ROLE OF CD34^{+} HEMATOPOIETIC STEM CELLS AND MESENCHYMAL STROMAL CELLS ON AVIAN INFLUENZA VIRUS (H5N1) PATHOGENESIS

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (MICROBIOLOGY) FACULTY OF GRADUATE STUDIES MAHIDOL UNIVERSITY 2013

Thesis
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was submitted to the Faculty of Graduate Studies, Mahidol University
for the degree of Doctor of Philosophy (Microbiology)
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ACKNOWLEDGEMENTS

First and foremost, I would like to express my deepest gratitude and appreciation to my advisor, Assistant Professor Arunee Thitithanyanont, for giving me invaluable advice, guidance, and support throughout my study. Without her guidance and persistent help this dissertation would not have been possible.

My great appreciation goes to my co-advisors, Professor Prasert Auewarakul and Dr. Sathit Pichyangkul, for all of comments and good suggestions.

I sincerely appreciate Assoc. Prof. Suradech Honging for providing bone marrow and cord blood samples. My deep gratitude goes to all of staff at Clinical Research Center, Ramathibodi hospital for providing flow cytometer facility.

I would like to thank many of Pr 615 laboratory's members; Ms. Suwimon Wiboon-ut, Ms. Pumaree Kanrai, Mr. Prasit Na-Ek for technical support, encouragement, and friendship. Working with all of them was a terrific experience, and also sharing the lunch table and the conversation during the lunch time are memorable. My special thanks go to Mr. Chokdee Wongborisut for helpful advice and technical assistance in MSCs isolation and culture, Ms. Nantarat Chantawat for always being on my side, and my dearest friends for sources of laughter, joy, and friendship. A lot of thanks are also given to all members of the Department of Microbiology for warmth and kindness.

Furthermore, I would like to acknowlede the Thailand research fund (TRF) for financial support for all my study years.

Finally and most importantly, I thank my parents who always support, care, and trust in me. Their greatest love provided my inspiration and was my driving force. I would like to dedicate this work to my lost Grandpa and Grandma who left us too soon. I hope that this work makes you proud.

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ABSTRACT

Avian influenza A H5N1 virus continues to cause a global threat to human health. Major complications of H5N1 infection are the systemic spread with uncontrolled replication and host cytokine responses leading to gross pathology-like sepsis. The clinical data suggests almost half of the fatal cases were associated with hyperactivation of cytokines which might be correlated with reactive hemophagocytosis. Moreover, the presence of abnormal hematologic findings such as lymphopenia, thrombocytopenia, and pancytopenia were diagnosed in severe cases. These increase interest in exploring bone marrow (BM) dissemination, which direct infection of BM cells remains elusive. The BM compartment contains two stem cell types, which are hematopoietic stem cells (HSCs) and mesenchymal stromal cells (MSCs) with possessing stem-like and immunomodulatory properties. Our investigation demonstrated that CD34^{+} HSCs and MSCs were susceptible to H5N1 infection. H5N1 virus could productively infect and induce cell deaths in both cell types. By contrast, these aspects were not found in human influenza virus infection. Reverse genetics analyses demonstrated that hemagglutinin played a role in the infection of MSCs. We also investigated host response following H5N1 infection of CD34^{+} HSCs and MSCs. We found that inflammatory cytokines were not induced in H5N1-infected cells. We next investigated whether infection affects the immunomodulatory function of MSCs. We noted a consequent dysregulation of MSCs-mediated immune modulation from high cytokine and chemokine production in H5N1-infected MSCs/Monocytes co-culture. These findings provide a better understanding of H5N1 pathogenesis in terms of host tropism and systemic spread.

KEY WORDS: H5N1/SYSTEMIC INFECTION/BONE MARROW/ HEMATOPOIETIC STEM CELL/MESENCHYMAL STROMAL CELL

108 pages

การศึกษาบทบาทของ CD34^{+} HEMATOPOIETIC STEM CELLS และ MESENCHYMAL STROMAL CELLS ต่อการเกิดพยาธิสภาพของไวรัสไข้หวัดนกสายพันธุ์ H5N1

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บทคัดย่อ

เชื้อไวรัสไข้หวัดนก (H5N1) เป็นเชื้อที่มีความรุนแรงในการก่อโรค และ มีการระบาดอย่างต่อเนื่องในหลายทวีปทั่วโลก การติดเชื้อ H5N1 ในผู้ป่วยส่วนใหญ่จะเป็นแบบแพร่กระจาย เนื่องจากมีการตรวจพบปริมาณเชื้อที่สูงในอวัยวะต่างๆรวมทั้งในกระแสเลือด จากผลการตรวจวิเคราะห์ทางโลหิตวิทยาทั้งในผู้ป่วยติดเชื้อที่มีอาการรุนแรง พบความผิดปกติของทั้งรูปร่างและจำนวนของเม็ดเลือด นอกจากนี้จากการศึกษาการเกิดพยาธิสภาพในไขกระดูกของผู้ป่วยที่เสียชีวิต พบแอนติเจนของไวรัสในเซลล์ต้นกำเนิดในไขกระดูก หลักฐานต่างๆเหล่านี้ ทำให้เกิดสมมติฐานที่ว่า เชื้อ H5N1 อาจมีการแพร่กระจายเข้าไปสู่ไขกระดูก เนื่องจากไขกระดูกเป็นแหล่งสำคัญที่ประกอบไปด้วยเซลล์ต้นกำเนิดสำหรับสร้างเซลล์เม็ดเลือดและเนื้อเยื่อชนิดต่างๆ ดังนั้นการที่เซลล์ต้นกำเนิดถูกรุกรานโดยไวรัส อาจจะทำให้เกิดพยาธิสภาพที่รุนแรงในไขกระดูกได้ สมมติฐานดังกล่าวได้นำไปสู่การศึกษาในหลอดทดลองโดยเริ่มต้นจากการแยกเซลล์ต้นกำเนิดซึ่งมี 2 ชนิด คือเซลล์ต้นกำเนิดเม็ดเลือด (Hematopoietic Stem Cells; HSCs) และ เซลล์ต้นกำเนิดที่ไม่ใช่เม็ดเลือด (Mesenchymal Stromal Cells; MSCs) ผลการทดลองเบื้องต้นพบว่า ไวรัสสามารถเข้าและเพิ่มจำนวนใน HSCs และ MSCs และยังสามารถทำลายเซลล์ทั้งสองชนิดนี้โดยเหนี่ยวนำให้เกิดการตายแบบ apoptosis ปัจจัยหนึ่งที่ทำให้เชื้อ H5N1 เข้าสู่เซลล์ได้ดีคือ Hemagglutinin ที่อยู่บนผิวของไวรัส นอกจากนี้ยังได้ศึกษาการตอบสนองของเซลล์หลังจากได้รับเชื้อเข้าไป โดยการทดสอบปริมาณการหลั่ง cytokine พบว่า HSCs และ MSCs ไม่สามารถผลิต cytokine ได้ แต่อย่างไรก็ตาม เซลล์ MSCs ซึ่งมีคุณสมบัติลดการตอบสนองของภูมิคุ้มกัน สามารถหลั่งสาร cytokine และ chemokine ในปริมาณสูงเมื่อเลี้ยงร่วมกันกับเซลล์ในระบบภูมิคุ้มกัน (ในการศึกษานี้คือโมโนไซต์) ในสภาวะที่มีเชื้อ H5N1 จึงเป็นไปได้ว่าเชื้อ H5N1 สามารถเปลี่ยนคุณสมบัติของการลดระบบคุ้มภูมิกันมาเป็นการทำให้เกิดปฏิกิริยาตอบสนองทางภูมิคุ้มกันที่รุนแรงมากขึ้น การศึกษานี้สามารถอธิบายการเกิดพยาธิสภาพของไวรัสไข้หวัดนกในไขกระดูก ที่อาจนำไปสู่สภาวะเม็ดเลือดต่ำอย่างผิดปกติและการอักเสบจากการหลั่ง cytokines ได้

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LIST OF ABBREVIATIONS

%	Percent
/	Per, in, or
+	Plus
×	Multiplication
AMV	Avian Myeloblastosis Virus
Arg	Arginine
Asn	Asparagine
BM	Bone marrow
˚C	Degree Celsius
CB-MSC	Cord blood-derived mesenchymal stem cell
cDNA	Complementary DNA
CO_2	Carbondioxide
CPE	Cytopathic effect
cRNA	Complementary RNA
DTT	Dithiothreitol
DAPI	4',6-diamidino-2-phenylindole
DC-SIGN	Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin
dNTP	Deoxyribonucleotide triphosphate
DMEM	Dulbecco's Modified Eagle's Medium
dsRNA	Double-stranded Ribonucleic acid
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
Gln	Glutamine
Glu	Glutamic acid
HA	Hemagglutinin

LIST OF ABBREVIATIONS (cont.)

HSCs	Hematopoietic stem cells
ICAM-1	Intercellular Adhesion Molecule 1
IFN	Interferon
IL-6	Interleukin 6
IL-8	Interleukin 8
IP-10	Interferon-inducible protein-10
Leu	Leucine
Lys	Lysine
M protein	Matrix protein
MCP-1	Monocyte chemotactic protein-1
MDCK cells	Madin-Darby Canine Kidney Epithelial Cells
MEM	Minimum Essential Medium
MIP-1β	Marcophage Inflammatory Protein-1β
MSCs	Mesenchymal stem cells
MoDCs	Monocyte-derived dendritic cells
M.O.I.	Multiplicity of infection
min	Minute
ml	Minlilitre
mM	Millimolar
mRNAs	Messenger Ribonucleic acids
NA	Neuraminidase
NEP	The nuclear export protein
NP	Nucleoprotein
NK cells	Natural killer cells
ng	Nanogram
nm	Nanometer
PBMCs	Peripheral Blood Mononuclear Cells
PB-MSC	Peripheral blood-derived mesenchymal stem cell

LIST OF ABBREVIATIONS (cont.)

PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PE	phycoerythrin
PFU	Plaque forming unit
RER	Ribosomes bound to the ER
RIG-I	Retinoic acid inducible gene-I
RNA	Ribonucleic acid
RNase	Ribonuclease
RNP	Ribonucleoprotein
RT-PCR	Reverse transcription polymerase chain reaction
SA	Sialic acid
sec	Second
TNF-α	Tumor necrosis factor-alpha
U	Unit
UBC	Umbilical cord blood
ug	Microgram
ul	Microlit
uM	Micromolar
Val	Valine
VCAM-1	Vascular cell adhesion protein 1
WHO	World Health Organization

CHAPTER I
INTRODUCTION

Highly pathogenic avian influenza A virus H5N1 subtype is endemic in poultry that crossed the avian-human species barrier and has emerged as a highly fatal infectious disease in the human population with 60% mortality since 2003 from more than ten countries reported to the World Health Organization (1, 2). The pathologic process of H5N1 patients is presented with fever and respiratory symptoms including cough and shortness of breath (3). In addition, severe cases were characterized by fulminant viral pneumonia, acute respiratory distress syndrome, multi-organ failure and death (4).

Upon infection, the virus can spread from lung to other organs (5) and can also pass to the fetus (4, 5), causing a systemic disease leading to unusual high mortality. The fatal outcome of H5N1 virus infection was linked to a high viral load and hypercytokinemia (4, 6). H5N1 infection was also associated with hematologic abnormalities such as leucopenia, thrombocytopenia, pancytopenia and reactive hemophagocytic syndrome (7, 8). Furthermore, depletion of lymphocytes in spleen and peripheral blood was observed along with viremia (6). Interestingly, H5N1 viral antigen was found in hematopoietic cells in bone marrow (9), which supported an extra-pulmonary infection of H5N1 virus particularly in BM organ. In accordance with hematologic abnormalities in severe H5N1 patients, it is possible that H5N1 virus is capable of invading to BM and inducing BM suppression.

Bone marrow is an important source that all cells are generated, contains two types of progenitor cells which are hematopoietic stem cells and non-hematopoietic stem cells that can differentiate into blood cells (monocytes, macrophages, neutrophils, eosinophils, erythrocytes, platelets) and mesenchymal lineages (osteoblasts, chondrocytes, myocytes, adepocytes, tenocytes and neuronal cells), respectively (10). Hematopoietic stem cells (HSCs) can be isolated from either blood or bone marrow. HSCs are capable of self-renewal, differentiating to a variety

of specialized cells, and mobilizing out of the bone marrow into circulating blood. CD34 is the most commonly used marker for HSCs (11, 12). Recently, umbilical cord and placenta were reported as rich sources for HSCs (13). There have been suggestions that umbilical cord blood contains stem cells that have the capability of developing cells of multiple germ layers (multipotent) or even all germ layers (pluripotent), e.g., endoderm, ectoderm, and mesoderm (14).

Mesenchymal stem cells (MSCs) are non-hematopoietic cells which can give rise to mesenchymal lineage. MSCs share key biological characteristics as accepted stem cells criteria; having the capacity for extensive self-renewal and for originating at least one type of differentiated descendant (15). MSCs were also isolated and cultured from many other species including mice, rats, cats, dogs, rabbits, pigs, and baboons (16)**.** MSCs are lacking of specific and unique markers when cultured *in vitro*. There is a general consensus that human MSCs express CD105, CD73, CD44, CD90, CD71, CD271 and Stro-1 as well as intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1. However, they do not express the hematopoietic markers CD45, CD34, CD14, CD31, and the co-stimulatory molecules such as CD80, CD86 and CD40, which discriminate MSCs from HSCs, endothelial cells, monocytes, and erythrocytes respectively. In BM, MSCs act as supporting cells that regulate normal hematopoiesis processes including growth, maturation, differentiation and survival of HSCs (17). Due to differentiation property, MSCs can regenerate wound or damaged tissues by differentiating into that damaged phenotypes (18). MSCs also exert immunomodulatory activity by suppressing NK, T, B and monocyte-derived dendritic cells (MoDCs) proliferation and function (19-21). This property appears to be more important in therapeutics for suppressing the immune response invoked in diseases such as tissue injury, transplantation, and autoimmunity (22).

Due to BM contains hematopoietic and non-hematopoietic stem cells (23) which are precursors of all cells and tissues, it was hypothesized that H5N1 virus was able to disseminate BM tissue and use stem cells as targets for dysregulation of both hematopoiesis and mesengenic processes resulting in hematologic abnormalities. In addition, H5N1 virus might induce hyper-inflammatory cytokines secretion as it has been known as a potent cytokine inducer. We, therefore, investigate the susceptibility

of BM stem cells to H5N1 infection which the outcome following infection possibly contributes to BM suppression.

CHAPTER II
OBJECTIVES

1. To isolate and characterize two stem cell types derived from cord blood and bone marrow
2. To investigate the susceptibility of $CD34^{+}$HSCs and MSCs to avian influenza viruses compared with human influenza viruses (H1N1 and H3N2)
3. To examine the contribution of the viral surface glycoproteins HA in the infection of MSCs
4. To study the outcome of H5N1 infection in HSCs and MSCs
5. To investigate the role of HSCs and MSCs on H5N1 pathogenesis

CHAPTER III
LITERATURE REVIEW

3.1 The influenza virus classification and nomenclature

Influenza viruses are enveloped RNA viruses which belong to the family of Orthomyxoviridae. According to the antigenic differences in Nucleoprotein (NP) and matrix (M) proteins, influenza viruses can be classified as type A, B, and C. Influenza A viruses infect multiple species of mammals, including humans, horses, pigs, ferrets and birds, and they are maintained in wild waterfowl and shore birds. Influenza A viruses are considered to be the most virulent group. Influenza A viruses are further divided into subtypes based on the antigenicity of their two surface proteins hemagglutinin (HA) and neuraminidase (NA). To date, 16 HA subtypes (H1-H16) and 9 NA subtypes (N1-N9) of influenza A virus have been identified (24). All these subtypes are present in aquatic birds and ducks which are the natural hosts and reservoirs of influenza viruses in nature. However, only three HA (H1-H3) and two (N1-N2) subtypes have established in human. The host range of different influenza viruses is specified by different forms of sialic acid presented on the cell glycoproteins. Avian viruses preferentially bind to avian cells while human viruses preferentially bind human cell receptors. However, porcine cells possess both human and avian type sialic acids on their surface and are susceptible to infection with both avian and human influenza viruses. Therefore, pigs are regarded as the mixing vessel where avian and human influenza viruses can reassort to generate novel pandemic strains of influenza (25). Influenza B viruses infect mostly humans. They are quite common, but clinical disease is usually less severe than influenza A (26). Influenza C virus has been identified in humans. They are associated with mild common cold-like illness or no clinical symptoms. So far, no antigenic subtypes have been identified among the influenza B and C virus. According to the World Health Organization (1), the standard nomenclature for influenza viruses consists of type of virus (A, B, and C), the host origin (excluding human), the place of isolation, the strain number, the year of

isolation and each is separated by slash. Influenza A viruses the HA and NA subtypes are put in parenthesis. For example, a type A H5N1 virus isolated in Nakhonsawan, from an Asian open-billed stork in 2004 with a strain number BBD0104F, would be designated A/open-billed stork/Nakhonsawan/BBD 0104F/04 (H5N1).

3.2 Influenza virus structure, genes, and proteins

Influenza virus particles are roughly spherical with a diameter 80-120 nm, however, newly isolated strains are pleomorphic and contain long filamentous particles (27). Viral particles contain negative sense single-stranded RNA divided into eight segments for type A and B or seven segments for type C. The outer surface of viral particle is a lipid envelope which is derived from host cell membrane. For influenza A and B, two distinct types of spikes, hemagglutinin (HA) and neuraminidase (NA) glycoproteins, reside on the surface of virion. Influenza C virus, by contrast, contains one type of glycoprotein on membrane called hemagglutin-esterase-fusion protein (HEF), which performs of both HA and NA activity. The HA spike has rod-shaped character and protrudes from the viral envelope as trimer, while the NA spikes are mushroom-shaped tetramer with stalk (28). The length of HA and NA spikes that protrude from the viral surface is range from 10 to 12 nm. The mean ratio of HA and NA is 5:1. HA is a type I glycoprotein (containing an N-terminal ectodomain and a C-terminal anchor) involving in viral attachment to terminal sialic acid residues on host cell glycoproteins and glycolipids. It is the main target for host immune system and changing in antigenicity of HA can make the virus escape from host immunity. NA is type II glycoprotein (containing N-proximal anchor and C-terminal ectodomain), transmembrane protein containing a head domain which is enzymatically active and a stalk region which is attached to the membrane. Activity of NA is to facilitate the release of progeny viruses from infected cells by catalyzing the cleavage of glycosidic linkages on host cell and viral surface. In mucous of respiratory tract, removal of sialic acid can lower the viscosity and facilitate the virus to access to the epithelial cells. Besides HA and NA proteins, M proteins are encoded from viral RNA segment 7. M1 forms dimer underneath the viral envelope and interacts with both RNP and the cytoplasmic tails of the surface glycoproteins to maintain the

structure of the virion. M2 protein is another integral protein that decorates the surface envelope. M2 protein has an ion channel activity which will be activated by the low pH in endosome resulting in the influx of proton into viral particle. Increased intra-virion acidity is required for the uncoating process during the early stage of viral replication (29). The genome of influenza virus is segmented, consisting of 8 single-stranded negative sense RNA molecules which encode for 11 proteins. The RNA segments are contained within the viral envelope in tight association with the nucleoprotein (NP) and three subunits of viral polymerase (PA, PB1, and PB2), which together form the ribonucleoprotein (RNP) complex. The matrix protein (M1 and M2) lies inside the lipid envelope and plays an important role in viral assembly (30). Another RNA segment consists of non-structural gene which encodes NS1 and the nuclear export protein (NEP), which supports nuclear export of the viral genome by alternative RNA splicing. NS1 is expressed in high amounts in infected cells but it has not been detected in virion. NS1 can repress IFN synthesis by inhibiting the activation of interferon regulatory factor3 and NF-κB signaling pathway, whereas NEP involves in nuclear export of RNA by acting as an adaptor molecule between viral RNP complex and the nuclear export machinery of the infected cell.

3.3 Replication cycle of influenza A virus

Influenza A virus binds to target cell through interaction between HA molecules and sialic acid (N-acetyl neuraminic acid) receptors on the host cell surface. After binding, the virus is then internalized to a host cell endosome via receptor-mediated endocytosis. Acidification of the vesicle (pH 5.0) triggers the fusion of the viral and endosomal membranes. The low pH induces a conformational change in HA0, which is cleaved by trypsin-like enzymes into HA1 and HA2 leading to expose the HA2 fusion peptide. This fusion peptide inserts itself into the endosomal membrane, bringing both the viral envelope and endosomal membranes to contact with each other. The acidic environment of the endosome is also important for open up the M2 ion channel, whose transmembrane domains form a channel that acts as a proton-selective ion channel. Opening the M2 ion channels acidifies the viral core by producing proton influx into the inside of virion. This mechanism causes the

dissociation of the viral ribonucleproteins (vRNPs) from the matrix (M1) protein. The vRNP is free to enter the host cell's cytoplasm, then transported to nucleus. As influenza virus genome is made up from negative sense strands of RNA, it is first converted into a positive sense RNAs which are messenger RNAs (mRNAs) and complementary RNAs (cRNAs) to serve as a template for the production of viral RNAs. The viral mRNAs are primed by 5' capped fragment derived from newly synthesized host mRNA and polyadenylated at the 3' end. This functional viral mRNAs are then transported to cytoplasm where translation occurs. The mRNAs encoded for HA, NA, M2 are translated by ribosomes bound to the ER (RER) and the protein enter the host cell secretory pathway, where HA and NA are glycosylated. Other proteins are translated by free ribosomes in cytoplasm. The viral polymerase protein (PA, PB1, and PB2) and nucleoproteins (NP) are imported into the nucleus, where they participate in the synthesis of full length (+) strand RNAs and then (–) strand genomics RNAs. The M1 and the NS protein are also transported into nucleus to shut down viral mRNA synthesis after binding to the (-) stranded viral RNAs. The M1 together with NS2 (nuclear export protein or NEP) exports vRNPs to the cytoplasm. The final assembly step occurs at the host cell plasma membrane exposing the newly synthesized HA, NA, and M2 proteins. The vRNPs are incorporated into the particle as it buds out from the plasma membrane. The virions are initially aggregated at the host cell membrane, but the neuraminidase activity of NA protein cleaves these sialic acids, releasing infectious virions.

3.4 Influenza outbreaks

Influenza pandemic is caused by viruses that possess an HA molecule in which most of the human population lacks immunity. During the 20^{th} century, influenza viruses emerged to cause major pandemics three times; the 1918 Spanish flu, the 1957 Asian flu, and the 1968 Hong Kong flu. The 1918 Spanish flu caused an estimated 20-50 million people died. The 1957 and 1968 flu, 0.5-1 million people died. The causative viruses of these three pandemics were originated from avian influenza viruses.

3.4.1 *1918 Spanish flu*

The most devastating influenza epidemic spread in three distinct waves during 1918-1919, across Europe, Asia and North America, known as Spanish influenza. This pandemic caused by H1N1 virus. The Spanish influenza virus was extremely virulent and caused 50-100 million deaths. The majority of deaths during the 1918 pandemic were among young and healthy persons age 15 to 35 year old and 99% of deaths occurred in people younger than 65 years (31). The recovery of the genomic RNA of the 1918 from formalin-fixed autopsy specimens and frozen lung tissue from influenza infected patient who was buried in permafrost has enabled the complete sequence of all eight genomic segments of 1918 H1N1 virus (32). According to this investigation, it suggested that the 1918 virus was more likely an avian-like virus which adapted itself in humans through mutation.

3.4.2 *1957 Asian flu and 1968 Hong Kong flu*

The pandemic 1957 (Asian influenza H2N2) and 1968 (Hong Kong influenza H3N2) emerged in southeastern Asia as a result of a reassortment between avian viruses and circulating human influenza strains (33). The Asian 1957 pandemic was caused by H2N2, and it was the first pandemic virus that was isolated. Three genes (HA, NA, and PB1) came from avian viruses and others derived from circulating human influenza strains. Similarly, HA and PB1 genes of H3N2 Hong Kong came from avian strains in a background of human H2N2 circulating at that time (34). The H2N2 virus disappeared with the emergence of H3N2 virus. These two pandemics cause mild clinical disease. The death number of H3N2 pandemic was fewer than that of 1957 virus. This due to the change of HA from H2 to H3 while the N2 stayed the same. People previously infected with an H2N2 virus developed antibody to the N2 surface protein and may have some cross protection against the H3N2 virus (35). Although 1957 and 1968 pandemics caused high morbidity and mortality rate, they were much less virulent than 1918 influenza pandemic.

3.4.3 *H5N1 avian influenza virus*

In 1997, an H5N1 avian influenza virus emerged in Hong Kong and caused high lethality in poultry. Three chicken farms were separately affected; the

death rate exceeded 70% of the total 6,800 chickens. Moreover, this virus gains ability to infect human with 18 cases of human H5N1 infections were identified, 6 of which were deaths. All clinical cases had history of exposure to infected poultry. The outbreak was ended by the slaughter of all poultry in Hong Kong. Although H5N1 viruses have not been detected since the poultry slaughter in late 1997, its putative precursors continue to persist in the region which different reassortants continued to emerge from goose and duck reservoirs that contained the same H5 HA but had different internal genes. In 2002, a new genotype was generated and killed most avian species in Hong Kong nature parks. This genotype of H5N1 spread to humans in Hong Kong in February 2002 and killed one of the two infected persons and was the precursor of genotype Z which contains both NA and NS1 deletions (36). The Z genotype extended progressively to domestic poultry in Southeast Asia which included Thailand, Vietnam, Indonesia, Cambodia, Laos, and Malaysia. H5N1 also spread to Russia, Europe, Africa, and the Middle East during 2005 and 2006. Until November 29, 2011, the WHO had confirmed 571 cases from 15 countries worldwide with 335 deaths (2). However, the ability of H5N1 virus to cause a human pandemic is restricted because the H5N1 HA in the virus has its specificity for avian receptor (α2,3-SA) (37), whereas, human receptors (α2,6-SA) are distributed in human upper respiratory tract. Nevertheless, the mutation in the receptor binding site of H5N1 HA has been shown to change the binding specificity from α2,3 to α2,6 receptors (37-39). All the evidence may manifest the potential of H5N1 virus to infect humans and cause pandemic.

3.4.4 *Novel Swine-Origin Influenza A (H1N1) Virus*

Swine Influenza is an acute respiratory disease caused by influenza A viruses that circulate among pigs. Virus can occasionally infect other species including humans. In April 2009, a novel virus with subtype H1N1 began circulating in humans, causing the first pandemic of 21st century. This novel virus represents a quadruple reassortment of two swine strains, one human strain, and one avian strain of influenza (40). The initial outbreak occurred in Mexico, and it has been reported sporadically in humans. In June, 2009, the WHO raised the pandemic alert to the level 6, which is the highest level (41). The majority of cases have been mild and uncomplicated.

Secondary bacterial infections have contributed to severe cases and deaths. The risk of severe illness has been greatest in children under the age of 2 years and immunocompromised patients. The pandemic was declared to be over in August, 2010 (42). It is now clear that the flu appears to kill about one of 2000 people which is the least lethal in modern pandemic.

3.5 Viral pathogenesis and Pathogenic determinants of H5N1

H5N1 virus has caused the most numerous and severe human infections. The majority of human infections have been the result of exposure to H5N1 virus-infected poultry, whereas the evidence for person-to-person transmission is limited. The virus isolated to date from humans lacks of the ability for sustained transmission in humans. In general, patients infected with H5N1 viruses presented with fever, respiratory symptoms including cough and shortness of breath gastrointestinal symptoms including diarrhea (43), and in one pediatric case, neurological complications. Leukopenia and lymphopenia have been associated with a poor prognosis. Severe cases were characterized by pneumonia, multi-organ failure, and in some cases acute respiratory distress syndrome (44) and death. The average incubation period is 2–5 days, and for the fatal cases, mortalities are commonly associated with secondary bacterial infections and frequently occur in patients with old age. However, the highest case fatality rate among H5N1-infected patients is among individuals 10–19 years of age (45). Post-mortem examinations conducted among H5N1 virus victims have revealed diffuse alveolar damage with hyaline membrane formation, interstitial lymphocytic infiltrates, bronchiolitis, pulmonary congestion, hemorrhage, and alveolar damage with macrophage, neutrophil (4). Apoptosis in alveolar cells, leukocytes infiltration and lymphocyte depletion in lymphoid organs have been detected (46). Reactive histiocytes with hemophagocytic activity have been noted in the spleen, lymph node, bone marrow, lungs, and liver (7). Both hypercellular and hypocellular bone marrow have been reported (7, 47). Hemophagocytosis, a complication characterized by excessive activation of phagocytosis of erythrocytes, platelets, and leukocytes, and one which is thought to be cytokine-driven, has been observed among the limited H5N1 fatal case studies (7). This symptom is correlated with abnormal

hematologic findings in H5N1 patients with severe disease which often have lymphopenia, thrombocytopenia, and increased levels of serum aminotransferases (6, 48, 49). Fatal H5N1 virus disease was associated with high viral load in the pharynx, more pronounced cytokine dysregulation, and more frequent detection of viral RNA in plasma compared with non-fatal H5N1 cases (6). There are several reports of extrapulmonary infection since viral RNA was detected in multiple non-respiratory organs including intestines, liver, spleen, brain and vertical transmission to a fetus suggesting widespread viral dissemination but more data are needed to confirm dissemination of H5N1 viruses in humans (3, 7, 50, 51). The isolation of H5N1 viruses from plasma suggests that the blood is a route of dissemination from primary infected (respiratory tract) sites to other organs. However, specific pathological changes were present only in the respiratory system (52). Identification of virulence determinants of disease would help in better understanding of the disease caused by influenza viruses observed in humans. Many viral genes can contribute to the pathogenesis of H5N1 virus. Multiple molecular determinants affecting virus virulence have been identified

3.5.1 Hemagglutinin

3.5.1.1 Hemagglutinin cleaveage site: HA cleavability is a critical determinant of HPAI H5N1 influenza virus pathogenicity in poultry and mammals. In the viral replication cycle, HA (HA0) is cleaved into disulfide HA1 and HA2 by host protease. This mechanism generates a fusogenic domain at the amino terminus of HA2 which mediates the fusion between the viral envelope and host endosomal membrane (53). Thus, the cleavage of HA is a prerequisite for initiating infection and is an important determinant for viral infectivity and dissemination of influenza virus. The low pathogenic H5N1 influenza virus which contains one arginine at the cleavage site is cleaved by an extracellular trypsin-like protease that is restricted in respiratory and intestinal organs, resulting in mild localized infections (54, 55), whereas HA of highly pathogenic H5N1 virus which contains polybasic amino acid at the cleavage site is cleaved by ubiquitous intracellular protease in a wide range of tissue distribution, enabling the virus to infect many cell types outside the respiratory and gastrointestinal tract and causing systemic infections (56).

3.5.1.2 Hemagglutinin receptor specificity: the HA protein is important determinant for host range because it is involved in host-cell receptor recognition. Human influenza viruses preferentially recognize glycoprotein or glycolipid receptors terminating with α2,6-linked sialic acid (SA) which are found predominantly on epithelial cells of human airway. Whereas avian influenza viruses have a binding preference for cell receptors containing terminal sialic acid that connects to galactose residue via α2,3 linkage. This type of receptor is found on epithelial cells in the intestine and respiratory tract of birds (57, 58). All avian H5N1 viruses isolated from Hong Kong in 1997, including the human isolates preferred to recognize α2,3 likage containing receptors but not to α2,6 linkage containing receptors (57). Recently, α2,3-linked sialic acids were identified on ciliated cells in human tracheobronchial epithelium which avian viruses can infect these cells (59, 60). The distribution of SA in human airways is α2,3-linked sialic acids are in lower repiratory tract while α2,6-linked sialic acids are in the upper respiratory tract (61). Thus, this can restrict the virus to transmit efficiently from human to human. However, some H5N1 isolates in 2003 and 2004 have HA mutations that allow binding to both types of α2,3 and α2,6 linkages. Those mutations, Gln182Arg, Asn192Lys, Leu129Val and Arg134Val demonstrated that avian influenza H5N1 virus could be adapted to the human-type receptor (62, 63). First two mutations were observed in human isolates but not found in any avian isolates indicating that these mutations may be selected during an early phase of human infection. Besides, the virus isolated from the 1918, 1957 and 1968 pandemics preferentially recognized α2,6-linked sialic acids, although their HA were from an avian virus (33). This indicates that conversion of receptor specificity to α2,6 likage is an alteration that might be necessary for the generation of a virus with pandemic potential.

3.5.2 Neuraminidase

Neuraminidase contains sialidase activity which is responsible for the virus binding, releasing and spreading from infected cell by cleaving the surface sialic acid. To obtain high pathogenicity, the receptor binding properties of HA should be compatible with the cleavage specificity of NA because virus needs the cleavage of the receptor by NA to bud out from the cell surface (64). NA can cleave sialic acid in

both α2,3 and α2,6 linkages. There was one evidence showing that α2,6 cleavage activity has been increased during N2 avian influenza virus evolution in humans, thus, giving the benefit to human virus which its HA has α2,6 linkages receptor specificity (65). The NA stalk is an important determinant since its sequence and length vary in viral strains. A short stalked NA is not efficient in releasing progeny viruses because of the difficulty for active site of enzyme in accessing its substrate (66). In addition, NA stalk deletion can decrease viral growth in eggs and can reduce virulence in mice (67). However, H5N1 avian influenza virus that having a short NA stalk are highly virulent in poultry, meaning a long stalk may not necessary for virulence in chickens (68). The H5N1 virus isolated during 1997 outbreak contain 19 amino acid deletions in stalk region. It was suggested that hyperglycosylation at the globular head of HA (decreased the affinity of the virus for cell receptor) combined with 19 amino acid deletion in the stalk of the NA (decrease its ability to release the virus from the cells) might be required for the adaptation of H5N1 from wild aquatic birds to poultry and raises the possibility the chickens may be a plausible intermediate host in zoonotic transmission. This effect suggested that virulence of avian influenza requires both effective binding of the virus to the receptor and efficient release of virus from infected target cells, however, this was achieved by compensating changes in affinity of HA and activity of NA to gain balance of these two molecules.

3.5.3 Polymerase proteins

Changes in the viral polymerase complex, especially in PB2, are important for the adaptation of avian influenza viruses to replicate in mammalian hosts. Two H5N1 avian influenza viruses from humans in 1997, which have polybasic amino acid at cleavage site of HA, were varied in their virulence in mice. The A/Hong Kong/483/97 was able to cause fatal systemic infection in mice but A/Hong Kong/486 /97 causes a non lethal respiratory infection. Mutational analysis by reverse genetic system revealed that a mutation at position 627 from Glu to Lys in the PB2 protein influenced the outcome of infection in mice (69). The presence of lysine also led to more aggressive viral replication by enhancing replication in mammalian cells. Interestingly, H5N1 virus with E627K in PB2 can reverse the cold-sensitivity of mammalian system, resulting in efficient replication of H5N1 virus at lower

temperature. This suggests the significance of PB2 protein in efficient human to human transmission by increasing viral replication in upper respiratory tract. PB1 could provide an advantage to the influenza virus in the analysis of replication efficiency of reconstituted RNPs (70). Experiment in mice was conducted and found that Lys at position 198 and Ile at position 317 of chicken and human H5N1 in Hong Kong 1997 isolates were associated with high pathogenicity (71). Moreover, an amino acid change in PB1-F2 at position 66 from Asn to Ser was correlated with high pathogenicity. This position also found in H5N1 (HK/97) virus and 1918 H1N1 virus (72).

3.5.4 Non-structural protein

NS1 is associated with the virulence of influenza viruses as it is thought to cause cytokine dysregulation which may play an important role in the pathogenesis of viral infection. The NS1 was shown to up-regulate the expression of pro-inflammatory cytokines in mice and directly contributed to the virulent phenotype. NS1 also protects virus from antiviral effect of IFN signaling. NS1 inhibits the activation of host innate immune response by sequestering dsRNA and by interacting with RIG-I, a cellular sensor of virus infection, leading to the suppression of IFN-α/β response (73, 74). Moreover, NS1 interacts with components of cellular pre-mRNA processing machinery including cleavage and polyadenylate specificity factor (CPSF), thus it inhibits the 3'-end processing and the nuclear export of cellular mRNA. NS1 from H5N1 1997 isolates were shown to implicate in the virulence by helping the virus to resist anti-viral effects of interferons and tumor necrosis factor alpha. A previous study showed that pigs infected with recombinant human H1N1 influenza virus that carried the H5N1 NS1 gene which had glutamic acid at position 92 experienced greater and more prolonged viremia, fever, and weight loss than did pigs infected with wild-type (75). Also, Ala at a position 149 of H5N1 NS1 protein is critical for the pathogenicity of avian influenza virus in chickens by inhibiting interferon induction in chicken embryo fibroblast cells (76). Therefore, NS1 may serve as additional marker for the pathogenicity due to its ability to evade the innate immune response, resulting in high virulence and severe symptom in human infection.

3.6 Target cells and tissue tropism

Human influenza viruses replicate through entire the respiratory tract, with virus being recoverable from the upper respiratory tract and lower respiratory tract. Human influenza virus specific antigen is present in types I and II alveolar epithelial cells, as well as in intra-alveolar macrophages (27). In contrast, H5N1 virus preferentially infects cells in the lower respiratory tract where it attaches predominantly to type II pneumocytes, alveolar macrophages and non-ciliated cuboidal epithelial cells in terminal bronchioles (61, 77). Cells of the whole respiratory tract harbor α2,6 SA receptor for human influenza viruses, whereas cells of the lung alveoli express also the α2,3 SA receptor for avian influenza viruses. The infection of type-II pneumocytes and alveolar macrophages with H5N1 viruses may contribute to the severity of pulmonary lesions (77). Damage of type-II pneumocytes may impair their function, including re-epithelization after alveolar damage, ion transport and surfactant production. This may result in inhibition of tissue repair. Infection of macrophages may compromise their function in innate immune reactions and aggravate inflammatory responses to H5N1 infection. The restriction of H5N1 infection to human lower respiratory tract may contribute to inefficient human-to-human transmission of H5N1 viruses. One could speculate that, if H5N1 mutates to a human-adapted, virus easily bind to mammalian α2,6 SA receptor, infectivity will dramatically increase, due to ability of the virus to replicate in upper respiratory tract and easily spread to another host by sneezing and coughing. This notion is supported by the finding that earliest isolates in 1918 (78), 1957 and 1968 pandemic preferentially recognized α2,6 SA (79), even though their HAs were derived from avian viruses. Point mutations (“antigenic drift”) may be responsible for the adaptation of H5N1 virus to infect humans. The change of one amino acid of the H5 protein could be potentially sufficient to change the receptor specificity of H5N1 viruses (80). In addition, H5N1 viruses might overcome their inefficient transmission to humans by reassortment with human viruses (“antigenic shift”) as was the case for the pandemics of 1957 and 1968 caused by avian-human reassortant influenza viruses (81). In a comparative ferret model that parallels the efficient transmission of H3N2 human viruses and the poor transmission of H5N1 avian viruses in humans. Reassortants of human and avian influenza viruses were studied, H3N2 reassortant virus with avian

virus internal protein genes exhibited efficient replication but inefficient transmission, whereas H5N1 reassortant virus with four or six human virus internal protein genes exhibited reduced replication and no transmission. These findings indicated that the human virus H3N2 surface protein genes alone did not confer efficient transmissibility and that acquisition of human virus internal protein genes alone was insufficient for the H5N1 virus (A/Hong Kong/486/97 strain) to develop pandemic capabilities, even after serial passages in a mammalian host (82). These findings suggest that reassortant viruses would likely need more genetic changes, such as ones that make the 1957 and 1968 strains better able to bind to human respiratory tract epithelial cells. In addition, recent investigations demonstrated that ex vivo cultures of human nasopharyngeal, adenoid and tonsillar tissues could be infected with H5N1 viruses in spite of an apparent lack of α2,3 SA. This implies that there might be other binding sites on the epithelium that mediate virus entry. Due to similar replication ability in the alveolar epithelium between human H1N1 and avian H5N1 viruses, it might be concluded that the increased severity of human H5N1 influenza could not be explained purely on the basis of a differential tropism of H5N1 to the lower respiratory tract epithelium (83).

3.7 Human cytokine response in H5N1 infection

Inflammation is widely thought to contribute to severe influenza pathogenesis. Specifically, dysregulated cytokine production is positively correlated with clinical severity of HPAIV H5N1 infection and infection with reconstructed 1918 pandemic influenza virus (H1N1) in animal models. Importantly, the degree or magnitude of dysregulated cytokine production fails to correlate with viral titers in both animal models (51, 84) and H5N1 influenza in humans (85, 86). H5N1 viruses are believed to induce a “cytokine storm”, a condition characterized by dysregulated production of pro-inflammatory cytokines which has repeatedly been observed in infected human from the earliest H5N1 outbreaks in 1997 onwards (85). H5N1-induced hypercytokinemia has also been observed in mouse models (87) and in cell culture (88). In these H5N1 studies, levels of cytokines, including IP-10, RANTES, MIG, MCP-1, IL-8, IL-10, IL-6, IFN-γ, TNF, MIP-1α and soluble IL-2, were elevated compared with other influenza strains. Severe H5N1 infected cases present with

pneumonia, multi-organ failure, and acute respiratory distress syndrome (44), a symptom that is characterized by alveolar hemorrhage and massive mononuclear cells infiltration in lung (89, 90). This symptom is a cytokine-driven condition. The multi-organ involvement is also associated with dysregulated immune response resulting in extrapulmonary organ failure including heart and kidney. One of severe manifestations in fatal cases is hemophagocytosis, a condition with excessive mononuclear phagocytosis of erythrocytes, platelets and leukocytes. This characteristic is thought to be a cytokine-driven symptom associated with elevated levels of sIL-2r, IL-6, IFN-γ, and TNF-α (7). The significance of each elevated cytokine in influenza cytokine dysregulation is not well defined. For example, Peiris *et al.* found significantly elevated levels of IP-10 and MIG, but no significant differences in levels of MCP-1, RANTES and IL-8 in patients with H5N1 virus infection compared with patients infected with other influenza viruses (91), while Perrone *et al.* did not observe significantly elevated levels of IP-10 or MIG, but rather significantly elevated levels of MIP-1α, KC (mouse IL-8), IL-1α, IFN-γ and IL-6 in HPAIV-infected mice (87). Cheung *et al*. demonstrated that H5N1/97 human isolate induced much higher pro-inflammatory cytokine gene expression, especially TNF-α and IFN-β in human macrophage than did H3N2 and H1N1 human influenza viruses (85). This finding emphasizes the role of macrophages and their ability to produce cytokines and chemokines in response to H5N1 infection. In addition, H5N1/97 and H5N1/04 human isolates were capable of inducing high levels of IP-10, IFN-β, IL-6 and RANTES production and also with mRNA expression compared with human H1N1 virus. Therefore, dysregulated host immune response is responsible for severe pathogenicity caused by H5N1 infection.

3.8 Systemic infection

Avian influenza (H5N1) virus is known to infect and replicate in lower respiratory tract of human due to its widely distributed sialic acid receptors in those tissue. Not only local infection, H5N1 virus can spread to internal organ. The mechanism of systemic spread has not been established, some studies suggested that DC-SIGN, a member of the C-type lectin family could serve as vehicle for systemic

spread via interaction with HA which in turn, facilitating capture and attachment of virus particles to dendritic cell (92). In addition, Yen L.H. *et al*, identified that a minor change in receptor binding domain on HA might modulate the virulence of H5N1 virus by affecting the spread of virus from respiratory tract to other tissues (93). The H5N1 dissemination in infected patients was examined by the immunohistochemical localization of viral antigen in other tissues beside lung and respiratory tract. Interestingly, viral nucleoprotein was detected in the small and large intestinal epithelial cells, hematopoietic cells in BM, glial cells and neurons of the brains, although viral culture was found only in the lung and pathology in multiple extrapulmonary organs was not much changed (52). Since fatal cases of H5N1 patients showed reactive hemophagocytosis in post-mortem biopsy, accompanied with abnormal hematologic findings such as leucopenia, lymphopenia and thrombocytopenia (7), this finding indicated that H5N1 virus disseminated to BM and altered normal BM environment. However, the direct infection as well as BM suppression mechanism is still elusive.

3.9 Bone Marrow microenvironment

Bone marrow (BM) is the soft tissue residing in the cavities of the bones housing the essential pluripotent precursor cells for the living organism. BM contains at least two kinds of stem cells. One population is hematopoietic stem cells (HSCs) which have the role of renewing the elements (monocytes, macrophages, neutrophils, eosinophils, erythroblasts, erythrocytes, megakaryocytes, platelets) in the blood. A second population is bone marrow stromal stem cells (also called mesenchymal stem cells. These non-hematopoietic stem cells make up a small proportion of the stromal cell population in the bone marrow, and can generate bone, cartilage, fat, cells that support the formation of blood, and fibrous connective tissue *in vivo* and *in vitro*.

3.9.1 Hematopoietic Stem Cells (HSCs)

Hematopoietic stem cell (HSC) is the common ancestor of all types of blood cells. It is one of the best-characterized stem cells in the body. It can be isolated from the blood or bone marrow. The unique capacity is to renew itself, differentiate to

produce all mature blood cell types, and mobilize out of the bone marrow into circulating blood. Identifying and characterizing properties of HSCs began with studies in mice, which laid the groundwork for human studies. The classic source of HSC is bone marrow. The formidable challenge is the number which is about 1 in every 10,000 to 15,000 bone marrow cells thought to be a stem cell. In the blood stream the proportion falls to 1 in 100,000 blood cells. In the late 1980s and early 1990s, physicians began to recognize that the human umbilical cord and placenta were rich sources of HSC. This tissue supports the developing fetus during pregnancy, is delivered along with the baby, and, is usually discarded. Since the first successful umbilical cord blood transplants in children with Fanconi anemia, the collection and therapeutic use of these cells has grown quickly. There is a substantial amount of research being conducted on umbilical cord blood to search for ways to expand the number of HSC and compare and contrast the biological properties of cord blood with adult bone marrow stem cells. There have been suggestions that umbilical cord blood contains stem cells that have the capability of developing cells of multiple germ layers (multipotent) or even all germ layers, e.g., endoderm, ectoderm, and mesoderm (pluripotent). Hematopoiesis or a blood cell synthesis is a dynamic process that requires the replenishment of more than 7×10^9 blood cells (leukocytes, erythrocytes and platelets) per body weight (kg) per day. Homeostasis of the hematopoietic system is considered to occur by the capacity of hematopoietic stem cells (HSCs) to undergo differentiation and self-replication (self-renewal) processes to replenish the pool of HSCs throughout life. The CD34 antigen has become the major positive marker for human hematopoietic stem and progenitor cells. Among non-hematopoietic tissues, CD34 is expressed on endothelial cells of small vessels. The biological function of CD34 on hematopoietic cells is poorly understood. HSCs in human fetal liver, cord blood, and bone marrow express CD34 antigen about 0.5–5%. $CD34^+$ cells harbor all *in vitro* clonogenic potential.

3.9.2 Mesenchymal Stromal Cells (MSCs)

Mesenchymal stem cells (MSCs) are non-hematopoietic cells which can give rise to both mesenchymal and non-mesenchymal lineage. They are rare, about 0.01%-0.001% of total bone marrow cells (94). They reside in various postnatal

organs and tissues. MSCs share key biological characteristics as accepted stem cells criteria; having the capacity of extensive self-renewal and originating at least one type of differentiated descendant (15). However, conflicts and misunderstandings about biological properties and scientific name of MSC might occur. To clearly define the terminology, Horwitz, E.M. and colleagues suggested that the fibroblast-like plastic-adherent cells, regardless of the tissue from which they are isolated, be termed 'Multipotent mesenchymal strem cells' whereas 'Mesenchymal stromal cells' specification can be used when these cells were compatible with stem cell criteria (95). MSC is an acronym that widely used for both cell populations. MSCs are typically isolated from the mononuclear layer of the BM after separation by density gradient centrifugation. The mononuclear cells are cultured in medium with 10% fetal calf serum. After culture in 2-3 passages, MSCs adhere to the tissue culture plastic, have fibroblast-like morphology and differentiate into bone, cartilage and fat cells *in vitro* (96). MSC were also isolated and cultured from many other species including mice, rats, cats, dogs, rabbits, pigs, and baboons (16). *In vitro* cultured MSC are lacking of specific and unique markers. There is a general consensus that human MSC do not express the hematopoietic markers CD45, CD34, CD14, CD31, and the co-stimulatory molecules such as CD80, CD86 and CD40, which discriminate MSCs from HSCs, endothelial cells, monocytes, and erythrocytes respectively. However, they express CD105 (SH2), CD73 (SH3/4), CD44, CD90 (Thy-1), CD71, CD271 (low-affinity nerve growth factor receptor) and Stro-1 as well as intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1. These expressed markers vary from species, tissue source and culture condition. MSC from other species do not express all the same molecules as those on human cells; for example, although human and rat MSC have been shown to be $CD34^-$, some articles reported variable expression of CD34 on murine MSC (23). It is generally accepted that all MSCs are devoid of the hematopoietic marker: CD34, CD45, and CD14 (23). Peripheral blood and Cord blood-derived mesenchymal stem cells (PB-MSC and CB-MSC) described in normal individuals, express many of the same markers as bone marrow-derived mesenchymal stem cells (BM-MSC), and also they differentiate down the osteoblastic and adipogenic lineages (97-99). However, these appear to be a different population from fibrocytes, which can differentiate into myofibroblasts and migrate to tissue but

express CD34 and CD45 whereas MSC obtained from every source are negative in both markers (17, 23). In BM, MSC act as supportive cells to regulate normal hematopoiesis process including growth, maturation, differentiation and survival of hematopoiesis cells (17). Due to differentiation property, MSC can regenerate wound or damaged tissues by differentiating into that damaged phenotypes (18). Another important function is that MSC exert immunomodulatory activities by suppressing proliferation and function of NK, T, B and monocyte-derived dendritic cells (MoDCs) (19-21).

CHAPTER IV
PART I
MATERIALS AND METHODS

4.1 Umbilical cord blood and bone marrow samples

Umbilical cord blood (UCB) was obtained from full term newborns and collected in sterile collection bags containing anti-coagulant citrate-phosphate dextrose (CPDA). BM samples were aspirated from posterior superior iliac spine of healthy volunteers. The $CD34^+$ expressing Hematopoietic Stem Cells ($CD34^+$ HSCs) were isolated from both UCB and BM sources, whereas, Mesenchymal Stromal Cells (MSCs) were BM derived. Stem cells were isolated no later than 15 h after collection of the material. All samples for the present study were obtained with written informed consent from all the volunteers as per the approval of Institutional Review Board of Ramathibodi Hospital at Faculty of Medicine, Mahidol University, Thailand.

4.2 Isolation and culture of cells

4.2.1 $CD34^+$ Hematopoietic Stem Cells

Mononuclear Cells were isolated from both UCB (UCBMCs) and BM (BMMCs) by Ficoll-Hypaque density centrifugation using IsoPrep (Robbins Scientific, Canada). $CD34^+$ HSCs were isolated from both UCBMCs and BMMCs using $CD34^+$ MACs Microbeads (Miltenyi, Gladbach, Germany) following the manufacturer's instructions. The purification of $CD34^+$ HSCs was determined by staining of isolated cells' surface marker by phycoerythrin (PE)-conjugated anti-CD34 monoclonal antibody (Miltenyi Biotec) for 30 min at 4°C. Cells were washed in PBS and analyzed by Flow Cytometry equipped with an argon laser tuned at 488 nm of BD FACS CantoTM Flow Cytometry (Becton-Dickinson Pharmingen, Heidelberg, Germany). The percentage of $CD34^+$ HSCs obtained ranged from 98% to 99% in all of

purified CB and BM samples. Cells expressing >98% $CD34^+$ antigen were cultured in Stemline II media (Sigma, USA) supplemented with specific hematopoietic growth factor cocktail that included 50 ng/ml stem cell factor, 50 ng/L rh-IL-6 and 50 ng/L rh-IL-3 (R&D Systems).

4.2.2 Mesenchymal Stromal Cells

BMMCs were separated by density gradient centrifugation with IsoPrep. Briefly, 10 ml of heparinized bone marrow samples were mixed in with an equal volume of Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Life Technology, Rockville, Md., USA) and centrifiuge at 1000 g for 30 min at room temperature. The interface mononuclear cells were collected and washed twice with DMEM. Total cell count and viability were evaluated by 0.2% Trypan blue. A total of 2×10^6 cells/ml of BMMCs were cultured in DMEM complete medium supplemented with 10% fetal bovine serum (FBS) and 100 U/ml Penicillin and 100 µg/ml Streptomycin at 37°C, 5% CO2. On 72 hours of cultivation, non-adherent cells were discarded and this process was repeated every four days. Upon 90% confluent, MSCs were trypsinized by 0.05% trypsin-EDTA and passaged for the next expansion. For MSCs surface marker staining, cells were incubated at 4ºC for 30 min with the following cell-specific antibodies; CD105-PE, CD90-PerCP.Cy5.5, CD73-APC (e-bioscience), CD34-APC (Miltenyi Biotec); CD14-PerCP (Becton-Dickinson Pharmingen, Heidelberg, Germany), washed twice with PBSA (PBS+0.1%BSA), and examined using flow cytometry. Osteogenic and adipogenic differentiation were performed as previously described (100).

4.3 Lectin staining

To identify sialyoligosaccharides reactive with SAα2,3Gal- or SAα2,6Gal-specific lectins, CD34+ HSCs were washed to remove culture medium and MSCs were trypsinized from culture flask. Cells were incubated with 50 µl of 1:200 dilution of FITC-conjugated with elderberry bark lectin [1 µg/ml; specific for SAα2,6galactose (Gal)/N-acetylgalactosaminide (GalNac)] or 1:100 dilution of biotinylated-Maackia amurensis lectin (5 µg/ml; specific for the SAα2,3Gal) for 30 min at 40C. After three

washes with ice cold PBS, cells were incubated with 1:25 of fluorescein-conjugated streptavidin (Boehringer Mannheim Biochemicals) for 30 min at 4ºC and then, after three additional washes in ice cold PBS, samples were resuspended in 2% formaldehyde in 1XPBS. The expression of sialic acid on target cells was examined using BD FACS CantoTM Flow Cytometry (Becton Dickinson). BD FACS Diva version 3.1 software was used for data analysis.

4.4 Influenza virus infection of HSCs and MSCs

The different subtypes of influenza A virus: H5N1; A/open-billed stork/Nakhonsawan/BBD0104F/04, A/open-billed stork/Nakhonsawan/BBD2316F/05 and A/open-billed stork/Nakhonsawan/BBD3009M/05 and human influenza A viruses; H1N1 (A/WS/33) and H3N2 (A/Hong Kong/8/68) were propagated and quantified as previously described (101). These virus strains were used throughout this study. For $CD34^+$ HSCs, cells were washed three times with serum free growth media and adsorbed virus for 1 hour with each virus types at a multiplicity of infection of 1 and 10. All experiments with H5N1 virus were performed inside the bio-safety level 3 facility. Following adsorbtion, cells were again washed with serum free growth media and cultured in Stemline II media supplemented with specific hematopoietic growth factor cocktails. Simultaneously, MSCs were plated one day prior to infection. After the cultures attained 100% confluent growth, cells were washed with serum free growth media and adsorbed for 1 hour with virus MOI 1 and 10. Following adsorption, cells were washed with serum free growth media and cultured in complete media. For H1N1 and H3N2 virus infection, the same media was used but containing 0.02 ug/ml of TPCK trypsin (Sigma-Aldrich) was placed into the wells. All infection experiments were performed in three independent experiments.

4.4.1 Nucleotide sequence accession numbers

The H5N1 virus sequence data from Asian open-billed stork described in this thesis were submitted to GenBank with accession numbers DQ989958 to DQ989965 and EF112206 to EF112378.

4.5 Detection of influenza A viral antigen

4.5.1 Fluorescence microscopy

An immunofluorescence assay was performed on 2.5×10^4 cells grown on coverslips. At 24 hours p.i., cells were fixed with 4% paraformaldehyde in PBS for 1 hour at room temperature and then washed three times with PBS. Cells were permeabilized with Cytofix/Cytoperm reagent (Becton Dickinson) for 2 min at 4ºC in the dark. After that, cells were additional washed with PBS. To detect viral nucleoprotein (NP), $CD34^+$ cells were stained with 1:250 dilution FITC-conjugated NP antibody (green) (Chemicon, Temecula, CA), while MSCs were stained with FITC-conjugated anti-NP antibody which Evan's blue was included for counterstaining (DAKO Diagnostics, Denmark) for 30 min at 4ºC. $CD34^+$ cells were counterstained with 1:10 dilution phycoerythrin (PE)-conjugated anti-CD34 monoclonal antibody (red) (Miltenyi Biotec) for 30 min at 4ºC. Cells were triple washed with PBS. The stained cells were analyzed using a laser scanning confocal microscope (LSM 510 Meta, Zeiss, Germany).

4.5.2 Flow cytometry

All infected cells were harvested at indicated time points. Cells were centrifuged at 800 rpm for 5 min and the supernatant was discarded. Cells were washed once with PBS solution and then fixed and permeabilized with Cytofix/Cytoperm reagent (Becton Dickinson). Then, fixed cells were washed once with PermWash (Becton Dickinson) and incubated with 1:500 dilution FITC-conjugated NP antibody (green) (Chemicon, Temecula, CA.) for 30 min at 4ºC. These cells were triple washed with PBS to remove excess antibody. Cells were resuspended in 500 µl of 3.7% formaldehyde prior to analyze by flow cytometry. The percentage of NP-positive cells was assessed using BD FACS CantoTM Flow Cytometry (Becton Dickinson). BD FACS Diva version 3.1 software was used for data analysis.

4.6 Real-time PCR

Infected MSCs were harvested at various time points, and cells were washed for three times with PBS to remove free virions. Total viral RNA was extracted by use of RNeasy Mini kit (QIAGEN, Germany) following the manufacturer's instructions. cDNA was synthesized from viral genomic RNA using oligodT primers and AMV reverse transcriptase (Promega, USA) at 42 ºC for 1 hour. The RT reaction contained 1 µg of total RNA template, 1X AMV RT buffer (Promega, USA), 0.005M DTT, 1mM dNTP, 0.5 µg Oligo dT, 8U RNase inhibitor (Promega, USA), and 8U AMV reverse transcriptase (Promega, USA). These cDNA samples were used as template. Real-time PCR of M gene of H5N1 virus was performed using the SYBR green system as previously described (101). Briefly, PCR amplification was performed in a final volume 20 µl using 10X buffer, 0.4 µl of 10mM dNTP mix, 0.5 µl of each primer (10 mM), 1.2 µl $MgCl_2$ (25 mM), 0.5 µl of SYBR Green dye (1:1000), 0.1 µl of the HotStarTaq DNA Polymerase (QIAGEN, Germany), 12.8 µl of H_2O and 2 µl of cDNA template. The amplificatiion of viral M gene was performed in condition as follow: denaturation at 95°C for 15 min, 40 cycles 95°C for 30 sec, 45°C for 30 sec, and extension at 72°C for 30 sec. Gene copies were quantitated on the basis of a SYBR green fluorescence signal by Rotor-Gene 3000 (Corbett Robotics). The standard curve was generated using serial dilution of plasmid (from 1-10^7 copies) containing the respective cloned gene target. The results were standardized due to the variable quantities of RNA and cDNA, they are expressed as numbers of target genes.

4.7 Plaque assay

MDCK cells were grown overnight in MEM media supplemented with 10% FBS, 100 U/ml Penicillin and 100 µg/ml Streptomycin. Afterwards, the confluent monolayers of MDCK cells were inoculated with 10-fold dilutions of supernatant of infected cells and incubated at 37°C for 1 hour. The inoculum was removed, and cells were washed and overlaid with plaque assay medium containing 0.6% Oxoid Agar. After 2 days at 5% CO_2 and 37°C, MDCK cells were fixed by adding 3.7% formaldehyde for 1 hour at room temperature, and then the agars were

removed and stained with 1.25% Crystal Violet. Finally, visible plaques were counted and the virus titer was calculated as plaque forming unit per milliliter (PFU/ml).

4.8 Generation of recombinant virus

4.8.1 Construction of HA expression plasmid

The full length HA fragment of H5N1 virus names A/open-billed stork/Nakhonsawan/ BBD0104F/04 was amplified using universal primers as follows forward primers: TATTCGTCTCAGGGAGCAAAAGCAGGGG and reverse primers: ATATCGTCTCGTATTAGTAGAAACAAGGGTGTTTT (102). PCR reaction was carried out in MJ mini gradient Thermal cycle (Bio-Rad, USA) using RT-PCR kit (QIAGEN, Germany). The PCR reaction contains: 1 µg of viral RNA, 5x One Step RT-PCR Buffer, 10 µM dNTP, 10 µM of each forward and reverse primer, one Step RT-PCR Enzyme Mix and RNase-free water. To make HA expression plasmid, plasmid pHW2000 was cut with BsmBI and the full length HA fragment was inserted. Then, integrity HA insertion was confirmed by nucleotide sequencing at Macrogen Inc., Korea. The PCR sequences are provided in table 4.1. Sequences were analyzed by using MEGA 4.0 software.

Table 4.1: Sequencing primers: HA full length

Primer	Sequence
H5 -F1	5'-AATCTGTCAAAATGGAGAAAATAGTGCTT-3'
H5 -F2	5'-TTTTGGTACTGTGGGGGATTCACCATCC-3'
H5 -F3	5'-AGGCAATAGATGGAGTCACCAATAAGGTCA-3'
H5 -R1	5'-TGGTGAATCCCCCACAGTACCAAAAGATCT-3'
H5 -R2	5'-ATTGCTATGGTGGTACCCATACCAACCATC-3'
H5 -R3	5'-AACTTACAAATTTAAATGCAAATTCTGCAT-3'

4.8.2 Generation of reverse genetics virus

One day before transfection, 1.3x 10^5 of MDCK cells and 1.3x10^5 293T cells were seeded in 6 well-plate. On the day of transfection, eight pHW2000 plasmids containing PB2, PB1, PA, NP, NA, M and NS fragment of influenza virus A/Puerto Rico/8/34 (PR8) (H1N1) and HA fragment of H5N1 virus from Asian open-

billed was prepared in one tube (1 µg each). Then, transfection medium, Optimem (Gibco, NY, USA) with antibiotics, was added to the final volume of 182 µl, and 18 µl of TransIT-LT (Mirus Bio, WI, USA) was added. After 45 minutes at room temperature, 800 µl of Opti-MEM was added to transfection solution. During incubation, the cells were washed with Opti-MEM for 2 times. After adding the transfection solution to washed cells, the cells were incubated for 7 hours at 37 ºC 5% CO_2. Next, the transfection solution was discarded, then 1 ml of Opti-MEM was added and cells were incubated at 37 ºC 5% CO_2 for 24 hours. Thereafter 1 ml of Opti-MEM with TPCK trypsin (2µg/ml) was added and incubated at 37 ºC 5% CO_2 for another 48-72 hours. Harvest the supernatants if cytopathic effect (CPE) was observed.

4.8.3 Infection of MSCs with reverse genetics virus

MSCs were plated one day prior to infection. After the cultures attained 100% confluent growth, cells were washed with serum free growth media and adsorbed for 1 hour with MOI 1 of wild type H5N1, RG-HA/PR8 (01-040204 HA and internal gene of A/Puerto Rico/8/34 (PR8)) and RG-PR8. Following adsorption, cells were washed three times with serum free growth media and cultured in complete media. For reverse genetics virus infection, the same media was used but containing 0.02 ug/ml of TPCK trypsin (Sigma-Aldrich) was placed into the wells. This infection experiment was performed in two different donors.

CHAPTER V
PART I
RESULTS

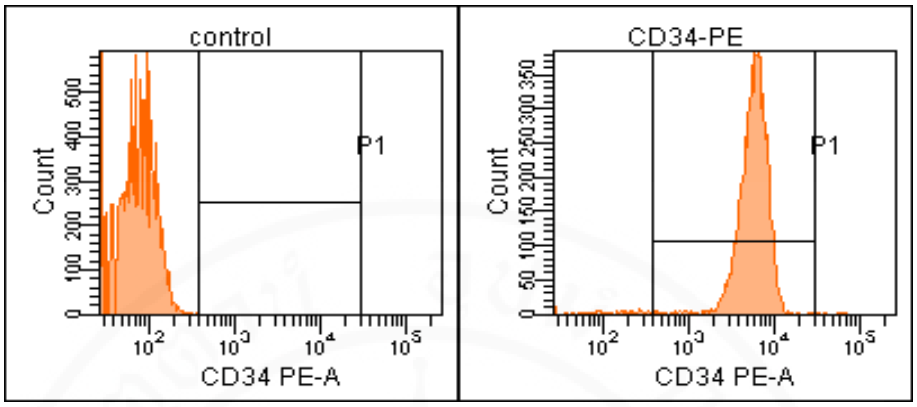
Unlike human influenza virus, avian (H5N1) virus can spread to extra-pulmonary organs. To study bone marrow (BM) dissemination, it is important to explore target cells in BM. Stem cells are likely to be H5N1 target, thus, an *in vitro* model was set up to investigate the susceptibility of BM cells to H5N1 infection. HSCs and MSCs were isolated from BM and cord blood (CB) by different techniques. The identity testing of each cell was examined. The receptor distribution was done to predict the susceptibility. The infectivity and efficiency of replication were determined by increasing of viral antigen positive-cells and infectious particle production, respectively.

5.1 Characterization of $CD34^+$ HSCs and MSCs

As BM is known as a rich source of progenitor cells, BM tissue contains two main stem cell types: Hematopoitic and Non-hematopoietic stem cells. Hematopoietic Stem Cells (HSCs) give rise to circulating blood cells as well as cells in immune system. Mesenchymal Stem/Stromal cells belong to non-hematopoietic stem cells group which can differentiate to mesengenic tissues. From this issue, it is reasonable to isolate HSCs and MSCs from BM, Recently, an umbilical cord blood (CB) was reported as another rich source of HSCs which the number and characteristics were comparable to BM derived (103). In this study, HSCs were isolated from both BM and CB sources, whereas MSCs were obtained from BM only. These two stem cell types are different in phenotypes and general surface markers. HSCs were isolated by using CD34 microbeads as it is a specific marker for HSCs. Separating cells that passed through magnetic beads system were suspension cells with rounded shape. $CD34^+$ cells were cultured in specific media supplemented with

growth factors. To test the purity of isolation, HSCs were detected by fluorescence-conjugated anti-CD34 monoclonal antibody and analyzed the expression of CD marker by flow cytometry. As shown in Figure 5.1A and 5.1B, the purity of HSCs isolation from both BM and CB sources was 99.8%, and 98.9%, respectively. The right shifted of histogram compared with unstained control indicated that isolated HSCs expressed CD34. The high percentage of fluorescence intensity marked a purity of isolated cells. This implied that using magnetic beads system in cell isolation gives a high purity. Thus, isolated $CD34^+$ HSCs could be used in next experiments. For MSCs, BMMCs were allowed to attach on culture flask. Attached cells with fibroblast shape were once sub-passaged before used. To test the characteristics of MSCs, cell surface marker staining was performed to identify the existing of this cell type. Although MSCs have no specific markers, there have been a number of studies for surface marker characterization. The most commonly reported positive markers are CD105, CD90, CD44, CD73 and CD29, and the most frequently reported negative markers are CD34, CD14, CD45 and CD31. To characterize surface markers of MSCs, staining of three or more of each positive and/or negative marker is accepted (104-106). In this study, CD105, CD90, and CD73 were selected as positive markers, whereas, CD14 and CD34 were negative ones. Early passage of MSCs was trypsinized and incubated with fluorescence-conjugated monoclonal antibodies. The expression of surface marker was examined by flow cytometry. Expectedly, MSCs were positive for CD105, CD90, and CD73 as shown in right shifted histogram with high percentage of fluorescence intensity; 79.7%, 80.9%, and 90.4%, respectively (Figure 5.2A). In contrast, CD34 and CD14 expression were no detectable (Figure 5.2B). This result indicated that the surface marker expression of isolated MSCs was corresponded with MSCs criteria and could be used in next experiment as well.

A.



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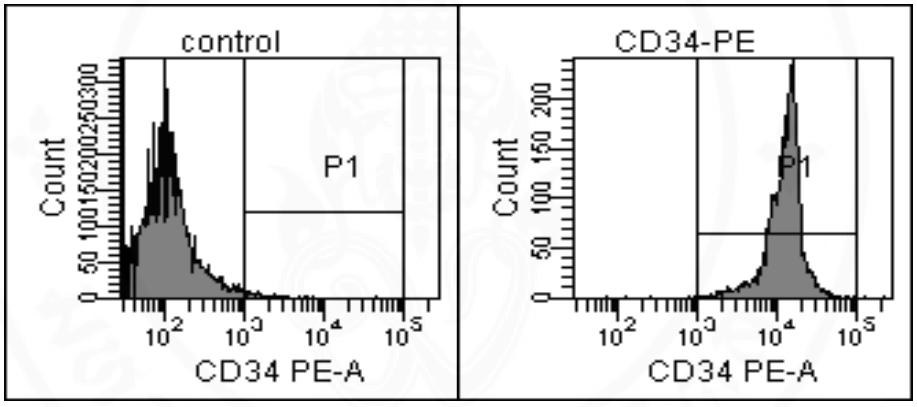
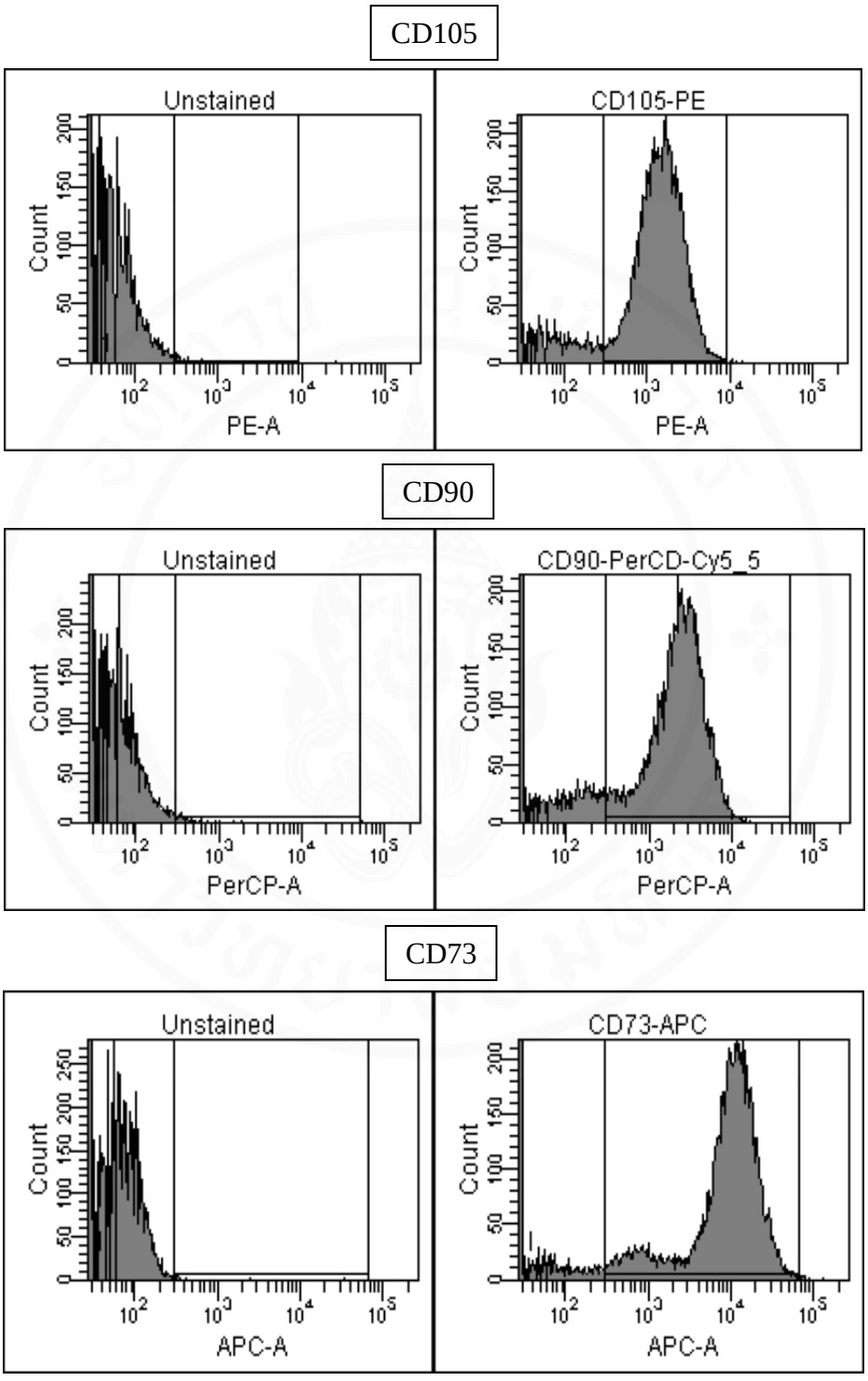


Figure 5.1: Purity of CD34$^+$ HSCs isolation. CD34$^+$HSCs were isolated from BMMCs and UCBMCs by magnetic microbeads system. Separated CD34$^+$HSCs from both sources were incubated with PE-conjugated anti-CD34 for 30 min at 4ºC in the dark and analyzed by flow cytometry. **(A)** purity of BM-derived was 99.8% and (**B)** purity of CB -derived CD34$^+$ HSCs reached 98.9%. Left panel was unstained control and right panel was antibody staining.

A.



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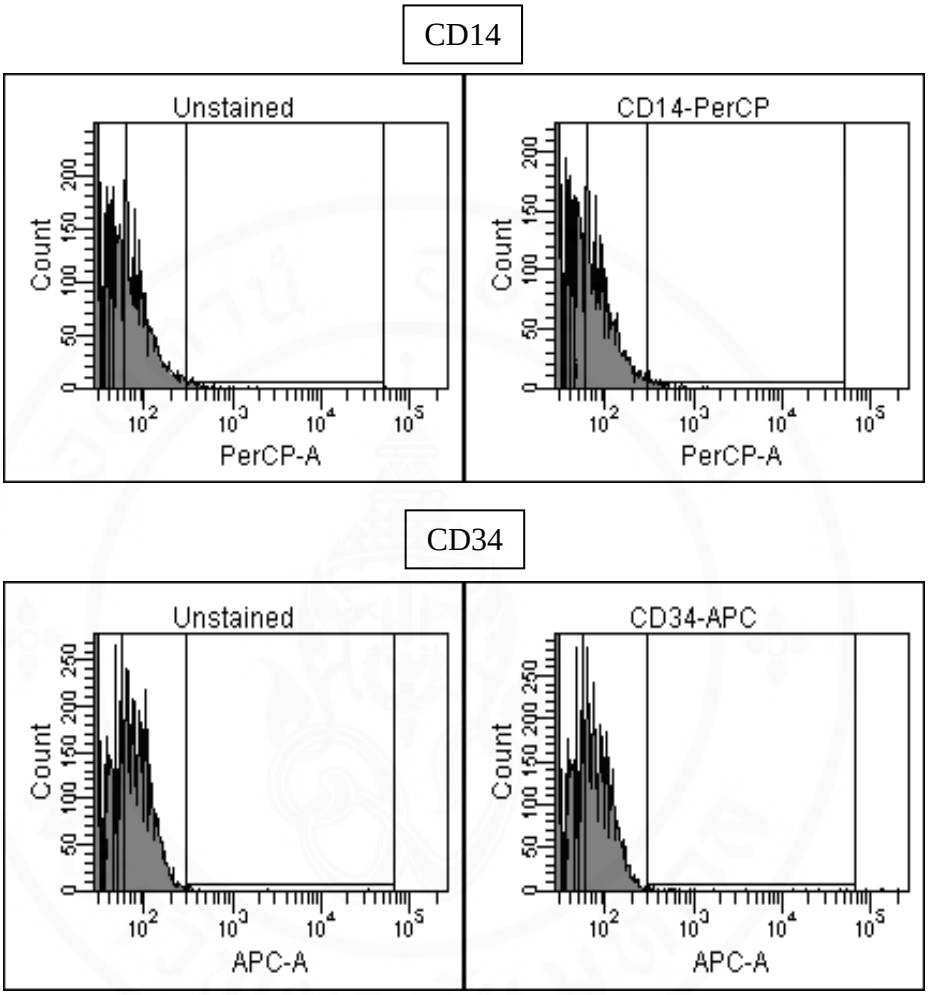

Figure 5.2: Surface markers characterization of isolated MSCs. BMMCs were allowed to attach on culture flask. Early passage of MSCs was trypsinized to test surface marker expression. Isolated MSCs were stained with **(A)** positive surface markers; CD105, CD90, and CD73, and also with **(B)** negative surface markers; CD34 and CD14. Samples were analyzed by flow cytometry. Left panel was unstained control and right was antibody staining.

5.2 Detection of α-2,3 and α-2,6 sialic acid (SA) on $CD34^+$ HSCs and MSCs surfaces

Sialic acid (SA) on cell surface was widely recognized as influenza virus receptor. SA linked to galactose residue (SAα2,3-Gal) was distributed in avian gastrointestinal tract, and human lower respiratory tract, whereas, SAα2,6-Gal was expressed in human upper respiratory tract. Influenza virus infection is initiated due to interactions between the HA protein and SA-containing glycans on the surface of host cells. Expression of SA can predict the susceptibility of host cells to invading virus. Bronchial epithelial cells equally expressed α-2,3 and α-2,6 SA. Therefore, epithelial cells were susceptible to both human (H1N1) and avian influenza (H5N1) viruses (60). Similarly, lung endothelial cells were α-2,3 more abundant than α-2,6 SA, they were susceptible to H5N1 virus which possess α-2,3 binding preference, whereas resistant to H1N1 which preferably bind to α-2,6. To determine the susceptibility of BM cells to influenza virus infection, we first examined the distribution of SA receptors on the cell surface using lectin staining. We stained isolated $CD34^+$ HSCs and MSCs with *Maackia amurensis* (107) and lectin *Sambucus nigra* (108) lectin specific for SAα2,3-Gal and SAα2,6-Gal, respectively and analyzed using flow cytometry. We found that $CD34^+$ HSCs and MSCs expressed receptors for both avian (SAα2,3-Gal) and human (SAα2,6-Gal) (Figure 5.3). However, the Mean Fluorescence Intensity (MFI) of MAA was about four times higher than SNA in $CD34^+$ HSCs indicating that $CD34^+$ HSCs might be more susceptible to H5N1 than H1N1 infection. MSCs showed positive staining of SNA and MAA with similar MFI as 113 and 101, respectively indicating that MSCs might be equally susceptible to both human and avian influenza viruses. Regardless of a number of SA molecules, $CD34^+$ HSCs and MSCs were able to be infected with systemic H5N1 virus.

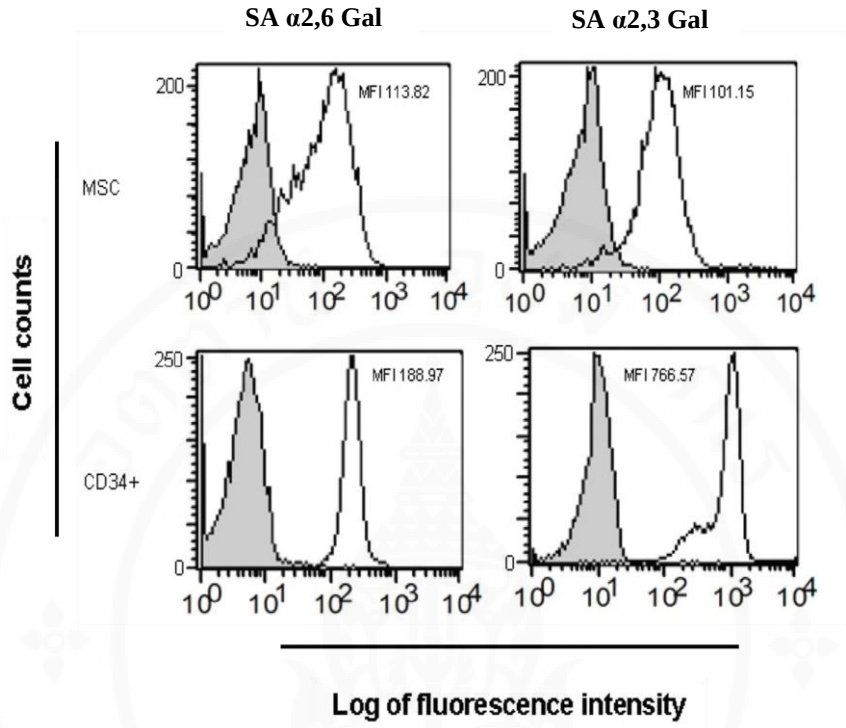

Figure 5.3: Sialic Acid (SA) on the surface of various cell types. $CD34^+$ HSCs, and MSCs were incubated with SNA and MAA which specific for α2,3 and α2,6 SA, respectively, and then analyzed for fluorescence intensity on a FACScan fluorospectrometer (Becton Dickinson). The profile shown depicts cell number as a function of log fluorescence intensity of SAα2,3Gal (α2,3; *Maackia amurensis* agglutinin)- and SAα2,6Gal (α2,6; *Sambucus nigra* agglutinin)-specific lectin-reactive oligosaccharide on the cell surface. Left panel was SAα2,6Gal and right panel was SAα2,3Gal. Grey histogram represented unstained control and White represented antibody staining.

5.3 Virus characteristics

A/open-billed stork/Nakhonsawan/BBD0104F/04, the virus used in this study, was isolated in Thailand from the Asian open-billed stork. Whole genomic nucleotide sequencing and molecular basis characterization were previously performed. For HA analysis, the motif of polybasis amino acid residues; RERRRKKR, IERRRKKR, and REKRRKKR were found. These motifs could define a high pathogenic feature of H5N1 avian influenza virus. The receptor binding pocket maintains amino acid residues Gln222 and Gly224 and other amino acids relevant to receptor binding were identical to those of A/Goose/Guangdong/1/96. Therefore, these Asian open-billed H5N1 viruses preferentially bind to cellular receptor with 2,3-NeuAcGal linkages. For NA, the virus contained 20 amino acid deletions at positions 49-68 in the stalk region. The residues of sialic acid binding site were conserved. Additional N-glycosylation site at position 156 of HA was detected as same as other genotypes Z viruses (109). In non-structural genes, there were five amino acids deletion which confer resistance to cytokines. Moreover, Ala at position 149 of NS1 is also critical for antagonistic induction of interferon in chicken embryo fibroblast cells (76). A substitution at a position 31, Ser31Asn (serine to asparagine) was found, this position was involved with resistant to amantadine (110). However, this H5N1 strain had no Lys627 mutation in the PB2 protein that was associated with increased virulence of influenza viruses. BBD0104F/04 strain had been used to study the susceptibility of dendritic cells which virus could infect and replicate in human primary monocyte-derived DCs (101). These findings suggested that H5N1 virus from avian isolates is genetically similar to H5N1 virus isolated from infected humans, thus it would be able to use in the study of viral tropism in human.

5.4 Susceptibility of HSCs and MSCs to H5N1 infection

Influenza virus infection of stem cells may acutely impair BM homeostasis and contribute to the pathogenesis of severe disease. Here, we investigated whether H5N1 virus could infect BM cells, CB-derived $CD34^{+}$ HSCs grown on coverslips were infected with live and heat-killed avian influenza virus H5N1 (A/open-billed

stork/Nakhonsawan/BBD0104F/04), and human influenza viruses; H1N1 (A/WS/33) and H3N2 (A/Hong Kong/8/68) at MOI of 10 for 24 hours (h). Mock infection was used as a control. Exogenous protease for cleavage of HA protein was added in experiment of human influenza viruses infection. After 24 h post infection (h p.i.), cells were fixed, permeabilized and stained with anti-NP FITC. $CD34^+$ HSCs were then counterstained with anti-CD34 PE. Samples were analyzed the localization of cells and viral antigens by confocal microscopy. The expression of viral antigen was observed in a few $CD34^+$ HSCs when infected with live H5N1 virus as seen in green localized in red cell surface (10% infection rate), whereas heat-killed H5N1 as well as two human influenza viruses were negative for viral antigen detection similar to mock infection (Figure 5.4A). In addition, flow cytometry technique was used to evaluate NP-positive cells which could determine the percentage of infection. Isolated $CD34^+$HSCs from BM and CB were infected with H5N1 virus at MOI 1 and 10. Kinetics of virus infection in $CD34^+$HSCs was observed at various time points (0-36 h p.i.). Greater number of NP-positive cells (12-16%) was detected and peaked at 12 h p.i. in $CD34^+$ HSCs infected with high MOI of virus and decreased to 6-9% at 24 h p.i. At low MOI, percentage of infection was 4-6% at 24 h and peaked at this time point (Figure 5.4B). The level of NP-positive cell at 24 h p.i. detected by flow cytometry was correlated with immunofluorescence staining which both techniques showed about 10% infection rate. To avoid the effect of strain variation, CD34+ HSCs were infected with different strains of H5N1; A/open-billed stork/Nakhonsawan/BBD0104F/04, A/open-billed stork/Nakhonsawan/BBD2316F/05, and A/open-billed stork/Nakhonsawan/BBD3009M/05 at MOI 10. After 24 h p.i., cells were stained for NP antigen. We found that there was no different of percentage of infection among three strains (range 9% to 12%) (Figure 5.4C) suggesting that there was no strain variation and the susceptibility to H5N1 of $CD34^+$ HSCs was not strain specific. To test the susceptibility of BM-derived MSCs, the same approaches used in $CD34^+$HSCs were performed. MSCs were infected with Mock (control), live and heat-killed avian influenza virus H5N1, and human influenza viruses; H1N1 and H3N2 at MOI 10. The intracellular viral antigen was evaluated in 24 h p.i. by confocal microscopy. Surprisingly, we observed the majority of MSCs were susceptible to live H5N1 virus after 24 h after infection as shown in green (70-80%) in Figure 5.5A,

whereas, there was a few NP-positive cells in human influenza viruses infection (3%), and negative in Mock and heat-killed H5N1 virus infection. We also used flow cytomery technique to analyze frequency of infected cells at various time points (0-36 h p.i.). MSCs were infected with H5N1, H3N2 and H1N1 at MOI 1 and 10. We found that the percentage of infection in MSCs infected with high and low MOI reached 60% and 30%, respectively at 12 h p.i. and slightly decreased until 36 h p.i. (Figure 5.5B). Correlating with obtained data from immunofluorescence technique, MSCs showed a limited infection with human influenza viruses which were 5% infection rate even using high MOI (Figure 5.5C). In addition, MSCs were infected with H5N1 with varying MOIs (1-0.0016) to define the sensitivity of cells to virus infection. At 12 h p.i., real-time PCR was performed to determine level of M gene expression. We observed that H5N1 infection of MSCs was dose dependent. The lowest MOI that virus could not enter cells at 12 h is 0.0016 (Figure 5.5D). Taken together, these data revealed the stem cells tropism of H5N1 virus which both $CD34^{+}$ HSCs and MSCs were directly infected by H5N1 virus, with MSCs being more susceptible.

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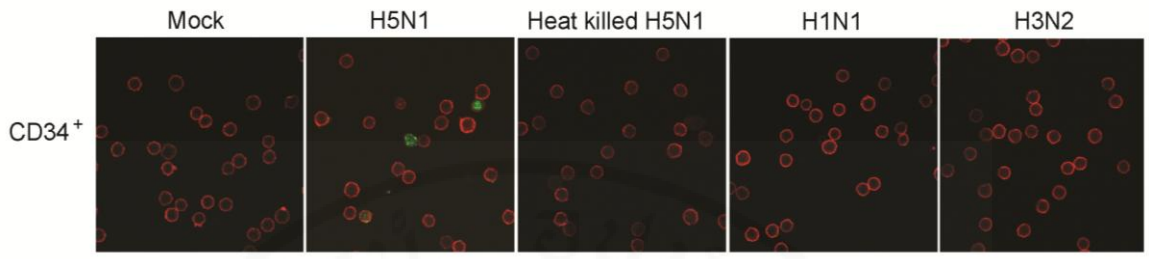

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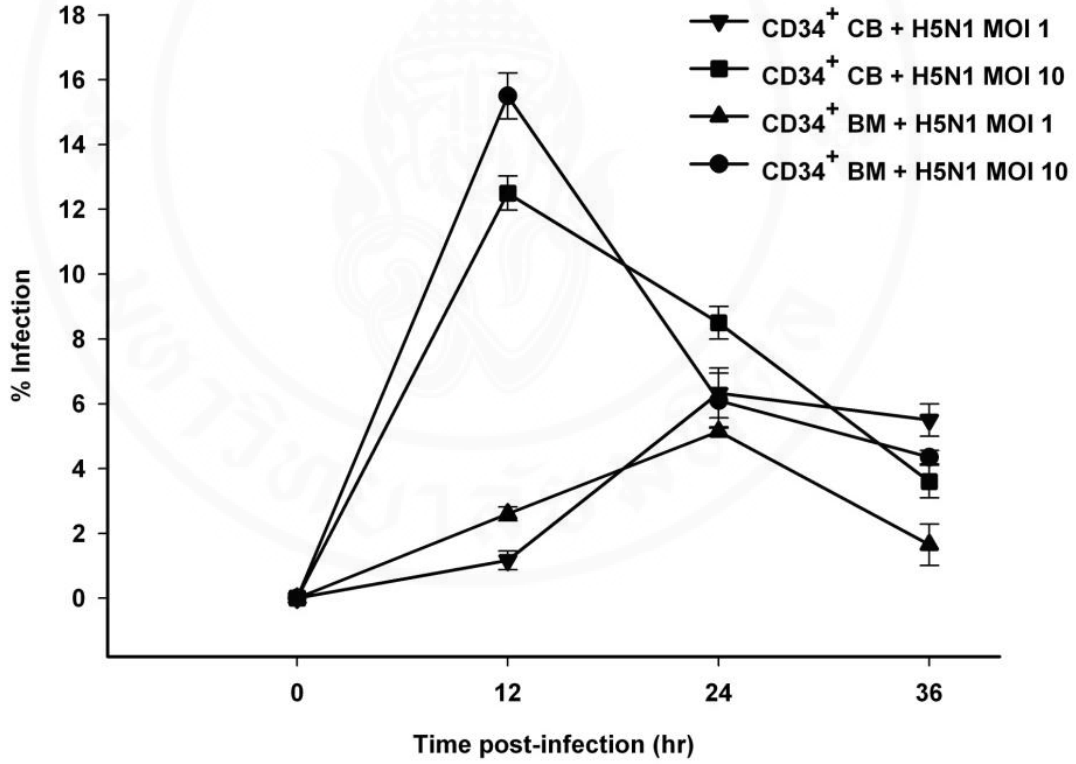

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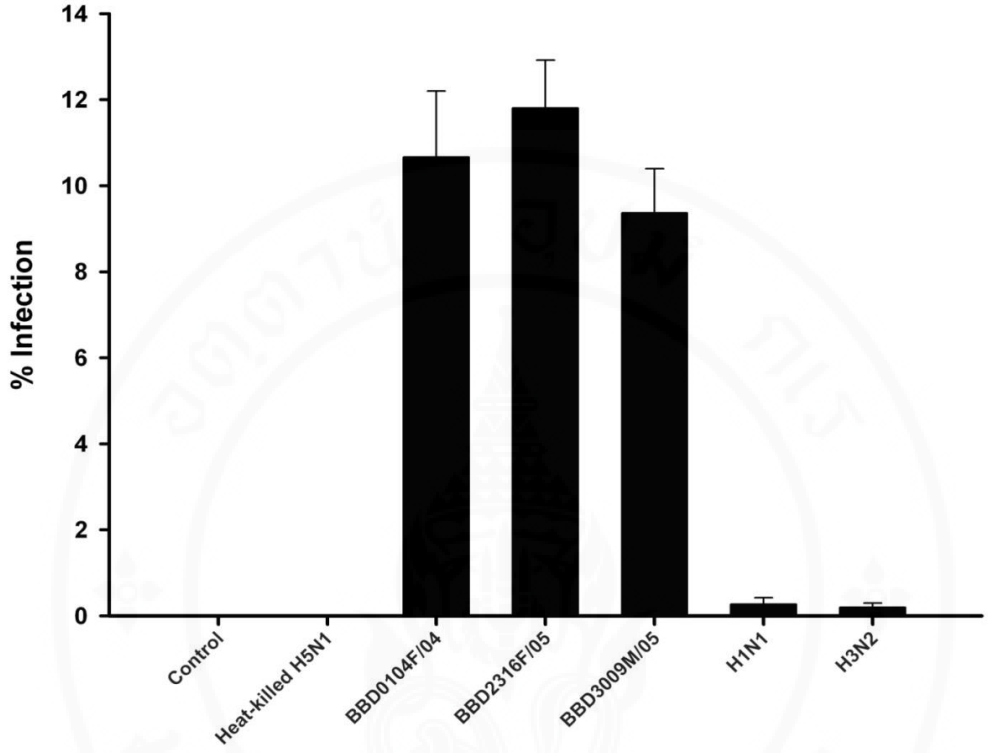
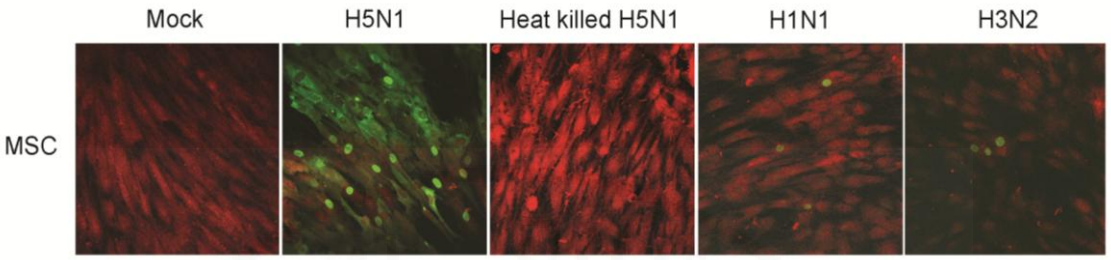


Figure 5.4: H5N1 infection in CD34$^+$ HSCs. CB-derived CD34$^+$ HSCs were infected with both live and heat-killed avian (H5N1) and human influenza viruses (H1N1 and H3N2) at MOI 10 for 24 hours. Cells were fixed and permeabilized for intracellular staining of Influenza nucleoprotein (NP; green). CD34$^+$ cells were counterstained with anti-human CD34-PE (red)**. (A)** Co-localization of viral antigen and cell surface marker was examined by confocal microscopy. **(B)** CD34$^+$ HSCs derived from both BM and CB were exposed with either high (MOI 10) or low (MOI 1) dose of H5N1 virus. The expression of viral nucleoprotein was analyzed by flow cytometry. **(C)** CD34$^+$ HSCs were infected with different strains of H5N1 virus; BBD0104F/04, BBD2316F/05 and BBD3009M/05, all are avian isolates, and human influenza viruses. Mock and heat-killed virus infected was used as a control of experiment. At 24 h after infection, cells were subjected to monitor viral nucleoprotein expression by flow cytometry. The error bars represent SD from three different donors.

A.



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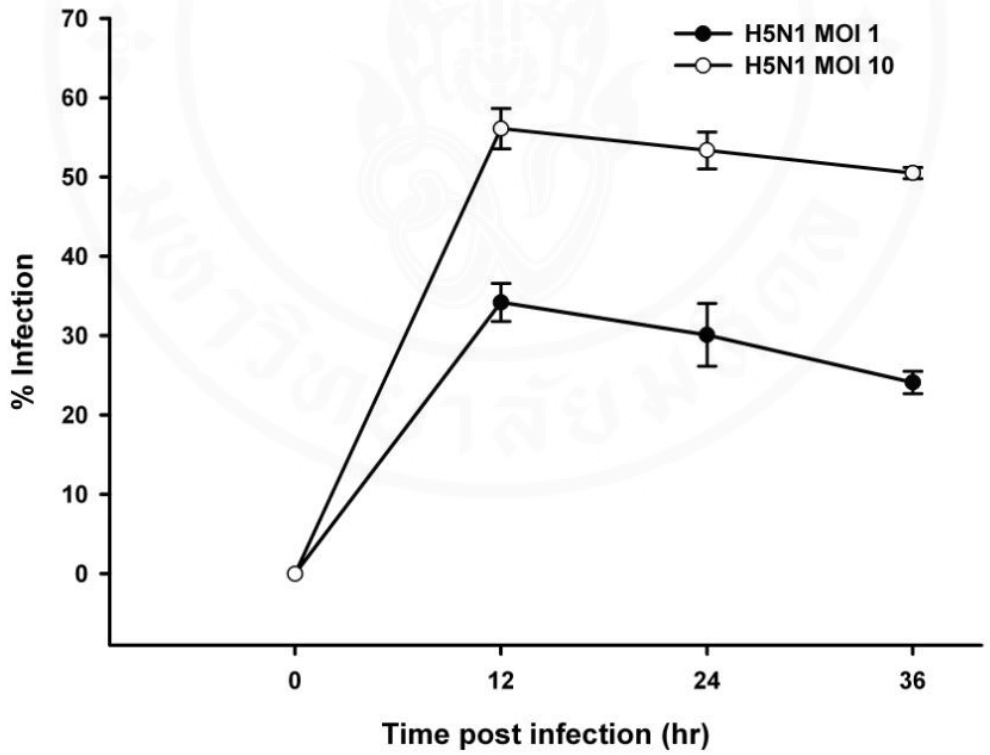

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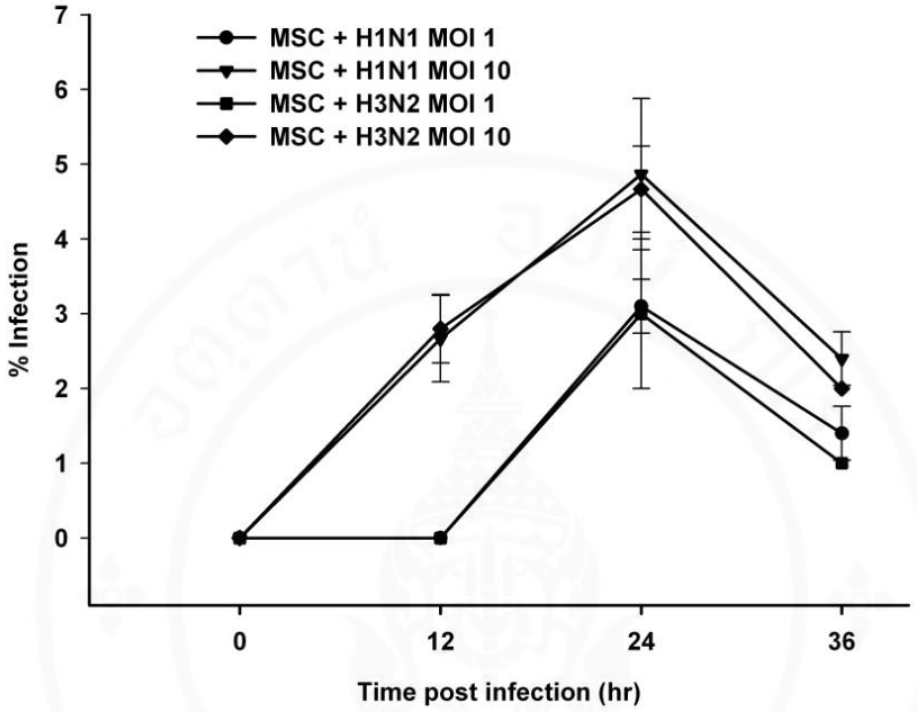

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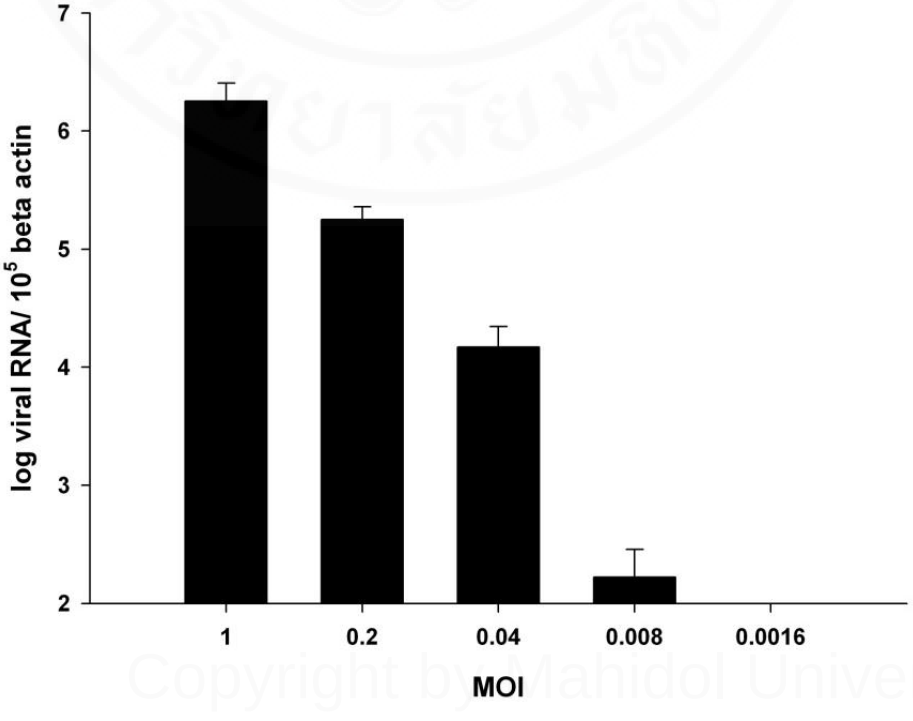


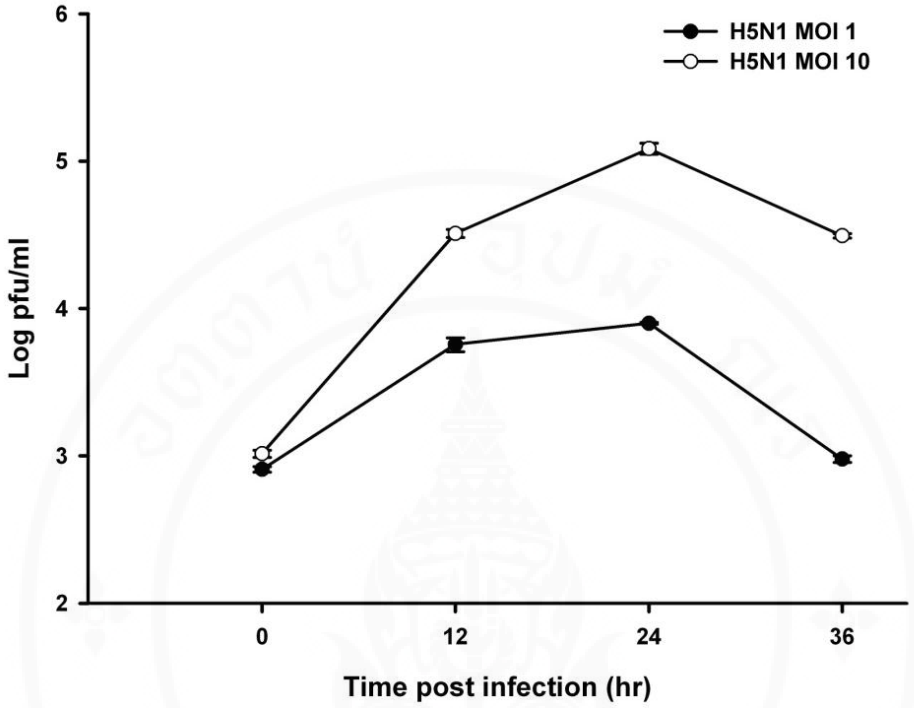
Figure 5.5: Highly susceptible of MSCs to H5N1 infection. (A) The ability of avian influenza H5N1 to infect MSCs was compared with human influenza viruses. Cells were incubated with H5N1; live or heat-inactivated, H1N1 and H3N2 at MOI 10. After 24 h, cells were stained and examined by confocal microscopy for the expression of viral antigens (green) with Evan's blue (red) as a counterstain. **(B, C)** Percentage of infected MSCs at high and low MOI of H5N1 virus and human influenza viruses were determined by flow cytometry. **(D)** Real-time PCR of H5N1 M gene in MSCs after infection with H5N1 virus at various doses (five-fold decreased) at 12 h p.i. The error bars represent SD from three independent experiments.

5.5 Replication kinetics of H5N1 virus in $CD34^+$ HSCs and MSCs

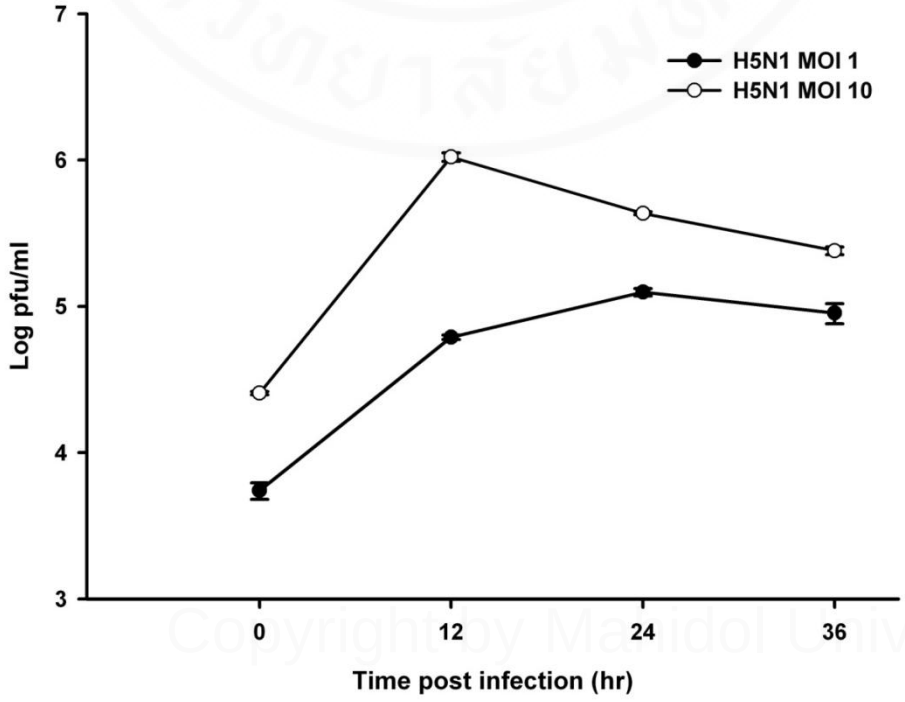
According to susceptibility to H5N1 infection of $CD34^+$ HSCs and MSCs, we next investigated the replication kinetics of H5N1virus in these two targeted cells. $CD34^+$HSCs were infected with H5N1 virus MOI 1 and 10. Culture supernatants were collected at various time points (0-36 h p.i.) to evaluate new infectious particles production by plaque assay. We observed that infectious virions from $CD34^+$ HSCs infected with H5N1 MOI 10 were produced more than one log from incubation initiated to 12 h p.i. Viral titers were increased onward and peaked at log 5 titers (pfu/ml) at 24 h p.i. and declined at 36 h p.i. For low MOI, less than one log of virus progenies was increased from 0 h to 12 h p.i., and seemingly stable until 24 h p.i. Similar to MOI 10 infected, viral titers were reduced at 36 h p.i. (Figure 5.6A). To examine viral output in MSCs, same incubation time as well as the technique was performed. Expectedly, viral titer in MSCs was higher than $CD34^+$ HSCs. Viral production of MSCs infected with high MOI of virus was more than one log increased from start incubation, peaked at 12 h p.i. and then slightly decreased through the end of incubation. The pattern of increasing of viral titer in low MOI infected was similar to high MOI but titer was lower and slower increased than high MOI which reached the peak at 24 h p.i. (Figure 5.6B). An MOI of 0.008 (one virus particle per every 120 target cells) was sufficient to infect MSCs to generate at least two-log new H5N1 virus particles at 36 h after infection (Figure 5.6C). Conversely, human influenza A viruses

were not able to replicate in MSCs even at a high MOI (data not shown). The replication kinetics of H5N1 virus in both $CD34^+$ HSCs and MSCs was correlated with the percentage of infection suggesting that H5N1 virus could efficiently infect and replicate in MSCs and, to a lesser extent in $CD34^+$ HSCs.

A. CD34^{+}HSCs



B. MSCs



C. MSCs

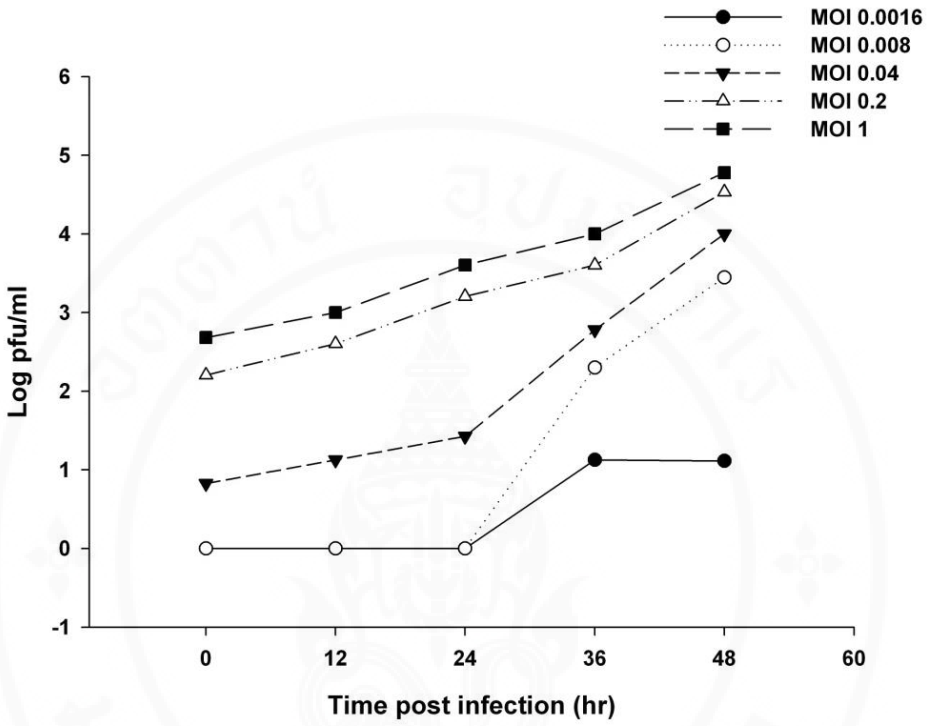


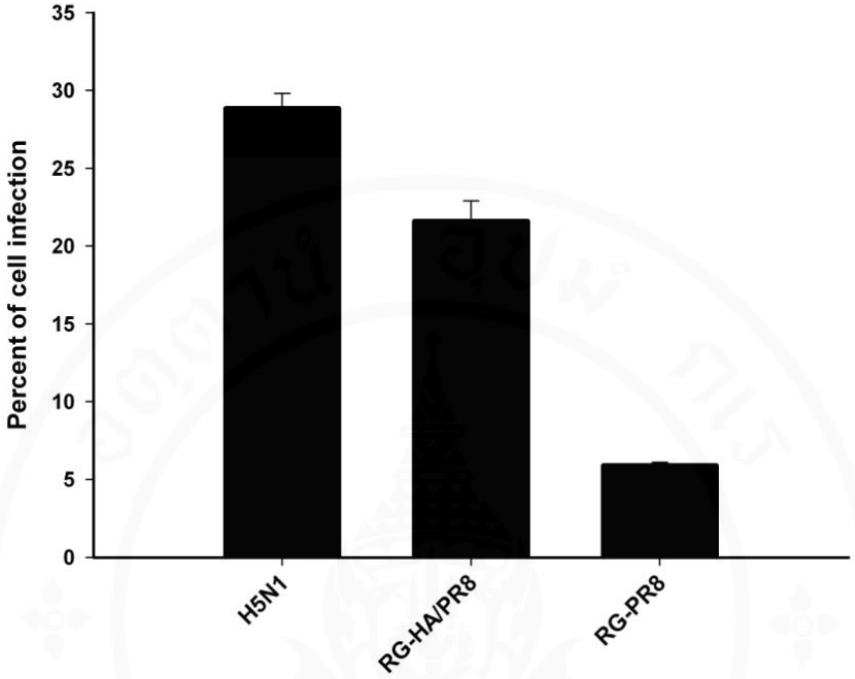
Figure 5.6: Replication of H5N1 virus in CD34$^+$ HSCs and MSCs. Supernatants of **(A)** CD34$^+$ HSCs and **(B)** MSCs infected with H5N1 virus (BBD0104F/04) at MOI 1 and 10, also **(C)** MSCs infected with various MOI of H5N1 virus were collected at different time points after infection and then titrated on MDCK cells to measure virus production by plaque assay. Data are expressed as log plaque-forming units per milliliter of supernatant. **(A, B)** The error bars represent SD from three different donors. **(C)** Data shown is from one representative donor of three different donors.

5.6 Role of hemagglutinin in H5N1 infection in MSCs

The characteristic of invading into stem cells is unique to highly pathogenic avian influenza strains. Viral protein could be responsible for increased virulence. Viral surface glycoprotein hemagglutinin (HA) is a receptor-binding and fusion protein which is required for virus entry. In order to investigate the contribution

of HA in the infection of MSCs, the reverse genetics (RG) virus containing HA of H5N1 was constructed. HA gene of H5N1 virus (A/open-billed stork/Nakhonsawan/BBD0104F/04) was reassorted into A/Puerto Rico/8/34 (PR8) backbone, RG-HA/PR8 was an abbreviation of the recombinant virus. MSCs were incubated with wild-type and RG-HA/PR8 at MOI of 1, then washed and incubated for 24 hours. Controls including mock infection and RG-PR8 virus which was used as a backbone. Using NP staining method, the percentage of infection of RG-HA/PR8 virus was comparable with wild-type H5N1 virus, although the infectivity of recombinant virus could not reach the level of wild-type virus. In contrast, a minimal infection was seen in RG-PR8-infected MSCs even in the presence of exogenous trypsin (Figure 5.7A). Culture supernatants were also collected to assess viral replication kinetics using standard plaque assay. In parallel with percentage of infection, the viral titer of RG-HA/PR8 virus was not different with wild-type H5N1 virus at every time point (Figure 5.7B). The replication kinetics of RG-PR8 was about one log lower than wild-type virus which was not correlated with its infectivity in Figure 5.7A. Our results suggested that HA of H5N1 virus played a role in productive infection of MSCs. However, the combination of multiple determinants is still required for efficient infection and replication as wild-type H5N1 virus.

A.



B.

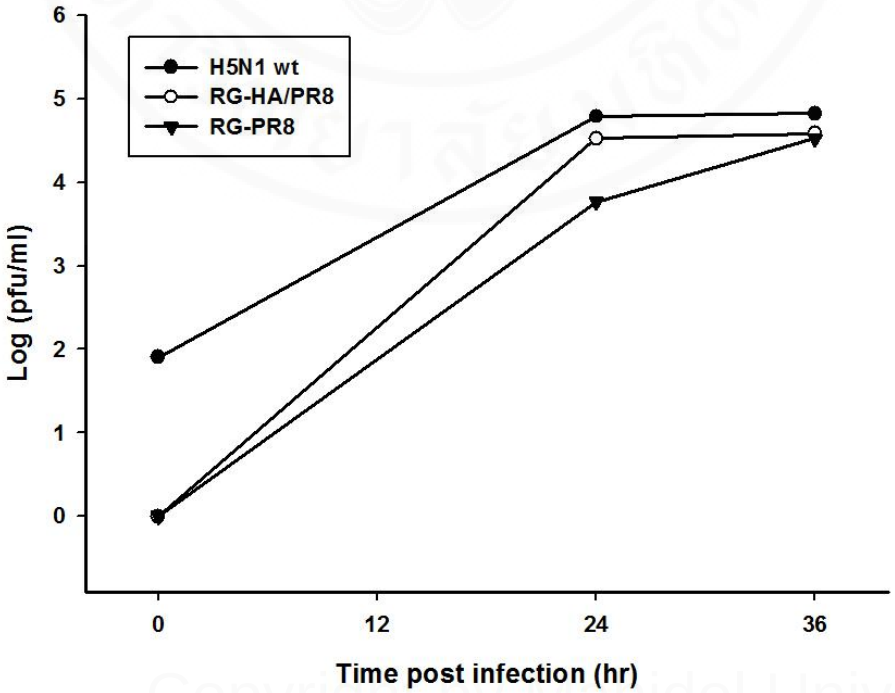


Figure 5.7: The contribution of H5 HA in MSCs tropism.

Recombinant virus (RG-HA/PR8) was constructed by using reverse genetics technique. MSCs were infected with H5N1 wild type virus (BBD0104F/04) and RG-HA/PR8 at MOI 1. **(A)** At 24 hours post infection, cells were stained for viral nucleoprotein, and then examined by flow cytometry to determine the percentage of infection. The error bars represent SD from two different donors. **(B)** Supernatants were collected to measure viral output by plaque assay. Data shown are from one representative donor of two.

CHAPTER VI
PART I
DISCUSSION

Influenza has long been recognized as a significant public health problem for century. Since 1997, avian influenza H5N1 virus has emerged as zoonotic human pathogen which causes high mortality rate among infected individuals and spread to large part of the world. However, human to human transmission is poorly occurred. To date, there were a few effective antiviral drugs and/or vaccines available. Also, the productive strategies for prevention of this infection were not successfully established. Therefore, many of H5N1 infected patients developed to a severe disease. Beginning with the main site of infection, H5N1 virus causes a pneumonia, and then progress rapidly to acute lung injury (ALI), in turns, acute respiratory distress syndrome (44). In addition, multi-organ dysfunction syndrome (MODS) was associated with fatal cases of H5N1 patients.

Even though there are a number of studies of H5N1 pathogenesis both *in vitro* and *in vivo* studies, a basis for H5N1 virulence remains unclear. From clinical manifestations of patients, H5N1 virus was not likely to cause localized infection. Evidence for proposing systemic spread capacity of H5N1 virus is;

(1) *In vitro* and animal model studies implied that viral dissemination is the cause of disease severity (44, 111, 112).

(2) Laboratory investigations suggested that viral-induced cytokine dysregulation results in hyperinflammation, ARDS and multi-organ failure (85, 88).

(3) Postmortem study showed a detection of viral genomic in extra-pulmonary organs. Moreover, the staining of viral nucleoprotein antigen by immunohistochemistry in the small and large intestinal epithelial cells, hematopoietic cells in bone marrow, glial cells and neurons of the brain, and lymphocytes revealed a systemic spread of H5N1 virus (52).

(4) Viral RNA in blood was present in fatal H5N1 cases and associated with high pharyngeal viral loads (6).

(5) The hematologic abnormalities, reactive hemophagocytosis, and hypo/hypercellular bone marrow were the major characteristics of H5N1 with severe cases (7).

These data support the systemic infection of extrapulmonary organs. An evidence of viral antigen detection in hematopoietic cells was consistent with hematologic abnormalities. Whether hematopoietic cell is a target of H5N1 remains questionable. Also, the mechanism of hematologic abnormalities has not been described. Due to the origin of blood cell synthesis takes place in bone marrow, an area with plenty of stem/progenitor cells. We hypothesized that H5N1 infection results in the destruction of BM homeostasis contributing to BM suppression. It is therefore important to investigate the viral tropism in BM which may be a cause of H5N1-mediated BM suppression.

Whole cells residing in BM, peripheral blood, and tissues are primarily originated from stem cells. The stem cells are divided to two groups; one is Hematopoietic Stem Cell (HSCs), a precursor of blood cells and the other is Non-Hematopoietic Stem Cell, mesenchymal stem cell (MSCs), a mesengenic progenitor cell of bone, cartilage, tendon, muscle, marrow, fat, and dermis. In this study, we focused on two stem cell populations which are HSCs and MSCs. Whether they are primary targets for H5N1 infection and the outcomes following infection produce deleterious effects are experimenting.

Previous studies of direct infection of $CD34^+$ HSCs and MSCs by persistent viruses, such as Measles virus (MV) (113) and Cytomegalovirus (CMV) (114, 115) showed a virus-mediated immunosuppression. Latent CMV exploited hematopoietic myeloid progenitor cell as a reservoir for escape an immune response. MV infection of $CD34^+$HSCs and stromal cell disrupted a hematopoiesis which was a proposed mechanism of immune suppression. However, the data of acute viral infection of stem cells was limited. As BM stem cells were more likely to be targets of invading acute H5N1 virus, we deliberated the investigation of susceptibility of two stem cell types to H5N1 infection. The study was initiated by isolation of HSCs and MSCs from BM which is an abundant source of stem cells. Alternatively, HSCs were

also obtained from umbilical cord blood (CB) which is non-invasive source and easily accessed without affecting with fetus and mother (103, 116, 117). The number and characteristics of CB-derived HSCs are similar to BM derived. The isolation and culture techniques used in this study are generally accepted. We used CD34 magnetic microbeads selection to separate $CD34^+$HSCs from mononuclear cells of BM and CB. Although there are many subsets of surface markers, CD34 is the specific marker present in most immature stem/progenitor cells (118, 119). The efficiency of purification was very high (almost 100%) indicating that we obtained a homogeneous population of $CD34^+$HSCs. To maintain $CD34^+$HSCs *in vitro*, the selective medium supplemented with growth cytokines were important for keeping stem cells alive in the progenitor state but not driving the differentiation of cells. For MSCs, BM mononuclear cells were allowed to attach with culture flask. Staining of MSCs surface markers indicated that isolated cells acquired MSCs characteristics, no fibroblast or macrophages contaminated.

H5N1 strain from avian isolates used in this study was phylogenetically clustered in the same group with highly pathogenic H5N1 viruses isolated from chickens, cats, tigers, and humans. Moreover, this strain was able to infect and replicate in monocyte derived dendritic cells (DCs) suggesting that virus might use migratory routes of DCs to escape immune response and dissemination to other organs. Using different techniques including flow cytometry, quantitative RT-PCR, plaque assay, immunofluorescence and confocal microscopy, we demonstrated that $CD34^+$ HSCs and MSCs were susceptible to H5N1 infection, although virus had less efficiency in infecting and replicating in $CD34^+$ HSCs. Infection of $CD34^+$ HSCs was not strain specific which implied that human-isolated H5N1 strain might show the similar result. Differential sources of $CD34^+$ HSCs did not affect the susceptibility to H5N1 infection. For MSCs, unexpected high infectivity and replication efficiency of H5N1 virus was observed. The viral titers in MSCs culture supernatants were comparable to those detected in human bronchial/tracheal epithelial cell culture supernatants (data not shown). These findings suggested that H5N1 virus had BM cells tropism. $CD34^+$ HSCs and MSCs could serve as a key amplifier of virus within BM. H5N1 virus may use two stem cell types as a source for viral distribution to downstream differentiated cells. Furthermore, infection of $CD34^+$ HSCs and MSCs

may promote the entering of virus into blood circulation and/or other tissues due to the mobilization characteristics of stem cells which are able to migrate to circulating blood and other organs. Previous studies demonstrated that genomic of CMV was detected in infected $CD34^+$ cells which were differentiating to form colonies of committed cells in methylcellulose culture. This indicated that viral progenies of CMV from $CD34^+$ cells were transferred during cells being differentiated (120). The other demonstrated that CMV infection impaired the process of adipogenic or osteogenic differentiation of MSCs suggesting that newly synthesized cells had an inefficient function and CMV particles could be distributed to descendants along differentiation process (114). Based on these data, H5N1 infection may interfere with hematopoiesis process, contributing to disrupt homeostasis in BM.

As the distribution of Sialic acid (SA) is one determinant of host tropism, thus, to examine SA distribution would confer a better understanding a host tropism and viral pathogenesis. Generally, H5N1 virus preferentially binds to sialic acid linked to galactose by an α2,3 linkage, whereas, human influenza virus binds to sialic acid linked to galactose by an α2,6 linkage. We therefore observed the SA distribution on both isolated $CD34^+$ HSCs and MSCs. SAα2,3Gal and SAα2,6 were equally expressed on both cells surface based on lectin staining which was inconsistent with susceptibility results of stem cells to H5N1 virus and human influenza viruses. It is possible that infection of stem cells is a unique property of H5N1 virus. As a matter of choice, SA is not sole receptor determining host tropism. Whether H5N1 virus enters stem cells via SA and/or alternative receptors remains challenging.

Productive infection and replication of H5N1 in MSCs are important findings that need to be explored the key viral virulence mediating in high pathogenicity. MSCs have clearly highly susceptibility to H5N1infection than human influenza viruses. It is associated with binding capacity of virus to cellular receptors. Hemagglutinin (HA) is responsible to receptor binding and entry to host cell, it is known to play a critical role in virulence (121). Previous studies demonstrated that HA represented a crucial factor in determining H5N1 tropism for human endothelial cells (122) and was important for induction of pro-inflammatory cytokines in human epithelial cells *in vitro* (123). Reverse genetic analysis indicated that HA of H5N1

virus played role in efficient infection and replication. However, HA might not be the sole determinant for producing high infectivity as wild-type.

Taken together, our results highlight a unique capacity and remarkable BM tropism of H5N1 virus that virus could directly infect and replicate in stem cells. These findings may help explain a severe pathogenesis resulting from H5N1 systemic infection.

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CHAPTER VII
PART II
MATERIALS AND METHODS

7.1 TUNEL assay for Apoptosis detection

Following virus absorption of 1×10^5 cells/well of 24-well plate at an MOI 10, $CD34^+$ HSCs and MSCs were cultured with specific media as previously described for 16 hours. Apoptosis was detected by terminal deoxynucleotidyl transferase (TdT) to label free 3'OH ends in genomic DNA with TMR red-dUTP using in situ cell death detection kit (Roche Applied Science, Indianapolis, Ind.) according to the manufacturer's instruction. Parallel samples were treated with DNaseI as a positive control. Briefly, 16 h after infection with H5N1, H1N1, or H3N2, cells were fixed with 4% paraformaldehyde in PBS for 1 hour at room temperature. After that, cells were three times washed with 1XPBS. Fixed cells were incubated in the labeling reaction mixture containing terminal deoxynucleotidyl transferase enzyme, TMR red-conjugated nucleotide for 1 hour at 37ºC and then cells were washed with PBS several times. To detect viral antigen after TUNEL staining, cells were incubated at 4ºC for 30 min with FITC-conjugated anti-NP antibody. DAPI (4',6'-Diamidine-2'-phenylindole dihydrochloride) (Roche Applied Science, Indianapolis, Ind.) was incubated at 37 °C in the dark for 15 minutes to visualize cell nuclei. Cells were then washed and examined with a Zeiss LSM 510 META confocal laser scanning microscope (Zeiss MicroImaging, Thornwood, NY, USA). The FITC and DAPI signal were excited at the wavelength of 488 nm and 405 nm, respectively.

7.2 Coculture of MSC and $CD14^+$ monocyte

$CD14^+$ monocytes were isolated as previously described (101). Briefly, Peripheral Blood Mononuclear Cells (PBMCs) were obtained by centrifugation using IsoPrep at 1000g for 30 min. PBMCs were washed twice with serum free media and

incubated with CD14 microbeads (Miltenyi, Gladbach, Germany) for 30 min at 4ºC. $CD14^+$ monocytes cultured in RPMI 1640 (Gibco, Life Technology, Rockville, Md., USA) supplemented with 10% FCS, 2mM L-glutamine and 1% Pen/Strep. 6×10^4 MSCs were seeded in 24-well flat-bottom plate in RPMI 1640 supplemented with 10% FCS and 1% Pen/Strep at 37°C, 5% CO_2 overnight. Freshly isolated $CD14^+$ monocytes 3×10^5 cells were added to each well (MSCs:Monocyte ratio 1:5). Confluent monolayers of coculture as well as MSCs and $CD14^+$ monocytes alone were washed 1X with serum free media and then, absorbed with H5N1 virus at MOI of 0.04, incubated at 37°C for 1 hour. Untreated cells were used as control. Following absorption, inoculum was removed, cells were washed three times with serum free media and 1 ml of RPMI 1640 media supplemented with 10%FBS was placed into wells and continued incubation at 37°C for 24 hours.

7.3 Cytokine assay

7.3.1 Quantitative analysis of mRNA expression by real time RT-PCR

The level of IFN-α, IFN-β, IP-10 and MxA expression from infected $CD34^+$ HSCs and MSCs was quantified by real-time PCR using the SYBR system. Total RNA was reverse transcribed into cDNA by reverse transcription reaction at 42ºC for 1 hour and used OligodT as a primer. Specific primers for each cytokine genes were provided in table 7.1. The PCR amplification was performed in condition as follows: 95°C for 15 min, 40 cycles of denaturation at 95°C for 30 sec, annealing 55°C for 30 sec, and extension at 72°C for 30 sec. Gene copies were quantified on the basis of a SYBR green fluorescence signal by Rotor-Gene 3000 (Corbett Robotics) using beta-actin as a control. The RNA copies were calculated based on the standard curve generated with serial dilution of plasmid (from 1-10^7 copies) containing the respective cloned gene target

7.3.2 Measurement of cytokine production by ELISA

The concentration of IL-6, IFN-γ, MCP-1 and MIP-1β in supernatant of H5N1-infected $CD34^+$ HSCs, MSCs and coculture were quantified by Bio-Plex Pro Human Cytokine assay (Bio-Rad, Hercules, CA) following the manufacturer's instructions. Supernatants from mock-infected cultures were used as a control. The assay was performed by using beads specific for human cytokines and detected by fluorescently conjugated monoclonal antibodies in duplicate against a standard curve. The shown data were calculated by Bio-plex manager 5.0 software.

Table 7.1: Primers for Cytokine gene amplification

Genes	Forward primer	Reverse primers	Product size
B-actin	5' CCTGGCACCCAGCACAAT 3'	5' GGGCCGGACTCGTCATAC 3'	144 bp
IFN-α	5'AGAATCACTCTCTATCTGAAAGAGAA GAAATA 3'	5' TCATGATTTCTGCTCTGACAACCT 3'	73 bp
IFN-β	5' AAACTCATGAGCAGTCTGCA 3'	5' AGGAGATCTTCAGTTTCGGAGG 3'	168 bp
MxA	5' TTCAGCACCTGATGGCCTATC 3'	5' TGGATGATCAAAGGGATGTGG 3'	70 bp
IP-10	5' GAAATTATTCCTGCAAGCCAATTT 3'	5' TCACCCTTCTTTTTCATTGTAGCA 3'	68 bp

CHAPTER VIII
PART II
RESULTS

8.1 Apoptosis induction of $CD34^+$ HSCs and MSCs by H5N1 virus

H5N1 infection of $CD34^+$HSCs resulted in less cytopathic effects (CPE) and cell deaths than observed in MSCs under light microscope. To determine the impact of H5N1 infection on the survival of stem cells, BM and CB-derived $CD34^+$HSCs and BM-derived MSCs were infected with live and inactivated H5N1 viruses and human influenza viruses with MOI 10. After 24 h, cells were counted for viability. We found that 60% of $CD34^+$HSCs and 40% of MSCs were viable as compared with mock (Figure 8.1). Therefore, we hypothesized that this was due to apoptosis of these cells. In order to investigate the fate of $CD34^+$ HSCs and MSCs after infection, $CD34^+$ HSCs grown on coverslips were infected with H5N1 virus, whereas MSCs were infected with both avian and human influenza viruses at MOI of 10. Mock infection and DNase-treated cells were used as negative and positive control, respectively. At 18 h p.i., apoptotic cells were characterized by positive terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) staining (Red) (In Situ Cell Death Detection Kit, TMR red, Roche), infected cells were detected by viral nucleoprotein staining, and analyzed the co-localization of viral antigen and apoptotic signal by using confocal microscopy. TUNEL assay confirmed that H5N1 virus could induce apoptosis in both $CD34^+$ HSCs and MSCs (Figure 8.2A and 8.2B). Virus-induced cell deaths were seen in almost all H5N1-infected MSCs but not in those infected with human influenza viruses. The apoptosis was also observed in $CD34^+$ HSCs, but most of apoptotic signals were detected in surrounding uninfected cells, only a few ones were seen in infected cells (Fig. 8.2A). The apoptosis result of both $CD34^+$ HSCs and MSCs were correlated with the viability data in Figure 8.1. These findings indicated that induction of apoptosis in MSCs was a

direct result of H5N1 infection, while it was an indirect effect in $CD34^+$ HSCs. Whether indirect effect caused by paracrine factors should be further investigated.

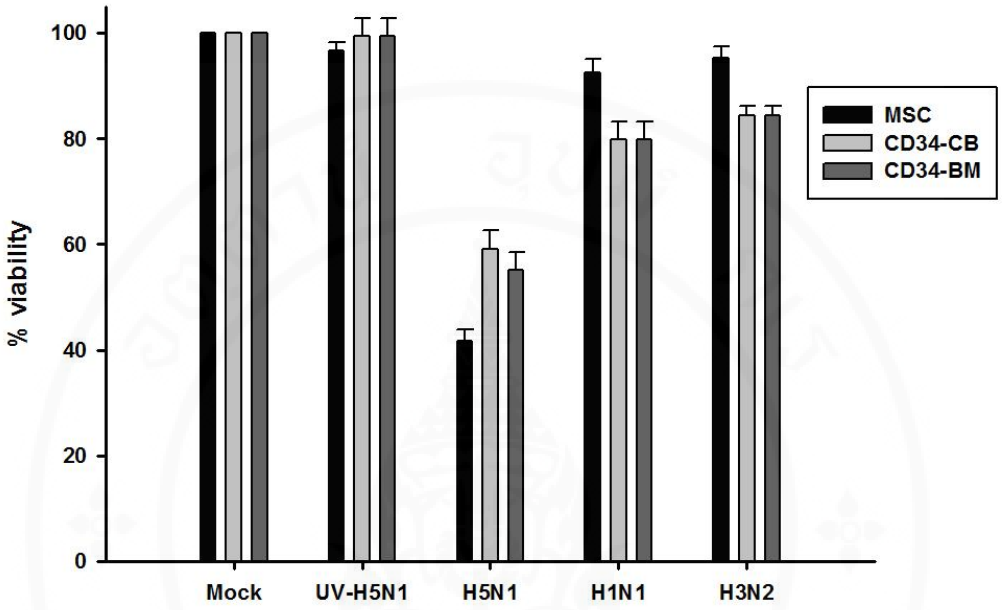
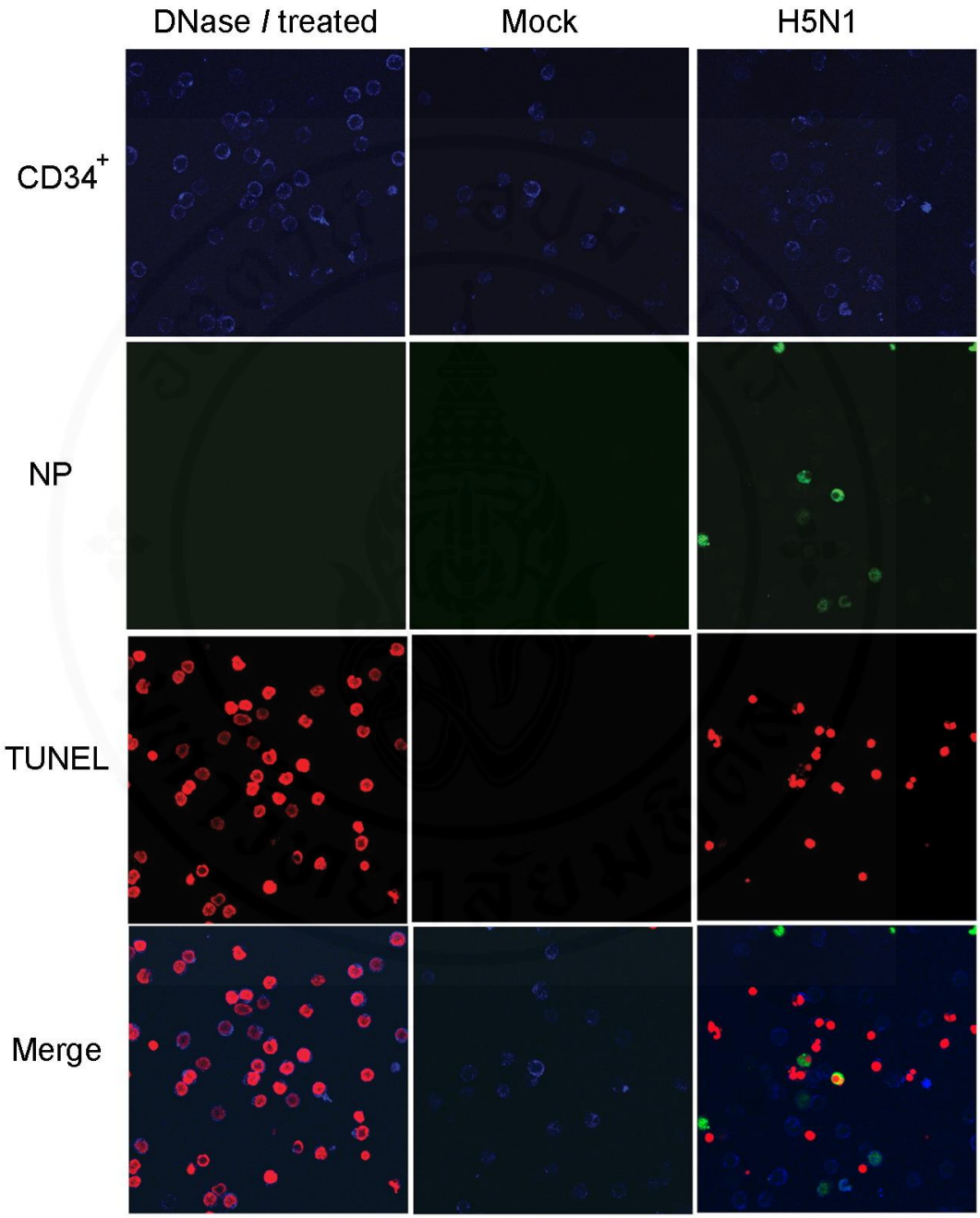


Figure 8.1: Viability of $CD34^+$ HSCs and MSCs after influenza virus infection. BM and CB-derived $CD34^+$ HSCs and BM-derived MSCs were infected with UV-inactivated and live H5N1 (BBD0104F/04), H1N1, and H3N2 virus MOI of 10 for 24 hours. Control is mock infection. The number of viable cells was obtained by comparing with mock. The error bars represent SD of three independent experiments

A.



B.

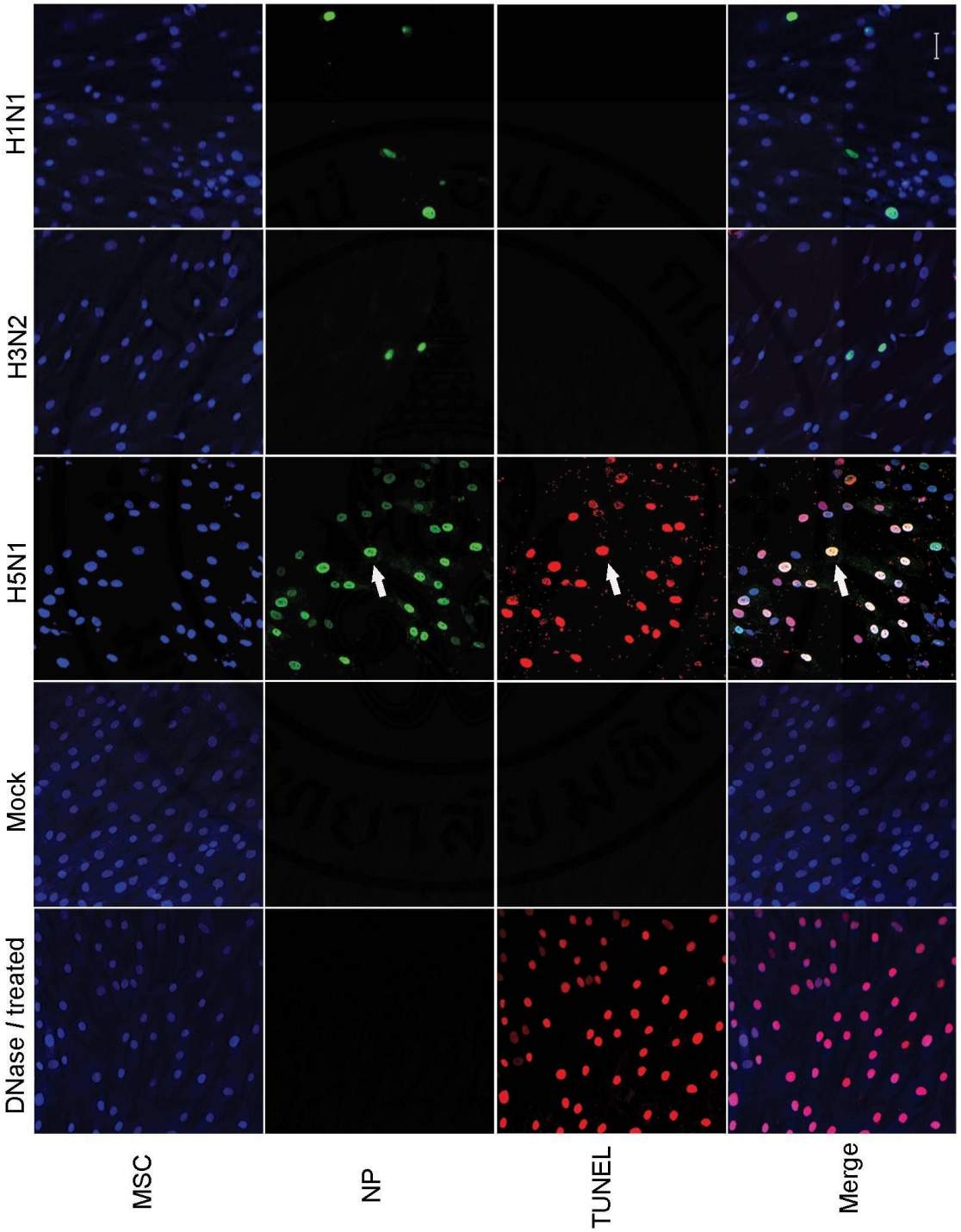


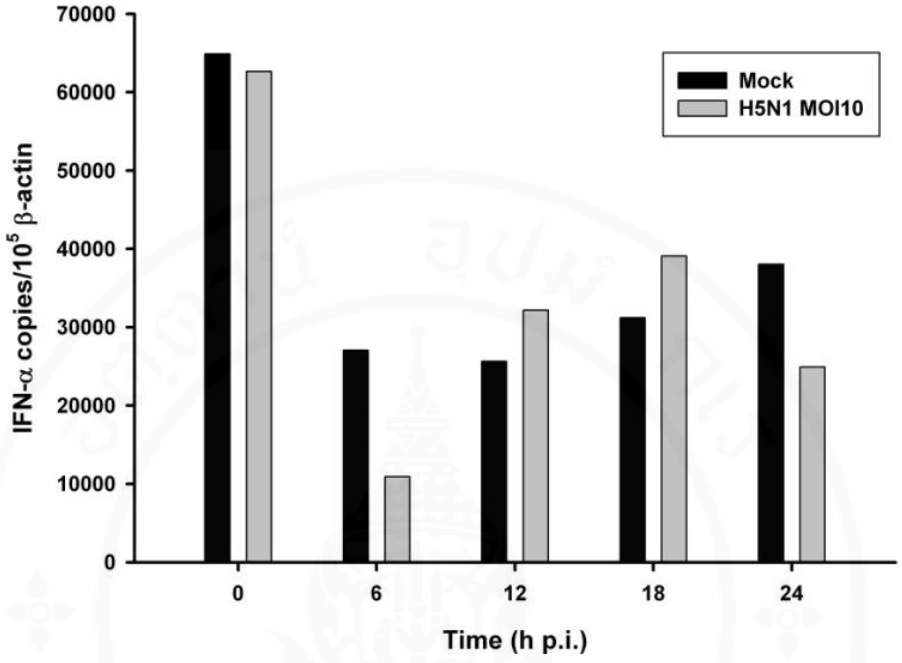
Figure 8.2: Apoptosis detection in H5N1-infected CD34$^+$ HSCs and MSCs. (A) CD34$^+$ HSCs were infected with H5N1 virus at MOI 10, whereas **(B)** MSCs were infected with avian and human influenza viruses at the same MOI as CD34$^+$ HSCs. Parallel samples were treated with DnaseI which breaks double stranded DNA as a positive control. After 18 hours post infection, cells were fixed, permeabilized and multiple stained using Terminal deoxynucleotidyl transferase–mediated dUTP-biotin nick end-labeling (TUNEL) staining (red), FITC-labeled anti-Nucleoprotein (green) and DAPI (blue). Arrows in B. indicate that cells expressed intracellular viral antigen and apoptotic signal simultaneously. Data shown are from one representative donor of three different donors.

8.2 Host innate response of influenza-infected CD34$^+$HSCs and MSCs

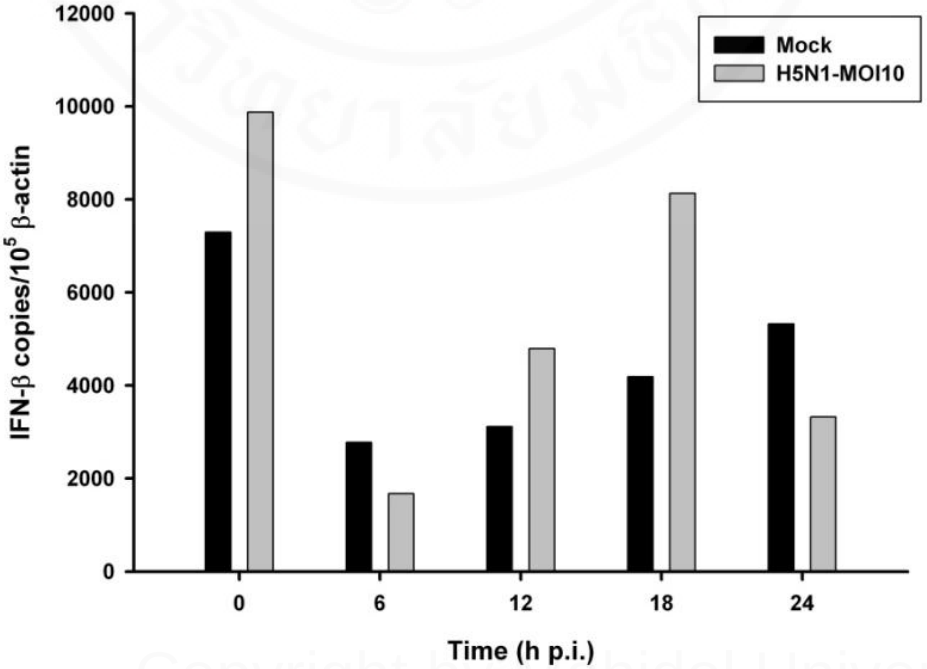
Once H5N1 virus infects target cells, it has a capability of activating host innate response which can be either protection or immunopathology from hyper-inflammatory cytokines production. We then investigated host response to infection with H5N1 in CD34$^+$ HSCs and MSCs. Interferon (IFN) and Mx1 (an interferon-stimulated antiviral gene) are well characterized components of the innate immune response directed against viral infections. We therefore examined the expression levels of IFN signature genes: IFN-α, IFN-β, MxA, and IP-10 in CD34$^+$ HSCs and MSCs by Real time RT-PCR. CD34$^+$ HSCs were infected with H5N1 MOI 10 because we could not see any changes when infected cells with MOI 1, whereas, MSCs were infected with both avian (H5N1) and human influenza viruses (H1N1 and H3N2) at MOI 1. Mock infection was used as a control. Cells were harvested at various time points (0, 6, 12, 18, 24 h) to perform RNA extraction, reverse transcription and real-time PCR by using specific primers for each cytokines. IFN-α and IFN-β genes from H5N1-infected CD34$^+$ HSCs were expressed in the same pattern which were not different from control cells at any time points, but were slightly increased in time-dependent manner (6 h to 18 h p.i.). Both gene expressions were dropped after 18 h p.i. (Figure 8.3A and 8.3B). Surprisingly, MxA and IP-10 were strongly triggered in H5N1-infected CD34$^+$ HSCs in every time points over mock infection. However, the expressions were slightly decreased after 18 h p.i. same as IFN-α and IFN-β (Figure 8.3C and 8.3D).

For MSCs, we compared the expression levels of cytokine genes by H5N1 and human influenza viruses. Real-time RT-PCR was performed at 0, 6 and 24 h p.i. Consistent with replication data, IFN-α, IFN-β, and MxA expression levels were up-regulated significantly higher than human influenza viruses (H3N2 and H1N1) and mock infected at 24 h p.i. (Figure 8.4A-8.4C). Conversely, IP-10 expression was undetectable in infected MSCs (data not shown). Together, these findings demonstrated that H5N1 infection resulted in a substantial induction of innate immune response in CD34$^+$ HSCs and MSCs.

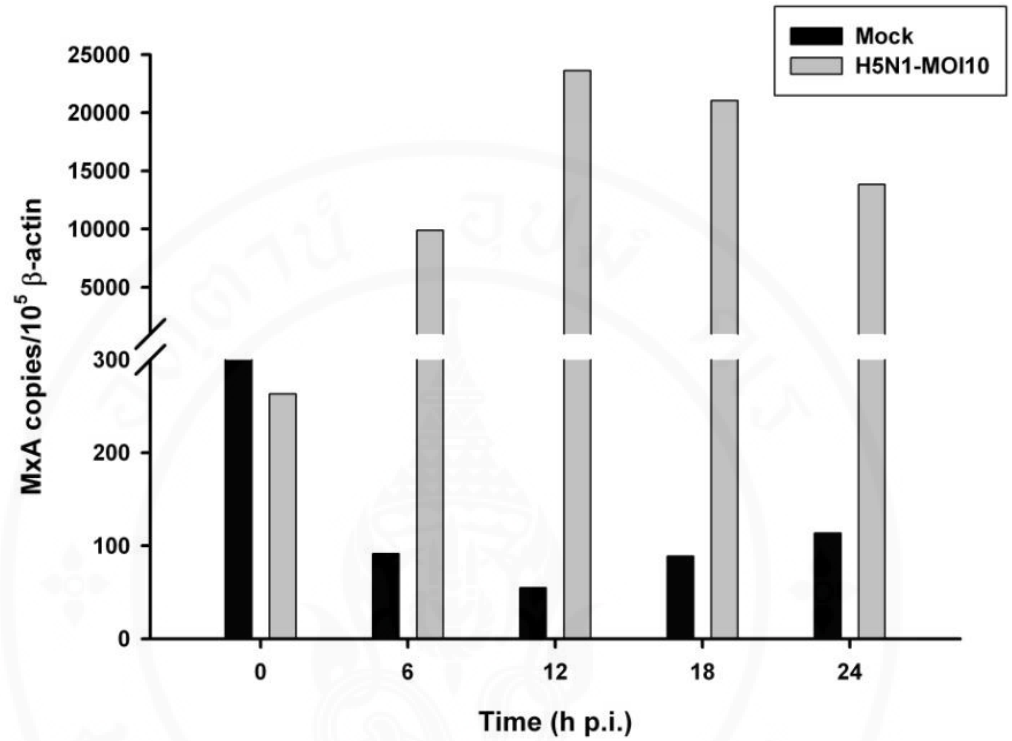
A. CD34^{+} HSCs-IFN-α



B. CD34^{+} HSCs IFN-β



C. CD34^{+} HSCs MxA



D. CD34^{+} HSCs IP-10

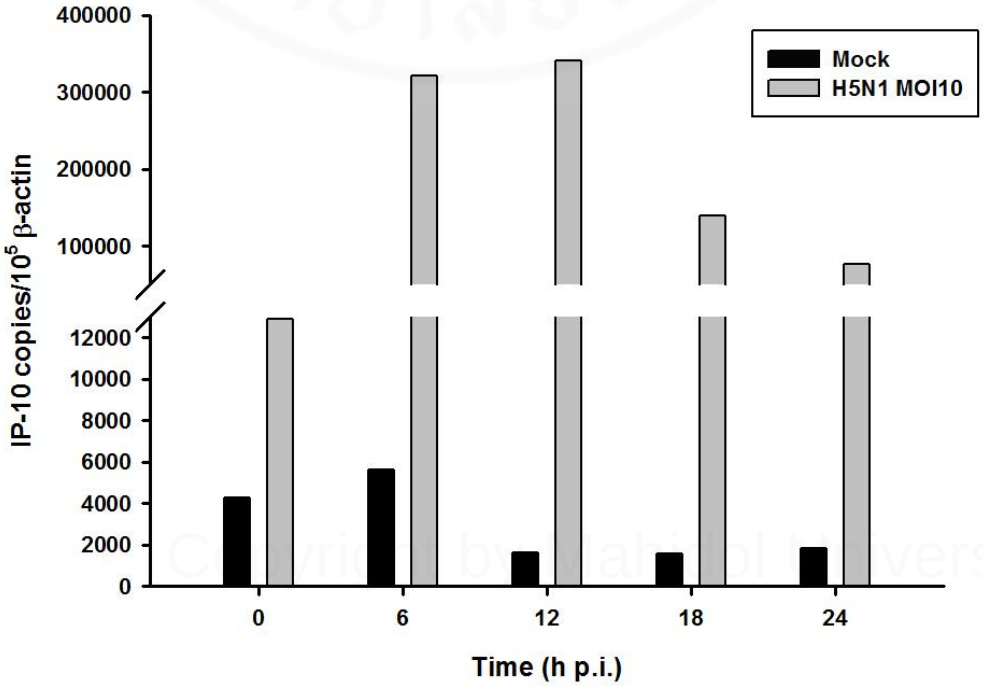
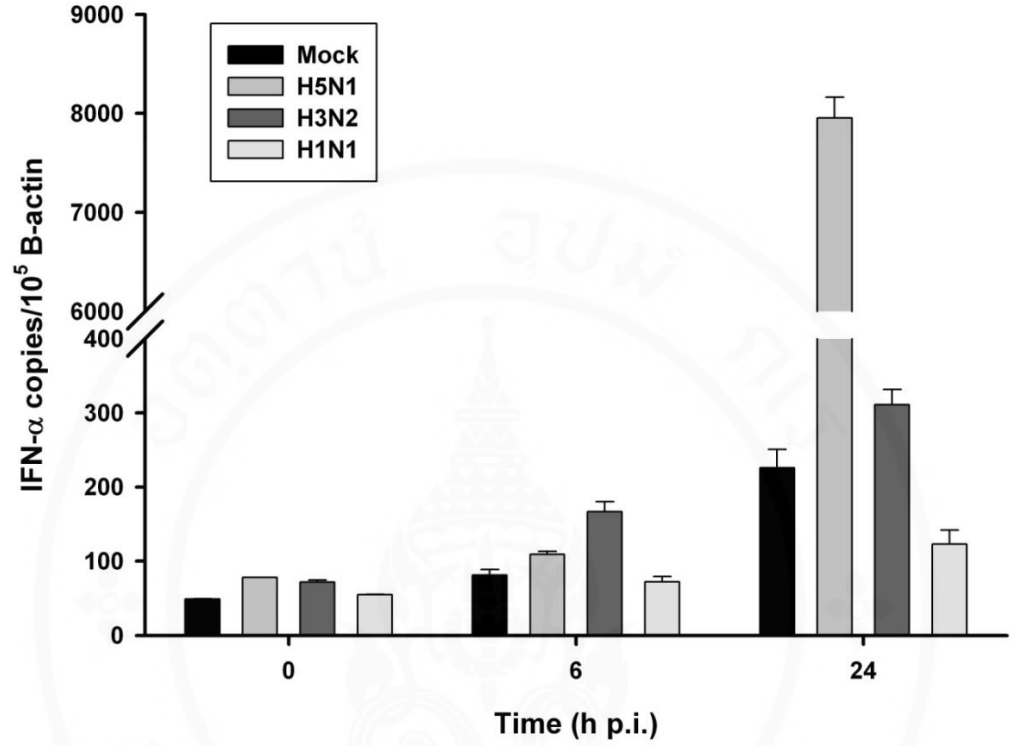
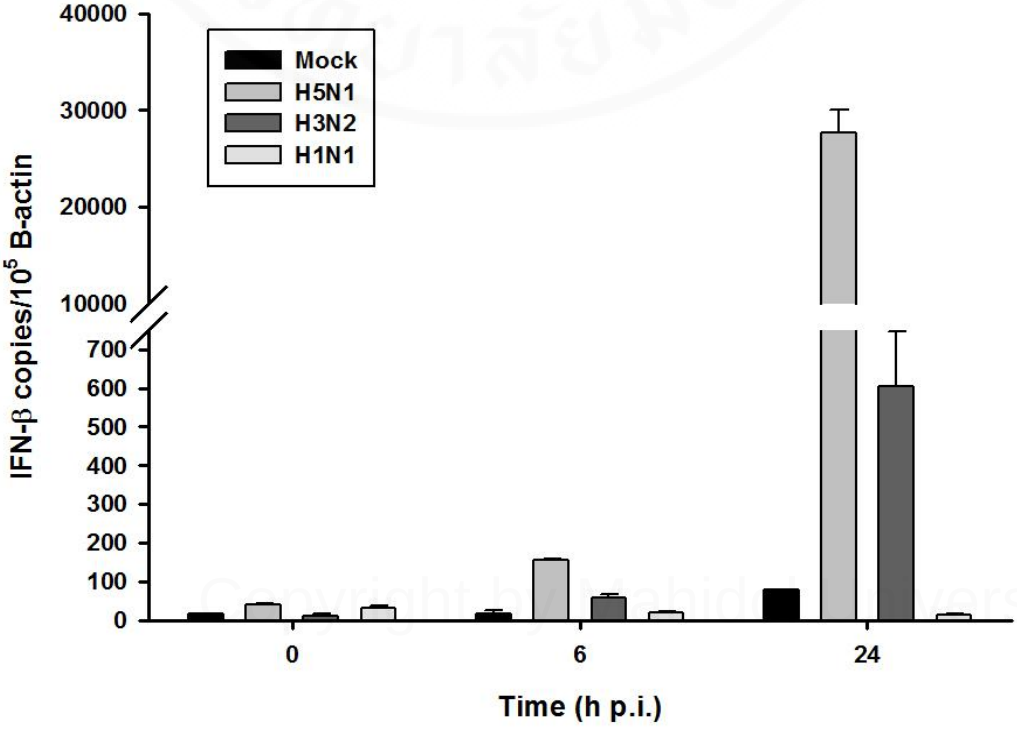


Figure 8.3: mRNA Expression of IFN-*α*, IFN-β, MxA and IP-10 in CD34^{+} HSCs following H5N1 infection. Cells were infected with H5N1 (BBD0104F/04) MOI 10. At indicated time points, RNA of uninfected and infected cells were extracted and reverse transcribed. **(A)** IFN-α, **(B)** IFN-β, **(C)** MxA and **(D)** IP-10 were amplified by using specific primers. Real-time PCR was performed to examine cytokine gene expression as a copy number, using beta-actin as a control. Data shown are from one representative donor. The experiments have been repeated several times with similar results.

A. MSCs-IFNα



B. MSCs-IFN-β



C. MSCs-MxA

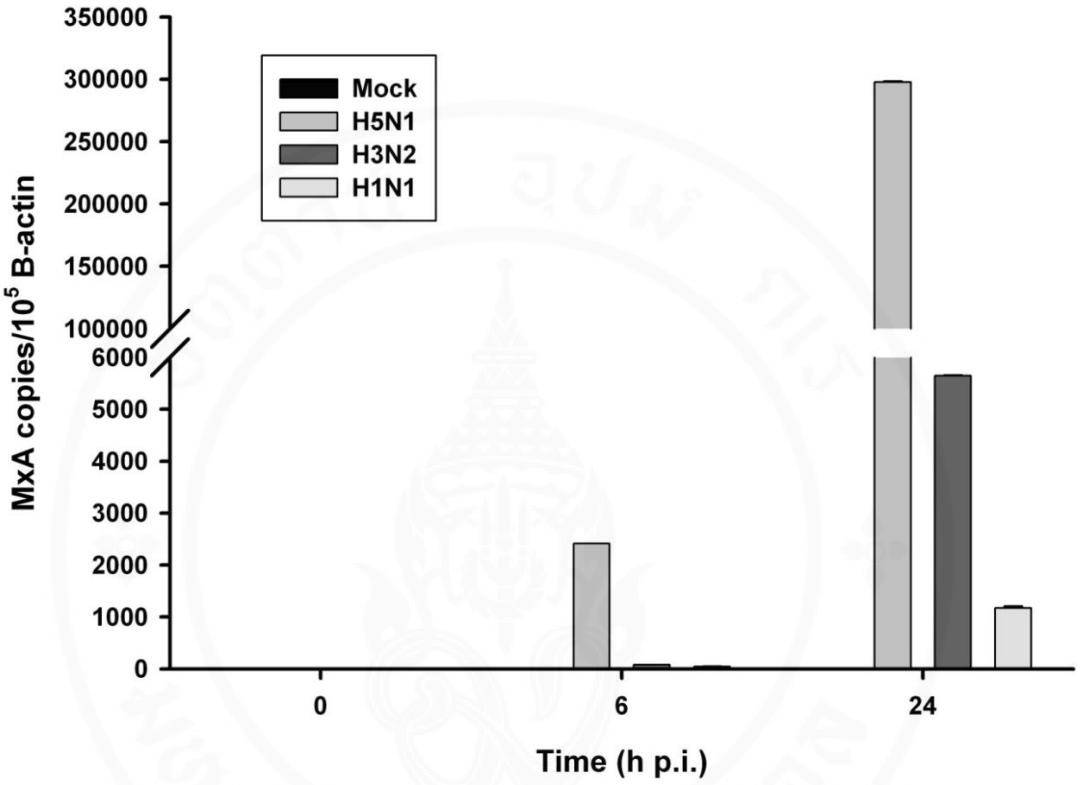
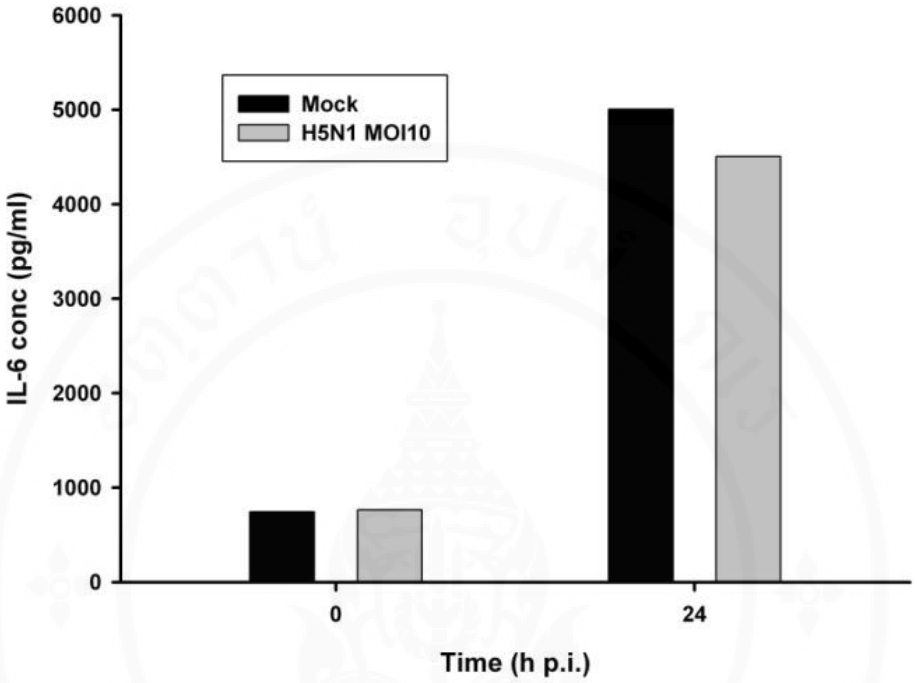


Figure 8.4: mRNA Expression of IFN-α, IFN-β and MxA in MSCs following H5N1 infection. Cells were infected with H5N1 (BBD0104F/04), H3N2 and H1N1 at MOI of 1. At indicated time points, cells were subjected to perform RNA extraction, cDNA synthesis, and quantified mRNA expression of **(A)** IFN-α, **(B)** IFN-β, and **(C)** MxA using real-time PCR. The graph shows the means and standard deviations (SD) of mRNA copies expressed per 10^5 β-actin copies from three representative donors.

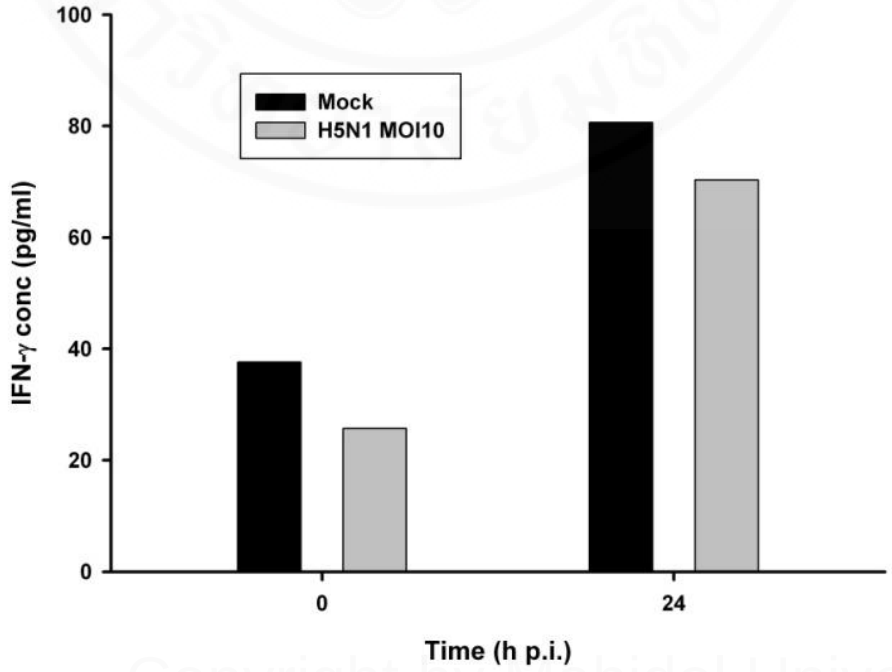
8.3 Cytokines and chemokines production by influenza-infected CD34$^+$HSCs and MSCs

As H5N1 virus has been reported a potent cytokine inducer, hyperactivation of pro-inflammatory cytokines following infection may cause inflammation in surrounding tissues. In addition, some chemotactic proteins or chemokines secreted by infected cells would trigger the influx of other immune cells to the site of infection. Thus, it is important to investigate the differential host response in the protein level. We then explored the potential of H5N1 infected CD34$^+$HSCs and MSCs in producing cytokines. Culture supernatants were collected in CD34$^+$HSCs with being infected with H5N1 at MOI 10, and MSCs infected with H5N1, H3N2 and H1N1 at MOI 1. Secreted cytokines in the supernatants of both mock and H5N1-infected cells were measured at 0 and 24 h p.i. by Bio-plex Pro-human cytokine. The amount of cytokines were analyzed and displayed as output data by using Bio-Plex manager 5.0 software. As shown in Figure 8.5A and 8.5B, the production of IL-6 and IFN-γ were not triggered by H5N1 infection in CD34$^+$ HSCs at 24 h p.i. as seen in the level of H5N1 infection was comparable with control. However, chemokines; MCP-1 and MIP-1β which play role in the recruitment of immune cells to infected areas were significantly up-regulated at 24 h p.i. (Figure 8.5C and 8.5D). For MSCs, IL-6 level in H5N1 infection was comparable with mock and human influenza virus infection (Figure 8.6A). Nevertheless, IFN-γ (Data not shown) and MCP-1 (Figure 8.6B) of H5N1-infected cells were higher than mock but not different to human influenza virus infected. These findings suggested that H5N1 virus could not induce a large amount of cytokines secretion in CD34$^+$ HSCs and MSCs. However, H5N1-infected CD34$^+$ HSCs but not MSCs were able to produce chemokines such as IFN-γ, MCP-1 and MIP-1β. Therefore, the inflammation in BM tissue may occur from more immune cells recruited.

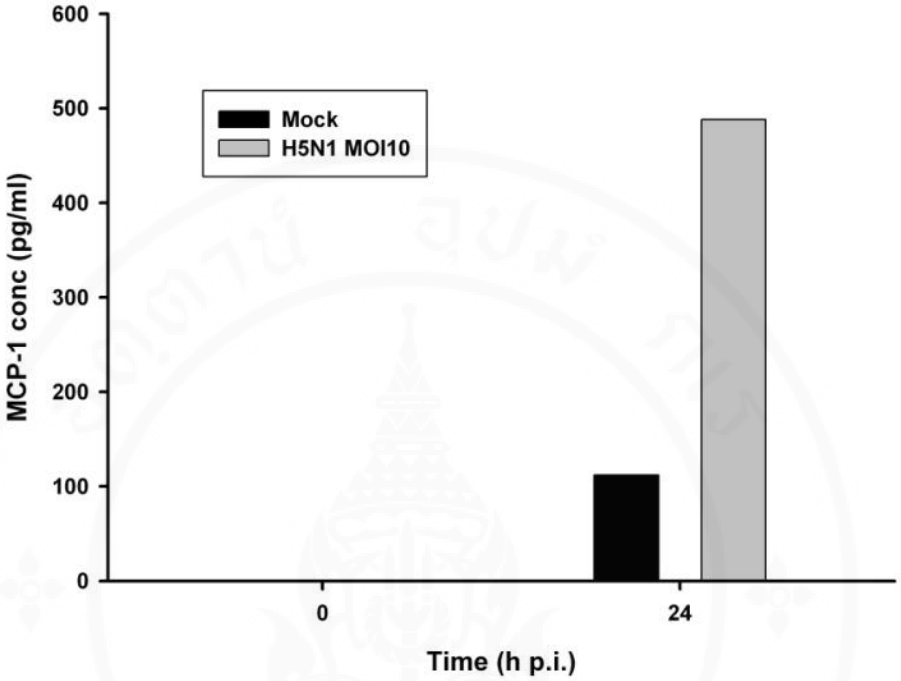
A. CD34^{+} HSCs-IL-6



B. CD34^{+}HSCs-IFN-γ



C. CD34^{+}HSCs-MCP-I



D. CD34^{+}HSCs-MIP-1β

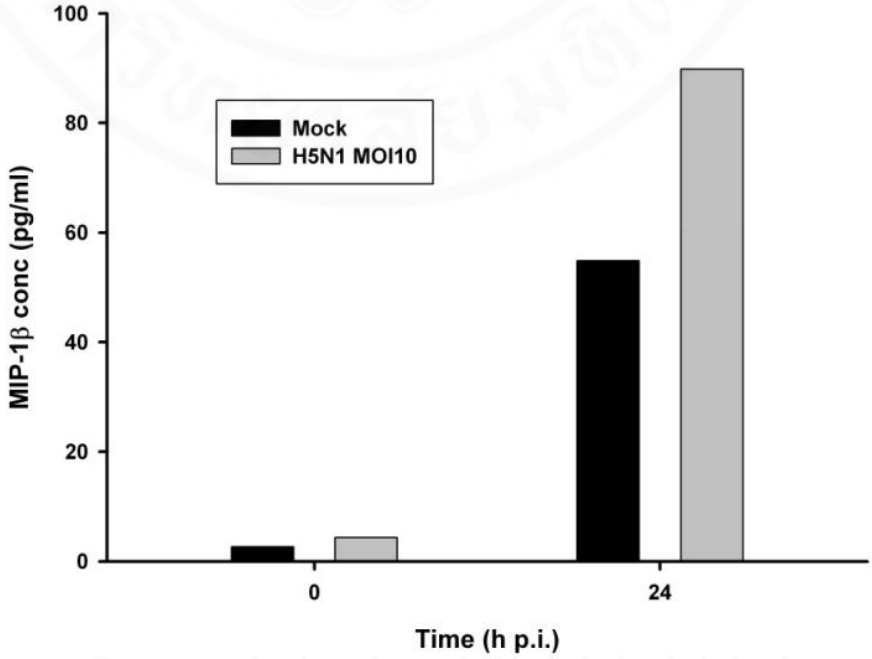
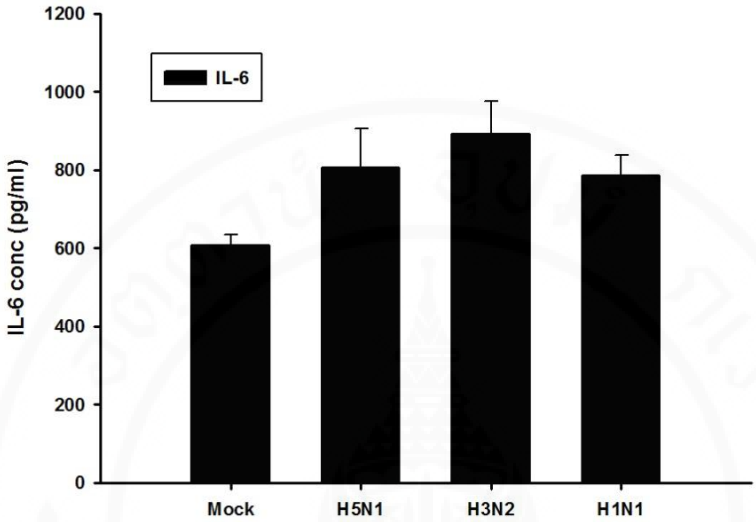


Figure 8.5: Evaluation of cytokine production in H5N1-infected CD34^{+} HSCs.
Cytokine and chemokine protein secretion of **(A)** IL-6, **(B)** IFN-γ, **(C)** MCP-1 and **(D)** MIP-1β in CD34^{+}HSCs infected with mock (black bars) and H5N1 virus (BBD0104F/04) MOI of 10 (grey bars). Culture supernatants were collected at 0 and 24 h postinfection. Each cytokine was measured using the Bio-Plex Pro-human cytokine assay and analyzed by Bio-Plex 5.0 software program. Data shown are from one representative donor.

A. MSCs-IL-6



B. MSCs-MCP-1

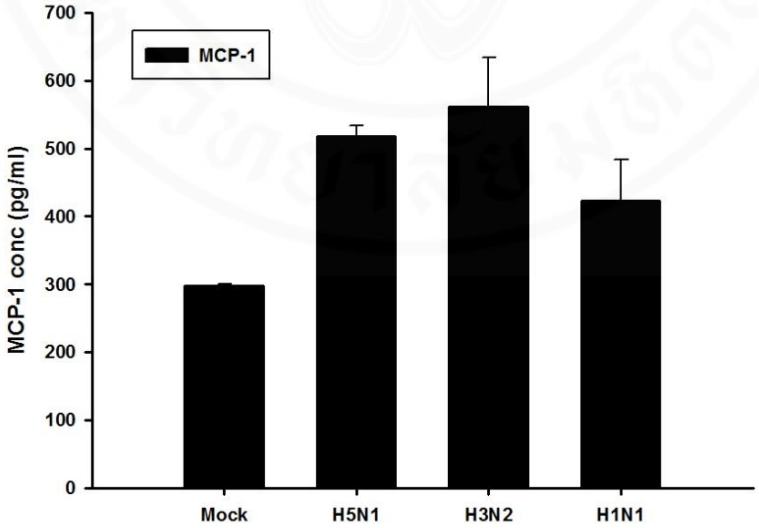
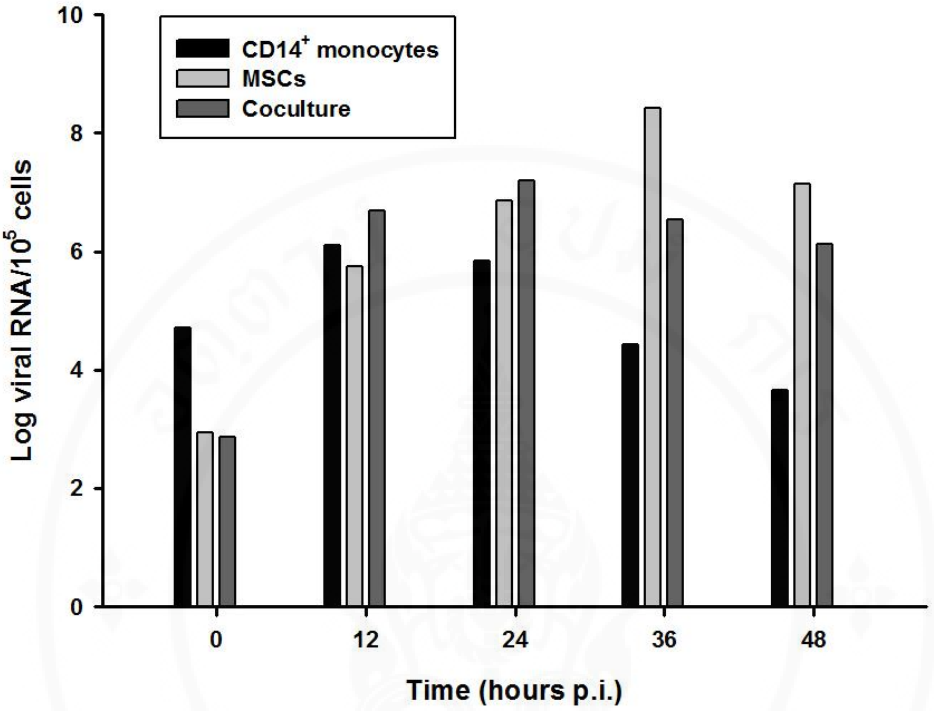


Figure 8.6: Evaluation of cytokine and chemokine production in influenza-infected MSCs. Cells were infected with H5N1 (BBD0104F/04), H3N2 and H1N1 at MOI 1. At 24 h after infection, culture supernatants were collected to measure **(A)** IL-6, and **(B)** MCP-1 by Bio-Plex assay (Bio-Rad Laboratories, Inc., Hercules, CA). Obtained results were analyzed by using Bio-Plex 5.0 software program. Data are mean cytokine secreted ± SD from two different donors.

8.4 Alteration of MSCs-mediated immune modulation by H5N1 virus

Considering MSCs are non-immune cells that possess an immunomodulative activity. This is crucial for exploring the role of MSCs in H5N1 pathogenesis. Here, we hypothesized that H5N1 virus could alter MSCs-mediated immune modulation. Since MSCs have been shown to suppress the differentiation of Monocyte-derived Dendritic cells (MoDCs) via either cell contact or paracrine factors. We designed an experiment by infecting H5N1 virus in coculture of 1:5 ratio of MSCs and $CD14^+$ monocytes at MOI 0.04. Real-time RT-PCR was used for detecting viral gene expression at 0, 12, 24, 36 and 48 h p.i. Based on M gene expression, a relatively similar infection rate was observed in coculture system and MSCs alone (Figure 8.7A). In addition, the replication kinetics of virus in coculture was also comparable with cells alone (Figure 8.7B), indicating that co-culture of MSCs and monocyte did not enhance infectivity and replication efficiency of H5N1 virus. We also analyzed the alteration of cytokine profiles of infected coculture in comparison with MSCs alone and $CD14^+$ monocytes alone. At 24 h p.i., culture supernatants were collected to determine cytokine production. Cytokines and chemokines were measured by using 17-plex Pro Human Cytokine Assay. We found that IL-6 was not significantly different between mock and H5N1-infected MSCs, whereas, barely detectable in infected $CD14^+$ monocytes. Chemokines; MCP-1 and MIP-1β were not significantly different between mock and H5N1-infected MSCs as well, but were increased in infected $CD14^+$ monocytes. Expectedly, these cytokine and chemokine levels (IL-6, MCP-1, and MIP-1β) were significantly up-regulated in infected MSCs/monocytes coculture compared with its control and much higher than MSCs or $CD14^+$monocytes alone (Figure 8.8A). In contrast, some cytokines and chemokines such as IL-1β, IL-8, and GM-CSF levels were not significantly different between infected coculture and non-coculture cells. H5N1-infected coculture produced higher IL-8 than cultured cells alone but the level was comparable with mock infection (Figure 8.8B). These findings suggested that coculture of MSCs and monocytes did not enhance the infection of H5N1 virus. However, coculture cells secreted high level of pro-inflammatory cytokine (IL-6) and chemokines (MCP-1 and MIP-1β).

A.



B.

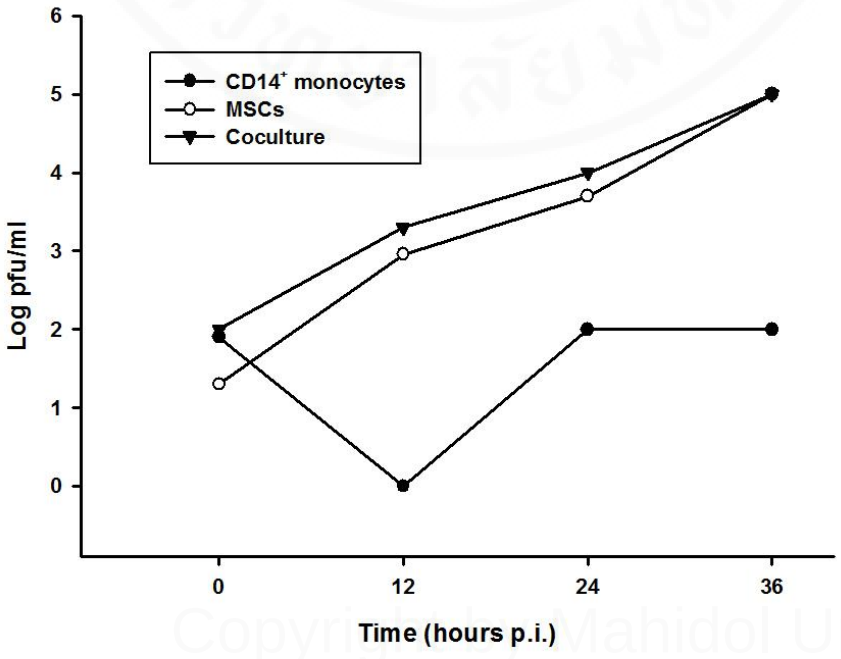
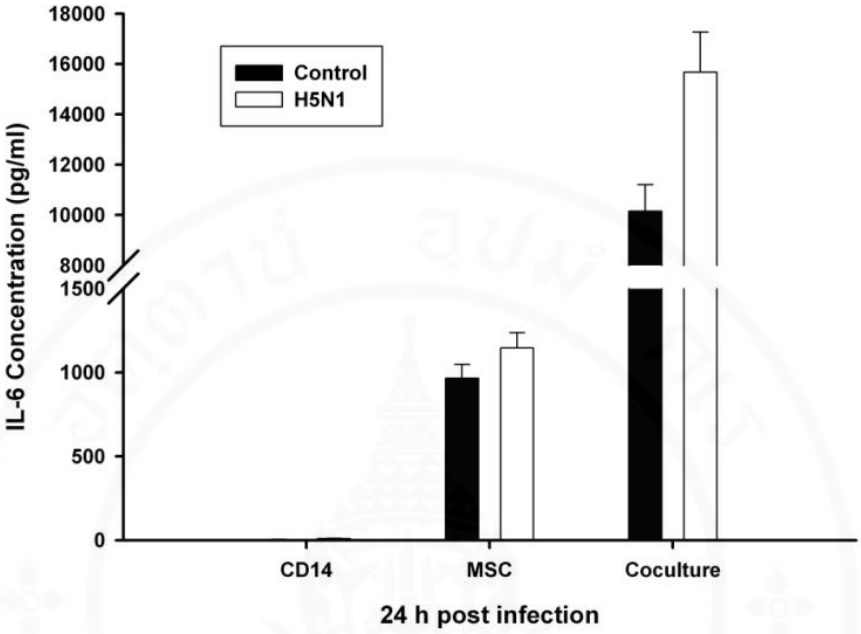
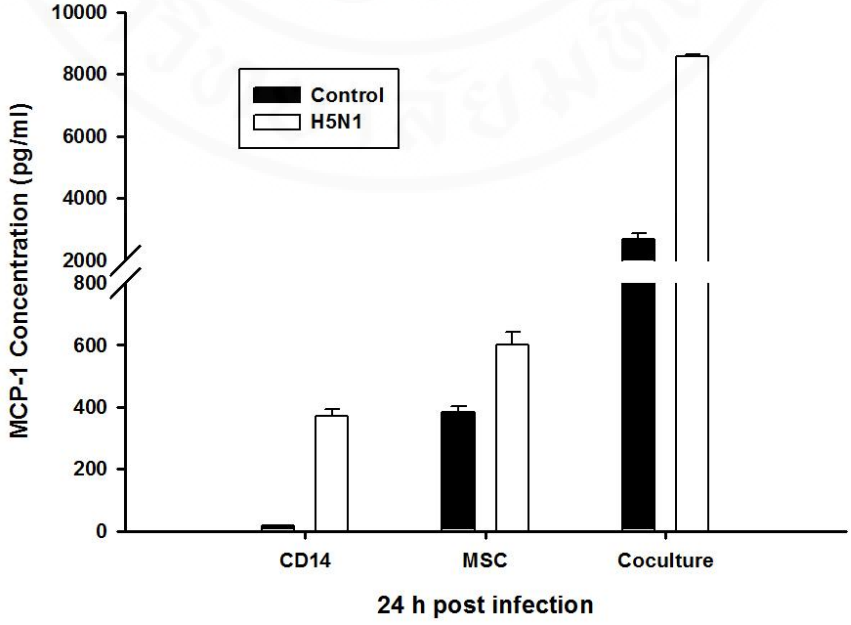


Figure 8.7: Susceptibility of MSCs/CD14$^+$ monocytes coculture to H5N1 infection. MSC were plated overnight in 24-well plate with completed RPMI medium. Then, freshly isolated primary human CD14$^+$ monocytes were seeded on MSCs in 5:1 ratio. MSCs alone and CD14$^+$ monocytes alone, and CD14$^+$ monocytes/MSCs coculture were infected with H5N1 virus (BBD0104F/04) with MOI 0.04. Untreated cells were used as a control. **(A)** Mock and infected cells were harvested at indicated time points in order to determine viral gene expression by quantitative real-time RT-PCR. **(B)** Culture supernatants were collected to examine the replication kinetics and viral output by plaque assay. MSCs and CD14$^+$ monocytes were derived from different donors. Data shown are from one representative experiment.

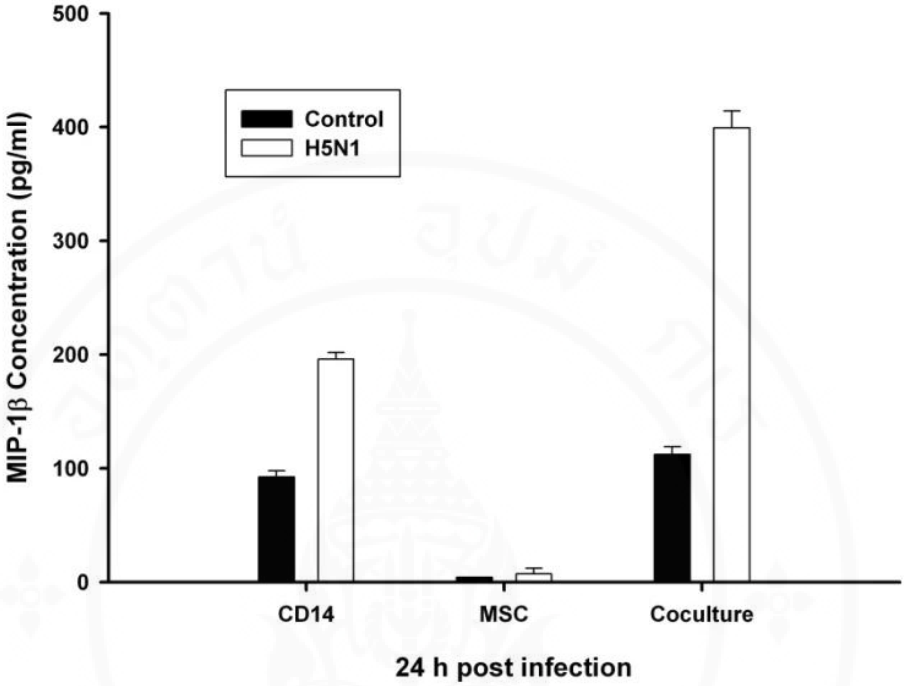
A. **IL-6**



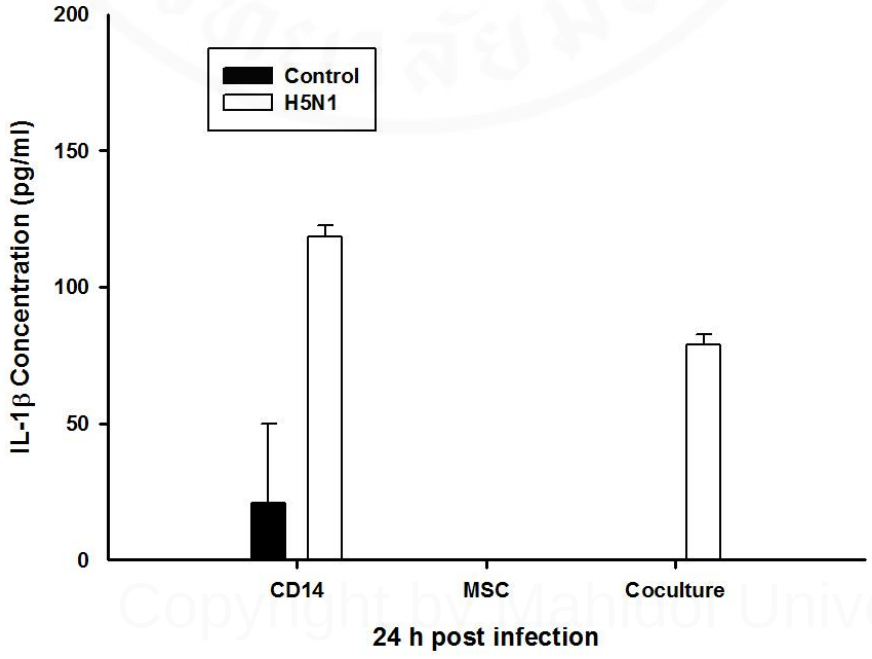
MCP-1



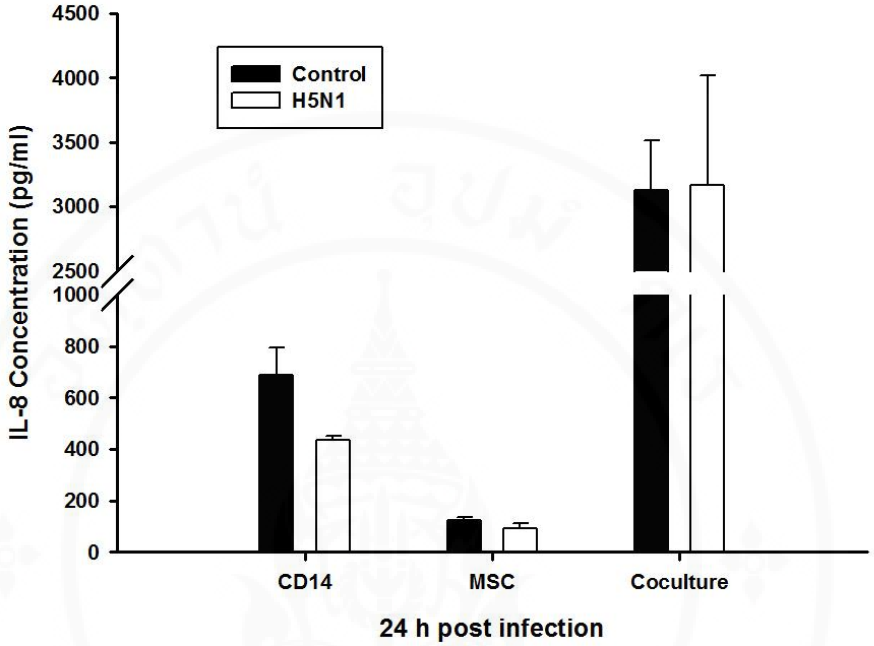
MIP-1β



B. **IL-1β**



IL-8



GM-CSF

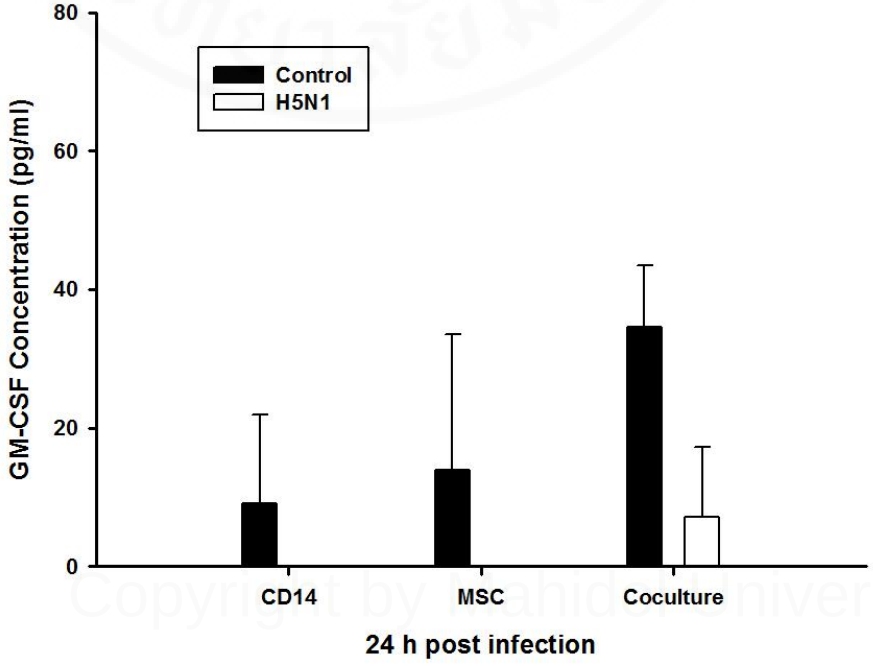


Figure 8.8: Cytokine and chemokine productions from MSCs/CD14$^+$ monocytes coculture after influenza virus infection. Cytokines and chemokines; **(A)** IL-6, MCP-1 and MIP-1β and **(B)** IL-1β, IL-8, and GM-CSF from supernatants of infected CD14$^+$ cells and MSCs alone and MSCs/CD14$^+$ cells coculture with H5N1 (BBD0104F/04) MOI 0.04. At 24 h after infection, each cytokine was measured in triplicate by using Bio-plex cytokine assay. MSCs and CD14$^+$ monocytes were derived from different donors. Data are shown as mean of cytokine secreted ± SD from triplicate measurements.

CHAPTER IX
PART II
DISCUSSION

Our findings of susceptibility of $CD34^+$HSCs and MSCs to H5N1 prompted us to investigate the outcomes of infection which were needed to elucidate the important interaction of stem cells and pathogen. This may help to better understand a severe pathogenesis of systemic infection of H5N1 virus.

H5N1 infection of $CD34^+$HSCs and MSCs induced cell-killing observed by prominent CPE, reduced cell viability and positive TUNEL signal. Half reduction of relative cell viability of H5N1-infected cells revealed cell death after infection. Positive TUNEL reaction indicated that H5N1 virus induced cell death by apoptosis. Not only did H5N1 infect MSCs efficiently, it also induced apoptosis of infected cells. The localization of virus antigen and apoptosis signal implied that apoptosis was caused by virus infection. For $CD34^+$ HSCs, the number of TUNEL positive-cells was correlated with the percentage of viable cells but not with the infection rate. Unexpectedly, apoptosis signals in H5N1-infected $CD34^+$ HSCs were not localized with viral antigen. The presence of apoptosis in surrounding cells may be induced by paracrine factors. Alternatively, apoptosis of uninfected cells might be caused by entering of virus into cells without causing any effects. Factors involved with apoptosis of uninfected $CD34^+$ HSCs need to be investigated in future studies. For MSCs, this phenomenon was exclusive for only H5N1 virus since there was no apoptosis signal in cells infected with human influenza viruses.

Studies demonstrated that apoptosis played a major role in H5N1 pathogenesis. Infection of epithelial cells and lymphocytes has been shown to induce apoptosis *in vitro* (124, 125). Increased apoptosis in human lymphocytes leading to severe lymphopenia was caused by either direct infection or over-activation of cytokine response (125). The apoptosis of $CD34^+$HSCs and MSCs either caused by direct or indirect infection probably affects progenitor cell numbers and also a

potential for renewal and ability to differentiate into specialized cells. Due to $CD34^+$ HSCs and MSCs are primitive progenitor cells that contain self-renewal ability and generate the descendant cells in hematopoisis and mesengenic process, the reduction of precursor cells may perturb these two processes which eventually decrease the number of differentiated cells in all lineages. Dysregulation of hematopoiesis has been observed in depleted stromal and $CD34^+$ populations by MV infection which significantly impaired the repopulation of lymphoid precursors resulting in MV-induced lymphopenia (113). Our data supported abnormal hematologic findings, like low peripheral blood counts, thrombocytopenia, and pancytopenia which are prominent clinical features in severe H5N1 patients. Nonetheless, our result was inconsistent with previous study (126). Uiprasertkul M. *et al.* suggested that BM was not likely responsible for the leukopenia and thrombocytopenia since there was no viral RNA detected as well as no increased of apoptotic cells in BM of fatal H5N1 cases (127). It is possible that there might be a loss of replication-competent virus during samples storage, and also host immune response could limit the spread of virus.

Innate immune responses are especially critical for controlling influenza virus replication. IFNs play roles in first line defense against infection. However, the viral non-structural protein NS1 has the capacity to counteract the IFNs system in order to replicate and escape the innate immune response. Here, we analyzed the host responses of $CD34^+$ HSCs and MSCs directed against viral infection by observing the expression of; IFN-α, IFN-β, MxA, and IP-10. H5N1-infected $CD34^+$HSCs did not express IFN-α and IFN-β mRNAs when compared with mock. Although IFNs were not induced in infected $CD34^+$ HSCs, MxA which is an interferon-inducible protein, was strongly up-regulated. It is possible that MxA was activated directly by H5N1 virus in the absence of IFNs secretion. However, this issue has not been described. Similarly, IP-10, an interferon gamma-induced protein, was also activated, despite a low amount secreted of IFN-γ (Figure 12B). Virus might directly activate IP-10 mRNA expression independent of IFN-γ. As IP-10 played role in the recruitment of other immune cells to the site of infection, thus, $CD34^+$HSCs were more likely to have a capability of recruiting other cells via chemokines production instead of being pro-inflammatory cytokines secretor. In contrast to $CD34^+$HSCs, IFN-α and IFN-β mRNAs were significantly up-regulated in MSCs. This was due to an active

replication of H5N1 virus strongly induced a host innate response. Also, MxA mRNA expression level was increased; however, it is not known whether MxA was activated by IFNs production or virus itself. IFNs signaling pathways are essential for viral clearance. Nonetheless, they would be able to contribute a severity of disease by mediating a link to amplify cytokine cascades and acting synergistically with other inflammatory cytokines, leading to hyper-inflammation (128) (129).

Pathogenesis of H5N1 infection is a complex consequence of broad tissue tropism, systemic replication and inflammatory response. Hypercytokinemia or cytokine storm is believed to be a major cause of multi-organ dysfunction. H5N1-induced pro-inflammatory cytokines were generated by various immune cells as they coordinate an attack on invading pathogens. If this response is out of control, it can cause fatal inflammation and damage to tissues. Cytokine dysregulation has been observed in H5N1 studies in mouse model and cell culture, level of cytokines including IP-10, RANTES, MIG, MCP-1, IL-8, IL-10, IL-6, IFN-γ, TNF, MIP-1α and soluble IL-2 were up-regulated compared with other influenza strains (7, 85). In the present study, we observed cytokine production from culture supernatants of $CD34^+$ HSCs and MSCs. Due to there was no report of an appropriate method for virus inactivation which would not interfere with Bio-Plex ELISA system. RIPA cell lysis buffer was chosen for inactivation of virus in supernatants since host cell membrane and virus envelope share a same property, so virus could be destroyed by this lysis buffer. Unfortunately, cell lysis buffer did affect with some cytokines beads in Bio-Plex ELISA system. Thus, we failed to evaluate the alteration of those cytokines. However, we could measure the production of IL-6, IFN-γ, MCP-1, MIP-1β because these cytokines were not affected by lysis buffer as seen in lysis buffer-treated cytokine standard did not change compared with untreated (data not shown). IL-6 and IFN-γ which function primarily to induce inflammation, were not up-regulated in H5N1-infected $CD34^+$HSCs, whereas MCP-1 and MIP-1β which are chemoattractors for recruitment of monocytes, macrophages, T cells, B cells, NK cells and immature dendritic cells into infected areas (87, 129) were significantly increased, it is possible that $CD34^+$ HSC itself is not a cytokine producer because it is non-immune cell, but it is capable of secreting chemokines for recruiting other immune cells to eradicate invading viruses. For MSCs, the level of IL-6, IFN-γ and MCP-1 in H5N1 infection

was not different from H1N1 and H3N2 infection. Based on the different infectivity rate between H5N1 and human influenza viruses, we suggested that MSCs were not likely to be a cytokine producer cells because MSCs could not secrete both cytokines and chemokines, despite supporting a productive infection of H5N1 virus.

In normal conditions, anti-inflammatory cytokines are immunoregulatory molecules that control pro-inflammatory response. In pathological conditions, insufficient control of anti-inflammatory response over pro-inflammatory may occur (130). MSCs exert a profound immunomodulatory action that is considered for potential clinical therapy of immune disorder diseases. MSCs have been shown to interact with $CD14^+$ monocytes and inhibit antigen presenting cell maturation and function (19, 21, 131). Furthermore, they modulate cytokine production of dendritic and T cell subsets (19). However, evidence that MSCs may be dysfunctional in disease is controversial. For MSCs' immune modulation, an adequate amount of IL-6 from coculture of MSCs with $CD14^+$ monocytes plays role in inhibiting dendritic cell differentiation from monocyte (21). IL-6 is pro-inflammatory cytokine elevated in H5N1 infection. The plasma level of IL-6 correlated with pharyngeal H5N1 load (6). Our result demonstrated that cytokine profiles (IL-6, MCP-1, and MIP-1β) from coculture of MSCs with $CD14^+$ monocytes were up-regulated, although, the infectivity was not enhanced. This effect was not seen in cultured MSCs alone. Thus, a possible explanation is that H5N1 may subvert MSCs-mediated immune modulation by skewing immune modulator toward enhancer as evidence of significantly increased of IL-6 in infected coculture compared with mock infection. Increased IL-6 is likely to promote the inhibition of DCs maturation contributing to reduce the number of antigen presenting cells. Alternatively, elevated IL-6 may enhance inflammation relating with previous studies (6, 88, 132). Moreover, leukocytes, monocytes and blood-derived macrophages may be recruited to BM via chemokines such as MCP-1 and MIP-1β secreted by infected coculture in order to amplify inflammatory response as previous reports about H5N1 pathogenesis (132, 133). The reduction of GM-CSF in H5N1-infected monocytes, MSCs, and monocytes/MSCs coculture (Figure 8.8B) might result in a decrease of growth factors of granulocyte and macrophage lineage populations (134). This could possibly support a cytopenia issue particularly in myeloid lineage of H5N1 pathogenesis.

The interaction of MSCs and monocytes is more active in responding to H5N1 infection. Thus, MSCs can be involved with H5N1-induced inflammation indirectly. Considering excessive pro-inflammatory cytokines; IFN-γ, TNF-α, soluble IL-2 receptor, IL-1 and IL-6 associated with reactive hemophagocytosis syndrome (RHS) (135). High level of cytokines and chemokines production in H5N1-infected coculture is likely responsible to induce RHS in BM. RHS is characterized by activated macrophages which consequently engulf neighboring cells including hematopoietic cells (136). This can contribute to markedly hypocellular in BM. To *et al.*, previously documented that both hypocellular and RHS in BM were the most prominent pathologic features observed in H5N1 fatal cases (7). However, there is no available BM autopsy to confirm our investigations.

The limitation of this study is a difficulty in obtaining BM samples because there is a limited number of HLA matched donors with recipients. These days, taking actual BM is not a common way to donate. Most often, stem cells are taken from the donors' blood. Most of $CD34^+$ HSCs used in this study were obtained from umbilical cord blood (UCB) which is non-invasive source, thus, it is easy to collect for fresh $CD34^+$ HSCs isolation. The outcomes of H5N1 infection seen in UCB-derived $CD34^+$ HSCs such as viral production, apoptosis, and cytokine secretion could partially represent what is occurring in BM environment. The MSCs used in whole study were derived from BM source. The early passages were suitable for performing experiments because the characteristics of primary cells would change in every passage. In addition, MSCs have limited life span and slow in proliferation. Although, the cell lines would be preferable in terms of consistency of results from experiment to experiment and well characterized, primary cells are still a superior model of the *in vivo* situations.

In conclusion, we demonstrated that $CD34^+$ HSCs and MSCs were susceptible to H5N1 infection. Virus induced cell deaths by apoptosis with different consequences; (i) $CD34^+$ HSCs: an indirect effect in surrounding naïve cells, probably caused by paracrine/growth factors secreted from infected cells, (ii) MSCs: direct effect from virus infection that apoptosis pathway was possibly activated by either viral proteins or paracrine factors. Both $CD34^+$ HSCs and MSCs induced an innate antiviral response to infection but did not up-regulate pro-inflammatory cytokines

associated with hypercytokinemia. This was due to stem cells are not cytokine secretor. However, IL-6, MCP-1, and MIP-1β were much higher produced when MSCs were cocultured with $CD14^{+}$monocytes. We noted that H5N1 virus indirectly enhance an inflammation by changing immunosuppressive function of MSCs to immune activator. Our explorations provide an insight of systemic infection in BM organ that can explain the hematologic abnormalities, hyperinduction of cytokines and reactive hemophagocytosis in severe H5N1 patients.

CHAPTER X
CONCLUSION

Avian influenza A H5N1 virus is a highly virulent pathogen that causes more than 300 deaths from H5N1 virus all around the world (1). Unlike other influenza viruses, H5N1 induces systemic infection and multi organ failure through viral dissemination and hypercytokinemia. Abnormal hematologic findings and viral antigen detection in hematopoietic cells raise the concern of BM dissemination. Whether BM suppression is a result from BM infection is what we need to explore. Although, stem cells present in a small number, they have a potential regulation of BM homeostasis. We therefore investigated the susceptibility to H5N1 virus of two stem cell types in BM environment as well as the outcome of infection compared with human influenza viruses. Our results showed a unique virulence characteristic of H5N1 virus in invading BM stem cells. We demonstrated that H5N1 virus was capable of infecting and replicating in both human $CD34^+$ HSCs and MSCs *in vitro*. Hemagglutinin (HA) played role in infection and replication of H5N1 in MSCs. We also found a substantial loss in infected MSCs and surrounding uninfected $CD34^+$ population suggesting that H5N1 infection caused cell death either by direct effect from infection or indirect effect from paracrine factors, respectively. Innate immune response was likely triggered by productive replication of H5N1 virus. However, both stem cells are non-immune cells, thus, they could not produce various of cytokines and chemokines. Interestingly, we noted a dysregulation of immunomodulation activity in MSCs with up-regulation of IL-6, MCP-1, and MIP-1β in the presence of monocytes, contributing to amplify cytokine signaling pathways from the influx of immune cells, and cytokine-driven hemophagocytosis. These events can contribute to impaired BM homeostasis which infected cells could not maintain self-renewal and differentiation properties, possibly leading to BM suppression. The major troubles of this study were from donor variations and passage dependent that caused fluctuating results less susceptibility in late passage.

Taken together, these findings supported the systemic infection and abnormal hematologic findings found in severe H5N1 patients and provide a better understanding of H5N1 pathogenesis. However, investigation of the susceptibility of BM stem cells in BM autopsy of H5N1 fatal cases should be performed to confirm a systemic spread. Also, profound molecular mechanism of H5N1 infection in MSCs needs to be further investigated.

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APPENDIX

1. Electrophoresis buffer

Tris base	3.0	gm
Glycine	14.4	gm
SDS	1.0	gm
Distilled water was added to	1000	ml

2. 1X Phosphate-buffered saline solution (PBS) pH 7.4

10X PBS	50.0	ml
Distilled water was added to	1000	ml

3. 10x TBE Buffer

Tris base	54.0	gm
Boric acid	27.5	gm
0.5 M Na_2EDTA pH8.0	20	ml
Distilled water was added to	500	ml

4. 0.5M Na_2EDTA

Na_2EDTA	9.3	gm
Deionized distilled water	50	ml

5. Bacterial agar plate

LB Broth, Miller	12.5	gm
American Bacteriological agar	7.5	gm
Deionized distilled water	500	ml
Autoclave at 121˚C 15 min		

6. Reagents for plasmid purification (alkaline lysis method)

6.1 Resuspension buffer (P1)

Tris base	3.03	gm
$Na_2EDTA.2H_2O$	1.86	gm
Add deionized distilled water to	500	ml

Adjust pH to 8.0 with HCl		
RNase A	0.05	gm
Store at 2-8 ˚C		

6.2 Lysis buffer (P2)

NaOH pellet	4.0	gm
Deionized distilled water	475	ml
20% SDS	25	ml
Store at RT		

6.3 Neutralization wash buffer (P3)

CH_3COOH	29.45	gm
Deionized distilled water	50	ml
Adjust pH to 5.5 with glacial acetic acid		
Adjust the volume to 100 ml with water		
Store at 2-8˚C		

BIOGRAPHY

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