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PATHOGENESIS OF INFLUENZA A VIRUS: INHIBITION OF MONOCYTE DIFFERETIATION INTO DENDRITIC CELL

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ABSTRACT OF DISSERTATION

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A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Agriculture
at the University of Kentucky

By

Saikat Boliar

Lexington, Kentucky

Director: Dr. Thomas M. Chambers, Associate Professor of Veterinary Science

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Dendritic cells (DC) are a heterogeneous population of hematopoietic cells that play a versatile role in orchestrating immune responses against an array of invading pathogens, including influenza virus. These cells reside in lymphoid organs as well as in non-lymphoid tissues such as mucosal surfaces of respiratory and gastro-intestinal system. Recent investigations have suggested that in the steady state, dendritic cells are derived mainly from bone marrow precursor cells without a monocytic intermediate whereas during inflammation or infection, monocytes readily differentiate to generate monocyte derived dendritic cells (MoDC). The ability of virus infected monocytes to differentiate into MoDC was investigated and the results demonstrated that *in vitro* infection of monocytes with influenza virus impaired their development into MoDC. It was also observed that influenza infection of monocytes, pre-treated with GM-CSF and IL-4 for DC differentiation, was minimally-productive and non-cytopathic. In spite of successful viral genome transcription, viral protein synthesis was restricted at an early stage. However, despite of the limited replication, influenza virus infected monocytes failed to develop the distinctive DC- like morphology when cultured with GM-CSF and IL-4 as compared to their mock infected counterparts. Infected cells, after 4 days in culture, expressed reduced amounts of CD11c, CD172a (myeloid marker), CD1w2 (CD1b) and CCR5. Influenza virus infected monocytes also retained substantial non-specific esterase activity, a characteristic for monocytes and macrophages. Antigen presentation capability of infected cells was also affected as indicated by decreased endocytosis. Production of IL-12, a pro-inflammatory cytokine and IL-10, a reciprocal inhibitory cytokine, was coordinately modified in influenza virus infected monocytes in order to arrest their differentiation into DCs. At least limited viral replication was necessary to impede the differentiation process completely. However, viral NS1 protein activity, as evidenced with a mutant influenza virus, was not essential for this inhibition. This identified a new strategy by influenza virus to interfere with DC differentiation and evade a virus specific immune response.

KEYWORDS: Influenza virus, equine, monocyte, dendritic cell, differentiation.

Saikat Boliar

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September 21st, 2009

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Influenza is a term that was appropriated from Italian in the mid-1700s to describe a disease that was believed to be the "influence" of miasma (bad air) and stars. The causative agent of such "bad influences" and the pandemic that swept the world in 1918 could only be identified in 1931, when Dr. Shope was able to reproduce an influenza like disease in healthy pigs by inoculating them with nasal wash from infected ones (1). Since then influenza virus has been the focus of intense research which has helped garner a very detailed knowledge about the molecular biology, pathogenesis and immune responses of the virus.

1. Influenza virus

Influenza virus is a member of the viral family *Orthomyxoviridae* that includes negative sense, single strand RNA viruses. The etymology of *orthomyxoviridae* can be traced back to two Greek words: *orthos* (standard or correct) and *myxa* (mucus) which thereby adeptly characterizes the ability of these viruses to bind and infect through the host mucus membrane (2). This family of RNA viruses consists of five genera, namely *Influenzavirus A*, *Influenzavirus B*, *Influenzavirus C*, *Thogatovirus* and *Isavirus* (3). The first three genera each have one species or type: Influenza A virus, Influenza B virus and Influenza C virus respectively. Theses three types of influenza viruses – A, B and C are

distinguished based on the antigenic differences in their internal proteins, nucleocapsid (NP) and matrix (M) protein. Influenza A virus has a broad host range that spans both mammalian and avian species whereas influenza B virus can infect humans and seals, and influenza C virus is contained within humans and swine. The genus *Thogotovirus* has two species – Thogoto virus and Dhori virus. They mainly infect ticks but serological evidence suggests that humans and other mammals are also susceptible. These tick borne viruses are known to cause a febrile illness and encephalitis in humans. Infectious salmon anemia virus is the only known virus within the genus *Isavirus* which has been isolated from Atlantic salmons (3).

1.1. Influenza A virus

Influenza A virus is the primary causative pathogen for the seasonal, highly contagious, acute respiratory illness in humans and other mammals. Influenza A viruses are sub-divided into various sub-types on account of their antigenic identity of two major surface glycoproteins – hemagglutinin (HA) and neuraminidase (NA). Sixteen different HA and 9 NA variants have been identified so far (4). Wild aquatic birds (waterfowl, ducks, geese, swan, gulls, terns etc.) are considered the natural reservoir for all subtypes of influenza A virus. In wild aquatic birds, these viruses are benignly adapted and cause only asymptomatic infection. Viruses in these reservoir hosts are suggested to be in an evolutionary stasis, which is imperative for perpetual maintenance of a consistent viral gene pool. However, over the years, different subtypes of influenza A virus have successfully jumped from their natural reservoir hosts to other avian and

mammalian species to establish clinical diseases (5). All currently known subtypes of influenza A virus and their natural hosts are listed in table 1.1.

Table 1.1: Natural hosts of influenza A viruses. The table indicates the subtypes of hemagglutinin (HA) and neuraminidase (NA), and the hosts they have been identified in.

Subtype	Predominant host	Subtype	Predominant host
H1	Human, pig, birds	N1	Human, pig, birds
H2	Human, pig, birds	N2	Human, pig, birds
H3	Birds, human, pig, horse, dog*	N3	Birds
H4	Birds	N4	Birds
H5	Birds, (human)	N5	Birds
H6	Birds	N6	Birds
H7	Birds, horse, (human)	N7	Horse, birds
H8	Birds	N8	Horse, birds, dog*
H9	Birds, (human)	N9	Birds
H10	Birds		
H11	Birds		
H12	Birds		
H13	Birds		
H14	Birds		
H15	Birds		
H16	Birds		

Adapted from Lamb RA, Krug RM. *Orthomyxoviridae*:the viruses and their replication. In: Knipe DM, Howley PM, Griffin DE *et.al*, editors, *Fields Virology*. 4th edition, 2001

* Science 2005; 310: 482–485

1.2. Equine influenza virus

Equine influenza virus is one of the predominant causes of respiratory diseases in horses worldwide (6). The first isolate of equine influenza virus was isolated from an outbreak in Eastern Europe in 1956 and was indentified as an H7N7 subtype (influenza A/equine/Prague/56). Subsequently, in 1963, another subtype (H3N8, influenza A/equine/Miami/63) was isolated from horses in Miami, USA (7). Both subtypes cocirculated among horses for about fifteen years, during which (around 1970s) equine influenza viruses of the H7N7 subtype underwent reassortment with their H3N8 counterparts and acquired all the internal genes (i.e. PB2, PB1, PA, NP and NS) except matrix gene (8). However, the H7N7 virus is believed to have ceased circulating after 1977 since when no new isolates of this subtype has been identified from horses (9). The H3N8 virus continues to circulate and is responsible for all recent equine influenza outbreaks. Based on their antigenic and genetic variability, H3N8 equine influenza viruses are considered to have evolved into two divergent lineages: “American” and “Eurasian” (10). Although initially they were grouped based on their geographical distribution, some viruses from the American lineage were later isolated from the United Kingdom. Equine influenza viruses from the American lineage have again been sub-divided into three sub-lineages: South American, Kentucky and Florida (11). In 2004, H3N8 equine influenza viruses from the American lineage, in an unprecedented manner, were found to cross species-barrier to infect dogs (12). Since then some other H3N8 equine influenza viruses from the European lineage have also been isolated from pigs which have raised a serious concern of probable host-expansion of these viruses (13).

2. Structure of influenza A virus

2.1. Morphology

Influenza A viruses are small, pleomorphic particles. Spherical virions range from 80 to 120 nm in diameter while filamentous particles can measure up to several micrometers in length. Fresh isolates are generally filamentous which changes into almost entirely spherical morphology following several passages (14). The virion, under electron microscope, shows two distinct layers – an evenly spaced outer zone (100 Å) covering a densely packed inner material (60-100 Å) (15). The outer zone or envelope consists of a host-derived lipid bi-layer within which the two surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA) remain embedded. M2 ion channels also protrude through this envelope. Within the lipid envelope M1 matrix proteins form the inner shell which encompasses the viral RNA genome. Each viral RNA segment remains encapsidated by multiple nucleoproteins (NP) in a helical symmetry to form the ribonucleoprotein (RNP) complex. Three RNA dependent RNA polymerase proteins (PB2, PB1 and PA) stay attached to the end of each viral RNA segment of the RNP complex (16).

2.2. Viral genome and encoded proteins

The genome of Influenza A virus (about 13.6 kb) consists of 8 single strand negative sense RNA segments which altogether encode for eleven viral proteins.

2.2.1. PB2

The PB2 gene of influenza A virus is 2341 nucleotide long and encodes for a 85.7 kDa polypeptide that constitutes one of the basic components of the heterotrimeric viral polymerase complex (16). The PB2 subunit plays a crucial role in viral mRNA transcription. It binds to the 5'-cap I structure (m7GpppNm) of host cell pre-mRNA which is later cleaved, in a process called "cap snatching", to prime viral mRNA synthesis (17). The role of PB2 polymerase in viral RNA replication, although essential, is yet obscure.

2.2.2. PB1

The PB1 gene of influenza A virus is the same in length as PB2 and it encodes the second basic subunit (MW 96.5 kDa) of the viral polymerase complex. It serves as a backbone that can bind to the other two polymerases as well as nucleoprotein (18, 19). PB1 is the catalytically active RNA dependent RNA polymerase which is involved in transcription initiation and elongation of messenger RNA (mRNA), complementary and viral RNA (cRNA and vRNA, respectively) in a sequence specific manner (17, 20). Other than the polymerase activity, PB1 also performs endonucleolytic cleavage of cellular mRNA to generate capped RNA primers for initiation of viral mRNA synthesis (21). Recently, a novel protein christened PB1-F2 has been identified to be expressed from an alternate open reading frame (+1 ORF) of PB1 gene. This viral protein (about 87 amino acids) localizes to the mitochondria and is emerging as an important player in influenza virus pathogenesis through induction of apoptosis (22).

2.2.3. PA

The third and the only acidic subunit (MW 84.2 kDa) of the viral polymerase complex is encoded by the PA gene which has a length of about 2233 nucleotides (16). Although particular functions of this polymerase protein have not been fully characterized, PA is known to interact with PB1, possess weak protease activity and play some yet unidentified role in both viral transcription and replication (18, 23, 24).

2.2.4. HA

The fourth largest gene (1778 nucleotides) in influenza genome encodes for a membrane glycoprotein (MW 61.5 kDa monomer), hemagglutinin or HA (16). This major surface antigen of influenza virus is responsible for receptor binding and membrane fusion during viral entry into the cell. Each HA polypeptide has three sub-domains: receptor binding, vestigial esterase and fusion domain (25). Biological activation of HA requires three posttranslational modifications: (a) cleavage of the amino terminal signal peptide (14-18 a.a.); (b) glycosylation and palmitoylation; and (c) proteolytic cleavage of precursor HA0 into two disulfide-linked subunits, HA1 and HA2 (26). During maturation, HA molecules also homotrimerize into a rod like structure with a globular head and a stalk. The globular head accommodates the receptor binding site of the antigen. Although sialyloligosacharides on cell membranes, in general, are recognized by all HA subtypes, its host specificity somewhat depends on whether the sialic acid residue has a α-2,3 or α-2,6 linkage to galactose. While most avian and equine influenza viruses favor the N-acetylneuraminic acid α-2,3-galactose (NeuAcα2,3gal) linkage, human viruses

preferentially bind to the N-acetylneuraminic acid α-2, 6-galactose (NeuAcα2,6gal) linkage (27). Tracheal epithelial cells of pigs express both types of receptors which render them a suitable "mixing vessel" for generation of avian-human reassortant influenza viruses with pandemic potential (28). Once HA binds to its receptors on the cell membrane, the virions are endocytosed into intracellular endosomes. In the acidic pH of endosome, HA undergoes conformational changes to expose the N-terminal fusion peptide of the HA2 subunit which brings viral and endosomal membranes together to enable fusion (29). This membrane fusion allows release of viral RNP complex into the cytoplasm to be imported into the nucleus for replication.

2.2.5. NP

The NP gene (1565 nucleotides) encodes a highly basic, single-strand RNA binding protein (MW 56.1 kDa) called nucleoprotein (16). NP encapsidates viral RNA molecules through their sugar-phosphate backbone to aid in their transcription and replication (30). Assembly of NP with viral RNA also provides structural organization to the RNP complex. This polypeptide contains at least two nuclear localization signals (NLS1, a.a. 3-13 and NLS2, a.a. 198-216) and thereby plays a decisive role in intracellular trafficking of RNP in and out of the nucleus (31, 32). NP also interacts with several viral and cellular proteins. In addition to self-oligomerization, NP binds to the viral polymerases (PB1 and PB2) and the matrix protein M1 (19, 33, 34). Cellular proteins that have, so far, been indentified to interact with NP include importin alpha, F-actin, CRM1, BAT1/UAP56 and Mx protein. Importin alpha, actin and CRM1 have functional

association with NP for cellular trafficking of RNP complex (35-37). On the other hand, UAP56 and Mx protein, through interactions with NP, up- and down-regulate viral RNA synthesis, respectively (38, 39).

2.2.6. NA

The NA gene (1461 nucleotides) encodes for the second surface glycoprotein (MW 50 kDa monomer) of influenza virus, neuraminidase (16). The major function of this protein is enzymatic cleavage of sialic acids from cell surfaces to allow release of newly synthesized progeny virions. It cleaves the α-ketosidic linkage that binds terminal NeuAc5 to its sugar moiety (40). NA polypeptide is synthesized as a 454 a.a. monomer which oligomerizes to form a mushroom shaped tetrameric protein. The box shaped head contains the catalytic activity of the enzyme while the N-terminal stalk anchors it into the viral membrane (41). Apart from releasing progeny viruses, it also removes sialic acid residues from the carbohydrates on viral membrane glycoproteins to prevent virus self-aggregation (42). It has also been suggested to remove mucins to lower viscosity and allow the virus access to the epithelial cells. However, it does not play any role in attachment, replication or assembly of the virus (43).

2.2.7. M

The M gene (1027 nucleotides) encodes two viral proteins: the matrix protein M1 (MW 27.8 kDa) and ion channel protein M2 (MW 11 kDa). M1 is the most abundant viral protein that underlies the lipid envelope to provide structural rigidity. It is also

believed to interact with the cytoplasmic tails of HA and NA (44). M1 binds to vRNPs through its C-terminal domain for their nuclear-cytoplasmic transport (45, 46). It also acts as a driving force for recruiting and assembling viral and host components during budding of the virus (47). The second protein, M2, is expressed from a spliced mRNA derived from M RNA segment. It is a type III integral membrane protein with a short ectodomain, a transmembrane domain and an endodomain or cytoplasmic tail (48). M2 primarily functions as a tetrameric ion channel that allows influx of protons from the acidic endosome, leading to conformational changes and eventually uncoating of viral RNP complex (49).

2.2.8. NS

The smallest gene, NS (890 nucleotides), also encodes two viral proteins: NS1 (MW 26.8 kDa) and NEP (formarly NS2, MW 14.2 kDa). Although NS1 is not incorporated as a structural component into the virion, it is abundantly synthesized during virus replication. NS1 is a multifunctional protein which not only assists in viral replication but also defends against cellular anti-viral responses. It is synthesized as a 230-237a.a. long polypeptide which likely forms dimers (50). NS1 protein is known to interact with the viral replication complex (RNP) and its role has been suggested in replication of vRNA as well as selective enhancement of viral mRNA translation (51-53). NS1 protein is functionally divided into two domains: N-terminal RNA-binding domain (RBD, a.a. 1-73) and C-terminal effector domain (a.a. 74-230) (54, 55). The RBD can nonspecifically bind to dsRNA and thereby prevent activation of several transcription factors (NF-kB, IRF-3,

JNK/AP-1) required for IFN-α/ß induction (56-58). More recently, it has been shown that, RIG-I, a cytoplasmic sensor for pathogen associated molecular patterns like ssRNA, is also inhibited by NS1 to block interferon induction (59). The RBD of NS1 also plays a potential role in inhibiting another cytoplasmic, dsRNA binding antiviral protein: 2'-5' oligoadenylate synthatase (OAS/RNaseL) (60). On the other hand, the effector domain of NS1 antagonizes the immune response at the post-transcriptional level through interaction with a number of cellular proteins. It can bind to two essential components of the cellular pre-mRNA processing machinery, namely, the 30 kDa subunit of cleavage and polyadenylation specificity factor (CPSF 30) and the poly (A)-binding protein II (PABPII); and this leads to accumulation of defective cellular pre-mRNAs (uncleaved or with short poly-A tail) in the nucleus of infected cells (61, 62). Interestingly, however, inhibition of CPSF30 by NS1 does not affect influenza virus mRNA processing since polyadenylation of influenza mRNA is independent of this cellular protein. The effector domain is also known to inhibit activation of serine/threonine protein kinase R (PKR), an anti-viral protein that can diminish viral protein synthesis (63). Finally, in an attempt to thwart the development of adaptive immune response, NS1 protein inhibits maturation and migration of dendritic cells leading to dysfunctional T cell stimulation and decreased cytokine production (64).

The second protein, NEP (nuclear export protein), is expressed from a spliced mRNA of NS RNA segment and is present in virions in a phosphorylated form (65). The main function of this protein is, in association with M1, transport of viral ribonucleoproteins from the nucleus to the cytoplasm. A leucine/methionine rich

nuclear export signal in the N-terminus is thought to be vital for this function (66). As for the mechanism of nuclear export, NS2 protein has been shown to interact with several nucleoporins as well as the nuclear export receptor, Crm1 (66, 67). Recently, it has also been suggested to play a role in regulation of viral RNA transcription and replication (68).

3. Replication cycle of influenza A virus

One complete replication cycle of influenza A virus can be divided into several stages: attachment, entry, fusion and uncoating, genome transcription, viral protein synthesis, assembly and egress/budding. Viral attachment to cell surface receptors (sialic acid) is mediated by hemagglutinin (HA) protein which has been described in section 2.2.4. Following attachment, virions are endocytosed into cellular compartments. Several endocytic mechanisms including clathrin-coated pits as well as clathrin-caveolin independent pathways have been described for influenza virus entry (69, 70). Low pH within the endosome brings about conformational changes in HA protein leading to fusion of viral and endosomal membrane resulting in release of viral RNP complex into the cytoplasm. Viral RNAs along with their replicases are then actively transported into the nucleus. Nuclear localization signals on the nucleoprotein are essential for this transport (31, 32). Once inside the nucleus, negative sense viral RNA segments are transcribed into mRNAs in a cap-dependent manner. These mRNAs are thereafter transported out into the cytoplasm for viral protein synthesis. Nucleoprotein (NP) and NS1 protein are preferentially synthesized at the early stage of viral infection

while synthesis of HA, NA and M1 is deferred to a later stage (71). After certain cycles of viral protein synthesis, the transcription machinery switches from mRNA to cRNA (complementary to viral genomic RNA) and vRNA (viral genomic RNA) synthesis. Following viral replication, RNP complexes are assembled in the nucleus and are exported out to the cytoplasm by M1 and NEP (46, 66). In the final step, the viral genome and proteins are assembled on the plasma membrane and newly synthesized virions are released from the apical surface of epithelial cells.

4. Pathogenesis of influenza A virus

The outcome of influenza virus infection varies with different virus subtypes and the susceptible host. In wild aquatic birds which serve as the natural reservoir hosts for influenza, viral replication is largely restricted to the gastro-intestinal tract and the infection is generally asymptomatic (72). In other birds such as domestic poultry, turkey, quail and pheasant, influenza infection is normally non-fatal; with the exception of some highly pathogenic H5 and H7 subtypes, which have run havoc among chickens in recent years (73). In mammalian hosts (swine, equine, human), influenza virus is mainly a respiratory pathogen with disease severity ranging from asymptomatic to severe systemic fatal infection. The virus primarily infects and replicates in the ciliated columnar trachea-bronchial epithelium and then spreads to the lower respiratory tract or sometimes to systemic organs. Normal manifestations of the seasonal illness include fever, headache, cough, sore throat, anorexia and malaise. Pathogenesis of influenza virus, as described here, can mainly be ascribed to its ability to shut off host cell protein

synthesis, induce apoptosis and regulate several cellular transcription systems in order to avoid the host immune response. The PB2 polymerase protein, as mentioned in section 2.2.1., binds to host cell mRNA cap structures in the nucleus and cleaves them to prime viral mRNA synthesis (17). The decapped cellular mRNAs then get degraded. Nuclear export of cellular pre-mRNAs that escape "cap snatching" is blocked by NS1 protein by inhibiting their splicing (74). Productive replication of influenza virus in respiratory epithelium results in apoptotic cell death (75). At least three viral proteins, NA, NS1 and PB1-F2, are known to regulate apoptosis of infected cells (22, 76, 77). Influenza virus infection activates several transcription factors which in turn induce expression of a number of anti-viral cytokines and chmokines genes. Viral NS1 protein, as explained in section 2.2.8., plays an important role in regulating those cellular transcription systems. The NS1 protein is a known antagonist of IFN α/ß production (56). It also inhibits maturation and cytotoxic T cell stimulation of dendritic cells and thereby stalls the development of an adaptive immune response against the virus (64). However, apart from these general mechanisms of cellular pathogenesis, highly virulent influenza viruses possess many other features to develop severe fatal disease.

Highly virulent human and avian influenza viruses, which have the potential to cause a human pandemic, exhibit several other pathogenic characteristics. Experimental infection of monkeys with virulent H5N1 influenza virus (human isolate) produces severe lesions including necrotic broncho-interstitial pneumonia, damaged respiratory epithelium and intra-alveolar hemorrhage as well as necrotic lesions in lymphoid organs, liver and kidney (78). Virulence of these viruses has been attributed to

several factors. One major determinant of influenza virus pathogenesis is the cleavability of precursor hemagglutinin protein (79). Proteolytic cleavage of precursor hemagglutinin (HA0) protein into two disulfide linked subunits, HA1 and HA2, is a prerequisite for successful fusion of viral and host endosomal membranes and therefore is necessary for viral infection. Low pathogenic avian influenza viruses and most mammalian influenza viruses except H7N7 equine influenza viruses, have a single arginine residue at the cleavage site between HA1 and HA2 subunits (80, 81). This requires serine family proteases for activation, therefore restricting their replication to the respiratory tract (82). On the other hand, highly pathogenic avian influenza viruses possess multiple basic amino acid residues at the cleavage site which is recognized by several ubiquitous proteases such as furin and PC6 (80, 83). This allows them to infect systemic organs other than lungs and thereby enhances their pathogenicity. Viral neuraminidase (NA) has also been suggested to play a role in it virulence. Lack of a carbohydrate side chain on NA in some viruses such as WSN/33 (H1N1) allows them to sequester cellular plasminogen which in turn facilitates the cleavage of hemagglutinin (84). The high replication ability of the 1918 pandemic influenza strain in cell culture in absence of trypsin has also been attributed to its neuraminidase protein (85). Another virulence factor and pro-apoptotic protein, PB1-F2, from this pandemic strain not only enhances the pathogenecity of the virus but also exacerbates the subsequent bacterial infections (*Streptococcus pneumonia*) by increasing cytokines and chemokines release (86). Additionally, the carboxy-terminus of the NS1 protein from highly virulent H5N1 human isolates has been found to possess a PDZ binding motif (Glu-Ser-Glu-Val, ESEV)

which can interfere with cellular signaling pathways responsible for protein trafficking and maintenance of cell morphology (87). Pathogenesis of influenza virus is a profoundly studied field and several other viral and host factors are yet under investigation that may elucidate important aspects in the disease progression and virulence of this virus.

5. Immune response to influenza A virus

In the wake of a seasonal influenza infection, an immune-competent host generates a robust anti-viral response. This anti-viral immunity consists of both innate and adaptive responses that enable containment and clearance of the viral infection in about a week's period.

5.1. Innate immune response

The innate immunity is a relatively non-specific response that constitutes the first line of defense against an invading pathogen. This defense mechanism is considered primitive as it has remained conserved among mammals, lower vertebrates and invertebrates. However, its does not provide a memory or long-lasting protection. The innate immune system is comprised of physical barriers, soluble chemical factors and cellular components.

5.1.1. Physical barriers

The epithelial surfaces form the first physical barrier to any bacterial or viral infections. Mucins produced by respiratory epithelial cells trap bacteria or virus particles while movement of broncho-pulmonary cilia clears them out of the airway.

5.1.2. Soluble antimicrobial mediators

Pathogens that successfully breach this barrier are neutralized by soluble antimicrobial factors, such as lysozyme, lactoferrin and defensins. Human alpha-defensins, for instance, are short cationic antimicrobial peptides of neutrophils that can inhibit influenza virus replication (88). Another well characterized heat liable, enzymatic mediator of the innate immunity is the complement system. Its antimicrobial activity is primarily mediated through opsonization which activates neutrophils and mast cells to phagocytose and lyse complement bound pathogens. Its important role in anti-viral immunity was established when complement deficient mice were found to be more susceptible to influenza virus infection (89). Complement present in human serum can neutralize influenza virus and in vivo experiments show that in association with natural antibody (IgM), complement can provide protective immunity in influenza naïve hosts (90-92).

5.1.3. Pattern recognition receptors

The innate immune system maintains an evolutionarily preserved set of receptors that distinguishes self from non-self (microbial) antigens based on their highly

conserved structural features called pathogen associated molecular patterns (PAMPs). PAMPs can consist of sugar moieties, lipids, nucleic acids or combinations of any of these. The receptors that recognize them are, therefore, known as pattern recognition receptors (PRRs).

Collectins, the C-type lectin bearing members of the collagen family, are important soluble PRRs of the innate immune system. Among the nine members that have been identified so far, mannan-binding lectin (MBL) and surfactant proteins A and D (SP-A and SP-D) are the most studied for their role in host defense against infectious agents. MBL is secreted into the bloodstream mainly by the liver whereas SP-A and SP-D are predominant among alveolar spaces in the lung (93, 94). They, in general, bind to a variety of carbohydrate residues on pathogen surfaces and thereby activate cells to respond by way of agglutination, complement activation, opsonization and phagocytosis (95, 96). During influenza infection, human MBL can bind to both HA and NA on virus particles and thereby neutralize and contain the viral spread (97). Similarly, the protective mechanism of SP-A and SP-D in influenza infection involves direct virus neutralization, opsonization or enhanced uptake and hydrogen peroxide production by neutrophils (98, 99).

In the past decade, scientific knowledge about cellular sensing of a microbial invasion was revolutionized by the discovery of toll-like receptors (TLRs). The first toll protein was identified in *Drosophila* as a mediator of its antifungal immunity (100). This was soon followed by the characterization of its mammalian homologue in humans, hToll or currently recognized as TLR4 (101). Since then 13 receptors (TLR 1 -13) have

been discovered in mammals. Among them, TLR10 is expressed in humans but not mouse whereas TLR11 has only been found in mouse. Except for TLR3, 7, 8 and 9 which are found within endosomes, all others are expressed on the plasma membrane (102). All of these TLRs are type-I transmembrane glycoproteins with a leucine rich extracellular domain and a cytoplasmic domain containing toll/interleukin-1 receptor (TIR) homology. The extracellular structure binds to its respective ligands and the intracellular domain is responsible for passing on the activating signal downstream (103). TLR2 which forms heterodimers with TLR1 and TLR6 binds to tri- or diacylated lipopeptides respectively. TLR3 recognizes dsRNA molecules while lipopolysaccharide (LPS) acts as a ligand for TLR4. Bacterial flagellin binds to TLR 5. TLR7 and 8 primarily sense viral ssRNA molecules whereas unmethylated CpG containing bacterial DNA motifs bind to TLR9. Uropathogenic bacteria can be detected by TLR11. Specific ligands for TLR10, 12 and 13 have not been identified yet (102). Binding of specific microbial components to TLRs results in their activation. All of these receptors use a common signaling pathway. Following recognition of a microbial motif, toll like receptors form dimers – most of them form homodimers, but TLR2 uses TLR1 or TLR6 to form heterodimers. Based on the adaptor molecules used, their signaling pathways are generally classified into two categories: (a) MyD88 dependent pathway and (b) MyD88 independent pathway. All but TLR3 utilize the MyD88 dependent pathway to activate transcription factor NF-kB which in turn leads to expression of various inflammatory cytokine genes. On the other hand, TLR3 and in part TLR4 activation promotes expression of interferon genes through stimulation of a different transcription factor

called IRF3. However, activation of either pathway results in production of pro-inflammatory cytokines and IFN which in turn lead to the development of antigen specific adaptive immunity (104). TLR7 expressed on plasmacytoid dendritic cells can detect influenza virus genomic RNA (105). Similarly, TLR3 which is constitutively expressed in pulmonary epithelial cells mediates activation and production of cytokines during influenza virus infections (106). More recently, an important role of the TLR7-MyD88 signaling pathway has been suggested even for the development of a virus specific adaptive (CD8 + T cell) immune response (107).

While TLRs are efficient at detecting pathogens either on cell surfaces or inside endosomes, some other recently identified PRRs can sense PAMPs in the cytosol: RIG-1 (retinoic acid inducible gene -1), MDA-5 (melanoma differentiation associated gene -5), NOD-1/2 (nucleotide binding oligomerization domain-1/2) and other C-type lectins e.g. dectin-1 [reviewed in (108, 109)]. Although both RIG-1 and MDA-5 recognize dsRNA from viruses, RIG-1 was found to play a more important role in interferon signaling during influenza virus infection (110). Similarly, NLRs (NOD like receptors) can also recognize viral RNA and are necessary for activation of inflammasomes (multiprotein complex responsible for activation of inflammatory processes) as well as development of adaptive immunity (111, 112).

5.1.4. Cells of innate immune system

Cells of monocytic lineage such as macrophages, dendritic cells and natural killer cells constitute the cellular compartment of innate immune system.

5.1.4.1. Macrophages

Macrophages are important effector cells of the innate immune system. In steady state or inflammation, circulating monocytes differentiate to generate tissues macrophages. Depending upon their anatomical localization, macrophages have been given different names, for example, alveolar macrophages in the lungs, microglia in the CNS, Kupffer cells in the liver etc (113). These are very potent phagocytic cells and their primary function is maintenance of tissue homeostasis by clearing out cellular debris, apoptotic as well as necrotic cells. However, upon activation, they produce large amounts of inflammatory cytokines and chemokines. Alveolar macrophages have been found susceptible to influenza virus infection *in vitro* and the infection results in quick cytopathic death of these cells (114). Influenza infected macrophages show an increased transcription level for an array of anti-viral cytokines including IL-1 beta, IL-6, TNF-alpha and IFN α/ß (115). They also produce chemokines such as RANTES, monocyte chemotactic protein -1 (MCP-1) and macrophage inflammatory protein -1 alpha (MIP -1α) which further attract more mononuclear cells to the site of infection to aid in viral clearance (116).

5.1.4.2. Dendritic cells

Dendritic cells (DC) are a unique population of cells that play a pivotal role in molding immune responses against invading pathogens. These are a sparsely distributed heterogeneous population of hematopoietic leukocytes with diverse phenotypes and functions. In 1973, R. M. Steinman and Z. A. Cohn first observed a population of large

stellate cells with distinct morphological properties in mouse spleen and described them, due to their veiled structure, as “dendritic cells” (117). Apart from lymphoid organs (spleen, lymph nodes), DCs are also found in non-lymphoid tissues such as lungs, epidermis (langerhan’s cell), dermis (interstitial cell) etc. Some characteristic features of DCs are: [a] DCs are plastic adherent, low density cells with poor viability in culture. [b] They have few lysosomes and lack Fc receptors. [c] They are the most potent antigen presenting cells (APC) and efficient activators of T lymphocytes. A very small number of DCs pulsed with little amount of antigen can stimulate a large number of T cells. They can stimulate proliferation of both CD4+ and CD8+ T lymphocytes (118).

Because of their heterogeneity, the origin of DCs has been a highly debated topic in recent years (Figure 1.1). In the bone marrow, DCs can originate from either $Flt3^{+}$ (FMS like tyrosine kinase 3) common myeloid (CMP) or common lymphoid progenitor (CLP) cells (119, 120). CMPs and CLPs differentiate into macrophage-DC progenitors (MDP, $Flt3^{+}$M-$CSFR^{+}CX3CR1^{+}$) and common DC progenitors (CDP, $Flt3^{+}CD115^{+}$) respectively which enter into the bloodstream and later migrate into secondary lymphoid organs to give rise to DCs. However, in the periphery during inflammation or infection, monocytes can also differentiate to generate inflammatory DCs (121). In human and mouse, based on phenotypic surface markers expressed and specialized functions, DCs have been categorized into several sub-types. Broadly, in lymphoid tissues, DCs are often classified as either conventional DCs (cDC), potent antigen presenting cells or plasmacytoid DCs (pDC), producers of type I interferon (122).

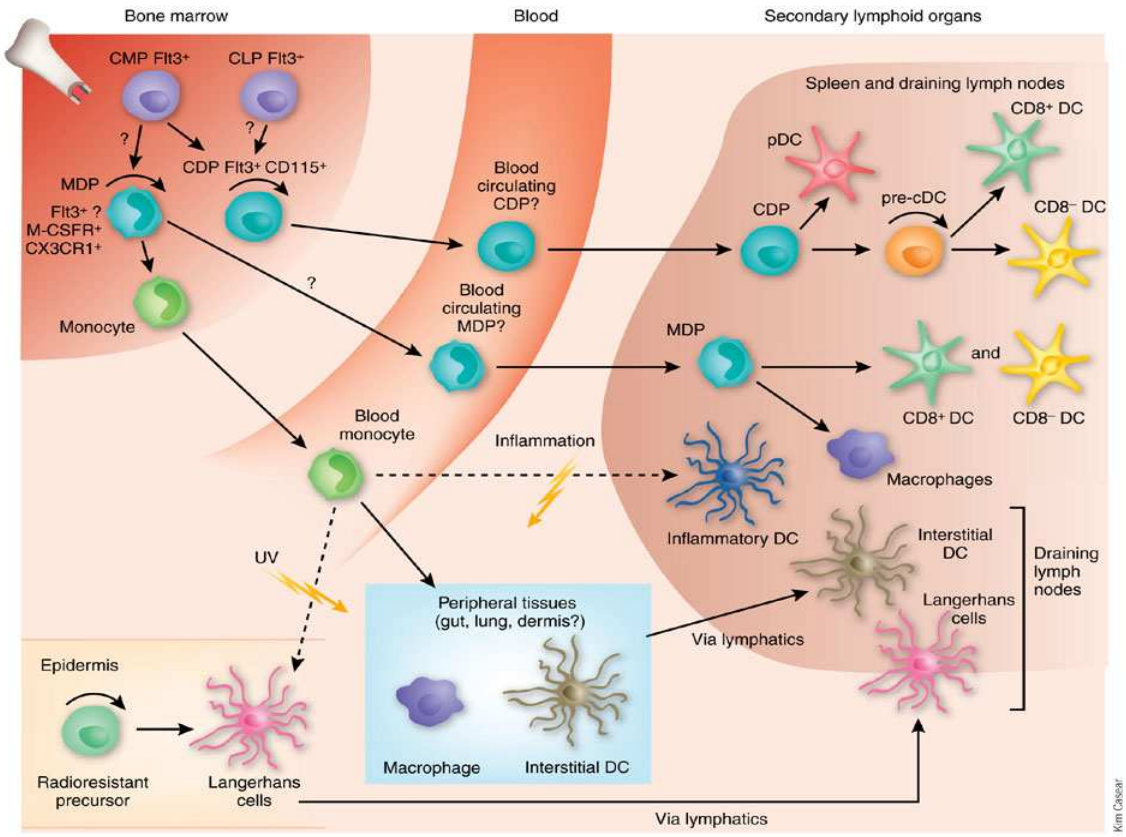

Figure 1.1: DC ontogeny in mice.

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Immediately after differentiation from hematopoietic precursors, DCs circulate throughout the periphery in “immature” forms and scan the body for any self antigen (tolerance) or invading pathogens (immunity). Once encountered, highly adept “immature” DCs capture the antigen by macropinocytosis, phagocytosis or receptor mediated endocytosis (123). This antigen capture initiates a series of phenotypic and biochemical changes in DCs called “maturation”. Maturation of DCs is characterized by reduced antigen capturing ability, increased surface expression of co-stimulatory molecules, i.e. CD68, CD83, CD86, MHC I, MHC II and production of cytokines and chemokines. Activation of the Interferon signaling pathway by microbial PAMPs has been shown to stimulate this maturation process (124). Initiation of maturation drives DCs to migrate to secondary lymphoid organs where they present the captured antigens to lymphocytes for proliferation and functional activation. Depending on the invading pathogen, DCs can present antigen on either MHC I or MHC II to a particular subset of lymphocytes. For intracellular microbes like viruses, DCs present antigens on MHC I which is recognized by CD8+ T cells. This results in vigorous proliferation of cytotoxic T cells that can in turn lyse infected cells. On the other hand, extracellular pathogens are taken up into endosomes from where they are presented on MHC II molecules leading to CD4+ T cell mediated immune response (125). While peptides mounted on MHC molecules provide the “signal 1” to T cells, “signal 2” or “co-stimulation” is delivered by interactions of CD80/86 with CD28 on T cells. Signal 3, a term coined very recently, is used by dendritic cells to promote T cell differentiation into specific effector cells, e.g. cytotoxic or Th1 or Th2 cells (126). Polarization of Th1 or Th2 type immunity is

determined by pathogen primed DCs through production of several DC derived polarizing factors. IL-12 primarily is considered to be the Th1 type cytokine whereas IL-4 (in absence of IL-12) drives helper cells towards Th2 type immunity (127). Th2 cells synthesize IL-5 and IL-4 which in turn stimulate B cells to produce appropriate antibodies against the invading pathogen.

Equine dendritic cells, when cultured *in vitro* from blood monocytes with GM-CSF and IL-4, exhibits similar morphological and cytochemical characteristics as of human DCs (128). They express similar pattern of phenotypic surface makers as human DCs and are also potent stimulators of T cells as observed in mixed lymphocyte reactions (129). When activated by maturation stimuli such as inactivated E.coli, immature equine DCs can mature; express maturation markers on their surface and produce enhanced cytokines as well (130).

The pivotal role of DCs in the innate and adaptive immune response makes them a major target for bacteria, viruses and other pathogens. Although the *in vivo* role of DCs in influenza infection is not completely understood, several *in vitro* studies have helped in unraveling some of the mysteries. It is not known whether influenza naturally infects DCs *in vivo*. However, *ex vivo* generated DCs of both human and mice origin can be successfully infected by influenza viruses (131, 132). However, the infection is non-productive – despite the synthesis of viral proteins, little progeny virus is produced. The infection is also non-toxic as infected DCs do not show any cytopathic effect or apoptosis (131). Influenza virus infected DCs can initiate a strong proliferation of cytotoxic CD8+ T lymphocytes (133). Activated DCs also secrete antiviral cytokines (IFN

α/ß) and promote a Th1 type immune response by stimulating IFN γ production by CD4+ T cells (134). The NS1 protein of influenza virus, as mentioned earlier, has been shown to inhibit the maturation of infected immature DCs and thereby prevent development of an adaptive immune response (64).

Table 1.2: CD antigens.

CD antigen	Cellular expression	Functions	Expression on DC
CD1b (CD1w2 homolog)	Cortical thymocytes, Langerhans cells, Dendritic cells	Antigen (lipid) presentation	+
CD4	Thymocyte subsets, TH1 and TH2 cells, monocytes, macrophages	Co-receptor for MHC class II molecules	+/-
CD8	Thymocyte subsets, cytotoxic T cells	Co-receptor for MHC class I molecules	+/-
CD11c	Myeloid cells	Binds fibrinogen	++
CD14	Myelomonocytic cells	Receptor for LPS and LPS binding protein	-
CD86	Monocytes, activated B cells, dendritic cells	Ligand for CD28 and CTLA4	+
CD172a	Myeloid cells	Adhesion molecule	+++
MHC I	Nucleated cells	Antigen presentation	++
MHC II	T cells, B cells, macrophages, dendritic cells	Antigen presentation	++

Adapted from Janeway's Immunobiology, 7th ed.

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5.1.4.3. Natural killer cells

Natural killer (NK) cells are a population of large granular lymphocytes that are particularly adept at lysis of target cells without prior sensitization. They are found abundantly in blood, liver, spleen, lymph nodes and other non-lymphoid organs such as pregnant uterus. They circulate in the body as "resting" cells containing a pool of constitutively expressed high levels of IFN γ, perforin and granzyme B mRNA transcripts (135, 136). However, these "resting" cells require activation by IFNα/ß or other pro-inflammatory cytokines (IL-2, IL-12, IL-15 and IL-21) to acquire their cytolytic effector functions (137). Following activation, they produce an array of cytokines (TNF-α, IFN-γ etc.) and cytolytic mediators (perforins, granzymes). Activated NK cells kill target cells either by creating pores on membranes with perforins and granzymes or by activating apoptotic signaling in the target cells (137). Since these naturally primed killer cells are broadly reactive and have the potential to kill harmful target cells as well as naïve host cells, their activity is strictly regulated through a series of activation and inhibitory receptors (138). The importance of NK cells in the innate immunity against influenza virus is evident by the large number of these cells that accumulate in the lungs of infected mice and any depletion or mutation of NK cell receptors can seriously exacerbate the illness (139, 140). NK cells recognize influenza HA proteins through direct interactions with its receptors called NKp46 and NKp44 (141, 142). Recent studies have also shown that use of an adjuvant that activates NK cells can boost the immunogenicity and protective efficacy of influenza vaccines (143).

5.1.5. Cytokine system

Cytokines are a diverse family of small proteins that are produced in response to different stimuli and exert their functions through binding of specific cellular receptors. They play an important role during viral infections.

Interferons are a type of cytokine that are produced during viral infections and are aptly named so because of their ability to interfere with viral replication. IFN-α and IFN-ß are type I interferons whereas IFN-γ is often called as type II. Although most nucleated cells can produce type I interferons, plasmacytoid DCs (pDCs) are considered as the specialized producers of these cytokines (144). They bind to a common cell-surface interferon receptor and activate transcription of several genes (ISG, interferon stimulated genes) through Janus family tyrosine kinase pathway. Several protein products of ISGs have anti-viral activities. For example, oligoadenylate synthetase (OAS) helps in degradation of viral RNA while protein kinase R (PKR) has an inhibitory effect on viral protein synthesis. Another ISG protein, Mx is associated with resistance against influenza infection (145). On the other hand, IFN-γ is primarily produced by T cells and NK cells and its function is to activate macrophages.

Interleukins (IL) constitute another group of cytokines that are mainly produced by leukocytes. A number of interleukins have been identified so far which can act locally or systemically to exert divergent effects on both innate and adaptive immune responses. Some of them such as IL-1, -6 and -12 act as “pro-inflammatory” cytokines whereas IL-10 exhibits an “anti-inflammatory” activity. They bind to their specific receptors and activate the JAK-STAT signaling pathway to modulate cellular functions.

5.2. Adaptive immune response

The adaptive immunity is an antigen-specific, cellular defense system that requires activation by the innate immunity to eliminate a microbial infection. Although it develops late into an infection, it can provide a strong memory or lasting protection against the same invading pathogen. The adaptive immune system is predominantly comprised of T and B lymphocytes.

5.2.1. T cells

T lymphocytes constitute an important effector arm of the adaptive immune response. Precursors of T lymphocytes develop from hematopoietic stem cells in the bone marrow and then migrate to the thymus for complete maturation (146). During an infection, APCs circulating in the periphery capture the antigen and bring them to the thymus where they present the antigen to T cells which in turn undergo “clonal expansion” to produce large numbers of progeny T cells. Depending on the surface molecule expressed on them, activated T cells perform different tasks to eliminate the invading pathogen.

5.2.1.1. CD8+ T cells

Antigens presented on MHC class I molecules can be recognized by CD8+ T cells. Once activated, they become cytotoxic T lymphocytes (CTL) which are programmed to kill infected cells. Several mechanisms including calcium dependent perforin exocytosis and Fas mediated apoptosis have been suggested as the means by which CTLs destroy

infected cells (147). During influenza infection, CTLs play a critical role in viral clearance. Initial activation of naïve CD8+ T cells in lymph nodes occurs within the first 3 days of infection. Antigen primed CD8+ T cells thereafter start to divide, acquire their cytotoxic activity and exit through draining lymphatic vessels to reach the lungs (148). Six days post infection, almost 70 % of lung-infiltrating lymphocytes are found to be cytotoxic. Most (90%) of these CTLs can recognize epitopes from either HA or NP viral protein (149). Viral antigen stimulated CTLs produce large amount of IFN-γ and TNF-α which help in recruiting leukocytes to the site of inflammation (148). Following viral clearance, memory CTLs down-regulate transcription of cytotoxic proteins and circulate in lymphoid (spleen) and non-lymphoid (skin/lungs) tissues (150). However, when re-exposed to the same antigen, memory CTLs can rapidly initiate their cytotoxic functions (151).

5.2.1.2. CD4+ T cells

CD4+ T lymphocytes are often referred to as helper T (T_h) cells as their primary function is to assist B lymphocytes in producing antibodies against microbial infections. These T lymphocytes recognize antigens presented on MHC class II molecules and depending on the type of pathogen, they can differentiate into either Th1 or Th2 phenotype. Th1 type CD4+ T cells produce IFN-γ and TNF-α, and stimulate cytotoxicity of macrophages and CTLs; whereas Th2 type cells secrete IL-4 and IL-5 and promote neutralizing antibody production by B cells (152). Influenza virus infection activates DCs to produce IL-12 which in turn drives CD4+ T cells towards Th1 phenotype. During

influenza virus infection, CD4+ T cells provide protection in a B cell dependent manner, although they themselves possess some cytotoxic (perforin) activity too (153). CD4+ T cells are also necessary for optimal recruitment of CD8+ T cells to the infected lungs and subsequent viral clearance by CTLs (154). Following recovery or live virus clearance activated CD4+ T cells progress to become memory cells. However the number of CD4+ memory T cells decline much faster than their CD8+ counterparts (155).

5.2.2. B cells

B cells are a lymphocyte subpopulation responsible for eliciting the humoral immune response during an infection. In mammals, B cells originate in the bone marrow and mature in secondary lymph nodes or spleen. Terminally differentiated B cells are called plasma cells whose primary function is to produce antibodies to specific antigens (156). However, they can also uptake, process and present antigens to T cells in an MHC restricted manner (157). Neutralizing antibodies produced by B cells during an influenza infection have an important protective role and are therefore the main targets of vaccine induced immunity. Development of humoral immunity to an influenza infection consists of an early rise in IgM antibody titer followed by their affinity maturation and immunoglobulin class-switing to IgG, IgA and IgE antibodies (158). Mice that are deficient in B cell and therefore incapable of producing are 50 – 100 times more susceptible to a lethal influenza infection and they show a higher mortality rate when challenged with a pathogenic PR8 strain of influenza (159, 160). On the other hand, passive transfer of HA-specific antibodies to SCID mice can protect them for an influenza

infection (161, 162). These experiments clearly suggest the significant contributions of B cells and anti-viral antibodies in recovery from an influenza infection. Following exposure to an influenza infection, two compartments of memory B cells develop. One subset is comprised of long lived plasma cells or antibody secreting cells (ASC) that localize in the bone marrow and continuously generate antibodies to maintain an optimal level of plasma antibodies for protection against re-infection. The other compartment is populated with quiescent memory B cells that localize in lymphoid tissues or lungs and require stimulation by a recall antigen to divide and differentiate into ASCs (163).

6. Research objectives

In the past decades, DCs have been the subject of extensive research because of their versatile role in organizing the immune system both in health and illness. An array of both endogenous (interferon, interleukin) and exogenous (bacterial, viral pathogens) agents have been investigated for their role in the induction or inhibition of DC maturation (164, 165). However, little is known about what dictates the differentiation of DCs from bone marrow progenitor cells or monocytes. It is plausible to presume that the local cytokine milieu plays a critical role in this process. In vitro, GM-CSF and IL-4 are widely used to facilitate generation of DCs from monocytes, whereas other cytokines e.g. IL-10 and IL-6 can block this differentiation pathway (166-169). Knowledge about the effects of microbial infections on this differentiation process is still limited.

Recently, a body of accumulating evidence suggests that during infection or inflammation, DCs differentiate more readily from monocytes than from bone marrow precursor cells (170, 171). Preferential production of monocyte chemo-attractant proteins over neutrophil chemo-attractant proteins has also been reported during influenza infection (172). The number of monocytes that migrate to the respiratory mucosa overwhelmingly exceeds the number of DCs (173). These findings underscore the importance of monocytes in the replenishment of APCs in airway mucosa during influenza infection. Therefore, it was hypothesized that virus-mediated impairment of monocyte differentiation into DC may contribute to influenza virus pathogenesis and also to evasion of host immune responses. Equine influenza viruses were used in an

equine monocyte model to determine the effects of the viral infection upon the differentiation of monocyte-derived DCs.

To test the experimental hypothesis, three specific aims were investigated: 1. Determination of the ability of equine influenza viruses to infect and replicate in equine monocytes when cultured in the presence of both eq.GM-CSF and eq.IL-4 for differentiation into DCs. 2. Characterization of morphologic, phenotypic and functional alterations of equine influenza virus infected monocytes with respect to their capability to differentiate into DCs. 3. Determination of the role of virus subtype and influenza NS1 protein in the virus mediated inhibition.

The experimental approaches that were used for evaluating aforementioned specific aims are as follows. In specific aim 1, the ability of equine influenza virus to replicate in equine monocytes was investigated by assessing the transcription of viral genomic RNA in infected monocytes and also by measuring viral protein (NP) synthesis and progeny virus production. Cytotoxicity of the virus was determined by its ability to induce apoptosis in infected cells. For specific aim 2, influenza virus infected monocytes were first evaluated microscopically for their morphologies and then analyzed by flow cytometry for phenotypic expression of DC surface markers. Functional inhibition due to the viral infection was measured by the endocytic ability and cytokine production by infected monocytes. Finally, the role of virus subtype and influenza NS1 protein in this process was examined in specific aim 3 by employing an H3N8 virus and another NS1-truncated virus.

CHAPTER II

MATERIALS AND METHODS

Purification of equine monocytes from PBMC

Isolation of equine monocytes from peripheral blood mononuclear cells (PBMC) and further culture for differentiation into DCs was carried out according to a previously published method (129). Horses were randomly selected from a population maintained at the University of Kentucky Maine Chance Farm and screened for presence of serum antibody against a panel of equine influenza viruses. A group of influenza sero-negative horses (n = 10, of mixed breed, age and sex) were finally selected for all future experiments. Briefly, blood was collected in heparin containing tubes or bottles and was placed at room temperature for 30 min for clear separation of plasma from red blood cells. PBMCs were isolated from this heparinized blood by Ficoll-Paque density gradient centrifugation. Blood plasma including the Buffy coat was carefully layered onto 10 ml of Histopaque – 1077 (Sigma Aldrich) in 50 ml conical tubes (Corning) and was centrifuged at room temperature (22°C) at 400g for 30 min without brakes (Beckman GS-6KR centrifuge, rotor type GH-3.7). A creamy white band of PBMCs that appeared at the interface between the plasma and Histopaque – 1077 was collected and washed twice with PBS (BioWhittaker, pH 7.4) by centrifugation at 500g for 5 min. PBMCs were then suspended in complete RPMI medium (cRPMI; RPMI-1640 containing 10% autologous horse serum, 2mM L-glutamine, 55µM ß–mercaptoethanol, 100 U/ml

penicillin-streptomycin and 0.25µg/ml amphotericin B) at a density of 10^7 cells per ml and plated in 100 mm tissue culture treated plates. For the autologous serum used in cRPMI medium, blood was collected in 10 ml tubes (BD vacutainer™), incubated at 37°C for 30 min and then centrifuged at 900g for 10 min. After 4 hrs of incubation at 37°C with 5% CO_2, non-adherent cells (mainly lymphocytes) were removed by gently washing the plates twice with warm PBS. In a few occasion, adherent cells were stained for CD14 (Big 10, Axxora Platform) and examined by flow cytometry (FACScan, Becton Dickinson) to determine the purity of monocytes among adherent cells.

To stimulate differentiation into DCs, purified monocytes were further cultured in cRPMI medium supplemented with eq.GM-CSF and eq.IL-4 (CHO cell supernatants containing recombinant eq.GM-CSF and eq.IL-4 were added at 10% each) at 37°C with 5% CO_2 for 4 days.

Preparation of virus stock

Equine influenza viruses that were used for different experiments are: (1) Influenza A/equine/New York/49/73, (H7N7 subtype); (2) Influenza A/equine/Kentucky/5/02, (H3N8 subtype) and (3) Influenza A/equine/Kentucky/5/02 NS1-73, a carboxy-terminally truncated virus (a kind gift from Dr. Peter Palese, Mount Sinai School of Medicine). The Kentucky/5/02 NS1-73 is a recombinant H3N8 subtype virus that expresses only the N-terminal 73 amino acid residues out of the full 219 amino acid long NS1 protein (174). Wild type viruses (NY/73 and KY/02, virus repository, OIE equine influenza reference laboratory, University of Kentucky) were propagated in

pathogen free 10-day-old embryonated chicken eggs whereas immune-incompetent 7-day-old eggs were used for the recombinant virus (KY/02 NS1-73) since this virus lacks proper anti-interferon activity. Viruses (0.1 ml per egg, 1:1000 dilutions in PBS) were inoculated into allantoic cavities and then incubated at 37°C for 72 hrs. Allantoic fluids were harvested and then clarified of any egg debris by centrifugation. Titration of the stock virus was done by 50% egg infectious dose (EID_{50}) assay as described by Reed and Muench (175). Stock virus was stored in 1 ml aliquots at – 80°C.

Where needed, viruses were inactivated by UV irradiation using a UV Stratalinker 1800 (Stratagene). Briefly, a thin layer of 1 ml virus was poured into a 35 mm plate, placed on ice and irradiated at 254 nm for 30 min at a distance of 10 cm. Proper inactivation was confirmed by inoculation of 0.1 ml of irradiated virus into a 10-day-old embyronated chicken egg. Although UV irradiated viruses could not replicate, they retained their receptor binding (cell attachment) capability as they could agglutinate chicken RBCs in the hemagglutination (HA) assays.

Infection of equine monocytes with equine influenza virus

Before infection, monocytes were washed twice with PBS to remove serum. The virus stock was diluted in serum free infection medium (cRPMI with 0.25 % bovine serum albumin replacing 10 % autologous horse serum plus 1µg/ml TPCK trypsin). Monocytes were infected with an infectious dose of approximately 5 EID_{50} units per cell or an equivalent amount of UV inactivated virus. Mock inoculums consisted of only the infection medium. After 1 hr of adsorption at 37°C, viral inoculums were removed; cells

were washed twice with PBS and then cultured in cRPMI medium with 1µg/ml TPCK-trypsin for 4 days or as otherwise mentioned.

Quantification of viral RNA levels in infected cells

To determine whether equine monocytes cultured in the presence of eq.GM-CSF and eq.IL-4 can support replication of equine influenza virus, viral genome transcription in infected cells was measured by relative real-time PCR assay. Purified equine monocytes were plated at 10^6 cells per ml and incubated overnight in cRPMI supplemented with eq.GM-CSF and eq.IL-4. The next day, cells were infected with equine influenza virus as described earlier. Following infection, cell samples were collected at different time points post-infection (p.i.) e.g. 0, 4, 8, 12 and 24 hrs. Total cellular RNA was extracted from collected samples using the RNeasy Mini Kit (Qiagen) following manufacturer's instructions. Possible DNA contamination was eliminated by in-column treatment with DNase I (RNase free DNase set, Qiagen). Taqman® one-step RT-PCR master mix reagents (Applied Biosystems) and an ABI Prism 7500 fast real-time PCR system (Applied Biosystems) were used to perform the assay. Five micrograms of extracted RNA were added to a total reaction volume of 20µl in a 96 well plate (Applied Biosystems). Primers and probe (TaqMan MGB), used in this experiment, were designed to amplify a 63bp region of the NP gene. The program for amplification consisted of a 30 min period at 48°C, then 10 min at 95°C followed by 40 cycles with the following conditions: 95°C for 15 sec and 60°C for 1 min. Threshold cycle (Ct) values of respective

samples were normalized by comparison with the ribosomal protein large PO (RPLPO) RNA level.

EqFlu NP forward primer: 5'- GAAGGGCGGCTGATTCAGA -3'

EqFlu NP reverse primer: 5'- TTCGTCGAATGCCGAAAGTAC -3'

EqFlu NP probe: 5'- CAGCATAACAATAGAAAGGA -3'

Quantification of viral protein synthesis in infected cells

Viral protein synthesis in infected monocytes was quantified by detecting NP protein levels using flow cytometry. Following infection of monocytes, samples were collected at 5 different time points p.i. (0, 4, 8, 12 and 24hrs). Cells were washed twice with PBS and then fixed with 2% paraformaldehyde in PBS for 1 hr at room temperatute. Cellular permeabilization was achieved by incubating samples in 0.5% saponin, 5% FBS in PBS solution for 15 min at 4°C. After washing twice with PBS, cells were first stained with the primary antibody (Mouse influenza A anti-NP, clone # M2110169, Fitzgerald Industries International Inc.) and then with a FITC-conjugated secondary antibody (Immunopure goat anti-mouse IgG, $F(ab')_2$, Pierce) at 37°C for 30 min each (1 µg of Ab per 10^6 cells). Subsequently, all samples were washed twice, resuspended in 400µl FACS buffer (PBS with 1% BSA and 0.1% sodium azide) and analyzed with by flow cytomerty (FACScan, Becton Dickinson) for their respective mean fluorescence intensities (MFI).

Detection of apoptosis in virus infected monocytes

Influenza virus infected or mock inoculated monocytes were further cultured in cRPMI medium with eq.GM-CSF and eq.IL-4 for 4 days. On day 4 p.i., cells were collected, washed in PBS and then resuspended in Annexin V binding buffer at the rate of 10^6 per ml. Next, 10^5 cells were used for staining with Annexin V and propidium iodide (PI) according to manufacturer's instructions (Annexin V-FITC apoptosis detection kit I, BD Pharmingen). After 15 min of incubation at room temperature, cells were analyzed by flow cytometry. When cells start to undergo apoptosis, phosphatidylserine (PS), a membrane phosphor lipid, translocates from inside to the outer side of the membrane. Annexin V then binds to PS on apoptotic cells in a calcium dependent manner. PI is used to separate viable cells from the non-viable ones.

Analysis of cell count, viability and average diameter

Vi-Cell ^{TM} (Beckman Coulter), an automated cell viability analyzer, was used to determine total cell counts, percent of viable cells and their average diameters. Briefly, cells were diluted in PBS at 1:10 ratio and then were run through the analyzer using the program specific for PBMC. This automated machine uses trypan blue exclusion method and video imaging to determine cell viability as well as the average diameter of the cells.

Analysis of cell surface marker expression by flow cytometry

Levels of expression of different cell surface markers for DC on mock- or influenza infected cells were examined by flow cytometry (176). On day 4 p.i., cells were

collected, washed twice with PBS and then resuspended in FACS buffer (PBS supplemented with 1.0% BSA and 0.1% sodium azide). Pre-incubation with purified horse IgG (ChromPure horse IgG, Jackson ImmunoResearch) for 30 min was used to block non-specific binding through Fc receptors. Cells were then incubated with either primary or fluorochrome-conjugated antibodies (1 µg of Ab per 10^6 cells) for 30 min at 4°C. The following antibodies were used: CD172a (DH59B, VMRD), CD1w2 (MCA2058PE, AbD Serotec), CD86 (IT2.2, Biolegend), MHCI (CVS22) and MHCII (CVS10, both CVS mAbs were gifts from Dr. Paul Lunn, University of Colorado). After labeling with primary antibodies, cells were washed twice with FACS buffer and then stained with FITC labeled secondary antibodies for 15 min at 4°C. Isotype-matched antibodies (BD Pharmingen) were used as negative controls. Cells were fixed with 2% paraformaldehyde in FACS buffer and then analyzed via flow cytometry (FACScan, Becton Dickinson). Cells were gated based on forward- and side-scatter profile and data were analyzed using CellQuest ™ software.

Quantification of cellular gene expression by real-time PCR

Cellular gene expression levels were quantified by two-step real-time PCR assays (RT-PCR). Total cellular RNA was extracted from infected cells with RNeasy Mini kit (Qiagen) as described earlier in this chapter. Then, following manufacturer's instructions, 1µg of the extracted RNA was reverse-transcribed into cDNA using Stratascript cDNA synthesis kit (Stratagene). Oligo-dT primers were used in the assay to selectively amplify cellular mRNAs. Gene-specific, intron-spanning primers and probes

used in these assays are listed below (Table 2.1). TaqMan® Fast Universal PCR master mix (Applied Biosystems) was added in 10µl of reaction mixtures in a 96 well plate and the fast amplification cycle (20 sec at 95°C, followed by 40 cycles of 3 sec at 95°C and 30 sec at 60°C) of an ABI Prism 7500 fast real-time PCR system was used to perform the experiments. Initially several house-keeping genes (RPLPO, ß-GUS, GAPDH, ß-2-microglobulin, phosphoglycerate kinas 1 and ß-actin) were tested and the expression level of RPLPO was found to be the most stable during influenza virus infection of monocytes. Therefore, RPLPO was applied to normalize the data. The relative expression levels of different genes were determined ($2^{-\Delta\Delta CT}$) by using the method of Livak and Schmittgen (177).

Table 2.1: Primers and probes for two-step RT-PCR assay.

Target gene	Sequences (5'-3')	References
RPLPO	Fwd: CTGATTACACCTTCCCACTTGCT Rev: AGCCACAAATGCAGATGGATCA Probe: FAM-AAGGCCTTGACCTTTTC-NFQ	
CD11c	Fwd: CTGATTTCCTGACCCACCTTCA Rev: ACCTCAGGCAGTCAGCAATG Probe: FAM-CTGTGCTGGACTGCTC-NFQ	
CCR5	Fwd: GCAGAGCAGCTGAGACATCT Rev: GGACTTGTCGTCTGATAATCCATCT Probe: FAM-CAACCCAGGAGGCCTT-NFQ	(176)
CCR7	Fwd: GTGGTGGCTCTCCTTGTCA Rev: AATCGTCCGTGACCTCATCTTG Probe: FAM-CAGGCACACCTGGAAAA-NFQ	(176)
IL-10	Fwd: AGGACCAGCTGGACAACATG Rev: GGTAAAACTGGATCATCTCCGACAA Probe: FAM-CCAGGTAACCCTTAAAGTC-NFQ	(176)
IL-12	Fwd: CTACACCAGCGGCTTCTTCAT Rev: GCTTCAGCTGCAGGTTCTTG Probe: FAM-CAGGGACATCATCAAACC-NFQ	(178)
TGF-ß	Fwd: CCCTGCCCCTACATTTGGA Rev: TGTACAGGGCCAGGACCTT Probe: FAM-CCTGGACACGCAGTACAG-NFQ	(176)
IFN-α	Fwd: GCTGCTCTCTGGGATGTGA Rev: TTTGTCCCAGGAGCATCAAGAC Probe: FAM-CCTGCCTCACACCCATAG-NFQ	(178)
TNF-α	Fwd: TTACCGAATGCCTTCCAGTCAAT Rev: GGGCTACAGGCTTGTCACTT Probe: FAM-CCAGACACTCAGATCAT-NFQ	(178)

Alpha-naphthyl acetate esterase (ANAE) assay

Mock or virus infected cells were collected at 4 days p.i., washed twice with PBS and then smeared onto an 8-well slide. The slide was air-dried, fixed and then analyzed for ANAE activity using alpha-naphthyl acetate (Sigma Aldrich) as the substrate as per the manufacturer's instructions and in accordance of previously published methods (179). Presence of ANAE was determined microscopically by the appearance of dark, black granulations at the site of enzymatic activity.

DQ-ovalbumin (DQ-OVA) endocytosis assay

DQ-ovalbumin (DQ-OVA, Molecular Probes) is a pH insensitive BODIPY-conjugated self-quenching dye which emanates fluorescence (505-515nm) upon protease degradation following endocytosis. On day 4 p.i., cells were collected, washed twice in PBS and then resuspended at 5 x 10^5 per ml in cRPMI medium. DQ-OVA (10µl, 1mg/ml) was added to 100µl of cell suspensions and incubated at 37°C or 4°C (negative control). After 1 hr of incubation, cells were washed thrice with ice-cold cRPMI media containing 10% autologous horse serum to remove unbound or non-specifically bound antigens. Cell samples were then resuspended in FACS buffer and evaluated by flow cytometry. The endocytic capacity of mock or virus infected cells was determined by the differences in mean fluorescence intensities between the treated and control samples.

Statistical analysis

Data were analyzed by the one-way analysis of variance (ANOVA) and the Tukey's multiple comparison tests using the GraphPad Prism 5 statistical software (GraphPad Software Inc.). A p value of less than 0.05 (95% confidence level) was considered to be significant.

CHAPTER III

RESULTS

The hypothesis of whether influenza virus infection hinders monocyte differentiation into DC was tested by evaluating the following results. In specific aim 1, transcription of viral genomic RNA and subsequent viral protein synthesis and progeny virus production in infected monocytes was quantified to assess whether equine influenza virus can replicate in equine monocytes cultured with both GM-CSF and IL-4. The ability to induce apoptosis in infected cells indicated viral cytotoxicity. In specific aim 2, microscopical examination for morphologies and analysis by flow cytometry for phenotypic expression of DC surface markers was done to evaluate the ability of influenza virus infected monocytes to differentiate into DCs. Functional inhibition due to the viral infection was measured by the endocytic ability and cytokine production by infected monocytes. Finally, an H3N8 virus (KY/02) and another NS1-truncated virus was used in specific aim 3 to evaluate if the viral inhibition is dependent on virus subtype and the NS1 protein function.

Proportion of monocytes in adherent cell population

Following isolation of PBMC, plastic adherent cells were analyzed by flow cytometry to evaluate the percentage of monocytes within the cell population. CD14 was used as the marker for monocytes and about 73.6% of the cells were found to be expressing CD14 on their cell surface. Therefore, this adherent cell population was used as the source of monocytes for further experiments.

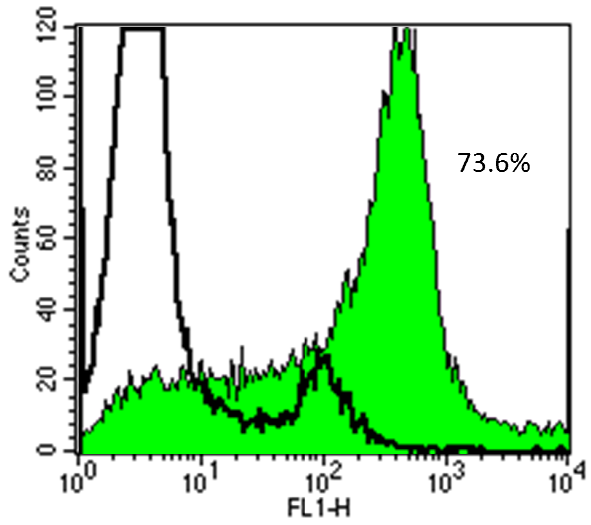


Figure 3.1: Percentage of monocytes in adherent cells. Adherent PBMCs were stained with monoclonal anti-CD14 antibody (Big 10, Axxora Platform) and then analyzed by flow cytometry. Empty black line represents the isotype control.

Influenza virus infection of monocytes

Monocytes, after overnight pre-treatment with GM-CSF and IL-4 in culture, were infected with influenza virus (NY/73, H7N7) at an infectious dose of 5 EID_{50} units per cell. To evaluate successful infection of monocytes with influenza virus, cells were evaluated for expression of viral nucleoprotein (NP) 24 hr p.i. About 59% (59.15 ± 2.87, N=5) of the adherent cells were found to express NP protein.

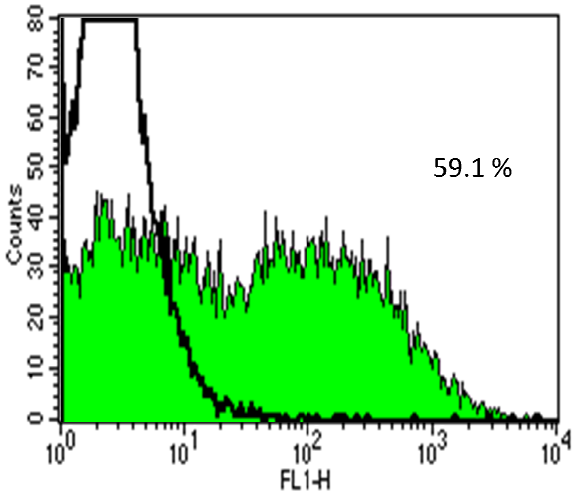


Figure 3.2: Percentage of cells positive for viral NP. Monocytes infected with NY/73 virus were cultured with GM-CSF and IL-4 for 24 hr and then stained intracellularly with mouse anti-influenza NP antibody. Empty black line shows the control of uninfected cells stained with anti-NP antibody. Figure is representative of 3 separate experiments.

Influenza viral RNA transcription in monocytes cultured with GM-CSF and IL-4

The ability of equine influenza virus (NY/73, H7N7 subtype) to replicate in actively differentiating monocytes was first evaluated by examining viral genome transcription. Before infecting with the virus, monocytes were pre-incubated overnight with GM-CSF and IL-4 to initiate the differentiation process. Upon entering into cells, viral genome transcription was evaluated by quantifying influenza virus nucleoprotein (NP) RNA levels at various time points post-infection (0, 4, 8, 12 and 24 hr p.i.). Monocytes in culture for DC differentiation allowed initiation of influenza genome transcription as viral NP RNA level continued to increase until 12 hr p.i. when it reached about 12-fold the amount of initial infectious viral RNA (Figure 3.3). However, after 12 hr p.i., viral RNA level started to decline. On the other hand, in UV-inactivated influenza infected cells, no significant increase in NP RNA level was observed which further confirmed successful inactivation of the virus through UV irradiation. However, detection of NP RNAs in UV-inactivated influenza infected cells showed that those viruses retained their cellular attachment and entry capabilities.

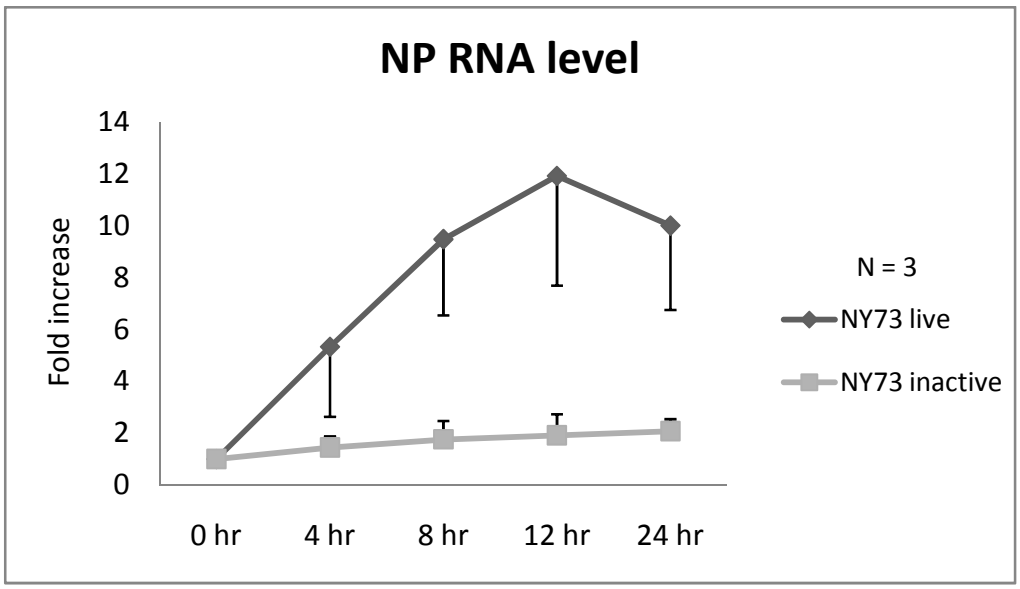


Figure 3.3: Viral genome transcription in influenza infected monocytes. Nucleoprotein (NP) RNA levels in UV-inactivated and live virus (NY/73) infected monocytes were measured by RT-PCR assays and were normalized based on RPLPO RNA levels. Results are expressed as fold-increase (mean ± SD) over 0 hr p.i.

Influenza viral protein synthesis in monocytes cultured with GM-CSF and IL-4

Since equine influenza virus (NY/73, H7N7) was able to transcribe its RNA genome in infected monocytes, we next evaluated whether it could translate its viral proteins. When virus infected monocytes were analyzed by flow cytometry for NP protein levels, an early restriction in viral protein synthesis was observed (Figure 3.4). Following infection, the virus started to synthesize viral NP protein which was evident by the sharp increase in mean fluorescence intensity (MFI) at 4 hr p.i. However, after this time point, NP protein level decreased continuously. At 24 hr p.i., fluorescence for NP protein was almost half that of at 4 hr p.i. This showed that monocytes stimulated for DC differentiation imposed an early inhibition in viral protein synthesis.

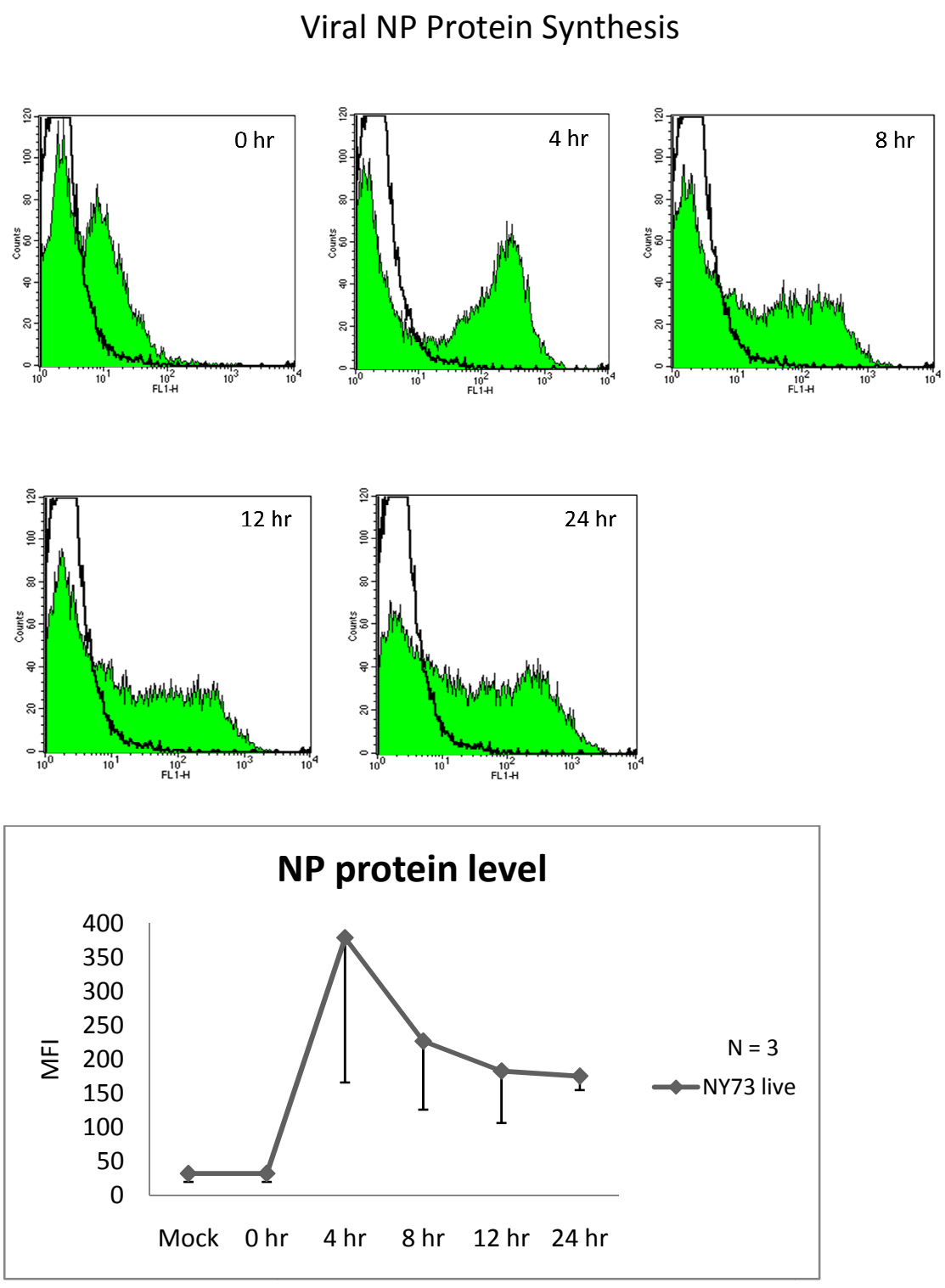


Figure 3.4: Viral protein synthesis in influenza infected monocytes. Monocytes were pre-treated overnight with GM-CSF and IL-4 before infection. Synthesis of viral nucleoprotein (NP) in live virus (NY/73) infected monocytes were detected by flow cytometry with mouse anti-influenza NP antibody. Results are expressed as mean fluorescence intensity (MFI) ± SD.

Progeny virus production in monocytes cultured with GM-CSF and IL-4

Ability of GM-CSF and IL-4 pre-treated monocytes to support influenza virus replication was further evaluated by quantification of progeny virus production by infected cells. Cell supernatants were collected from infected monocytes (2 $X10^6$ cells, MOI of 5 EID_{50} units per cell) at 0 and 24 hr p.i. and presence of live virus particles was measured by 50% egg infectious dose assay. Loose attachment of monocytes to the plate prevented vigorous washing of cell surface after infection which resulted in substantial titer at 0 hr p.i. However, when viral titer at 24 hr p.i. was compared to that of 0 hr, only 10-fold increase in EID_{50} titer was observed (Table 3.1). This is significantly less than what is normally observed with more permissible cells such as MDCK (2 X 10^6 cells, MOI of 5 EID_{50} units per cell; Table 3.1). This demonstrated that GM-CSF and IL-4 treated monocytes were non-permissive to influenza virus as only limited progeny virus production was achieved.

Table 3.1: EID_{50} titers of influenza virus infected monocytes and MDCK cells.

EID_{50} titer	0 hr p.i.	24 hr p.i.	Fold difference
Monocytes (GM-CSF & IL-4)	9.8 X 10^4	10.5 X 10^5	10.7
MDCK cells	2.81 X 10^5	6.58 X 10^7	234.1

Level of apoptosis of developing DCs following influenza infection

Having established that influenza virus infection of differentiating monocytes is abortive or minimally-productive, we next investigated whether this limited virus replication could inflict any toxic effect i.e. apoptosis in differentiating monocytes. Mock- or virus-infected monocytes were cultured for 4 days in cRPMI supplemented with GM-CSF and IL-4. Annexin-V (apoptosis) and propidium iodide (loss of cell membrane integrity) staining of cells revealed that the presence of GM-CSF and IL-4 in the culture media of mock-infected monocytes did not adversely affect their viability (86.7% vs 84.0%, data not shown). When influenza virus-infected monocytes were cultured with GM-CSF and IL-4 for 4 days, the level of apoptosis was significantly higher than in mock infected cells (10.3% vs. 1.8%, $p < 0.01$, Figure 3.5). However, considering the fact that an infectious dose of 5 EID_{50} units per cell succeeded at inducing apoptosis in only about 10.3% of the cells and the majority of cells (about 76.9%) were still viable based on trypan blue staining at 4 days p.i., it can be concluded that influenza virus infection did not induce substantial toxicity or apoptosis in developing DCs.

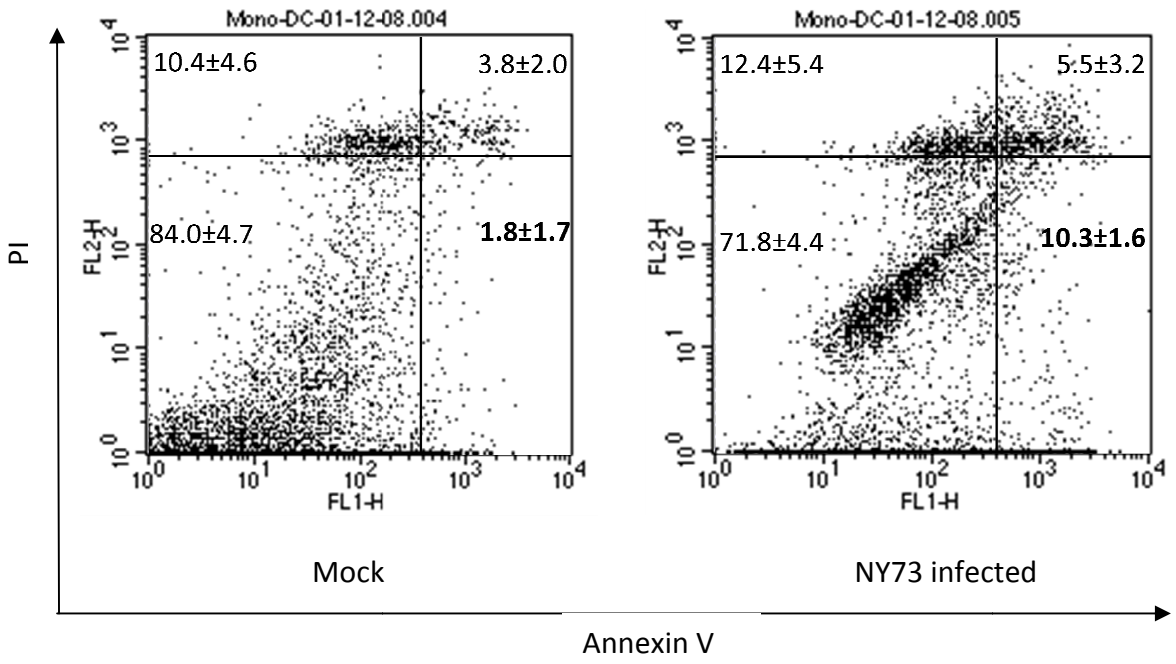


Figure 3.5: Apoptosis of influenza virus infected monocytes cultured in GM-CSF and IL-4. Mock or NY/73 influenza virus infected (MOI = 5 EID_{50} units/cell) monocytes at 4 days p.i. were stained with FITC-conjugated annexin V and propidium iodide (PI) and analyzed by flow cytometry. Percents of cells in four quadrants of the dot plots are shown as mean ± SD from 4 separate experiments.

Morphology of mock and influenza virus (NY/73) infected monocytes

The morphological distinction between mock- or influenza-infected monocytes was determined by inverted light microscopy (Figure 3.6). After 4 days of incubation, mock-infected monocytes showed typical DC-like appearances such as finger-like projections. Fully differentiated DCs were observed as loose floating clumps of large, viable cells. On the other hand, infected cells failed to acquire DC-like processes and many cells were found to float as a single cell or dense clumps of dark cells. Monocytes infected with UV inactivated virus exhibited DC morphology, although the proportion of DC-like cells was smaller than in the mock infected population (Figure 3.6). The average diameter, as obtained by Vi-Cell ^{TM} cell viability counter (Beckman Coulter), also indicated that influenza-infected monocytes did not increase significantly in size as compared to their mock-infected counterparts (Figure 3.7). Differences between live influenza infected monocytes and both mock and UV-inactivated virus infected cells were also significant (Figure 3.7). Analysis by flow cytometry, as well, revealed a lower degree of granularity for the virus-infected cell populations as most of the virus infected cells, similar to monocytes, were still congregated in the lower right quadrant of the dot plot.

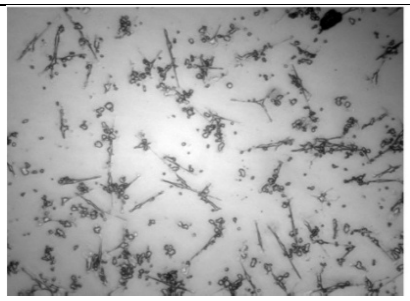
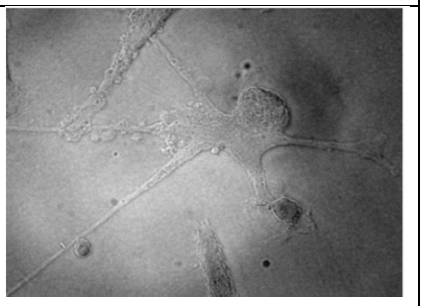
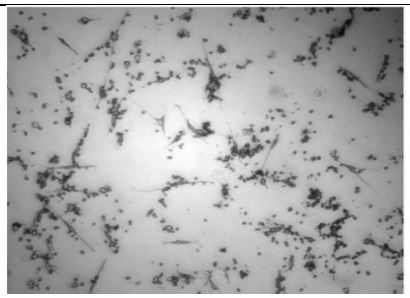
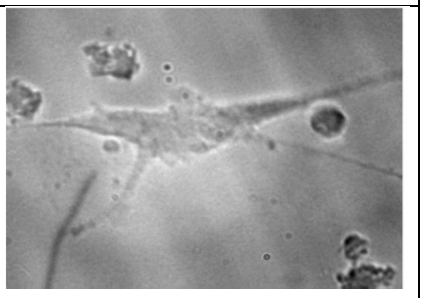
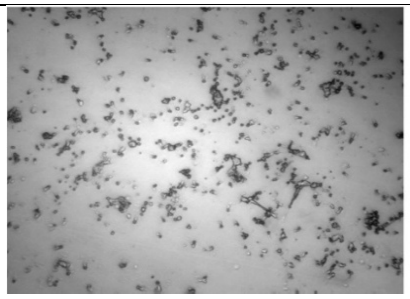
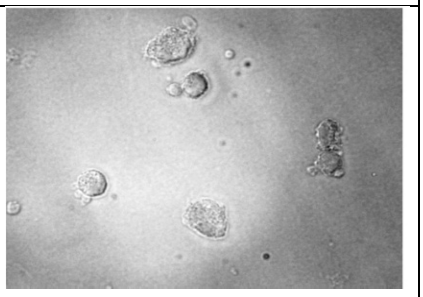
	10X	100X
Mock		
NY73 inactivated		
NY73 live		

Figure 3.6: Light microscopy images (10X and 100X magnifications). Mock, UV-inactivated and live influenza virus (NY/73) infected monocytes were cultured in cRPMI media supplemented with GM-CSF and IL-4 for 4 days and observed for DC morphology under a light microscope.

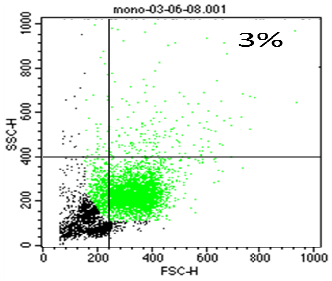
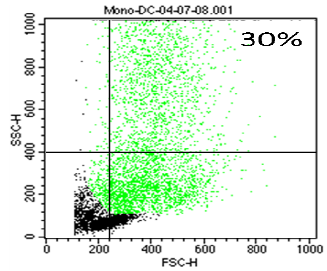
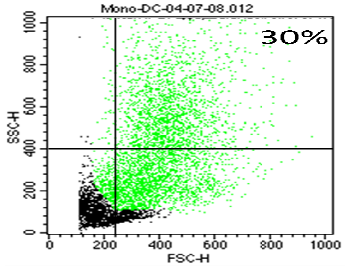
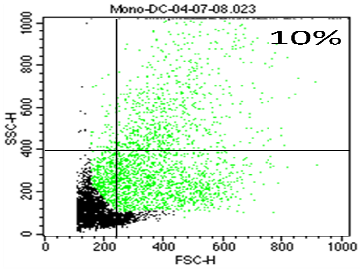
	Avr. Diam. (µm ± SD)	Dot Plot
Monocyte	9.02 ± 0.20*,†	
Mock	9.99 ± 0.17*,a	
NY73 inactivated	9.88 ± 0.21†,b	
NY73 live	9.02 ± 0.45a,b	

Figure 3.7: Average diameter and dot-plot analysis. Average diameters (mean ± SD, N=4) of monocytes, mock, UV-inactivated and influenza virus (NY/73) infected monocytes were measured with a Beckman Coulter ViCell ™ cell viability counter. A confidence interval of 95% was used to determine significant increases in diameters (*, †, a, b = $p < 0.05$). The lower panel shows flow cytometry dot plots (forward vs. side scatter). Cells with increased granularity tend to move towards upper right quadrant. Numbers in the figures indicate percents of cells within that quadrant.

Expression of DC surface molecules on influenza virus (NY/73) infected monocytes

Successful differentiation of monocytes into DCs was confirmed by analysis of a set of distinctive surface molecules. Monocytes were infected with 5 EID_{50} units per cell of live influenza virus or equivalent amount of UV-inactivated virus; cultured in presence of GM-CSF, IL-4 for 4 days and then compared with their mock-infected counterparts for relevant phenotypes. Differentiated DCs are characterized by high levels of CD11c, CD1b, CD86, MHC I and MHC II. Accordingly, mock-infected monocytes which effectively differentiated into DCs, up-regulated CD11c gene expression by almost 20-fold ($p < 0.05$) compared to non-stimulated monocytes (Figure 3.8). In contrast, CD11c expression on live influenza infected monocytes was nearly negligible ($p<0.05$ vs mock) and UV-inactivated virus treated monocytes exhibited only moderately (5-fold) increased CD11c gene expression (Figure 3.8). Similarly, CD172a (a myeloid marker) was found on 86% of mock–infected monocytes whereas only 66.1% and 36.2% of UV-inactivated or live virus treated cells were positive for this marker respectively (Figure 3.9). This expression level of CD172a on live virus infected monocytes was significantly lower ($p < 0.05$) than the mock cell population. Mock-infected cells also had a significantly higher ($p < 0.05$) number of CD1w2 (human CD1b homolog) positive cells than either of the virus infected cell populations (Figure 3.10). Influenza virus infected monocytes also failed to up-regulate expression of “immature” DC-associated chemokine receptor, CCR5 as efficiently as mock or UV-inactive virus treated cells (Figure 3.8). On the other hand, expression of CCR7, which is predominantly found at high levels on “mature” DCs, did not vary significantly among mock and virus treated cell populations (Figure 3.8).

However, percentage of cells expressing CD86, MHC I and MHC II were comparable among mock, UV-inactivated or live influenza virus infected cells (Figure 3.11). On the other hand, when the mean fluorescence intensities (MFI) were evaluated, these were considerably higher for both UV-inactivated and live virus infected cells as compared to mock samples (Figure 3.12). These results indicated that influenza virus infection of monocytes impeded expression of different surface antigens characteristic of DC differentiation. Since the phenotype of UV-inactivated virus treated monocytes, as evaluated from the surface antigen expression profile, lay intermediate between mock- and live influenza virus infected cells, it appears that at least limited viral replication was necessary for significant inhibition of monocyte differentiation into DCs.

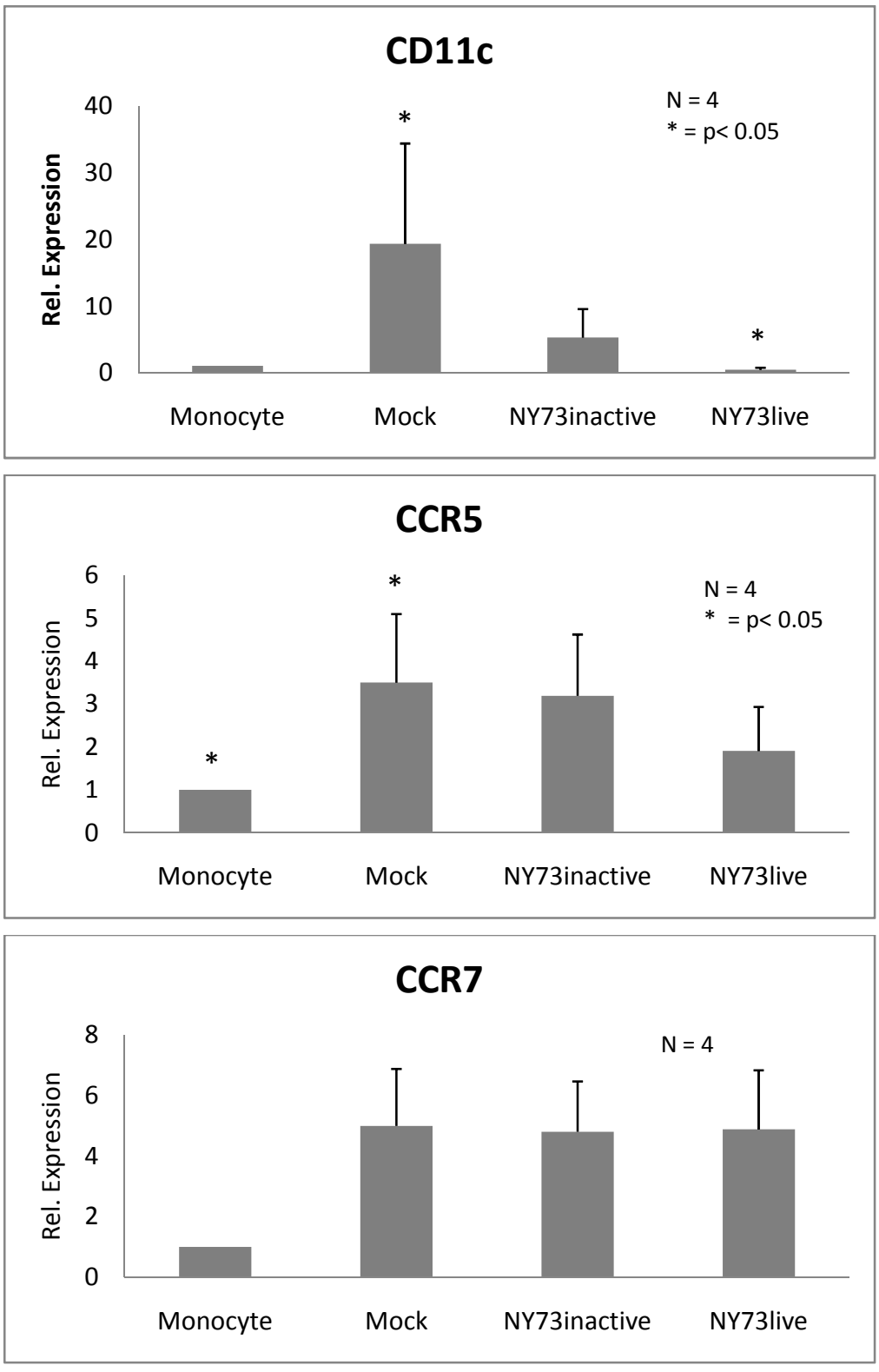


Figure 3.8: Gene expression of CD11c, CCR5 and CCR7. Relative RT-PCR was used to measure gene expression levels. Data were normalized based on house-keeping gene, RPLPO and shown as mean ± SD. A p value of 0.05 or less was considered significant.

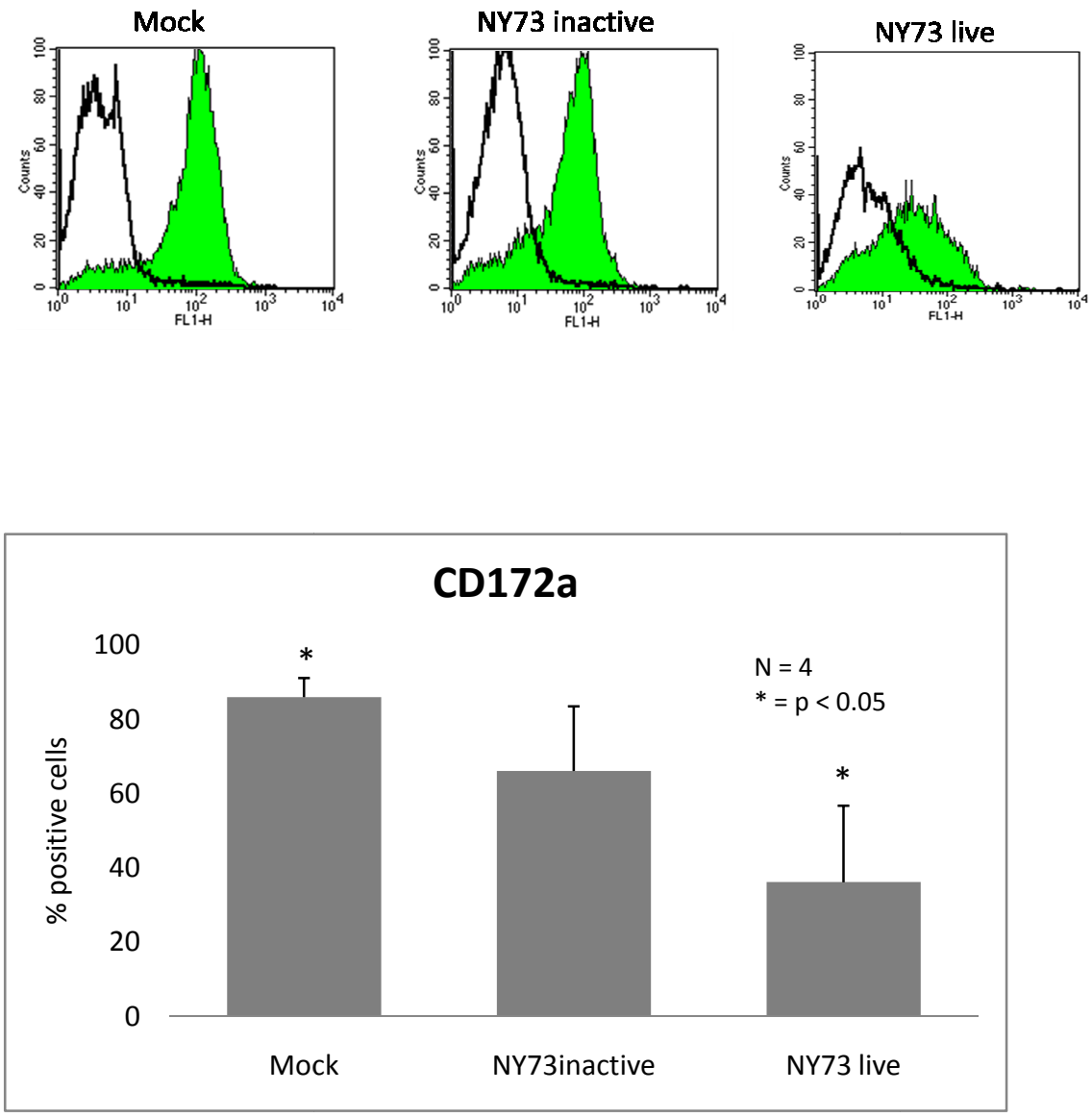
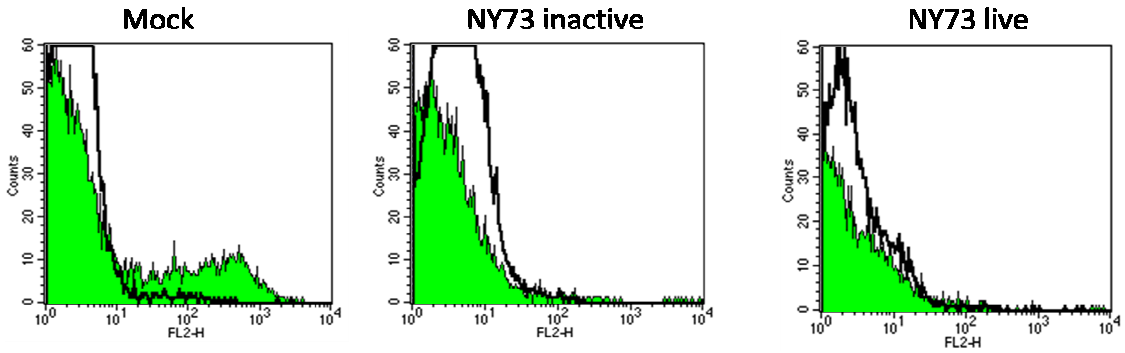


Figure 3.9: Surface expression of CD172a. Mock, UV-inactivated and live influenza virus (NY/73) infected monocytes were stained with anti-CD172a antibody (DH59B) at day 4 p.i. and analyzed by flow cytometry. Percentage of CD172a positive cells are shown as mean ± SD (N=4; *= p <0.05).

CD1w2



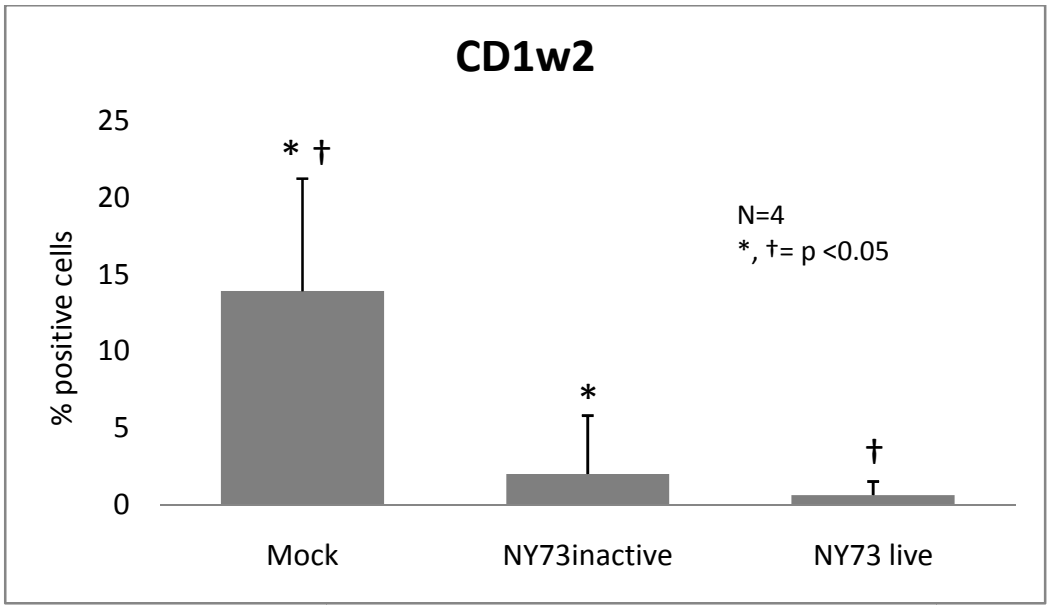


Figure 3.10: Surface expression of CD1w2. Mock, UV-inactivated and live influenza virus (NY/73) infected monocytes were stained with anti-CD1w2 antibody (MCA2058PE) at day 4 p.i. and analyzed by flow cytometry. Percentage of CD1w2 positive cells are shown as mean ± SD (N=4; *, †= p <0.05).

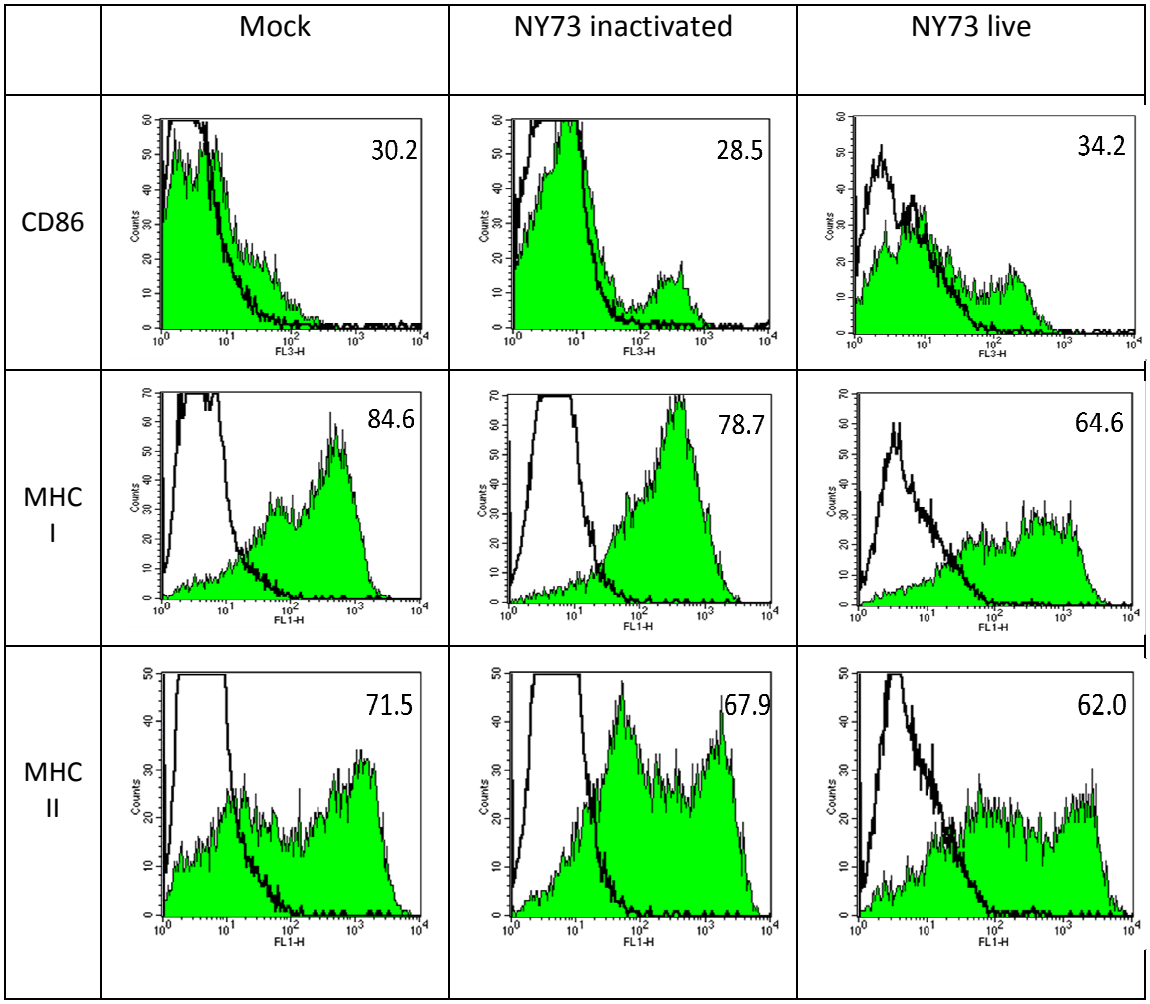


Figure 3.11: Histograms of CD86, MHC I and MHC II surface expressions on mock, UV-inactivated and live influenza virus (NY/73) infected monocytes. Empty black lines represent isotype matched controls. Mean percentage of positive cells from 4 separate experiments are labeled on the plots.

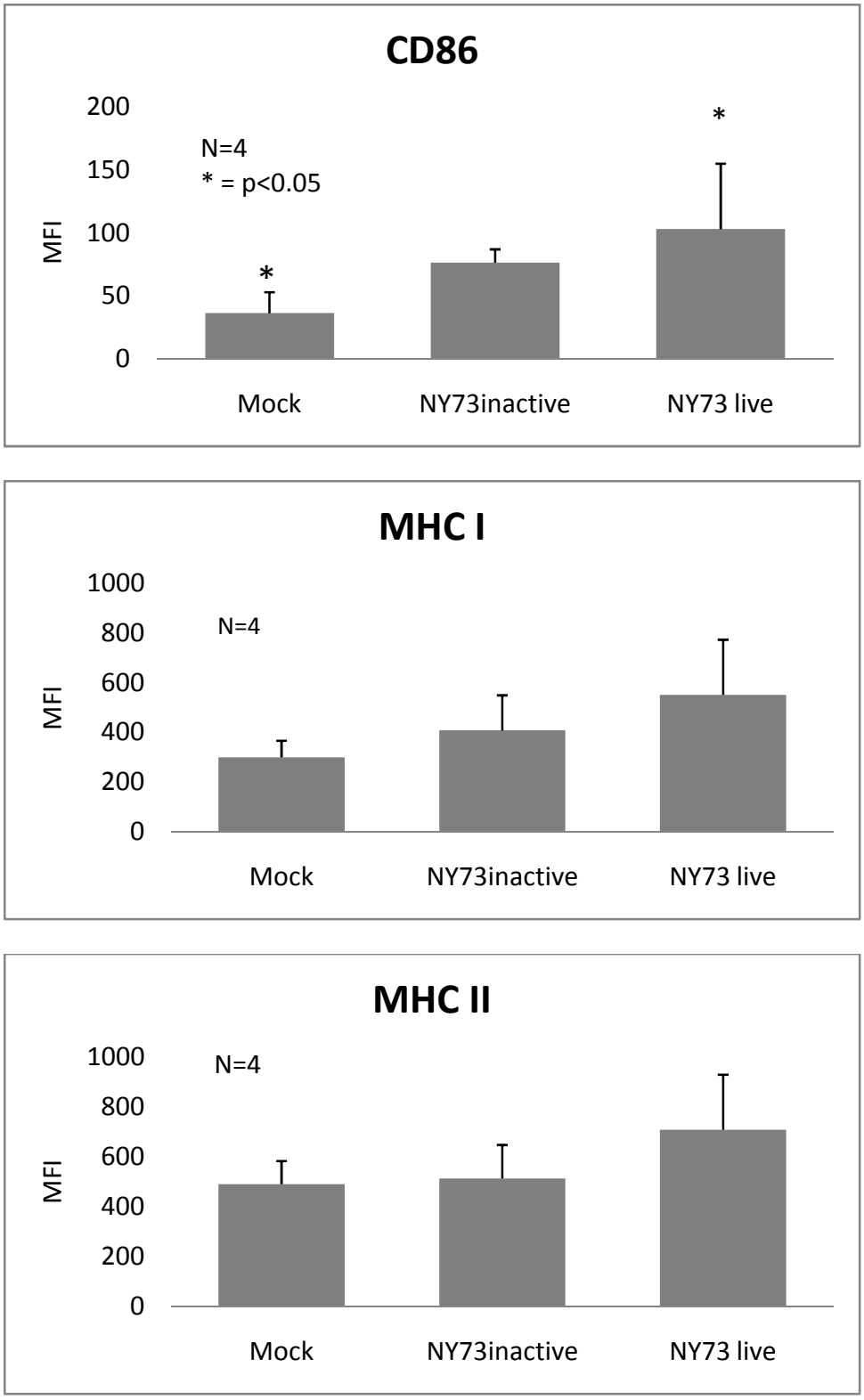


Figure 3.12: Mean fluorescence intensity (MFI) of CD86, MHC I and MHC II surface expressions. Data are shown as mean ± SD (N=4). A p value of less than 0.05 was considered significant.

Alpha-naphthyl acetate esterase (ANAE) activity of influenza virus (NY/73) infected monocytes

Dendritic cell differentiation from monocytes was further investigated by cytochemical staining for ANAE. This is a family of non-specific esterase enzymes which is found uniformly throughout the cytoplasm of monocytes and macrophages (180, 181). Mock infected monocytes which successfully differentiated into DCs, had very faint and focal ANAE staining mainly around the nucleus (Figure 3.13). Influenza virus infected cells retained considerable enzymatic activity as observed by the dense and diffuse ANAE staining throughout the cytoplasm. The staining for UV inactivated virus treated cells fell intermediate between mock- and live virus infected cells.

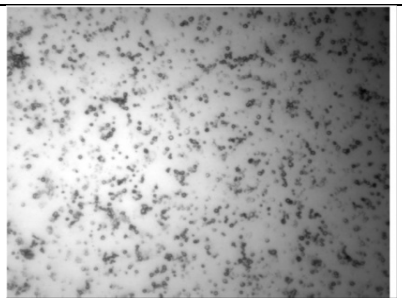
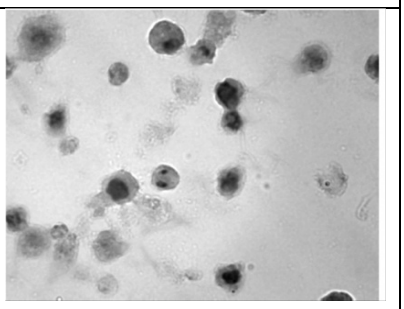
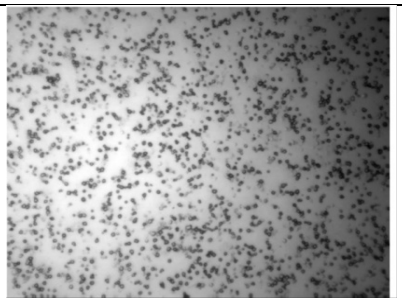
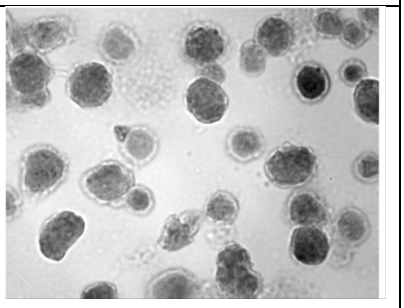
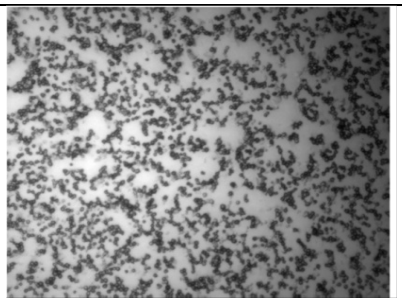
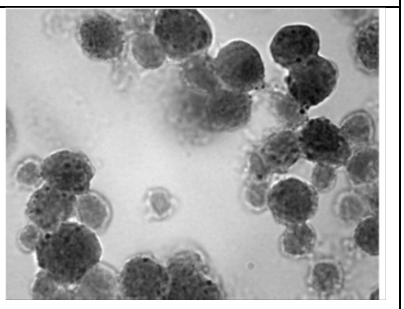
	10X	100X
Mock		
NY73 inactivated		
NY73 live		

Figure 3.13: ANAE activity in influenza infected monocytes. Mock, UV-inactivated and live influenza virus (NY/73) infected monocytes were cultured in presence of GM-CSF and IL-4 for 4 days and then stained for non-specific esterase. Dark black granulations are indicative of ANEA activity.

Endocytosis by influenza virus (NY/73) infected monocytes

The ability of influenza infected monocytes to acquire DC-like functions was evaluated by determining their antigen endocytosis capability. Mean fluorescence intensity of DQ-ovalbumin which liberates fluorescence upon endocytosis was used as an indication of antigen uptake ability. As shown in Figure 3.14, mock infected cells had significantly higher ($p<0.05$) endocytic function (high MFI), while live virus infected ones failed (low MFI) to uptake DQ-OVA through receptor mediated endocytosis. Consistent with other results, UV-inactivated virus treated monocytes showed a partially reduced endocytic activity compared to mock cells although significantly more than live influenza infected monocytes. These data suggest that live influenza virus infection successfully prevented monocytes from acquiring endocytic function.

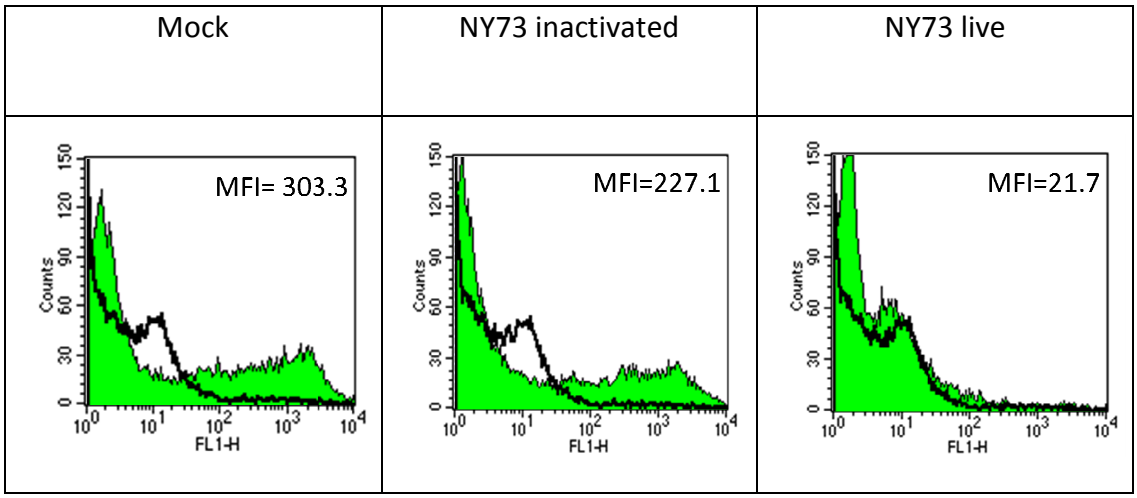
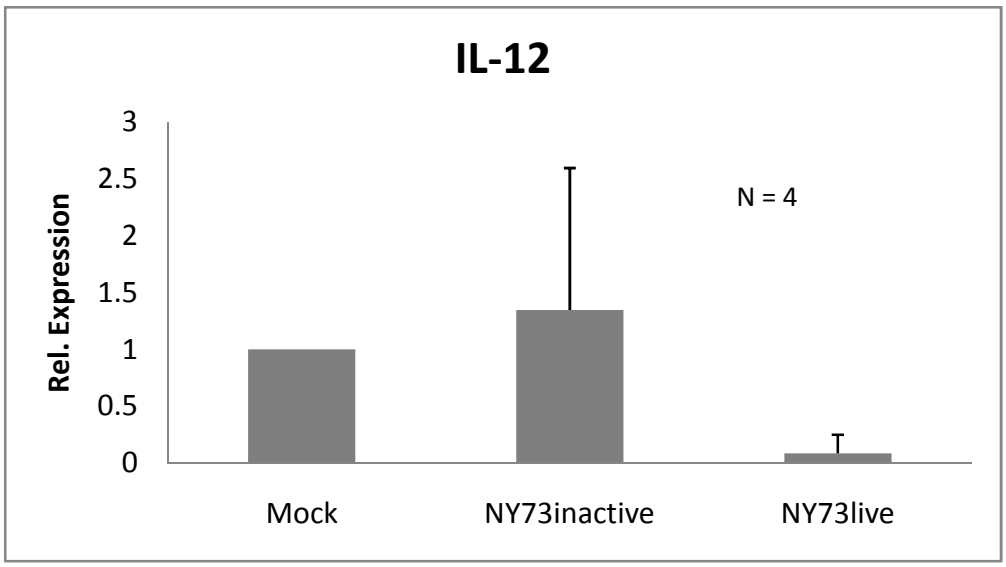


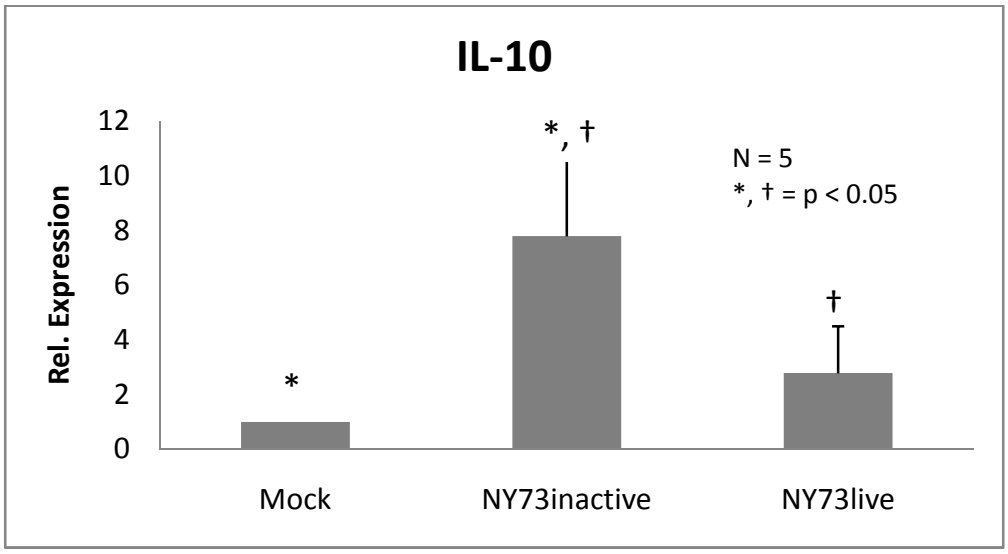
Figure 3.14: Receptor-mediated endocytosis of DQ-ovalbumin. Mock, UV-inactivated and live influenza virus (NY/73, H7N7) infected monocytes at 4 days p.i. were incubated with DQ-ovalbumin for 1 h at 4°C (black line) or 37°C (green). Level of antigen endocytosis was analyzed by flow cytometry and measured by the mean fluorescence intensity (MFI). Numbers in the figures indicate average MFI of 4 separate experiments.

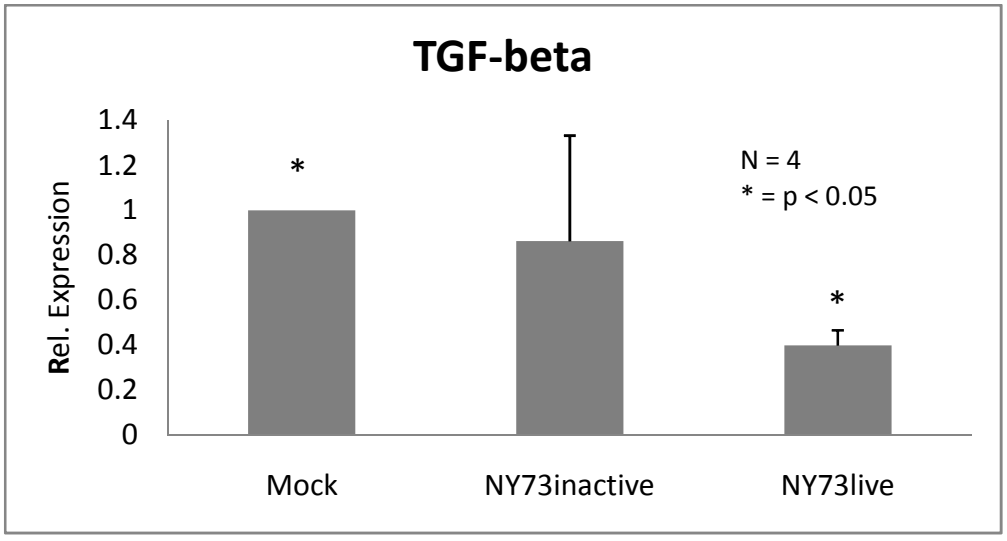
Cytokine production by influenza virus (NY/73) infected monocytes

Since cytokines play a critical role in monocyte differentiation into DCs, production of IL-10, IL-12 and TGF-ß which are known to influence DC development was analyzed. Mock or influenza virus infected cells were analyzed for their intracellular cytokine mRNA synthesis. Mock infected cells had considerably higher IL-12 mRNA level than live influenza virus infected monocytes (Figure 3.15). On the other hand, virus infection enhanced IL-10 mRNA transcription by 3-fold over mock infected cells (Figure 3.15). Another cytokine, TGF-ß which favors DC differentiation was also significantly down-regulated in influenza virus infected monocytes (Figure 3.15, $p < 0.05$). These data indicated that IL-12, a pro-inflammatory cytokine produced by DCs, was down-regulated in influenza virus infected monocytes whereas IL-10, a reciprocal regulatory cytokine to IL-12, was up-regulated which would be favorable for arresting monocyte differentiation into DCs.

Other inflammatory cytokines, however, were differently influenced by the viral infection. Live influenza virus infection significantly up-regulated gene expression of IFN-α and TNF-α as compared to mock infected monocytes (Figure 3.15, $p < 0.05$).


IL-12
Rel. Expression
3
2.5
2
1.5
1
0.5
0
N = 4
Mock
NY73inactive
NY73live


IL-10
Rel. Expression
12
10
8
6
4
2
0
*, †
*
†
N = 5
*, † = p < 0.05
Mock
NY73inactive
NY73live


TGF-beta
Rel. Expression
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
*
N = 4
* = p < 0.05
Mock
NY73inactive
NY73live

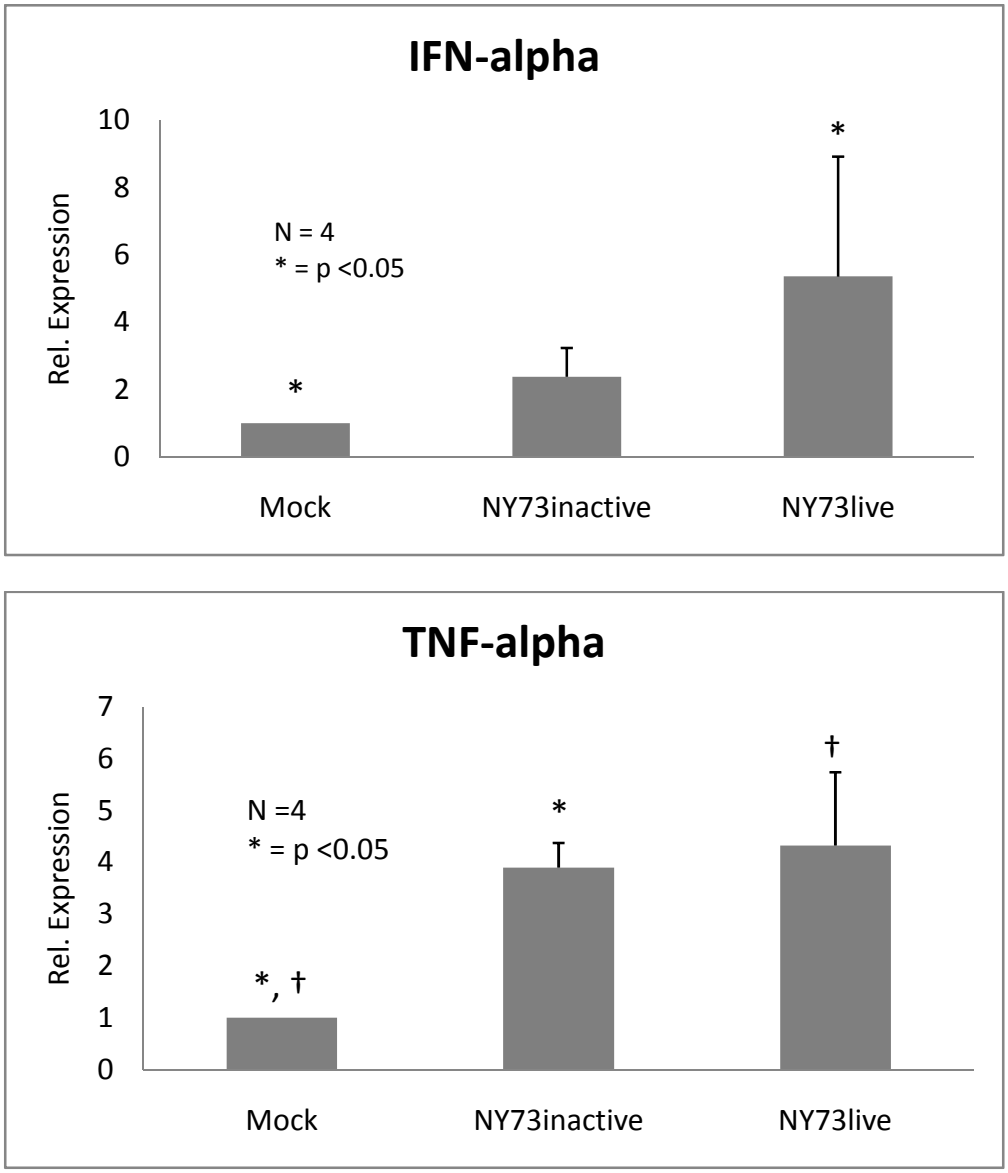


Figure 3.15: Cytokine production by influenza infected monocytes. Mock, UV-inactivated or live influenza virus (NY/73) infected cells were cultured in GM-CSF and IL-4 for 4 days and mRNA levels of IL-10, IL-12, TGF-ß, IFN-α and TNF-α were quantified by real-time PCR. RPLPO mRNA levels were used to normalize the data. Results are shown as mean ± SD. A p value of less than 0.05 was considered significant.

Expression of DC surface molecules on monocytes infected with KY/02 and a NS1-defective influenza virus

An H3N8 equine influenza virus (KY/02) and its NS1-truncated variant (KY/02 NS1-73) were utilized in order to investigate whether the viral inhibition is subtype-dependent and if viral NS1 protein plays any role in the process. Similar to the experiments with NY/73 virus, expression of DC markers such as CD11c and CD172a was measured. The mock infected cells, as expected, enhanced their expression of CD11c significantly ($p < 0.05$) with respect to monocytes. But its expression on both wild type and NS1-truncated virus infected monocytes was either comparable to or less than monocytes and significantly lower than mock infected cells (Figure 3.16, $p<0.05$). Similarly, fewer wild type virus (KY/02) infected monocytes expressed CD172a on their surface than mock infected cells. The expression of this myeloid marker was even lower in NS1-truncated virus infected monocytes (Figure 3.16). This demonstrated that both H3N8 wild type and NS1-defective influenza viruses were able to hinder monocytes from attaining distinctive DC phenotypes.

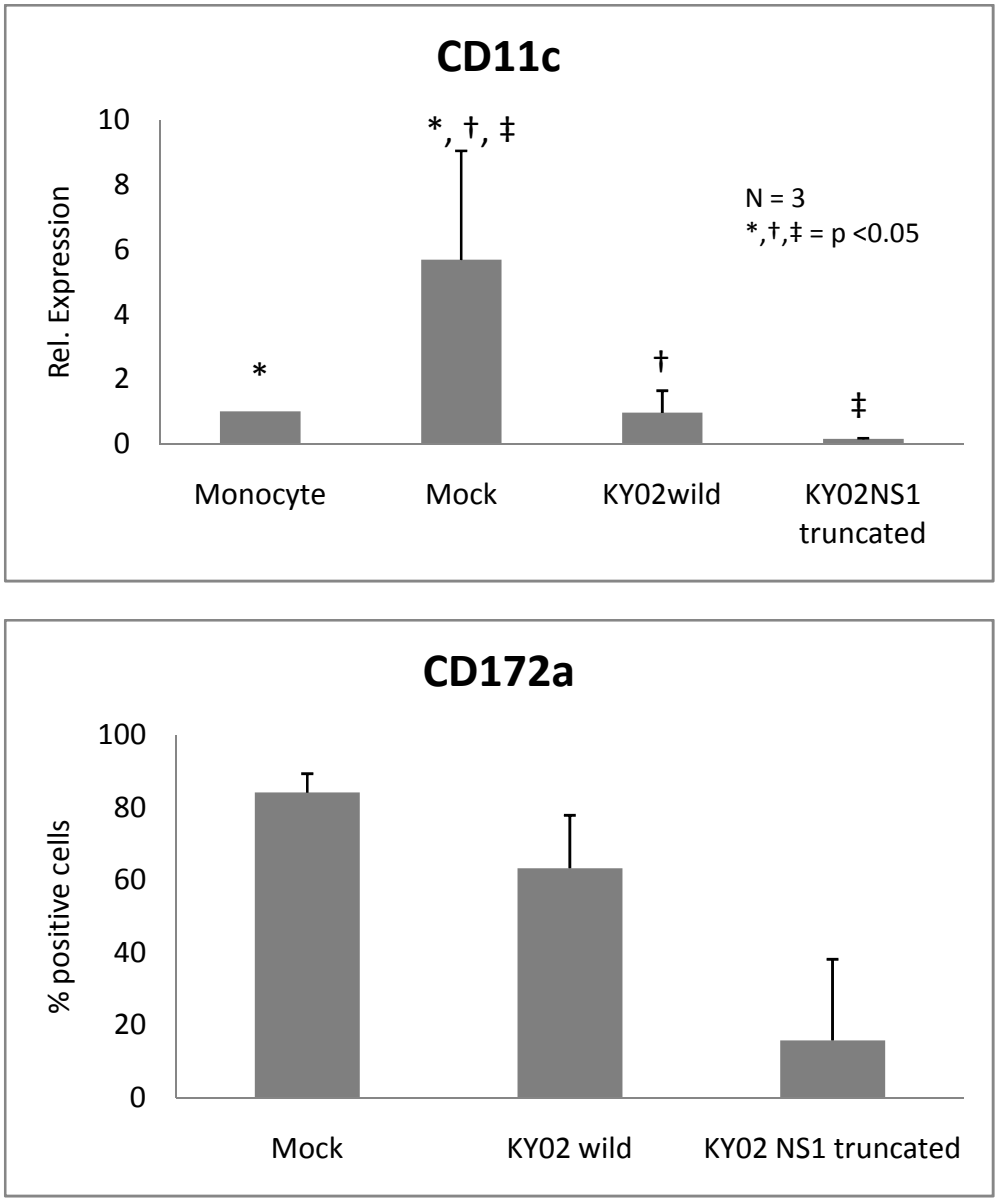


Figure 3.16: Expression of DC surface on H3N8 influenza infected monocytes. Relative RT-PCR was used to measure gene expression levels of CD11c. Expression levels were normalized based on house-keeping gene, RPLPO. Surface expression of CD172a was measured by flow cytometry. Data are shown as mean ± SD. A p value of 0.05 or less was considered significant.

Endocytosis by monocytes infected with KY/02 and a NS1-defective influenza virus

DCs are the most potent APCs and efficient endocytosis is a prerequisite for presenting antigens on MHC molecules. When KY/02 wild type and the NS1-truncated influenza virus infected monocytes were examined for their ability to uptake ovalbumin, both wild type and NS1-truncated virus infected cells showed considerably less endocytic function compared to mock infected monocytes (Figure 3.17). This indicated that H3N8 influenza virus, similar to H7N7 subtype, was also able to inhibit antigen uptake by monocytes and truncation of its NS1 viral protein did not hamper this inhibition by the virus.

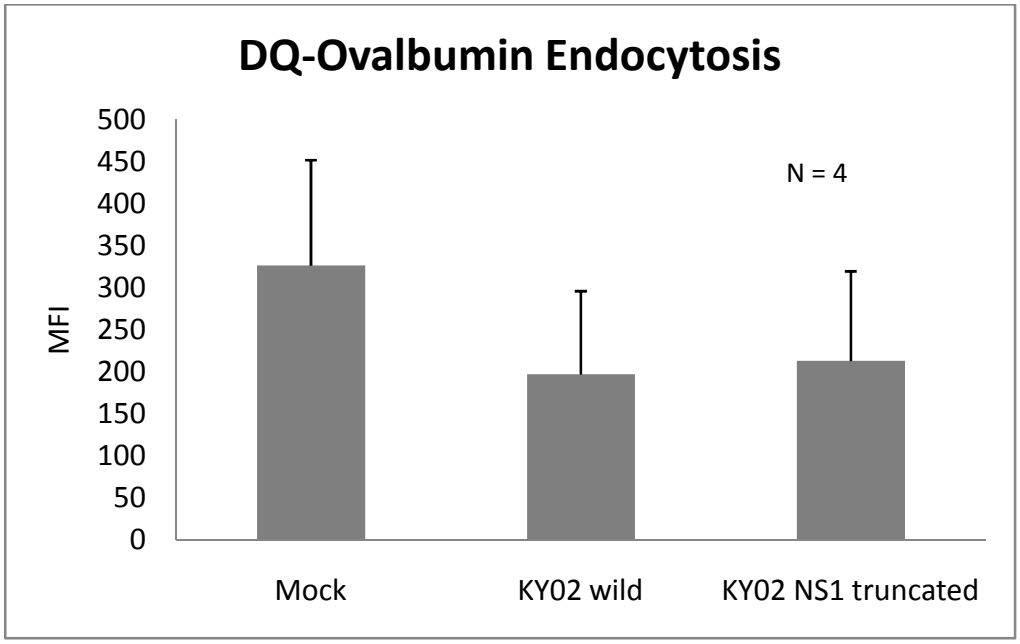


Figure 3.17: Endocytosis of DQ-ovalbumin by H3N8 influenza infected monocytes. Mock, KY/02 and NS1-truncated influenza virus (H3N8) infected monocytes at 4 days p.i. were incubated with DQ-ovalbumin for 1 h at 4°C (black line) or 37°C (green). Level of antigen endocytosis was analyzed by flow cytometry and measured by the mean fluorescence intensity (MFI). Results are presented as mean ± SD.

Cytokine production by monocytes infected with KY/02 and a NS1-defective influenza virus

As described in chapter I (section 2.2.8), NS1 protein of influenza virus is a strong antagonist of cellular interferon production. To evaluate whether truncation of NS1 protein affected this function, IFN-α productions by mock, wild type and NS1-defective influenza virus infected monocytes were compared. Not surprisingly, NS1-truncated influenza virus allowed infected monocytes to produce significantly more IFN-α (Figure 3.18).

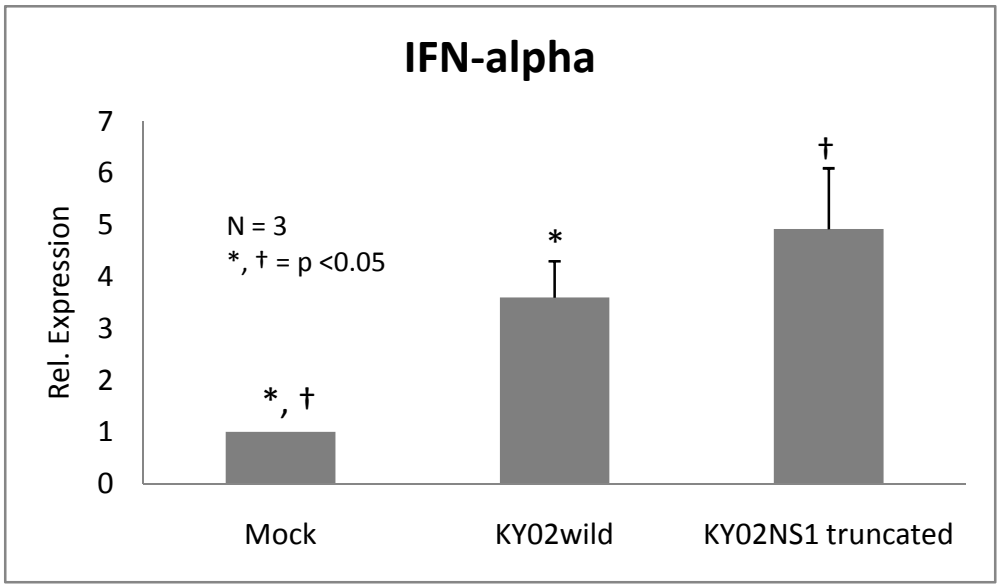


Figure 3.18: IFN-alpha production by H3N8 influenza infected monocytes. Relative RT-PCR was used to measure gene expression levels. Data were normalized based on house-keeping gene, RPLPO and shown as mean ± SD. A p value of 0.05 or less was considered significant.

CHAPTER IV

DISCUSSION

In spite of the extensive research done on influenza virus, our understanding of the mechanism of its pathogenesis at the cellular level still remains incomplete. Because of the segmented genome, it can undergo gene reassortment or antigenic shift which can be indicted for the three documented human pandemics in the last century (5). Again, its negative sense RNA genome requires viral RNA dependent RNA polymerase for its replication whose error prone properties enable the virus to introduce point mutations or antigenic drift which helps the virus in evading existing host antibodies. While these strategies counteract the humoral immune response, influenza virus has also evolved to escape the cell mediated immunity as well. For example, influenza virus NS1 protein inhibits T cell activation and IFN-γ production (64). However, only limited information is available on influenza virus mediated inhibition of the innate immunity. DCs play a vital role in innate immunity as well as activation of adaptive immunity and as mentioned in Chapter I, monocytes are a major source of these APCs, particularly during infections. Therefore, in this dissertation, the effects of influenza virus infection on monocyte differentiation into DCs were studied.

An equine virus-host model system has been utilized to evaluate the hypothesis. Since influenza virus infections of mice do not always simulate natural infections, the ability to examine the effects of equine influenza viruses on equine monocytes provides

a distinctive opportunity of studying a virus in its natural host. The availability of large amounts of blood from horses also satisfies the needs of the experiments, which could otherwise pose a difficulty in human or mouse model systems. The discrete features of two equine influenza subtypes (H7N7 and H3N8) also offered a prospect of investigating whether viral subtype differences play any role in the process. It is noteworthy to mention that equine-1 (H7N7) influenza viruses possess the basic amino acid containing highly cleavable HA proteins which is a phenotypic attribute of pathogenic avian influenza viruses. However, despite being highly virulent in mice, it only causes a mild disease in horses (81). This offers the unique chance to investigate the effects of a genotypically predicted highly virulent mammalian influenza virus on the differentiation of monocytes into dendritic cells. A NS1-defective equine influenza virus was also tested. As described in chapter I (section 2.2.8.), NS1 protein of influenza virus plays an important role in viral pathogenesis and immune-evasion. The NS1-truncated virus that was used in the experiments lacks the C-terminal “effector domain” which mediates several of its anti-immune activities (section 2.2.8). So, contrasting the effects of wild-type and NS1-defective viruses on DC differentiation could illuminate additional roles of this viral protein (NS1) in influenza virus pathogenesis.

During influenza virus infection, respiratory epithelial cells secrete an array of inflammatory cytokines and chemokines that lead to a massive influx of immune cells including monocytes into the airway mucosa. Microbial infections and allergens are known to stimulate increased GM-CSF production by airway epithelial cells (182, 183), that along with other cytokines can drive the priori uncommitted monocytes, now in the

inflammatory milieu, to differentiate into either macrophages or DCs in order to supply adequate APCs at the site of infection. Pre-treatment of cells with cytokines such as GM-CSF have been found to modulate the outcome of viral infections. GM-CSF is a potent cytokine which can stimulate cellular metabolism, RNA transcription and protein synthesis as well as endocytosis (184). Accordingly, it has been shown that influenza progeny virus production and cytotoxicity is increased when monocytes are cultured with GM-CSF alone leading them towards the macrophage lineage (185). Similar results are obtained when GM-CSF treated mononuclear phagocytes are infected with HIV (186, 187). IL-4, a Th2 type cytokine, also enhances replication of SIV when given to macaque monkeys (188). Although the effects of GM-CSF and IL-4 on virus replication have been demonstrated separately, the fate of virus infected monocytes cultured with both GM-CSF and IL-4 and thus differentiating towards DCs, was yet unknown.

While monocytes in the blood stream are highly unlikely to become infected with influenza virus, monocytes in the respiratory mucosa would come in contact with an enormous number of virus particles produced by infected airway epithelial cells. So in specific aim 1, the replication behavior of equine influenza virus in monocytes cultured in the presence of both GM-CSF and IL-4 was characterized. Adherent cells were used as the source of monocytes since about 73.6% of the population was consisted of CD14 positive cells (Figure 3.1). An infectious dose of 5 EID_{50} units per cell was chosen to ensure viral exposure to most of the cells. It was found that equine influenza virus successfully infected monocytes, as about 59% of the adherent cells expressed viral NP protein at 24 hr p.i. when analyzed by flow cytometry (Figure 3.2).

Since It is known from a published report that influenza virus can not readily infect lymphocytes, the reason for the other 41% of the cells not expressing viral NP protein could be traced to the fact that about 73.6% of the adherent cell population was monocytes (CD14 positive, Figure 3.1), while the rest would be comprised of primarily lymphocytes, which are non-permissive to influenza infection (189). Successful replication of the virus was also evaluated by its genome transcription ability, viral protein synthesis and progeny virus production. As shown in Figure 3.3, the live virus could transcribe its viral genome as opposed to the UV-inactivated virus. However, viral protein synthesis ceased very early at 4 hr p.i. This resulted in a very limited progeny virus production. The lack of cytotoxicity (Figure 3.5) of virus infected monocytes could be associated with the inefficient viral protein synthesis. This is in stark contrast to the infection of only GM-CSF pre-treated monocytes where influenza virus replicates productively and induces much higher rate of apoptosis (185). Similar results are also observed when equine monocytes are infected with equine influenza viruses (personal communication, Liang Zhang). The reason for this inhibition due to GM-CSF and IL-4 pre-treatment of monocytes is yet unclear. The most plausible explanation is that GM-CSF and IL-4 pre-treatment sets off monocyte differentiation towards DCs and stimulates production of cytokines (TNF-α, IFNs) which in turn can block influenza virus replication. In fact, GM-CSF and IL-4 pre-treated monocytes, when infected with influenza virus, expressed significantly higher levels of IFN-α and TNF-α than their mock infected counterparts (Figure 3.15). Several reports have shown that IFNs induce expression of anti-viral genes such as Mx and viperin which can inhibit influenza virus replication (145,

190). While Mx inhibits viral mRNA synthesis, viperin interferes with the fluidity of cellular lipid rafts and thereby prevents budding of progeny viruses from infected cells. TNF-α also possesses a strong anti-influenza activity (191). Pre-treatment of otherwise susceptible cells such as MDCK with TNF-α makes them resistant to viral cytopathic effects by inhibiting viral protein synthesis. Additionally, the reduced level of apoptosis in GM-CSF and IL-4 pre-treated monocytes could be due to the anti-apoptotic effects of IL-4. It can activate signal transducer and activator of transcription 6 (Stat-6) and phosphatidylinositol 3 kinase (PI3K) pathways which up-regulate anti-apoptotic protein Bcl-xL and thereby prevent cells from undergoing apoptosis (192, 193). So these results identify that influenza virus infection of blood monocytes culminate into two distinct outcomes depending upon whether those cells are differentiating towards macrophages or DCs.

Although influenza virus undergoes restricted replication in differentiating monocytes, it was further investigated if the viral infection had any morphological or functional effects on monocyte differentiation into DCs. When monocytes differentiate into DCs, they acquire a distinctive DC-like morphology with veils and large finger-like projections. In these experiments, mock and UV-inactivated virus infected monocytes acquired the DC-like morphology, but live influenza virus infected monocytes remained round and lacked dendritic processes (Figure 3.6). When cell diameter was measured, equine monocytes had an average diameter of 8.92µm. Mock and UV-inactivated virus infected monocytes, which successfully differentiated into DCs, had significantly increased their average diameter to 10.00µm and 9.88µm, respectively. On the

contrary, live virus infected monocytes failed to increase in size as their average diameter (9.03µm) remained close to that of monocytes (Figure 3.7). Live virus infected monocytes were also unsuccessful in increasing their cellular granularity as fewer cells moved to the upper right quadrant of the dot plot compared to both mock and UV-inactivated virus infected monocytes (Figure 3.7). Although the precise reason for the inability of influenza infected monocytes to attain DC-morphology is unclear, viral attachment and entry alone was not adequate to bring about the changes. Viral genomic RNA transcription and limited viral protein synthesis was required for the inhibition because UV-inactivated influenza infected monocytes successfully acquired the DC-like morphology. These results showed that live influenza virus infected monocytes failed to attain the morphological characteristics of DCs.

Apart from the morphological alterations, influenza virus infection of monocytes also modified expression of different DC surface molecules. CD11c, an integrin, is a specific marker for DCs and is expressed abundantly on myeloid and other types of DCs (194). Thus, mock infected cells up-regulated expression of CD11c significantly (20-fold, $p< 0.05$) over untreated monocytes (Figure 3.8). On the contrary, CD11c expression on live virus infected monocytes was almost negligible. Similarly, live virus infected monocytes failed to up-regulate expression of several other DC markers (CD172a, CD1w2, CCR5; Figure 3.8 and 3.9). Interestingly, surface markers on monocytes infected with UV-inactivated influenza virus, although considerably higher than live influenza virus infected cells, were moderately lower than mock infected monocytes. The expression of CD1w2 (human CD1b homolog), in particular, was remarkably low (Figure

3.9). However, this finding corroborates with a previous report that TLR7/8 agonists have an inhibitory effect on the surface expression of CD1 family members (195). Although UV-inactivated influenza viruses could not replicate their viral genome, they would retain enough genomic RNA to stimulate TLR7/8 in monocytes (Figure 3.3). Considerably enhanced expression (MFI) of CD86, MHC I and MHC II on both UV-inactivated and live influenza virus infected monocytes was also observed, which suggests an involvement of TLRs which when activated are known to stimulate expression of these co-stimulatory molecules (196). As suggested above, UV-inactivated influenza virus, possibly through activation of PAMPs such as TLRs, was able to partially modulate the phenotypic characteristics, but complete inhibition of appearance of DC-specific markers required at least limited viral replication as exemplified by live influenza infected monocytes.

Effects of influenza virus infection on monocyte differentiation were further characterized by cytochemical staining for ANAE. Splenic and tonsilar DCs are negative for ANAE, but monocyte derived DCs retain a little enzymatic activity, although the staining appears to be much more focal (197, 198). Accordingly, mock infected monocytes which successfully differentiated into DCs had retained very little enzymatic activity as compared to the substantial staining in live influenza infected monocytes. UV-inactivated virus infected monocytes, again, showed an intermediate phenotype (Figure 3.13). These results further demonstrated that live influenza virus infected monocytes failed to attain cytochemical characteristics of fully differentiated DCs.

An important finding was the ability of influenza virus to affect the antigen-capturing capability, the hallmark function of any APC including DCs. Antigen endocytosis by live influenza infected monocytes were significantly reduced compared to mock infected cells (Figure 3.14, $p < 0.05$). UV-inactivated virus infected monocytes showed similar endocytic capability to that of mock samples suggesting that functional inhibition of monocyte-derived DCs required active viral replication. This effect of influenza mediated inhibition of endocytosis is far-reaching since this would in turn prevent infected monocyte derived DCs from presenting the viral antigen to T cells and thereby blocking any subsequent activation of adaptive immune response.

Next, the mRNA expression of cytokines that are known to influence monocyte differentiation into dendritic cell was studied. IL-10 which is an anti-inflammatory and immunosuppressive cytokine which was synthesized at increased levels both in UV-inactivated and live influenza infected cells compared to mock infected monocytes (Figure 3.15). On the contrary, production of IL-12, an inflammatory cytokine, was higher in mock samples than in live influenza infected monocytes. TGF-ß also followed a similar pattern as of IL-12. Now, it is known that IL-10 inhibits development of DCs from monocytes while IL-12 and TGF-ß favors the differentiation process (168, 199). Additionally, differentiated DCs produce large amounts of IL-12 that in turn drive CD4+ T cells towards a Th1 type immune response which is necessary for clearance of influenza virus infection (200, 201). On the other hand, IL-10 has a detrimental effect on the host immune response and subsequent recovery from influenza infection (202). It also increases susceptibility to secondary bacterial infections (203). These results showed

that influenza virus infection of monocytes resulted in production of a coordinated cytokine profile that not only inhibited DC differentiation but also prevented development of a Th1 type immune response and recovery from virus infection.

These conclusions were further extended to include the other subtype (H3N8) of equine influenza virus and to address the possibility of a virus subtype specific inhibition. Since influenza virus NS1 protein has an established immune antagonistic activity and is also known to inhibit maturation of DCs (section 2.2.8, chapter I), an NS1-truncated virus was used to evaluate the role of this viral protein in this inhibition process. As evidenced in Figure 3.16, both the wild type virus (KY/02) and its NS1-defective variant inhibited phenotypic expression of DC surface markers. Both viruses were also able to block antigen endocytosis by infected monocytes (Figure 3.17). Since the NS1-truncated virus (NS 1 -73 a.a.), although lacks the effector domain but retains the RNA-binding domain (section 2.2.8), it is possible that only the RNA-binding domain was sufficient for the viral inhibition of DC differentiation. However, it is particularly important to note that the inhibition by NS1-defective variant was more enhanced than the wild type virus (Figure 3.16). This data not only proved that completely functional NS1 protein was not necessary for influenza virus mediated inhibition of monocyte differentiation, but also indicated to a possible mechanism of this viral inhibition. IFN-α/ß, when added to the culture media, have been shown to exhibit a negative regulatory effect on differentiation of fully functional DCs from monocytes (204). In these experiments also, NS1-truncated influenza virus infected monocytes synthesized more IFN-α mRNA than the wild type virus (Figure 3.18). Although no direct co-relation

could be established here, IFN-α produced by infected monocytes can act in an autocrine negative-feedback mechanism to inhibit their development into DCs. Nonetheless, these results demonstrated that the viral inhibition of monocyte differentiation is not specific for the H7N7 subtype as H3N8 subtype was also able to block DC development. It further showed that influenza virus can inhibit monocyte differentiation into DC independent of complete NS1 protein activity.

Although the heterogeneity of monocyte-derived DCs makes their precise characterization very intricate, experiments in this dissertation provided a general insight into the effects of influenza virus infection of monocytes that are cultured *in vitro* for differentiation into DCs. These experiments, taken together, demonstrated that equine influenza virus could successfully infect and transcribe its genomic RNA in GM-CSF and IL-4 pre-treated monocytes, although an early inhibition of viral protein synthesis prevented efficient progeny virus production. It also showed that infection with influenza virus prevented monocytes from differentiating into DCs. Although UV-inactivated virus imposed a partial inhibition, complete morphological and functional inhibition required at least limited viral replication. The mechanism of this inhibition, although unconfirmed in this dissertation, possibly involves TLR-mediated activation of cytokine (e.g. IFNs) productions. Since DCs are critical in initiating immune responses against viral infections, this inhibitory effect of influenza virus would block an early development of virus specific immune activation. For an acute infection like influenza, this will provide ample opportunities to the virus for efficient replication and transmission to other susceptible hosts. So, the experiments described in this

dissertation identify a new strategy by influenza virus to stall activation of an innate and subsequent adaptive immune response and thereby evade an anti-viral immunity.

Future research prospect

Findings of this dissertation research have shown that influenza virus infection of blood monocytes can inhibit their differentiation into DCs and have alluded to possible mechanism of this inhibition. However, the precise mode of this viral inhibition remains to be ascertained. It needs to be determined whether activation of TLR pathways plays any role in this process and if it does, which TLR in particular is resdponsible. Additionally, although it has been shown in this dissertation that activity of fully functional NS1 protein is not necessary for the viral inhibition, roles of other viral proteins such as HA and NA are yet to be defined. This offers an interesting avenue of future research.

Since at least a proportion of DCs develop from blood monocytes and influenza virus infection of monocytes inhibited their development into DCs; this would impair proper immune activation and thereby exacerbate the disease condition or delay recovery. Revelation of the molecular mechanism of this inhibition would be important because that knowledge can be further employed for improvement of vaccines. Prospects of different vaccine adjuvant which may help monocytes overcome virus mediated inhibition of DC differentiation could be investigated.

The results described in this dissertation may also serve as the basis for further research to better understand influenza virus replication as well as improvements of vaccines. Since only GM-CSF pre-treated monocytes are known to be permissive for influenza infection and the experiments here showed that GM-CSF and IL-4 pre-treatment inhibited viral replication, particularly at the stage of viral protein synthesis;

advanced research could be carried out to elucidate the precise molecular mechanism of this inhibition. Development of monocytes towards DCs may stimulate gene transcription and expression of cellular proteins (that may not be activated in macrophages) which may block viral protein synthesis. Such discoveries may lead to development of novel anti-viral drugs.

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Personal Information

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Education

B.V.Sc & A.H., 2004	West Bengal University of Animal and Fishery Sciences, Kolkata, India

Professional Training/Appointment

2004-present	Research Assistant (Laboratory of Dr. T. M. Chambers) University of Kentucky, Department of Veterinary Science
2004	Internship with veterinary hospitals, Kolkata, India

Awards and Honors

2007-present	Geoffrey C. Hughes Fellowship
2006	Induction to Gamma Sigma Delta Honor Society
2005	Graduate School Academic Year Fellowship, University of Kentucky
1997-2004	National Scholarship, India

Membership in Professional Society

2007-present	American Society for Virology (ASV)

Peer-reviewed Publications

1. **Boliar, S.**, Stanislawek, W., Chambers, T. M. 2006. Inability of kaolin treatment to remove nonspecific inhibitors from equine serum for the hemagglutination inhibition test against equine H7N7 influenza virus. J. Vet. Diagn. Invest. 18**:**264-7. (Won Best Brief Communication of the year award)
2. Lu, Z., Chambers, T. M., **Boliar, S.**, Timoney, P. J., Branscum, A. J., Reedy, S. E., Tudor, L., Dubovi, E. J., Vickers, M. L., Sells, S., Balasuriya, U. B. R. 2009. Development and evaluation of one-step TaqMan real-time reverse transcription PCR assays targeting NP, M and HA genes of equine influenza virus. J. Clin. Microbiol. (Submitted).
3. **Boliar, S.**, Horohov, D. W., Chambers, T. M. 2009. A new strategy of immunoevasion by influenza A virus: inhibition of monocyte differentiation into dendritic cell. (Manuscript in preparation).

Abstracts/Presentations

1. **Boliar, S.**, Chambers, T. M. 2007. Characterization of temperature sensitive (ts) phenotype of Flu Avert™ I.N. equine influenza vaccine virus. *26th Annual Meeting of American Society for Virology (ASV)*.
2. **Boliar, S.**, Horohov, D. W., Chambers, T. M. 2008. A new strategy of immune evasion by influenza virus: inhibition of monocyte differentiation into dendritic cell. *27th Annual Meeting of American Society for Virology (ASV)*.