 4^{o}C

7.3 Fungizone 1 mg/ml (10X)

Fungizone (Amphotericin B)	50	mg

Add sterile distilled deionized water to 50 ml. Aliquot 1 ml and kept at 4^{o}C

Reagents for electrophoresis assay

1. 50X TAE stock solution		
Tris base	48.4	g
Gracial acetic acid	11.425	ml
0.5 M EDTA (pH 8.0)	20	ml
Adjust the volume to 200ml with distilled water.		
2. 1X TAE buffer (working solution)		
50X TAE buffer	20	ml
Adjust the volume to 1000ml with distilled water.		
3. Agarose gel (1.5%)		
Agarose (powder)	1.5	g
TAE buffer	100	ml

Trypan blue 0.1% (Store at 4 ^{o}C)

0.4% Trypan blue	1	ml
NSS	3	ml

Protocol No MUVS-2009-30
(assigned by FVS-ACUC)

Documentary Proof of Ethical Clearance
The Faculty of Veterinary Science- Animal Care and Use Committee
(FVS-ACUC)
Mahidol University

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Protocol Title: ประสิทธิภาพของส่วนประกอบของไวรัสไข้หวัดใหญ่ชนิด H5N1 (HA และหรือ NA gene) recombinant vaccinia ในการกระตุ้น cell mediated cytotoxic immune response ในหนูทดลอง (Stimulation efficacy of influenza H5N1 viral gene (HA and/or NA) recombinant vaccinia of cell mediated cytotoxic immune response in mice)

Principal Investigator: มลวิภา วงษ์สกุล (Molvibha Vongskul)

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Approved by the Faculty of Veterinary Science- Animal Care and Use Committee
FVS-ACUC Review

Approval Recommended

.....October 27, 2009.....
Assoc. Prof. Kampon Kaeoket DVM, MSc, PhD
(Chair, FVS-ACUC Signature, Date)

Dean, FVS-MU

Approval Recommended

.....October 27, 2009.....
Wantanee Ratanasak DVM, MSc
(Dean, FVS-MU Signature, Date)

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