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ROLE OF VIRAL AND HOST FACTORS IN INFLUENZA VIRUS MEDIATED INHIBITION OF INTERLEUKIN-23

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ROLE OF VIRAL AND HOST FACTORS IN INFLUENZA VIRUS MEDIATED INHIBITION OF INTERLEUKIN-23

DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements of the degree of Doctor of Philosophy in the
College of Agriculture, Food and Environment
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By
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2014

ABSTRACT OF DISSERTATION

ROLE OF VIRAL AND HOST FACTORS IN INFLUENZA VIRUS MEDIATED INHIBITION OF INTERLEUKIN-23

Influenza virus is one of the major respiratory pathogens of humans as well as animals, including equines. There is an increasing evidence that bacterial infections are the most common cause of the death during influenza. In horses also, secondary bacterial pneumonia can lead to death, and surviving horses may take up to six months for the complete recovery resulting in heavy economic loss to the equine industry. Interleukin (IL)-23 mediated innate immune response has been shown to protect the host from various respiratory bacterial infections. However, studies to investigate the role of host and viral factors in the regulation of IL-23 are limited. Endoplasmic reticulum (ER) stress-induced transcription factor CHOP-10 and IFN-β has been shown to participate in the regulation of IL-23. Primary hypothesis for the current study was that influenza A virus (IAV) NS1 protein downregulates the IL-23 expression via inhibition of CHOP-10. In order to test our hypothesis, we infected the RAW264.7 cells - a murine macrophage cell line, and primary murine alveolar macrophage cells either with the wild type Influenza A virus (PR/8/34, PR8) or isogenic mutant virus lacking NS1 (delNS1). Quantitative analysis of mRNA expression revealed a significantly higher mRNA expression of IL23p19, IFN-β and CHOP-10 in delNS1 virus infected cells as compared the PR8 virus infected cells. Additionally, overexpression of CHOP-10 partially restored the expression of IL-23p19 in PR8 virus infected cells and knockdown of CHOP-10 resulted in downregulated expression of IL-23p19 in delNS1 infected cells. Taken together, these results suggest that IAV NS1 protein mediated inhibition of CHOP-10 expression leads to downregulation of IL-23 expression in macrophage cells *in-vitro*. Similar results were also observed *in-vivo* using IAV and *Streptococcus zoooepidemicus* (S. ze) co-infection model. In a co-infection mouse model delNS1 virus co-infection resulted in significantly higher expression of the IL-23 and IL-17. Considering the role of IL-23 in protection against respiratory bacterial pathogens, effect of exogenous supplementation of IL-23 was also investigated in the influenza and S. ze co-infection mouse model. We found that a single intranasal dose of recombinant murine IL-23 significantly improved the survival of mice co-infected with PR8 and S .ze. Overall, our study suggests that IAV infection subverts the IL-23 mediated respiratory innate immune response and restoration of IL-23 could protect from influenza-associated respiratory bacterial infections.

Keywords: Influenza virus, Co-infection, macrophage, interleukin-23, CHOP-10

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ROLE OF VIRAL AND HOST FACTORS IN INFLUENZA VIRUS MEDIATED INHIBITION OF INTERLEUKIN-23

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CHAPTER 1

Introduction and hypothesis

1.1. Introduction

Influenza is one of the most common viral respiratory disease of birds, humans, and animals including horses and is caused by influenza A virus (IAV). Each year, recurrent epidemics of influenza, commonly called seasonal influenza, result in 3-5 million severe cases and 250,000-500,000 deaths worldwide, leading to significant economic losses. Occasional pandemics further add to the quandary. Secondary bacterial infections were suggested to be major contributors in the pathology and mortality associated with human influenza almost a century ago and confirmed later [1]. Similarly, secondary bacterial infections are not uncommon in equine influenza. Despite significant progress in understanding the pathogenesis of influenza and the mechanism of influenza and bacterial synergy, bacterial co-infections still remain the major factor in determining the outcome of influenza. Therefore, the current study was designed to explore the possible mechanism that could contribute to increased susceptibility to secondary bacterial infection during influenza.

The first hypothesis was that influenza virus mediated inhibition of IL-23 leads to enhance susceptibility of the host to secondary bacterial infections. Cytokines are integral parts of the innate immune system, a broadly reactive defense mechanism which acts as the first line of defense against a variety of invading pathogens. Interleukin (IL)-23 is a recently identified cytokine that has been shown to be important in the respiratory innate immune response. Interleukin (IL)-23, in concert with IL-17, constitutes a newly identified innate immune pathway. The host IL-23/IL-17 pathway has been shown to play a critical

role in the clearance of many respiratory bacterial pathogens [2]. However, its role during influenza and bacterial co-infection has not been investigated. Thus, it was hypothesized that inhibition of respiratory IL23/IL17 pathway by influenza virus predisposes the host to secondary bacterial infection.

The second hypothesis was that influenza virus inhibits the expression of endoplasmic reticulum (ER) stress induced transcription factors. ER stress response, also called the unfolded protein response (UPR), is an evolutionary molecular cascade to maintain ER homeostasis and the protein folding capacity of the ER. However, recent studies implicate the ER stress response in other cellular and disease processes such as apoptosis and inflammation [3]. Since viruses rely heavily on the host cell protein synthesis machinery, the functional status of the ER may significantly affect viral replication and pathogenesis. Differential activation of ER stress pathways has been shown for multiple viruses including influenza. However, viral factors responsible for differential activation of ER stress response in influenza-infected cells are not known. C/EBP homologous protein-10 (CHOP-10) is one of the key mediators of ER stress. During prolonged ER stress, CHOP-10 mediates cell death by apoptosis. Although apoptosis could help in the pathogenesis of the virus, it could also prematurely terminate viral replication. CHOP-10 also acts as a transcription factor for several other genes. In human dendritic cells (DCs), induction of CHOP-10 was critical in IL-23 expression [4]. Therefore, it was hypothesized that influenza virus inhibits the CHOP-10.

The next hypothesis for the study was that influenza virus NS1 protein was the viral factor that mediates the inhibition of CHOP-10. In order to reduce the protein load during ER stress, eukaryotic initiation factor-2 alpha (eIF2-α) is phosphorylated to shut down

global protein synthesis in the cell. Two major cellular kinases, double-stranded-RNA (dsRNA)-activated protein kinase (PKR) and PKR-like ER kinase (PERK), are important in the phosphorylation of eIF2-α. Influenza virus NS1 is known to inhibit PKR that also results in inhibition of type-I interferons (IFN). Additionally, influenza virus infection has been found to not to activate PERK. Phosphorylation of eIF2-α, also leads to selective transcription of activating transcription factor 4 (ATF4) that leads to induction of CHOP-10. Inhibition of the kinases thus may favor influenza virus replication in two ways- I) by allowing viral proteins to be synthesized before the IFN response shuts down cellular protein synthesis; II) by delaying the onset of apoptosis induced by the endoplasmic reticulum (ER) stress-induced transcription factor CHOP-10. Therefore, it was hypothesized that influenza virus NS1 inhibits the expression of CHOP-10.

1.2. Hypothesis

Influenza virus NS1 protein mediated inhibition of CHOP-10 leads to an impaired IL-23/IL-17 immune pathway in the lungs and causes increased susceptibility to secondary bacterial infections.

1.3. Research objectives

In order to test the above hypothesis the study is performed with following objectives

1. **Determine the effect of influenza virus on ER stress**
2. **Determine the effect of NS1 mediated CHOP-10 inhibition on IL-23 expression**
3. **Determine the effect of restoration of respiratory IL-23 on pathogenesis of influenza**

Macrophages are important components of host innate immune defense. Besides DCs, macrophage cells are the only cells that secrete biologically active IL-23 [5]. Therefore, the hypothesis was tested in macrophage cells *in vitro* and in a mouse model *in vivo*. The overreaching goal of the study was to investigate role of the IL-23/IL-17 pathway in the horse. However, a mouse model for influenza as well as co-infection has been well established using the PR8 virus. Although there are areas that need further investigation, results of this dissertation provide evidence to support the major hypotheses. Secondary bacterial infections, similar to human influenza, are also common in equine influenza and it is possible that IL-23/IL17 pathway could be a general mechanism that leads to increased susceptibility during influenza and these results could be applied to equine influenza.

CHAPTER 2

Literature review

Influenza viruses are negative-sense single-stranded, segmented RNA genome viruses of family *Orthomyxoviridae*. The family *Orthomyxoviridae* consists of five, well-characterized genera, including Influenzavirus A, Influenzavirus B, Influenzavirus C, Isavirus and Thogotovirus, and recently discovered, still undescribed, sixth genus [6]. Each genus of influenza virus has one species or type - Influenza A virus, Influenza B virus and Influenza C virus, respectively. These three genera can be distinguished by antigenic differences in their nucleoprotein and matrix protein. Among the three types, only influenza A viruses (IAV) are further subtyped based on their surface glycoproteins hemagglutinin (HA) and neuraminidase (NA). All three influenza viruses can infect mammals. Whereas influenza B viruses have been shown to infect humans and seals and influenza C viruses infect human and pigs, IAVs can infect various mammals including equine.

2.1. Influenza A virus

IAV is the primary etiological agent of a highly contagious, acute respiratory disease of humans and animals that follows recurring seasonal epidemics of high morbidity and mortality, as well as worldwide pandemics. On the basis of two surface glycoproteins-HA and NA- influenza a viruses have been further subtyped into 16 well established HA subtypes and 9 NA subtype [7]. Recently, a 17th HA subtype has been identified from little yellow-shouldered bats captured at two locations in Guatemala. However, attempts of virus isolation in either cell culture or chicken embryos were unsuccessful [8]. Wild aquatic bird such as- waterfowl, duck, geese, swan, gulls, terns, etc. - are the natural reservoirs for all

the subtypes of IAV. In the wild aquatic birds, the virus is benignly adapted and does not cause symptomatic infection. In these reservoir hosts, virus is considered to be in an evolutionary stasis that establishes a perpetual viral gene pool. Different subtypes of IAV, however, have successfully jumped from their natural reservoir hosts to other avian and mammalian hosts and caused clinical disease [9].

2.2. Viral genome and encoded proteins

The genome of influenza virus is about 13.6 kb and consists of eight single-stranded, negative-sense RNA molecules. Each RNA segment is encapsidated with several nucleocapsid protein (NP) molecules forming a flexible rod-shaped ribonucleoprotein (RNP) that also has three viral polymerase protein- basic polymerase 1(PB1), basic polymerase 2 (PB2) and acidic polymerase (PA)- associated at the end of RNP [10]. The eight genome segments of influenza virus encode for the ten proteins of influenza virus (HA, NA, PB1, PB2, PA, NP, NS1, NS2, M1 and M2) that are found in all IAV. In addition to PB1, gene segment 2 also encodes another protein PB1-F2 that localizes to mitochondria and has been implicated in cellular apoptosis. Recently, an additional protein N40, an N-terminal truncated version of PB1, was discovered to be translated from fifth AUG codon (codon 40) of PB1 [11]. In 2012, PA-X, another new protein of IAV, was discovered which is synthesized from segment three that primarily encodes PA [12].

2.2.1. The surface glycoproteins

Influenza virus encodes two surface glycoproteins-HA and NA that have important roles in the host range, viral replication, and pathogenicity.

Hemagglutinin (HA)

Hemagglutinin is membrane glycoprotein (MW 61.5 kDa monomer) and the major surface antigen of influenza virus encoded by the fourth largest gene (1778 nucleotides) of the viral genome. The HA molecule is synthesized as homotrimeric spike of non-covalently linked monomers in the rough endoplasmic reticulum of the infected cells [13]. Native HA is synthesized as a single polypeptide (HA0) that is processed into two disulfide-linked subunits referred as HA1 and HA2. Depending on the strain, the cleavage of HA0 is carried out either by ubiquitously distributed furin proteases or tissue specific proteases such as trypsin [14] and HA cleavage is a crucial determinant of viral pathogenicity [14]. Influenza virus HA has two important functions- (I) facilitate viral attachment by binding to sialic acid-containing receptors on the host cell, and (II) mediate fusion between the endocytic vesicle and viral membrane during penetration, enabling release of the viral genome into the cytoplasm. Being a surface antigen, HA is also under constant immune selection pressure. Thus, mutations in HA may allow the virus to escape neutralizing antibodies by antigenic drift [15].

Neuraminidase (NA)

The NA gene (1461 nucleotides) of influenza virus encodes for neuraminidase protein -the second surface glycoprotein of influenza virus. The NA molecule is synthesized as a 454 amino acid (aa) monomer that oligomerizes to form a mushroom shaped trimeric protein. While the N-terminal stalk of NA anchors it to the viral membrane, the box-shaped head contains enzymatic activity to catalyze the cleavage of sialic acid [16]. The NA protein-mediated enzymatic cleavage of sialic acid from cell surfaces is critical for the release of progeny virus during the viral replication cycle. Additionally, it also

removes sialic acid residues from the carbohydrates on viral membrane glycoproteins and prevents virus self-aggregation [17]. Although NA does not have any direct role in the attachment, NA could also remove mucins and facilitate virus access to the epithelial cells [18]. NA has also been implicated in facilitating a secondary bacterial infection that will be discussed later in this chapter.

2.2.2. Polymerase proteins

The PB1, PB2 and PA proteins together form the polymerase complex of the influenza virus that provides RNA-dependent RNA polymerase activity for the virus [19, 20].The proteins are named on the basis of their isoelectric properties: whereas PB1 and PB2 are basic proteins PA is an acidic protein.

PB1 is a 96.5 kDa basic subunit of the polymerase complex encoded by segment 2 (2270 nucleotides) and serves as a backbone that can bind to the two other subunits of the polymerase complex as well as to NP (SB 18, 19). PB1 is a catalytically active subunit of RNA-dependent RNA polymerase that is involved in initiation and elongation of mRNA, complementary RNA (cRNA), and genomic RNA, in a sequence-specific manner [21, 22]. PB1, by endonucleolytic cleavage of cellular mRNA, also generates capped RNA primers for initiation of viral mRNA synthesis [23].

The coding region (2275 nucleotides) of genomic RNA segment 1 of influenza virus encodes the second basic subunit (PB2) of viral polymerase complex. The PB2 protein binds to the 5’-cap structure (m7GpppNm) of host cell pre-mRNA, which is later cleaved in a process called “cap snatching,” to prime viral mRNA synthesis.

Encoded by the segment 3 (2150 nucleotide coding region) of the influenza virus genome, PA is the third and the only acidic subunit of the viral polymerase complex. When

expressed individually, PA demonstrates proteolytic activity that affects co-expressed proteins and PA itself [24]. However, its specific roles in viral transcription and replication are poorly defined [24-26].

2.2.3. Nucleoprotein (NP)

The NP gene (1565 nucleotides) encodes a highly basic, single-stranded RNA binding protein with a molecular weight of 56.1 kDa. Nucleoprotein is the second most abundant protein of virion. After synthesis and post-translational phosphorylation in the cytoplasm, NP is transported into the nucleus of host cells where it binds to newly synthesized viral RNA [27, 28] and provides structural organization to the RNP complex. NP also interacts with other viral proteins including the viral polymerase proteins (PB1 and PB2) and the matrix proteins (M1) [29-31]. Interaction with cellular proteins such as importin –α, F-actin, CRM1, BAT/UAP56 and MX has also been observed. Whereas interaction of NP with importin-α, F-actin and CRM1 is critical for intracellular trafficking of RNP complexes [32-34], interaction with BAT1/UAP56 and MX protein up- and down-regulates viral RNA synthesis, respectively [35, 36]. NP is also a major target of immune cells as cytotoxic T lymphocytes non-specifically cross react with NP of all influenza virus subtypes [37].

2.2.4. Matrix protein

The M gene (1027 nucleotides) of influenza virus, by alternative splicing of overlapping reading frames, encodes two viral structural proteins- matrix protein 1 and 2 (M1 and M2). M1 is the most abundant protein present underlying the viral envelope. M1 is also suggested to interact with the cytoplasmic tails of HA and NA molecules [38]. M1 binds to the RNP through its C-terminal domain to facilitate their nuclear-cytoplasmic

transport [39, 40]. It also has critical roles in recruitment and assembly of viral and host components for the budding of the virus [41]. M2 protein is a type-III integral membrane protein with a short ectodomain, a transmembrane domain, and a cytoplasmic tail [42]. It forms a tetrameric proton channel that allows the acidification of the interior of the virion from the acidified endosomes leading to critical conformational changes and uncoating of the viral RNP complex. Therefore, M2 ion channel blockers have been used as influenza virus replication inhibitors.

2.2.5. Nonstructural proteins

Segment eight of the influenza viral genome is the smallest gene (890 nucleotides) and encodes two nonstructural protein- NS1 and NS2. The NS1 protein has a molecular mass of approximately 26 KDa and a strain-specific length of 230-237 aa [43]. However, a C-terminal truncated (15-30 aa) NS1 protein also exists in nature [44]. The NS1 protein of IAVs can be divided into two distinct functional domains. The N-terminal RNA-binding domain (RBD) consist of residues 1-73 that in vitro binds with low affinity to various RNA molecules in a sequence-independent manner [45, 46]. Residues 74-230 of NS1 protein form the C-terminal effector domain (ED) that mediates interaction with cellular proteins of the host, and also helps in stabilization of the RNA-binding domain [47]. Within host cells, functional NS1 exists as a homodimer stabilized by interaction between both the N-terminal RBD and C-terminal ED of NS1 [48]. Homodimerization of the RBD is also essential for its RNA binding function. A double-stranded RNA –binding pocket is formed by two antiparallel tracks of basic and hydrophilic residues from identical α-helices on either side of a deep cleft from two NS1 subunits [49]. Assembly of the dimeric-NS1 effector domain has not been completely understood. Based on the crystal structure of

human NS1 allele A, a strand-strand model that consisted of two NS1 effector domain monomers made up of seven β-strands and three α-helices was proposed [50]. In this model, each monomer β-sheet forms a crescent shape twisted structure around the central α-helix [50]. On the other hand, Hale at al., 2008 [51], proposed a helix-helix model and observed that a tryptophan residue located at the interface of monomers was critical for dimerization of the effector domain. However, it is important to note that effector domain structure published by Hale et al., was for an avian influenza virus allele B NS1, whereas the model by Bornholdt and Prasad [50], was based on allele A of NS1 of human influenza virus. Hence, it is conceivable that two alleles of NS1 might have a different structure and might have a variable effect on pathogenicity of influenza viruses. Also, it should be noted that full-length NS1 might have a different conformation, which might impact the functions of NS1. To date only two studies are available on the structure of full-length NS1. A crystal structure of H5N1 (A/Vietnam/1203/2004) NS1 was determined and it was found that NS1 forms a dimer through interaction of the RBD and two ED flank the RBD dimers. Also, the two domains of NS1 interact separately with respective domains of alternating RBD and ED dimers of neighboring NS1 molecules to form a higher-order chain like structure [52]. It should be noted, however that H5-NS1 in this study contains a 5aa deletion in the linker region and represents only a minority of influenza virus NS1. Recently, Carillo et al., determined the structure of full length NS1 from an H6N6 IAV (A/blue-winged teal/993/1980) that does not have five aa deletion, and thus may represent more common form of NS1[53]. The authors also studied the effect of linker region (LR) mutations on the three-dimensional structure of full-length NS1. The study revealed that length of the linker, composition of residue 71 and the mechanical hinge are critical determinants in the

structure of full-length NS1. Full-length NS1, depending on the strain, can adopt different structures. Depending on the orientation of the ED with respect to RBD there could be three possible conformations as “open," semi-open and closed conformation that might explain some of the strain variation in NS1 functions [53]. Although NS1 is not incorporated into the virion, it is abundantly present in virus-infected cells and is one of the most versatile protein of influenza virus. NS1 interacts with a variety of cellular proteins and plays critical roles in the replication and pathogenesis of influenza virus including inhibition of splicing and export of polyadenylated cellular mRNA cellular [54, 55], promoting translation of viral mRNA [56], and inhibiting cellular innate antiviral pathways [57-62]. NS1 protein also inhibits maturation and migration of dendritic cells resulting in dysfunctional T-cell stimulation and cytokine production [7]. The functions of NS1 will be discussed in detail later in this chapter.

Unlike NS1, NS2/NEP (nuclear export protein), expressed from an alternatively spliced mRNA of NS RNA segment, is incorporated in the virion in the phosphorylated form [63]. NEP, in association with M1, facilitates the transport of viral RNP complex from nucleus to the cytoplasm. NS2/NEP is also critical in the regulation of viral RNA transcription and replication [64].

2.3. Replication of influenza virus

Replication of influenza A viruses is a multistage process that includes attachment, entry, fusion and uncoating, genome transcription, viral protein synthesis, assembly and finally egress/budding of progeny virions (Figure 2.1).

2.3.1. Receptor binding and cell entry

Influenza virus attaches to sialic acid receptors through HA protein present on the surface of the virion. Following receptor binding, virions are endocytosed into a cellular compartment. Clathrin-mediated endocytosis is apparently major endocytic mechanism [65], however, clathrin- independent mechanisms [66, 67], and macropinocytosis [68, 69] has also been proposed for influenza virus internalization.

2.3.2. Membrane fusion and uncoating

The acidic pH of endosomal vesicles induces irreversible conformational changes in the HA protein that causes the fusion peptide (HA2 subunit) to insert in to the endosomal membrane. At this time, the M2 ion channel also facilitates acidification of interior of the virus particle that leads to the release of the viral RNP complex. The viral RNP complex consists of eight viral RNA segments wrapped around the nucleoprotein together with viral polymerases [70]. Subsequently, viral RNP is transported into the nucleus in a process mediated by nuclear localization signals on the nucleoprotein [71, 72].

2.3.3. RNA replication and translation

The trimeric viral polymerase complex, consisting of PB2, PB1 and PA subunits, transcribes viral genomic RNAs in to mRNA by a cap-dependent manner using 5’ cap structures derived from host mRNAs. Viral mRNAs derived from viral RNA segments M and NS are alternatively spliced to generate M1, M2 and NS1, NEP/NS2, respectively. Although host cell machinery performs the splicing, it is most likely regulated by NS1 [73, 74]. Translation of viral mRNAs is carried out by the host translation machinery, thus, during IAV infection, host cell protein synthesis is limited and viral mRNA translation is preferred [75-77]. Interaction of NS1 with cellular poly A binding protein II (PABII) [78-

80] and cleavage and polyadenylation specificity factor subunit 30 (CPSF30) [57, 81] has been shown to be important in the inhibition of host mRNA synthesis.

2.3.4. Virus assembly and release

Following synthesis in the cytoplasm, viral proteins are transported back to the nucleus where viral RNP complexes are formed and then exported out to the cytoplasm mediated by M1 [82-84] and NEP/NS2 [85-88]. The ER has an important role in the generation of mature influenza virions as it carries out important post translational modifications such as glycosylation (HA, NA and M2) and palmitoylation (HA and M2) of viral proteins [70]. Virus assembly requires transport of the viral proteins to the plasma membrane likely mediated microtubule organizing centers (mTOCS) [89], microtubules [89-91] and additional host factor that include coatomer I (COPI) proteins [92](147), Rab GTPase (Rab11A) [93-95] and HIV rev Binding proteins (HRB) [90, 96]. The assembly starts by association of HA and NA with lipid rafts at plasma membrane of the host cell (152-160). Assembly involves incorporation of eight viral RNP dictated by segment specific packaging signals in the viral RNAs (161, 162). The M1 and M2 proteins play critical roles in packaging [97, 98]. Viral M2 protein and NA proteins are critical in budding and release of the virions. The M2 protein, present in the raft periphery, mediates cleavage and particle release [99]. The NA protein cleaves sialic acid from host cells, and from the virion glycoproteins that leads to the virion release and prevents virion aggregation, respectively [70]. Additionally, the ER serves as an important site of post-translational modification of influenza viral proteins which could induce ER stress. However, functional ER is important in the replication of influenza virus. It is evident that

NS1, although not incorporated into the virion, plays critical roles in the regulation of influenza virus replication.

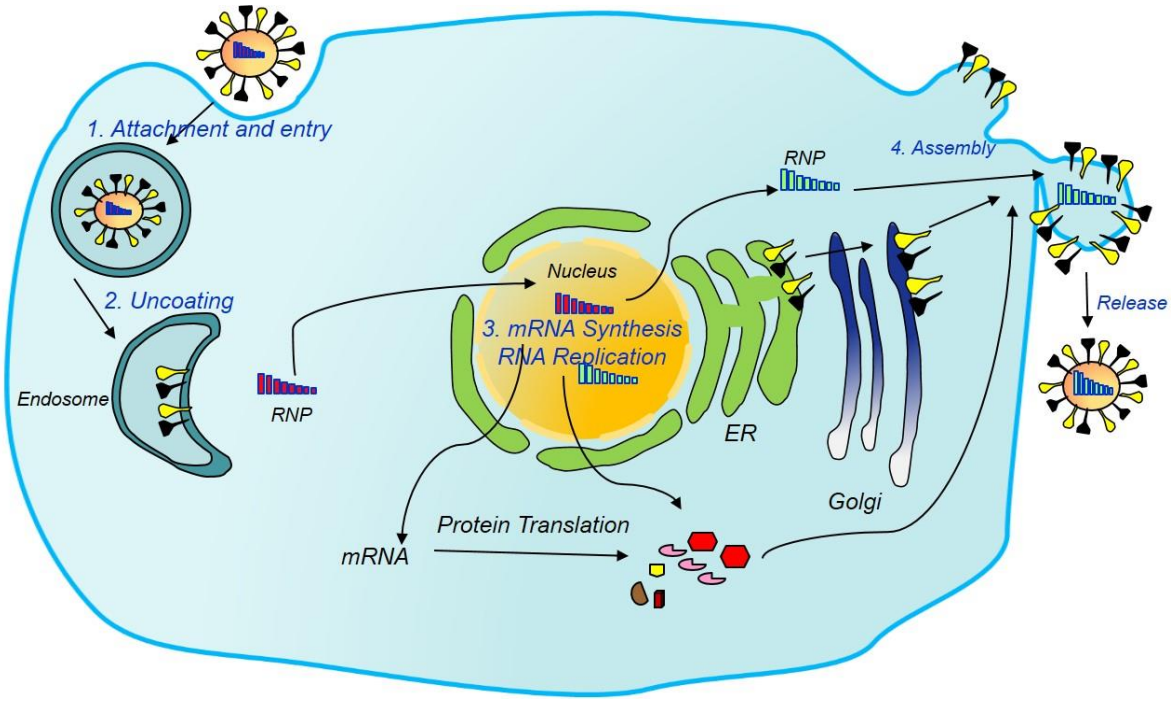


Figure 2.1. Simplified schematic representation of influenza virus replication cycle

2.4. Host innate defense mechanisms against influenza

An immunocompetent host during influenza virus infection mounts a robust anti-viral response to limit viral replication. Both, innate and adaptive immune systems participate in containment and clearance of viral infection in about a week. The innate immune response, not specific to the pathogen, is however the first and foremost barrier in acute influenza infection. An emerging theory is that besides reducing the pathogen burden (antiviral resistance), reducing the negative impact of infection on host fitness (disease

tolerance) could significantly affect the outcome of infection [100]. While disease resistance is important in controlling acute infections, disease tolerance can also protect the host from some acute and chronic infections even when resistance mechanisms fail to protect the host. In the case of infection of African green monkey and sooty mangabey with simian immunodeficiency virus (SIV), for example, despite high viral burden, clinical disease does not occur [101]. In the case of influenza and *Legionella pneumophila* co-infection impaired ability to tolerate tissue damage resulted in an increased susceptibility to bacterial infection; and promoting the tissue repair with amphiregulin treatment resulted in increased survival of the host without affecting the pathogen burden [102].

Innate immunity against influenza involves a concerted participation of various strategies such as physical barriers, soluble factors and immune cells. Pattern recognition receptor recognition of viral RNA as a foreign molecule leads to the secretion of type-I IFN mainly from macrophages, pneumocytes, conventional DCs and plasmacytoid DCs (pDCs) [103-105]. Type-I IFN, in turn, activates hundreds of genes collectively called IFN-stimulated genes (ISGs), in nearby cells resulting in the antiviral state. Viral infection, in addition to type-I IFN, also induces pro-inflammatory cytokine and chemokines. Chemokines recruit additional immune cells including neutrophils, monocytes, and natural killer (NK) cells to the lungs. Virally infected epithelial cells become the target of NK cells that mediate viral clearance [106]. By phagocytizing the virus infected cells, in concert with alveolar macrophages, recruited monocytes and neutrophils are an important mechanism of viral clearance [107].

2.4.1. Physical barriers

The respiratory epithelium is coated with large amounts of mucins that can trap the invading viral or bacterial pathogens. While mucins trap the pathogen, concerted beating of the broncho-pulmonary cilia expel the pathogen out of the airway. Loss of this mucociliary escalator system is one of the mechanisms of influenza pathogenesis that can pre-dispose the host to increased susceptibility to secondary infection [108].

2.4.2. Soluble antimicrobial factors

Pathogens that have successfully compromised the physical barrier are neutralized by antimicrobial factors such as lysozymes, lactoferrin, and defensins. In humans, alpha-defensins, neutrophil produced short cationic peptides, can inhibit the replication of influenza virus [109]. Another important soluble factor is the complement system. The complement is a heat labile, enzymatic mediator of the innate immune system that helps in the clearance of pathogens by increasing the phagocytic clearance of the pathogens. Complement present in human serum can neutralize influenza virus and *in vivo* experiments found that in association with natural antibodies (IgM), complement can provide protective immunity in influenza naïve hosts [110-112]. Furthermore, complement deficient mice were more susceptible to influenza virus infection [113].

2.4.3. Cytokine and chemokine system

Cytokines are a diverse family of small proteins that are produced in response to different stimuli for intracellular signaling and communicatios. They play an important role during viral infections. Interferons are a type of cytokine that are produced during viral infections and are aptly named because of their ability to interfere with viral replication. While IFN-α and IFN-ß are type-I IFNs, IFN-γ is often called a type-II IFN. Although

most nucleated cells can produce the type-I interferons, pDC are considered as the specialized producers of these cytokines [114]. pDCs bind to a common cell surface IFN receptors and activate transcription of hundreds of ISGs. Several protein products of ISGs have anti-viral activities. For example, while OAS helps in degradation of viral RNA, PKR has an inhibitory effect on viral protein synthesis. Another ISG protein, Mx is associated with resistance against influenza infection [115]. On the other hand, IFN-γ is primarily produced by T-cells and NK cells and its function is to activate macrophages. Interleukins, produced by leukocytes, are mainly regulators of immune cell differentiation and activation. 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. Chemokines are the largest and fast growing family of cytokines that signal by binding to one or more G-protein coupled receptors [116]. Depending upon the spacing of their first two cysteine residues, chemokines are classified into four types (CXC, CC, C, and CX3C). Chemokines function as chemoattractants to control the migration of cells, particularly those of the immune system, and contribute to innate and adaptive immunity [117]. Influenza virus infection induces the upregulation of several inflammatory cytokines and chemokines such as IL-1β, IL-6, TNF-α, CCL-2 (MCP-1), CCL3 (MIP1-α), CCL3 (RANTES) and CXCL10 (IP-10)[118]. Current literature has conflicting reports on the role of the cytokine and chemokine system during influenza infection. Some studies suggest that influenza virus infection triggers a robust inflammatory cytokine response or "cytokine storm" that is responsible for the pathogenesis of influenza while other studies identified a protective role.

2.4.4. Cells of innate immune system

Natural killer cells

Natural killer cells are a population of large granular lymphocytes with potent cytotoxic activity and robust production of inflammatory cytokines such as IFN-γ, tumor necrosis factor- alpha (TNF-α), and macrophage inflammatory protein-1 alpha (MIP-1α) [119-121]. While, NK cells are broadly reactive against a wide variety of pathogens including bacteria, virus, and intracellular parasites, their role during influenza is poorly defined. Two NK cells receptors, natural cytotoxic receptors (NCR) Nkp46 (NCR- in the mouse) and Nkp44, have been shown to recognize influenza virus HA on virus-infected target cells [122, 123]. Moreover, large numbers of NK cells accumulate in the lungs of infected host; and depletion or mutation of NK cell receptors resulted in increased morbidity and mortality [124, 125]. Furthermore, adoptive transfer of donor cells, even at <20% of the endogenous pulmonary NK cell content, resulted in restored protection [126]. Moreover, the differential activation of NK cells correlated with the pathogenicity of avian influenza viruses [127]. NK cells have also been shown to enhance both DCs [128] and the adaptive T-cell responses [129, 130] following influenza virus infection. Recently, it was found that type-I IFN, but not IL-12 or IL-18, as critical for NK cell expression of both IFN-γ and granzyme B in response to influenza infection [131].

Alveolar macrophage cells

Alveolar macrophages (AM) are one of the primary phagocytic and predominant antigen presenting cells in the lungs. During homeostasis, AM are relatively quiescent and have a regulatory cell phenotypes [132]. These homeostatic AM are less phagocytic and produce relatively low amount of cytokines [132]. Interestingly, homeostatic AM have also

been shown to suppress the induction of innate and adaptive immunity [133-136]. However, these homeostatic AM can be activated during influenza infection and convert into highly phagocytic cells that produce robust amounts of inflammatory cytokines including IL-6 and TNF-α [137]. Influenza infected macrophages also produce chemokines such as RANTES, monocyte chemotactic protein-1 (MCP-1), and MIP-1α which further recruit more mononuclear cells to the lungs to aid in viral clearance [138]. Besides their contribution to viral clearance, especially in the case of seasonal influenza, AM have also been implicated in influenza virus pathogenesis [139, 140]. Nevertheless highly pathogenic IAV was found to induce significant recruitment of AM to the lungs [141, 142]. However, studies using depletion of AM suggest that these cells are critical in the early protection during influenza. Depletion of AM prior to, but not, 3 or 5 days following influenza infection resulted in uncontrolled viral replication and a significant increase in mortality [143]. Likewise, pigs depleted of AM prior to influenza infection exhibited increased respiratory stress, reduction in lung TNF-α levels and increased IL-10. AM depletion also reduced numbers of virus-specific CD8+ T-cells in the lungs and led to a diminished antibody response [144]. Taken together, these observations suggest that AM may have both protective as well as detrimental roles, due to excessive inflammatory cytokine production- during influenza virus infection; and a balanced AM response is essential in controlling the influenza virus infection.

Dendritic cells

Dendritic cells are a unique population of cells that play a pivotal role in molding immune response against invading pathogens. DCs are one of the most potent antigen presenting cells (APC) and play a pivotal role in bridging the innate and adaptive immune

system following influenza virus infection. In the naïve homeostatic state, DCs are distributed throughout the respiratory tract including airway epithelium, lung parenchyma and the alveolar space of the lungs where they constantly survey for invading pathogens or foreign materials. While lung resident DCs constitute a heterogeneous population in terms of their surface phenotype and function, predominant DCs in the naïve lungs are airway and alveolar DCs characterized by expression of $CD11c^{+}MHCII^{+}CD11b^{neg}$ $CD4^{neg}CD8^{neg}$, and interstitial DCs characterized as being CD11c+MHC II+$CD11b^{hi}CD4^{neg}CD8^{neg}$ [145-149]. Pulmonary insult or infection results in a significant influx of $CD11c^{+}MHCII^{+}$ DCs in to the lungs increasing in the number of alveolar DCs and interstitial DC, as well as recruitment of other subsets such as inflammatory monocyte derived DC (MoDC), pDC, and CD8α+ DCs [139, 150-152]. While most influenza viruses can infect DC and lead to viral protein synthesis, infection is generally abortive and does not produce progeny virus [153, 154]. However, some highly pathogenic H5N1 viruses have been described to replicate in human and mouse DCs and result in cytopathic effects [118, 155]. Following infection by or encounter with IAV, DCs initiate production of proinflammatory cytokines and chemokines that can include IL-6, IL-12, TNF-α, IL-8, IP-10, RANTES, MIP-1β, and most importantly, type-1 IFN (IFN-α and IFN-β) [156]. Type-I IFN possesses potent antiviral properties and is critical to the control of IAV infection. However, influenza virus possesses mechanism to subvert this critical antiviral response by virtue of its NS1 protein, and it will be discussed later in the current chapter.

2.5. Pathogenesis of influenza

Depending on the virus subtype and host, pathogenesis and outcome of influenza virus infection is variable. In a natural reservoir host, such as wild waterfowls, influenza

virus replication is limited to the gastro-intestinal tract and does not cause clinical disease [157]. In general, influenza virus infection is also mild and non-fatal in other avian species such as domestic poultry, turkey, quail and pheasants. However, some H5 and H7 strains are highly pathogenic in birds and responsible for large scale outbreaks in recent years [158]. In mammals, including humans and equines, influenza virus causes a respiratory disease that could range from mild to highly fatal in severity. Uncomplicated cases of seasonal influenza in human are characterized by symptoms such as fever, headache, sore throat, malaise, anorexia and coughing. The virus primarily infects and replicates in ciliated columnar epithelial cells of the upper respiratory tract and subsequently infects cells of the lower respiratory tract. Macroscopic pathogenesis of influenza involves physical damage to respiratory epithelium, increased cellular infiltration and respiratory edema. The molecular basis of the pathogenesis is mainly due to shut down of host cell protein synthesis, apoptosis, inhibition of cellular anti-viral factors, and modulation of cellular signaling molecules that results in altered cytokine/ chemokine response. Modulation of respiratory cytokine and chemokine milieu by influenza virus also results in altered recruitment of the effector cells of the respiratory innate and adaptive immune systems. Influenza virus NS1 has a predominant role in the pathogenesis of the influenza virus and will be discussed below. Secondary bacterial infection also plays a major role in the pathogenesis and mortality associated with influenza and its role during influenza will be reviewed later in the current chapter.

2.6. Role of NS1 in pathogenesis and immunity to influenza

Although the NS1 protein is not a structural component of the virion, it is expressed at very high levels in the infected host cells and performs various functions critical for

pathogenicity and replication of IAV. NS1 has been shown to inhibit critical steps of the host innate as well as adaptive immune responses. NS1 has also been implicated as a critical factor in several other viral functions including control of viral replication [159], facilitation of viral mRNA translation [160], inhibition of host mRNA processing [161-163], and regulation of apoptosis [164, 165].

2.6.1. Antagonism of cellular antiviral mechanism

One of the well-studied functions of the NS1 protein of the influenza viruses is to inhibit host type-I IFN mediated antiviral defense. By using IAV with a truncated form of NS1 or complete deletion of NS1 (delNS1) it was shown that NS1 is critical in counteracting the host IFN response [166-168]. Whereas, delNS1 viruses replicated efficiently in IFN-deficient systems such as vero cells, these viruses were highly attenuated in an IFN-competent system [169]. The attenuated phenotype of these viruses might be due to potent induction of the IFN-α/β in IFN-competent system [167]. Several groups have investigated the mechanism of NS1 mediated inhibition of Type-I IFN, and it is now evident that, depending on the strain, NS1 anti-IFN activity could either be at pre-transcriptional or post-transcriptional level [170-172]. IFN antagonistic activity of NS1 protein of PR8 relies on blocking the dsRNA-and virus- mediated activation of key regulators of IFN-β mRNA transcription such as interferon regulatory factor-3 (IRF-3), NFκB and c-jun/ATF-2 that are essential for IFN-β mRNA synthesis [173-175]. Post-transcriptional inhibition of IFN-β by NS1 is mediated by its interaction with CPSF30 and PABPII. Studies using IAV Udorn/72 (Ud) found that the C-terminal effector domain of Ud-NS1 binds to two zinc finger domains of CPSF30 [176, 177] and interacts with PABPII [57] resulting in inhibition of polyadenylation and nuclear export of cellular mRNAs.

OAS and the PKR are the two major cytoplasmic anti-viral proteins. Besides their key roles in regulation of viral transcription and translation, both of these proteins have critical roles in other innate defense mechanisms of the host such as IFN-β induction and apoptosis [178, 179]. Influenza virus NS1 has been shown to directly inhibit OAS [60] as well as PKR [180]. A key event for the activation of OAS is binding of dsRNA, therefore RNA binding activity of NS1 could lead to inhibition of OAS [181]. Since RNaseL of the OAS pathway also participates in IFN-β induction [179], inhibition of OAS could also lead to suppression of IFN-β [174, 182]. Binding of dsRNA releases the auto-inhibition of PKR and leads to activation. Thus the dsRNA binding activity of NS1 was thought to result in competitive inhibition of PKR [183, 184]. However, Li et al., observed that an NS1 protein defective in dsRNA binding efficiently blocked the activation of PKR [185]. Moreover, NS1 was also reported to interact with PKR through a dsRNA–independent mechanism that involved critical role of residues 123-127 [180, 185]. Domain mapping studies suggest that NS1 binds to a linker region of PKR that prevents a conformational change otherwise required to release autoinhibition [185]. Additionally, to counteract the effect of PKR, influenza virus activates a latent chaperon related protein p58IPK that interferes with dimerization and activation domain of PKR [186, 187], and has been shown to prevent apoptosis [188]. Activation of PKR leads to phosphorylation of cellular eIF2-α that results in reduction of viral as well as cellular protein synthesis [178]. Inhibition of eIF2α is also critical in regulation of ER stress-induced transcription factor CHOP-10 and induction of CHOP-10 is a key factor in deciding the fate of infected cell. While at early time points it helps to restore the ER homeostasis, prolonged induction of CHOP-10 leads to apoptosis.

2.6.2. NS1 and RNA interference (RNAi) pathway

Although the role of mammalian RNAi pathway in innate anti-viral defense is not well established, NS1 has been suggested to antagonize putative RNAi-mediated innate anti-viral mechanisms [189]. Whereas NS1 inhibited the host RNAi pathway in drosophila and plant cell systems [190, 191], it has not been observed in the mammalian cells [192].

2.6.3. NS1 and apoptosis

Although the biological consequence of apoptosis during influenza virus infection is yet to be defined, apoptosis is widely accepted as a cellular antiviral mechanism to limit viral replication. While inhibition of apoptosis early during infection could promote events such as viral replication, later during infection apoptosis may help in the efficient release of progeny virus [193]. Influenza virus has been shown to possess anti-apoptotic activities [165, 194-196]. Conversely, certain viral protein such as PB1-F2 and NA have been shown to have pro-apoptotic activities [43]. The role of NS1 in apoptosis is controversial, and it has been shown to have both pro-apoptotic [164, 197] as well as anti-apoptotic activities [165, 196]. In MDCK cells, Zhirnov et al., observed that, as compared to the wild type PR8 virus, isogenic delNS1 virus induced higher level of apoptosis [165]. Although such conflicting results may be due to differences in the experimental setup, there is an emerging hypothesis that NS1 temporally regulates both early suppression and late induction of apoptosis [51]. Activation of PKR during influenza virus infection has also been reported to play a role in apoptosis [198]. Thus, direct binding and inhibition of PKR by NS1 could also suppress apoptosis [51]. Likewise, suppression of the pro-apoptotic activity of OAS/RNaseL [60] or the JNK/AP-1 stress pathway [173] could also contribute to anti-apoptotic activity of NS1. Influenza virus activates the PI3K/Akt pathway by the binding

of NS1 to the p85 subunit of PI3K [199-201] and by viral RNA via RIG-I [202]. Activation of the PI3/Akt pathway by NS1 limits the early induction of apoptosis [195, 200, 203].

2.6.4. NS1 and dendritic cell functions

Influenza virus NS1, in addition to its effect on innate antiviral defenses, also interferes with critical components of the adaptive immune response. DC are the critical sentinel cells of the adaptive immune system. Upon an encounter with foreign antigens, DCs undergo maturation, release proinflammatory cytokines/chemokines and migrate to lymph nodes where they present pathogen specific antigens to cytotoxic and helper T-cells [193]. Presentation of antigens by dendritic cells activates cytotoxic T-cells that directly kill the infected cells to eliminate the pathogen, whilst helper T-cells produce cytokines such as IFN-γ and TNF-β that augment the cytotoxic activity of T-cells. NS1 protein of influenza virus has been shown to reduce systemic and respiratory cytokines and prevent TNF-α mediated depletion of bone marrow lymphocytes [204]. Influenza virus NS1 has also been shown to interfere with the activation and maturation of DCs. Using a PR8 virus mutant lacking NS1 and Newcastle disease viruses engineered to express NS1, Fernandez-Sesma et al., found that NS1 inhibited expression of several genes crucial for the maturation, migration and T-cell stimulatory activity of DCs [205]. Dendritic cells infected with wild type PR8 virus failed to mature and did not induce secretion of IFN-γ from helper T-cells. Additionally, NS1 affected only a specific set of genes that mechanistically appear independent of IFN-β production. Important genes affected by NS1 included MIP-1β, IL-12p35, IL-23p19, RANTES, IL-8, IFN-α/β, and CCR7 [205]. Monocytes are important progenitor cells of DCs. Infection of equine peripheral blood monocytes with influenza virus inhibited the differentiation of monocytes into DCs in response to GM-CSF and IL-

4 [153]. Despite a non-productive infection, infected monocytes displayed a morphology, functional characteristics and cytokine profiles suggestive of arrested differentiation [153]. Reactivation of memory T-cells by bone marrow-derived DCs has been shown to be critical in protection against influenza [206] therefore, prevention of DC maturation by NS1 has important implications in viral clearance by the host.

2.7. Influenza and bacterial synergy

Although infection with some highly virulent influenza viruses alone can kill the host, influenza-associated death may also be due to exacerbation of physiologic stress from chronic health conditions or secondary bacterial infections. Among these, secondary bacterial infections appear to be the most common cause of death due to influenza, especially during pandemics [207]. Although *Streptococcus pneumoniae (S. pneumoniae)* has been the most common bacteria, other bacteria such as *S. aureus* and *H. influenzae* have also been linked to influenza-associated secondary bacterial pneumonia [207] [208]. The earliest associations between influenza and bacterial pathogens date back to the 17th century when French physician Laennec observed increased cases of pneumonia following an epidemic of influenza ("la grippe") in 1803 [209]. In 1935 Andrewes et al. were first to confirm secondary bacterial pneumonia following influenza infection where virus was recovered from a patient who was febrile and then developed pneumococcal pneumonia seven days into his convalescence and died [210]. The 20th century has seen at least three well-defined influenza pandemics- "Spanish flu" (1918-19, H1N1), Asian flu (1957, H2N2), and Hong Kong flu (1968-69, H3N2) resulting in 675,000 [211], 86,000 [212] and 56,300 [213] death, respectively, in the United States. The pandemic of 1918, which killed about 40-50 million people worldwide [214], mostly due to secondary bacterial pneumonia

[215-219], brought the focus on synergy between influenza and bacterial pneumonia. In the following years, several epidemiological and laboratory studies were conducted that supported secondary bacterial pneumonia as the major cause of death during influenza infections. In the investigation of an epidemic in Boston during the winter of 1943-44, Finland et al., [220] observed high titers of antibodies against IAV (PR8) from the cases of bacterial pneumonia during and immediately following the epidemic and concomitant with history of clinical influenza. They also isolated influenza virus from the lungs of three fatal cases and concluded that the severity of the pneumonia was due to preceding influenza infection [220]. In a study of an influenza epidemic in the Baltimore area during March-April of 1947, using virus isolation and serology, Maxwell et al. reported that 47% (17/36) of cases of lobar pneumonia in humans were due to simultaneous infection with influenza and pneumococcus [221]. Similar findings were reported by Tyrrell in an influenza outbreak in Sheffield in 1949 [222]. In order to determine what killed the patients during the 1918-1919 pandemic influenza, Morens et al., re-examined hematoxylin and eosin-stained sections recut from the lung blocks obtained from 58 victims of the 1918 outbreak available in the National Tissue Repository of the Armed Forces Institute of Pathology [223]. The authors also reviewed 1539 pathology and bacteriology research records that included 8398 individual autopsies from the 1918-1919 pandemic. From the histopathological, epidemiological and microbiological data they suggested that a synergistic association between influenza and bacterial pneumonia was responsible for the unprecedented mortality seen during the 1918-1919 pandemic [224, 225]. Based on gross pathology and bacterial isolation from blood only, 96% of 8000 cases reviewed showed secondary bacterial infection [225]. Even with the widespread availability of antibiotics,

more than two-thirds of fatal cases during the 1957 pandemic were associated with bacterial pneumonia [207]. Recent epidemiological studies also suggest that influenza infection predisposes the host to secondary bacterial infection. In a prospective study of lower respiratory tract infection of 154 children, Michelow et al., observed that 23% of children with identified pathogens showed influenza-associated secondary bacterial pneumonia resulting in heightened lung inflammation and disease severity [226]. Similarly, in an epidemiological study using binomial regression, Grabowska et al., reported a yearly increase of 12-20% in invasive pneumococcal disease (IPD) per influenza season, confirming a strong association between influenza and IPD [208]. The Center for Disease Control (CDC) evaluated the lung tissue from 77 fatal cases from the 2009 pandemic influenza (H1N1) and observed that concurrent infection of bacterial pathogens was evident in approximately one-third of the cases, approximately 50% of those cases were due to *S. pneumoniae* [227].

Animal models for secondary bacterial infection following influenza were attempted soon after the pandemic of 1918 [228]. In 1945, Mercedes and Torregrosa developed a mouse model for secondary bacterial pneumonia [229]. Using the mouse-adapted Influenza virus A / Puerto Rico/8/34 (PR8) and different strains of *H. Influenzae, S. Pneumoniae and S. aureus,* they observed that preceding influenza infection resulted in increased severity of bacterial pneumonia. Recently, McCullers et al., also developed a similar mouse model to study the synergistic relationship between IAV and *S. Pneumoniae*. They observed that influenza infection preceding the pneumococcal challenge increased the severity of pneumonia and resulted in 100% mortality in mice. On the other hand, pneumococcal infection preceding influenza infection resulted in protection from influenza

and improved survival [230]. Although the relationship between influenza and pneumococcus has been known for centuries [209], there are discrepancies that need to be resolved. While laboratory animal experiments support synergy between pneumococcus and influenza [231, 232] epidemiological data shows either limited association at best [233, 234] or no association [235]. Shreshta et al., developed a computer model that integrates weekly incidence reports and a mechanistic transmission model within a likelihood-based inference framework to define the nature, strength and temporal interaction between influenza and pneumococcal pneumonia. Using their model, they analyzed weekly reports of influenza and pneumococcal pneumonia from Illinois from 1989-2009 and observed that influenza infection increased the susceptibility for pneumococcal pneumonia by approximately 100 fold for a week following the initial influenza infection [236].

Mechanism of Synergy

Bacterial pneumonia during influenza could either be due to combined viral/bacterial pneumonia or a secondary complication following influenza. Combined viral/bacterial pneumonia has about a 10% mortality rate and is at least three times more common than primary viral pneumonia [237]. Furthermore, differentiation of primary viral or bacterial pneumonia is clinically challenging. Chest radiographs of patients with advanced cases of viral pneumonia usually show bilateral interstitial infiltrate similar to bacterial pneumonia [238]. Inflammatory markers also fail to distinguish between primary viral and bacterial pneumonia. Secondary bacterial pneumonia with a mortality rate of about 7%, on the other hand, is easily recognizable as it develops during the recovery phase of influenza [239, 240]. Pathogenesis of influenza-associated bacterial pneumonia is multifactorial and differs between concurrent bacterial infection and infection following

influenza. It is important to note that concurrent viral/bacterial infection is relatively more complex than post-influenza pneumonia. In combined viral/bacterial infection, both the bacteria and the virus elicit a host defense response that could affect growth of both or the other. On the other hand, pneumonia following influenza involves virus-induced changes to the host and host response to bacteria [241, 242].

Physical changes in respiratory epithelium

Increased bacterial adherence to the damaged respiratory epithelium due to influenza virus replication is the earliest explanation for increased susceptibility to bacterial superinfection [243, 244]. Bacteriology and histopathology of lungs from the fatal cases of hospital-acquired *S. aureus* pneumonia during 1957-58 influenza pandemic, showed increased bacterial adherence in the areas of the bronchial tree where influenza virus replication had denuded the epithelial layer [245]. Influenza virus infection can expose basal membrane components such as fibrin that facilitate attachment of bacteria. In mice infected with PR8 virus, and subsequently with *S. pneumoniae*, desquamation of tracheal epithelium caused exposure of the basal cell layer and exposed basal membrane component that favored the adherence of *S. pneumoniae* [246]. Additionally, influenza virus NA that cleaves terminal sialic acid from cell surface glycoproteins could generate alternate receptors, or virus-induced inflammation could activate some inactive cellular receptors for bacteria [209, 230]. In a tracheal organ perfusion system, exogenous administration of NA results in an increased number of receptors and adherence of *S. pneumoniae* [247]. Using a mouse model, McCullers and Bartmess found that influenza virus NA facilitates bacterial adherence by stripping sialic acid from the lung, exposing receptors for pneumococcus. Administration of selective NA inhibitor (Oseltamivir) improved survival

and morbidity from influenza independent of viral replication [248]. Pairs of otherwise isogenic influenza viruses generated by reverse genetics to express different N2 subtype NAs showed a differential attachment of *S. pneumoniae* and development of pneumonia proportional to the activity of expressed NA [249]. Besides NA, PB1-F2 of influenza has also been implicated in the susceptibility to secondary bacterial pneumonia. Mice infected with influenza viruses lacking PB1-F2 showed decreased susceptibility to secondary infection [207]. Mice infected with a viral strain engineered to express PB1-F2 protein of 1918 pandemic influenza were more susceptible to pneumococcal pneumonia [250]. A possible mechanism involves excessive lung damage and enhanced inflammatory response; however, the exact underlying mechanism is unknown [207]. Upregulation of cryptic cellular receptors might also contribute to increased bacterial susceptibility. Influenza virus infection induces inflammatory cytokines that result in upregulated expression of platelet activating factor receptor (PAFR) that can be used by pneumococci for attachment [251]. The role of PAFR in bacterial superinfection, however, is controversial. Influenza infected PAFR deficient mice infected with *S. pneumoniae* showed significantly reduced bacterial outgrowth in the lungs, reduced dissemination of the infection and prolonged survival [252]. However, antibody-mediated neutralization of PAFR had no effect on secondary bacterial infection in influenza-infected mice [230]. Additionally, reduced clearance of bacteria from the influenza-infected lungs could also predispose the host to increased bacterial burden. Influenza infection can result in decreased function of surfactant protein and increased mucinous secretions that together with fibrin and edema fluid and cellular infiltrate result in a dead space and ideal culture conditions for bacteria [244, 253]. Impairment of the mucociliary escalator mechanism has

also been observed during influenza [254, 255]. Substantial reduction in mucociliary velocity was observed in influenza-infected tracheas as compared to the non-infected tracheas [256]. Therefore, it appears that influenza infection results in decreased clearance of bacteria leading to increased bacterial burden in the lungs.

Defective cellular innate defense

Although, defects in recruitment or activation of immune effector cells, such as neutrophils, have also been implicated in enhanced bacterial superinfection during influenza infection, their role is controversial. While most studies observed a reduced early recruitment of neutrophils after subsequent bacterial challenge of influenza-infected mice, some studies found that neutrophil recruitment 24 hrs post bacterial challenge is either uninhibited or sometimes increased [242, 257-259]. Irrespective of levels of neutrophil recruitment in the lungs, defects in the bactericidal functions of these cells such as phagocytosis, respiratory burst, myeloperoxidase production and lysozyme production have been detected [257, 260, 261]. Macrophage cells are strategic resident immune effector cells in the lungs and act as immune sentinels for bacterial infections. Influenza and other respiratory viruses have been shown to inhibit chemotaxis, phagocytosis and microbicidal function of macrophage and monocytes. In the influenza infected individuals, monocyte chemotaxis was suppressed 40%-72% during acute infection and reverted back to normal by three weeks after recovery. Increased susceptibility to bacterial superinfection in influenza patients can also be due to the virus-mediated suppression of monocyte function [262]. In a mouse model, Kleinerman et al., found that, compared to the non-infected mice, influenza-infected mice showed 57% and 65% depression of total leukocyte and macrophage accumulation, respectively. On the other hand, bacterial pneumonia did

not suppress the macrophage response, suggesting that macrophage inhibition was specifically due to influenza virus infection [263]. In dual infected mice, bacteria were primarily bound to resident AM cells. Furthermore, depletion of alveolar macrophage cells with liposomal Clodronate™ resulted in bacterial outgrowth in lung tissue as well as in alveoli [264]. Defects in NK cell function has also been proposed to contribute to secondary bacterial infections. In the mouse model, preceding influenza infection resulted in impaired NK cell response to subsequent *S. aureus* infection. Adoptive transfer of naïve NK cells restored the impaired host antibacterial response. This NK cell dependent impairment of host antibacterial defense was due to reduced TNF-α production by NK cells that resulted in depression of macrophage activation [265].

Dysregulated cytokine response

A fine balance of pro-inflammatory and anti-inflammatory cytokines is central in determining the outcome of the immune response. A dysregulation of the cytokine and chemokine balance during influenza has been observed and suggested to promote tissue injury, as well as impair bacterial clearance. Increased production of pro-inflammatory cytokines such as TNF-α, IL-6, IL-1β and chemokines, commonly referred to as a cytokine storm, has been reported to contribute to the pathology of influenza infection of animals as well as humans [257, 266]. Conversely, Shahngian et al., observed that influenza infection resulted in decreased production of neutrophil activating chemokines such as MIP-2 and keratinocyte-derived chemokine [KC] [259]. Imbalance in the release of anti- and pro-inflammatory cytokines could also suppress the effector response of innate immune cells. Besides inflammatory cytokines, influenza virus also stimulates anti-inflammatory cytokines such as IL-10 [266]. IL-10 limits both systemic as well as respiratory

inflammatory cytokine response of the host [267]. Additionally, enhanced release of IL-10 also results in suppression of leukocyte activity and impaired antibacterial response in the lungs [268]. In a mouse model of post-influenza secondary pneumonia, Van der Slujis et al., [242] observed that higher IL-10 levels, at least in part, were associated with increased susceptibility to secondary infection with *S. pneumoniae*. Mice recovered from influenza infection showed 50-fold higher expression of IL-10 in their lungs as compared to the lungs of control mice. Furthermore, treatment with an IL-10 antibody before bacterial challenge reduced the bacterial outgrowth and lethality of secondary pneumonia as compared to the IgG1 control antibody treated mice [242]. However, studies with IL-10 knockout mice did not show any improved outcome after dual infection [269]. Transforming growth factor-β (TGF-β) is another important anti-inflammatory cytokine that has been implicated in influenza-associated susceptibility to secondary bacterial infections. TGF-β is synthesized as an inactive factor that needs to be cleaved to generate a biologically active form of TGF-β. Latent TGF-β remains linked with latency-associated peptide (LAP) attached non-covalently to the amino terminus of the immature TGF-β protein. Release of LAP from TGF-β is essential for its binding to cellular receptors [270]. Influenza virus neuraminidase is capable of processing the latent TGF-β to its active form *in vitro* resulting in increased serum TGF- β activity as early as day one post-influenza infection [271]. Although interferons including IFN-γ and type-I IFN (IFN-α and -β) are primarily believed to participate in antiviral immunity, recent studies also support their role in suppressing secondary bacterial infections. Sun and Metzger [231] reported that during influenza infection, pulmonary T-cell produced IFN-γ inhibits bacterial phagocytosis by alveolar macrophage cells and leads to increased susceptibility to secondary pneumococcal

infection. Whereas exogenous administration of IFN-γ mimics the effect of influenza infection and increases secondary bacterial susceptibility, neutralization of pulmonary IFN-γ restores antibacterial immunity. Also, mice deficient in either IFN-γ or IFN-γ receptor showed significant bacterial clearance as compared to wild type mice [264]. Type-I IFN such as IFN-α or IFN-β are soluble cytokines that act as the first line of defense against viral infections and establishment of antiviral state and activation of various immune cells [272] [273]. Influenza virus NS1 is a potent antagonist of IFN-α/β antiviral responses [193]. Studies using influenza viruses engineered to express either truncated forms of NS1 or lacking NS1 confirmed the role of this protein in counteracting host IFN response [169, 274, 275]. Influenza viruses lacking NS1 (DelNS1) induce large amounts of type-I IFN and are, therefore, highly attenuated in IFN-α/β competent system [193] and display pathogenicity only in mice lacking antiviral signaling components such as STAT-1 [276]. Influenza virus has also been shown to block IFN mediated anti-viral signaling. Expression of H5N1 NS1 in HeLa cells suppresses IFN signaling in part due to NS1-mediated inhibition of expression of the IFN receptor subunit [273]. Infection of *ex-vivo* human non-tumor lung tissue with H5N1 and H1N1 viruses resulted in downregulation of *ifanr1* expression. Furthermore, infection of human monocyte-derived macrophages with H5N1 and H1N1 viruses suppressed *ifnar1* and *ifnar2* expression [273]. Interestingly, IFN receptor null mice ($ifnr^{-/-}$) showed resistance to secondary infection with *S. pneumonia*e when compared to wild type control mice [259]. Mice deficient in IFN receptor produced significantly higher amounts of CXC chemokine resulting in greater recruitment of neutrophils in the lungs [259]. Some studies suggest that the type-I interferon-mediated suppression of TLR signaling through TRIF participates in regulating pro-inflammatory

cytokines and chemokines [277]. However, the mechanism underlying the IFN mediated suppression of chemokines is still poorly defined.

2.8. IL-23/IL-17 axis of innate immune response

IL-23 is a member of the IL-12 family of heterodimeric cytokines [278]. Functional IL-23 is composed of a unique subunit-IL-23p19 and p40 subunit which is shared with IL-12. Although IL-23p19 is expressed by a variety of tissues, it lacks any functional activity by itself. Indeed, only activated macrophages and DCs secrete the biologically active IL-23 heterodimer [279]. Since IL-23 also shares receptor IL12Rb1 with IL-12, it was expected to have similar roles as IL-12 in promoting T helper 1 (Th1) type responses. However, it has become evident now that IL-23 has different roles in regulating the immune response. Importantly, IL-23 is a key factor in the development and maintenance of a subpopulation of CD4+T cells called Th17 cells [280]. Based on the cytokine profiles and functional properties, Mosmann et al., [281] proposed two classes of helper T (Th) cells as Th1 and Th2 cells which participate in cell mediated immunity and humoral immune response, respectively. After about two decades, an IL-23 dependent subset of $CD4^{+}$T cells, distinct from Th1 and Th2 cells, characterized by IL-17 secretion was identified [282]. These Th17 cells do not express the T-bet or GATA3 lineage-specific transcription factors of Th1 and Th2 cells, respectively [283, 284].

Initial studies found the IL-23/IL17 immune axis to be a major contributing factor in the development of autoimmune diseases including experimental autoimmune encephalomyelitis (EAE) [285], collagen–induced arthritis (CIA) and inflammatory bowel disease (IBD) [reviewed by 286]. By studying the onset of EAE in IL-12p35, IL23p19 and IL-12/23p40 knockout (KO) mice, Cua et al., reported that IL-12/23p40 KO mice were

resistant but IL-12p35 KO mice were susceptible to EAE. They further observed that IL-23p19 deficient mice were resistant to disease, indicating that IL-23 was important in the development of EAE [287]. Likewise, it was found that IL-12p35 deficient mice were more susceptible to CIA following immunization with type II collagen in complete Freund's adjuvant, whereas IL-23p19 or IL-12/23p40- deficient mice did not develop the disease [288]. Recent evidence, however, also suggests a protective role of the IL-23/IL-17 axis in the innate immune responses. Exposure of macrophages and DCs to lipopolysaccharides (LPS) and other microbial products leads to the secretion of IL-23 from these cells [279, 289, 290], that induces a rapid release of IL-17 cytokines from Th17 cells [280, 291]. The IL-17 cytokine family consist of six members (IL-17 A, B, C, D and F), however; only IL-17A and IL-17F have been studied well and will be referred as IL-17 hereafter. Endothelial cells and macrophage cells express IL-17 receptor and apparently are the main targets of IL-17. IL-17 signaling in these cells results in induction of several pro-inflammatory cytokines, including IL-6, IL-1β, IL-8 and TNF-α, which mediate host defense; as well as several neutrophil chemoattractants such as CXCL-1, CXCL2, and CXCL5 that mediates neutrophil recruitment to the infection site. Thus, the IL-23/IL17 immune axis might be an important driving force in early immune responses against invading pathogens. IL-17 also induces cytokines for targeting other immune cells to the mucosal surfaces. These include CXCL9, CXCL10 and CXCL20 that possess chemotactic activity for lymphocytes and DCs, and CCL2 and CCL7 that recruit monocytes. Additionally, IL-17 also induces some antimicrobial peptides which can directly kill the pathogens [2]. Moreover, some of the chemokines such as CCL20 also exhibit antimicrobial activity [292].

2.9. IL-23/IL-17 axis and immunity against pathogens

A protective role of the IL-23/IL-17 immune axis has been reported against many bacterial and fungal pathogens [reviewed by 2]. IL-17 has been found to be critical for recruitment of phagocytes that leads to the clearance of *S. pneumoniae* from the mucosal surface of the nasopharynx [293]. Happel et al., found that IL23p19, IL-17 receptor (IL-17R) and IL12p35 deficient mice were more susceptible to infection following intrapulmonary inoculation with *Klebsiella pneumoniae.* They also observed that IL23p19 deficient mice had significantly lower IL-17A and IL-17F production in lungs, and despite the normal IFN-γ levels in the lungs, these mice showed significant mortality from a sublethal dose of bacteria (10^3 CFU). Notably, administration of recombinant IL-17 restored the protection against bacterial infection [294]. In a mouse model of streptococcus lung infection, inhibition of the IL-23/IL-17 axis by morphine resulted in diminished release of antimicrobial proteins S100A8/A9, reduced neutrophil recruitment and more severe streptococcal infection in the lungs [295]. Markel et al., found that exogenous administration of IL-17 or IL-23 had improved survival rates in pulmonary infection with live vaccine strain (LVS) of *Francisella tularensis*, albeit to a limited extent. On the other hand, antibody-mediated neutralization of IL-17 resulted in significantly higher mortality (66.6%) as compared to the control mice infected with sublethal LVS [296].

Although the role of the IL-23/IL17 axis against bacterial pathogens has received much attention, its role in viral infection is less explored, and there are conflicting findings. The role of IL-12, IL-23 and IL-17 on viral host defense in poxvirus infection was evaluated using vaccinia virus (VV) genetically engineered to express IL-12 (VV-IL-12), IL-23 (VV-IL-23) or IL-17 (VV-IL-17) [297]. It was observed that VV-IL-23 and VV-IL-

17 were less virulent in BALB/c mice as compared with VV-IL-12 virus. Additionally, in IFN-γ deficient mice infected with VV-IL-23 neutralization of IL-17 using anti-IL-17mAb resulted in a significant increase in viral titers [297]. These findings suggested a protective role of IL-23/IL-17 axis against VV. On the contrary, Patera et al., reported that VV-IL-17 was more virulent than the parental virus in mice, possibly due to altered generation of IgG isotype antibodies [298]. Similarly, contrasting findings on the role of the IL-23/IL-17 axis during influenza virus infection have also been reported. In mice infected with 100 PFU IAV (PR8), IL-17A and IL-17F was induced as early as two days post-infection [299]. When mice deficient in IL-17R were challenged with PR8 virus, they showed less weight loss and better survival rates than the wild type mice. Also, inflammation was less severe in the IL-17 deficient mice as compared with wild type mice, possibly due to reduced neutrophil infiltration and lower levels of IL-17 induced proinflammatory cytokines in IL-17R deficient mice [299]. In contrast, when c57BL/6 mice challenged with 100 PFU of influenza virus (PR8) for six days were subsequently challenged with 10^8 CFU of *S. aureus*, clearance of both the bacteria and virus were attenuated. In addition, IL-17R and IL-22 deficient mice had impaired bacterial clearance compared to the wild type mice. Furthermore, exogenous supplementation of IL-23 via an adenovirus expressing IL-23 resulted in decreased lung inflammation, increased IL-17A and Th17 chemokines, and increased clearance of bacteria and virus. Therefore, it appears that IL-23 overexpression helps to restore the bacterial immunity and prevent increased susceptibility to secondary bacterial infection [300]. These observations imply that the IL-23/IL-17 axis might have a detrimental as well as protective effect during influenza virus infection. Therefore, to

resolve this question, the relationship between the IL-23/IL-17 axis, influenza virus and secondary bacterial infection needs further investigation.

2.10. Regulation of IL-23 expression

Although IL-23 has protective roles during the early innate immune response, its chronic activation has been implicated in autoimmunity. Thus, a fine regulation of its synthesis and secretion is necessary. IL-23 is a heterodimeric cytokine and formation of biologically active IL-23 (p19/p40 heterodimer) requires synthesis of both the subunits within the same cell. Therefore, regulation of IL-23 specific p19 subunit is critical in determining secretion of biologically active IL-23. Transcriptional regulation of p40 (shared subunit) has been studied well, and it is now known that its expression is controlled by various transcriptional factors such as NF-κB, C/EBP, ets-2, PU.1 and AP-1 [301-305]. However, owing to the recent discovery of p19, very little is known about the transcriptional regulation of IL-23, or more specifically p19. Transcription factor NF-κB was found to be critical for the expression of *IL23p19* in dendritic cells [306] and macrophages [307]. Liu et al. demonstrated that the ERK pathway was essential for *IL-23p19* gene expression. They also identified an Ap-1 element in the *IL-23p19* promoter and established that AP-1 was required for the *IL-23p19* expression [308]. More recently, Goodall et al. investigated the role of the endoplasmic reticulum (ER) stress pathway in the expression of IL-23 in human dendritic cells. They found that ER stress-induced transcription factor CHOP-10 was crucial for the IL-23 expression [309]. Their study suggests that ER stress-induced by invading microorganisms could significantly affect the IL-23-IL-17 mediated host innate immune response.

2.11. Influenza virus and IL-23/IL-17 axis

The effect of influenza virus on IL-23 expression has not been investigated. In human primary macrophages, Pirhonen et al., showed that, unlike Sendai virus, influenza virus did not induce IL-23 [310]. Recently, Kudva et al., investigated inhibition of IL-23 by influenza virus. They found that when IFN-α receptor knockout (IFN-α$R^{-/-}$) mice were challenged by influenza virus and subsequently with *S. aureus*, preceding influenza virus infection did not inhibit IL-23 or IL-22 production in IFN-αR deficient mice. Therefore, they concluded that IAV induced type-IFN (IFN-β) is responsible for inhibition of the IL-23 in lungs [300]. However, Fernandez-Sesma et al., observed that, as compared to the dendritic cells infected with wild type virus, an NS1 deletion mutant influenza virus (PR/8/34 DeltaNS1) induced significantly higher expression of both IFN-β, as well as IL-23p19 [274]. Therefore, a different mechanism must exist for influenza virus mediated inhibition of IL-23. Considering the role of ER stress-induced transcription factor CHOP-10 on IL-23p19 expression, one such mechanism of influenza virus mediated suppression of IL-23-IL-17 axis could be inhibition of CHOP-10.

2.12. Influenza virus and ER stress response

Protein overload in the ER activates a signaling cascade collectively called the unfolded protein response (UPR), which tries to resolve increased protein load. IRE1, PERK and ATF6 are the sensors of ER stress and initiate downstream signaling events to reduce the protein overload and increase cell survival. Activated IRE1 has endoribonuclease activity, and it performs alternative splicing of XBP-1 mRNA with a frame-shift leading to a premature stop codon and production of the active XBP-1 transcription factor [311, 312]. Active XBP-1 then translocates to the nucleus and activates

transcription of ER chaperon genes involved in ER protein folding. ATF6 is an ER resident transmembrane protein which in the event of ER stress is cleaved by serine proteases in Golgi apparatus and translocates to the nucleus where it activates transcription of UPR target genes. PERK is an eIF2α kinase which helps in reducing ER overload by attenuating protein synthesis through phosphorylation of eIF2α [313]. Phosphorylation of eIF2α, however, results in preferential translation of ATF4 that activates downstream targets, including CHOP-10. There are two possible outcomes of the UPR pathway activation - either the cell resolves the protein overload issues and survives or if it fails to resolve the protein overload apoptosis ensues. Levels of CHOP-10 have a crucial role in determining the fate of the cells as its long-term induction results in apoptosis such as in the case of prolonged ER stress in the cell.

The effect of influenza virus on ER stress has been the focus of several studies. Indeed, it was found that IAV infection of primary tracheal epithelial cells of mice activated ATF6 and increased ERp57, but not CHOP-10 [314]. ERp57 is known to be involved in folding of hemagglutinin (HA) protein of influenza [315]; therefore, its upregulation might be favorable for influenza virus replication. Viruses rely heavily on cellular machinery for their replication. Therefore, unlike the induction of ERp57, induction of CHOP-10 might be detrimental to the virus replication. Thus, it is intuitive to speculate that influenza viruses might possess mechanisms to inhibit expression/activation of CHOP-10. Whether influenza virus infection inhibits the expression of CHOP-10 in IL-23 secreting cells such as macrophages is not known and needs to be investigated. NS1 is a multifunctional protein of influenza virus that has been shown to interact with viral RNP complex [316] and is important in viral replication and selective enhancement of viral mRNA translation [317,

318]. Influenza NS1 protein has also been shown to inhibit the induction of important host antiviral proteins- the type-I IFNs. Influenza virus NS1 protein, therefore, seems to be a suitable candidate for the viral factor involved in the modulation of the ER stress response pathway. Whether influenza virus infection inhibits the expression of CHOP-10 in IL-23 secreting cells such as macrophages is not known and needs to be investigated.

2.13. Equine influenza virus

Equine Influenza is a common respiratory viral disease of equids caused by IAV. Based on the reactivity of the HA and NA surface proteins, equine influenza viruses (EIV) have been divided into two subtypes, H7N7 and H3N8 respectively. Of the two EIV subtypes, only the H3N8 subtype is currently in the circulation and responsible for outbreaks of the disease and is endemic to the equine population in the United States and most of the world [319]. The disease is highly contagious and after an incubation period of 1-3 days the clinical picture is characterized mainly by high fever, a serous nasal discharge, dry, harsh nonproductive coughing and swelling of the submandibular lymph nodes [320]. Equine influenza itself is generally non-fatal except in donkeys [321] and mildly affected animals recover within 2-3 weeks. However, in severely affected horses it may take up to six months. Secondary bacterial infections such as *Streptococcus zooepidemicus*, *Pasteurella* and *Actinobacillus spp*. [322] often complicate the disease. In cases of secondary bacterial infections a second febrile response that is generally higher and of longer duration, typically develops 2-3 days after initial fever. In such cases nasal discharge becomes mucoid to mucopurulent; coughing and respiratory distress are more pronounced, and if untreated could lead to severe bronchopneumonia that could be fatal. Exercise and training need to be stopped as they could reduce the rate of recovery. Lost training and

performance due to influenza, therefore, heavily influence the economy of the equine industry. Secondary bacterial pneumonia may further delay the recovery and contribute even more to the losses due to equine influenza. Significant improvements, since the discovery of the virus, have been made in understanding the pathobiology of the virus and development of vaccines. However, despite the regular use of vaccines, outbreaks of equine influenza continue to occur [323, 324]. Thus, research on novel preventive and therapeutic interventions is highly sought after.

CHAPTER 3

Modulation of endoplasmic reticulum stress pathway by influenza virus

3.1. Introduction

Influenza A virus is the etiological agent of one of the most common respiratory diseases of birds and mammals that results in seasonal epidemics as well as occasional pandemics with significant mortality and economic losses. Each year, seasonal influenza epidemics result in 3-5 million severe cases and 250,000-500,000 deaths worldwide. In United States alone, influenza virus infection is estimated to result in 226,000 hospitalizations and 36,000 deaths [325, 326]. Antivirals such as neuraminidase inhibitors and M2 ion channel inhibitors have been used in the past with limited success. Thus annual vaccination is currently the most preferred method for control of influenza. Current vaccines for seasonal influenza are based on predicting the vaccine strain on the basis of surveillance data and carry an inherent risk of failure. Additionally, with current technology it would be difficult to prepare large amounts of vaccines in a limited time. Pathogenesis of influenza involves a complex interplay of host and viral factors. Despite decades of research there are still unresolved areas in the interaction of host cells and influenza virus. Further understanding of influenza virus and the host cell would be a valuable resource in developing new therapeutic and preventive modalities. The current study was designed to understand the interplay between influenza virus and the cellular ER stress response of host.

The ER stress response, also known as unfolded protein response (UPR), is an evolutionary conserved molecular cascade that helps to maintain ER homeostasis and protein folding capacity during ER stress. However, recent advances in the field suggest a

broader range of effects of ER stress response in multiple cellular and disease processes such as apoptosis, inflammation, and metabolism [327-333]. In mammalian cells IRE1, PERK and ATF6 are the sensors of ER stress that initiate downstream signaling events to reduce the protein load on the ER and the ER chaperone immunoglobulin heavy-chain binding protein (BiP) also known as glucose-regulated protein 78 (Grp78) acts as a master controller [334-336]. Activated IRE1 has endoribonuclease activity, and it performs alternative splicing of XBP-1 mRNA production of the active XBP-1 transcription factor [311, 312]. Active XBP-1 then translocates to the nucleus and promotes transcription of ER chaperone genes that are involved in ER protein folding. ATF6 is an ER resident transmembrane protein which in the event of ER stress activates transcription of UPR target genes including chaperone proteins. Activation of PERK leads to phosphorylation of eIF-2α at serine 51 that leads to the inhibition of general protein synthesis to reduce the protein overload in the ER [337].

Since viruses rely heavily on the host cell protein synthesis machinery, the functional status of the ER may significantly affect viral replication and pathogenesis. For example, influenza virus uses ER chaperone protein Erp57 for the folding and maturation of HA protein [315]. Therefore, differential regulation of ER stress could be important in the pathogenesis and replication of viruses [3]. Differential activation of ER stress response pathways has been reported for multiple virus [3, 314, 338]. Although differential regulation of the ER stress response by influenza virus has been shown in tracheal epithelial cells [314, 338], ER stress response of macrophages, important in the pathogenesis as well as control of influenza virus, has not been investigated. Moreover, viral factors involved in the differential activation of ER stress response have not been investigated.

CHOP-10, a key mediator of ER stress response, is a potent inducer of apoptotic cell death [339]. Influenza virus lacking NS1 (delNS1), as compared to the wild type virus, has been reported to induce significantly higher apoptosis. Therefore, the study was designed to test the hypothesis that influenza virus NS1 protein is responsible for differential activation of ER stress response and inhibits the expression of CHOP-10 in mouse macrophage. The hypothesis was tested using recombinant influenza virus lacking NS1 (delNS1) otherwise isogenic to PR8 virus, and Newcastle disease virus (NDV) engineered to express NS1 protein of PR8 virus. The results of study suggest that NS1, at least in part, mediates the inhibition of CHOP-10.

3.2. Materials and methods

RAW264.7 Cells: RAW264.7 cells (RAW hereafter) were used as a surrogate for murine AM. To make sure the observed results are not due to altered physiology due to culture conditions RAW cells used in the study were obtained from two different sources (ATCC and a kind gift from Dr. S. Straley at University of Kentucky). Cells were cultured in growth medium (RAW-GM) (high glucose (4.5g/L) and low sodium bicarbonate (1.5 gm/L) containing DMEM- supplemented with 10% fetal bovine serum (Hyclone) and penicillin-streptomycin solution (CellGrow) to give a final concentration of 100 I.U. penicillin and 100 μg/mL. For the subculture, cells were gently scraped with a cell scraper and resuspended in RAW-GM for seeding at appropriate density into the new culture vessels. For *in vitro* infection experiments, after inoculation cells were cultured in high glucose and low bicarbonate DMEM supplemented with 0.35% BSA and 1ug/mL trypsin (Sigma) (RAW-SFM).

For the mRNA expression studies, $1x10^6$ cells were seeded in 12 well tissue culture plates in duplicate/treatment and incubated overnight at 37°C/5% CO_2. Before infection cells were washed three times with pre-warmed phosphate buffered saline (PBS, pH 7.4) and inoculated with virus as desired multiplicity of infection (MOI) in 200uL final volume of inoculum. Virus was allowed to adsorb for 1hr at 37°C/5% CO_2. After adsorption, cells were washed three times in pre-warm PBS to remove unabsorbed virus. Cells were then fed with pre-warmed RAW-SFM containing 1ug/mL trypsin and incubated for the desired time at 37°C/5% CO_2.

Viruses: Wild type and recombinant PR8 virus lacking NS1 gene (delNS1) generated by reverse genetics (obtained from Dr. Peter Palese and has been described previously [276]). This virus replicates efficiently only in substrate or hosts deficient in the type-1 IFNs (IFN-α/β), therefore the viruses were grown in Vero cells that lack type-I interferon signaling. Vero cells were cultured in DMEM containing 10% fetal bovine serum (Hyclone) and 1X PSA solution (CellGrow) at 37°C/5% CO_2. For virus isolation cell were seeded in roller bottles and medium was supplemented with HEPES (CellGrow) at 20mM final concentration. After inoculation with virus, cells were cultured in DMEM supplemented with 0.35% bovine serum albumin (BSA), 1X PSA solution (CellGrow), HEPES at 20mM and 1ug/mL trypsin (Sigma). After 48 hrs cell culture supernatant was collected and clarified by centrifuging at 3000 RPM for 30 min at 4°C. Clarified virus was further concentrated by pelleting at 26000g for 2hrs at 4°C over 25% sucrose cushion. After centrifugation the supernatant was removed and pellet was resuspended in DMEM supplemented with 5% glycerol and stored at -80°C in single use aliquots. Viral titer was determined by performing TCID50 titer analysis in Vero cells.

Recombinant B1 vaccine strain of Newcastle disease virus expressing NS1 of PR8 virus (NDVB1-NS1) and parent virus (NDVB1) were a kind gift from Dr. Adolffo Garcia-Sastre (Mount Sinai, NY). Recombinant NDV viruses were generated by reverse genetics as described previously [340]. Design of the NDV constructs is given in figure 3.1. The viruses were grown in the 10 day old chicken embryos and allantoic fluid was harvested 48 hrs after inoculation. Allantoic fluid was clarified by centrifugation at 3000RPM for 30 min at 4°C. Virus was distributed as single use aliquots and stored at -80°C. EID50 titer was determined following method the method of Reed and Meunch [341].

RNA extraction and Real-time PCR: Total cellular RNA was extracted using Purelink® RNA mini kit (Life Technologies) with on column DNase digestion using Purelink® DNase (Life Technologies). At the desired time point, media was removed from the wells and cells were washed once with PBS. Cells were lysed in the wells with 600uL of RNA lysis buffer supplied with the kit. Cell lysates were homogenized using shredder columns (Qiagen) followed by RNA extraction protocol with on column DNase digestion supplied with the Purelink® RNA mini kit (Life Technologies). Total cellular RNA concentration and purity was determined by UV spectrophotometry using Nanodrop™ and a total of 1µg total RNA was reverse transcribed using high capacity RNA-to-cDNA© kit (Life Technologies) following protocol supplied by the manufacturer. Relative mRNA expression of the markers of different ER stress pathways (Table 3.1) was analyzed by quantitative real-time PCR using commercially available pre-validated Taqman© assays (Applied Biosystems) and Taqman® Universal Master Mix II no UNG (2X) (Applied Biosystems) following manufacturers recommendations. Briefly, 20 µL cDNA was diluted to a final volume of 100 µL and a 4.5 µL cDNA was used in real-time PCR reaction with

a final volume of 10uL with 1X final concentration of Taqman® assay and mastermix. Each sample was run in duplicate on ABI 7900HT sequence detection system (Applied Biosystems) following the standard protocol suggested by the assay manufacturer. Amplification data was exported to LinregPCR [342] for the calculation of efficiency of real-time PCR reactions. LinReg PCR efficiency calculations were based on the slope of linear regression line containing 4-6 data points. For relative quantitation, only the reactions with efficiency ranging between between 2± 0.2 (2 =100% efficiency) and R^2 (squared correlation coefficient) greater than 0.98 were used for data analysis. Relative expression was analyzed by the ΔΔCT method [343]. Murine GAPDH was used as an internal control and mock infection was used as a calibrator for relative expression analysis.

Western blotting: For immunoblotting, $5x10^6$ RAW264.7 cells were plated in a 60 mm tissue culture dish and incubated overnight. Cells were infected with the respective viruses at a MOI of 2 $TCID_{50}$/cell as described above. Two sets of cells were mock inoculated. While one of the mock inoculated cells served as negative control, the second one was treated with 0.025uM Thapsigargin (TG, T-7459, Life Technologies) for 12 hrs and served as positive control. At 20 hrs PI cells were washed twice with cold Ca^{++} and Mg^{++} free PBS (pH 7.4) and cells were harvested by gentle scraping. Scraped cells were resuspended in cold PBS and centrifuged at 600g/5min at 4ºC. The Supernatant was carefully removed, and cells were lysed by resuspending in 200 µL RIPA lysis buffer (Santacruz biotechnology) supplemented with Halt protease and phosphatase inhibitor cocktail™ (ThermoScientific Pierce) at 1X final concentration following manufacturer's recommendation. Cells were kept on ice during lysis. Total cell lysates were clarified by centrifuging at 16000g/5 min 4ºC. Total cell protein obtained was transferred to 1.5 ml

microcentrifuge tubes as single use aliquots. Protein concentration was determined using the BCA protein assay kit© (Thermoscientific Pierce) using the microplate procedure supplied with the kit. For immunoblotting, approximately 30 µg of whole cell protein was diluted 1:5 in denaturing protein loading buffer supplemented with protease inhibitors and boiled for 5 min at 95 ^{o}C, cooled and then loaded for electrophoretic separation. Proteins were separated by electrophoresis on 12% polyacrylamide gels (Laemmli, 1970), and subsequently transferred to nitrocellulose membranes by semi-dry electrophoretic transfer in Tris-glycine buffer (pH 8.3). The membranes were blocked for 1hr with PBS containing 5% non-fat dry milk (NFDM), 5% normal goat serum (NGS), and 0.05% Tween 20, followed by primary antibody (anti-Influenza NP (Fitzgerald, Concrod, MA) and anti-murine CHOP-10 (Santacruz biotechnology)) incubation for overnight at 4 ^{0}C in PBS containing 0.1% normal goat serum (Sigma) and 0.1% nonfat dry milk (Carnations, Nestle). After multiple washes, the membranes were incubated with peroxidase-conjugated anti-rabbit (IgG-HRPO) secondary antibody (Jackson Immunoresearch Labs, Inc.) for 1 hr. The membranes were then washed and incubated with Supersignal substrate (Pierce, Rockford, IL, USA) for chemiluminescence detection, and visualized with a FluorChem 8800 imaging system (Alpha Innotech, San Leandro, CA).

Immunofluorescence assay: Indirect double immunofluorescence staining was used to localize CHOP-10 and the influenza virus NP protein. For the immunofluorescence assay, $1x10^{6}$ cells were seeded into the 12-well tissue culture plates with coverslip inserts. Cells were infected with either PR8 or delNS1 virus at MOI of 2 $TCID_{50}$/cell. For a negative control, cells were mock infected with medium alone and for a positive control of CHOP-10, mock-infected cells were treated with 0.025uM TG during last 7 hrs of infection. At 20

hrs PI, cells were rinsed twice with cold PBS and then fixed with 4% paraformaldehyde for 10 min at room temperature. After washing once with cold PBS, cells were permeabilized with 0.2% Triton X-100 (Sigma) in PBS for 10 min at room temperature. Nonspecific antibody-binding sites were blocked by incubation of cells with 10% normal goat serum (Sigma) in PBS for 1hr. Mouse monoclonal primary antibody against influenza virus NP protein (Fitzgerald, Concord, MA) and polyclonal rabbit antibody against murine CHOP-10 (SC-575, Santacruz Biotechnology) were diluted in blocking solution at 1:100 and 1:50 dilution, respectively and reacted with cells for 1hr at room temperature. After washing three times for 5 min each wash, cells were incubated at room temperature for 30 min with FITC-conjugated goat anti-mouse IgG antibody (Pierce, Rockford, IL) and Texas red conjugated goat anti-rabbit IgG antibody (1:400 dilution in blocking buffer). Cells were washed three times with PBS, and then coverslips were carefully blotted to remove excess liquid. A small drop of Vectashield H-1200 (Vector labs) anti-fade medium containing DAPI was placed on the coverslips and coverslips were mounted on to clean glass slides. Slides were examined using Zeiss Axioplan-2 fluorescence microscope and images were captured with cytovision /Genus™ software application.

Statistical analysis: Statistical analysis of data was performed by one way ANOVA, and means of treatment groups were compared using Fishers least significant difference test using GraphPad (Prism6.0) software.

3.3. Results

NS1 inhibits expression of BiP/Grp78 (Hspa5): BiP/Grp78 has been suggested as the master regulator of the ER stress pathway therefore effects of influenza virus NS1expression on the induction of BiP were studied first. The hypothesis was that NS1

inhibits the ER stress response. So we expected that virus lacking NS1 (delNS1 and NDVB1) will induce a higher expression of BiP as compared to the respective parental viruses. In order to determine the role of NS1 on BiP mRNA expression, RAW cells were infected either with PR8 virus or delNS1 and either with NDVB1 virus or NDVB1-NS1 virus at MOI of $2TCID_{50}$/cell. Cells were incubated at 37 C/5% CO2 for 20 hrs after infection. Two sets of cells were mock infected to serve as controls. TG is a competitive inhibitor of Sarco/endoplasmic reticulum Ca++ [344] and a potent inducer of ER stress. Thus, one set of mock-infected cells was treated with TG (0.025uM) for the last 7 hrs of incubation to serve as a positive control, while mock-infected cells were used as a negative control and calibrator for relative expression analysis. Results of BiP mRNA expression are presented in Fig 3.2. Being a potent inducer of ER stress TG, as compared to all other treatments, induced significantly higher expression of BiP mRNA($P<0.0001$). While relative expression of BiP mRNA in the delNS1 virus treated cells was significantly higher than the PR8 virus ($P<0.0097$), it did not differ significantly from the mock-infected cells. Relative expression was significantly lower in the PR8 virus infected cells ($P= 0.0114$) as compared to the mock-infected cells, as well as TG treated cells. These results suggest that virus infection resulted in the inhibition of ER stress, however it was more pronounced in the wild type PR8 virus. When compared to the mock-infected cells, neither NDVB1 nor NDVB1-NS1 showed any significant difference ($P>0.05$). However, relative expression of BiP mRNA was significantly lower in the NDVB1-NS1 infected cells as compared to the NDVB1 infected cells ($P= 0.0312$). Together these data suggest that influenza virus NS1protein causes downregulation of BiP protein in the RAW264.7 cells.

Effect of NS1 on IRE1 Pathway: The IRE1 mediated branch of ER stress response was evaluated by determining the relative expression of total XBP1 (unspliced) mRNA expression as well as by determining the relative expression spliced form of XBP1 mRNA in cells treated as above. Results are presented in Fig 3.3. As compared to all other treatments, TG treated treatment resulted in a significantly higher expression of total as well as spliced XBP1 mRNA ($P<0.0001$). DelNS1 virus induced a significantly higher expression of Total XBP1 mRNA as compared to the PR8 infected ($P = 0.0056$) or mock infected cells ($P =0.005$), suggesting that NS1 does inhibit XBP1 mRNA expression. However, neither PR8 nor mock cells showed a significant difference in the mRNA expression of spliced XBP-1. Although statistically not significant ($P= 0.0507$), expression of spliced XBP-1 mRNA was approximately two-fold higher in the cells infected with delNS1 virus as compared to the PR8 virus infected cells. As compared to the mock-treated cells, both NDVB1 and NDVB1-NS1 induced significantly higher expression of total XBP ($P=0.0031$ and 0.0079, respectively). While NDVB1, as compared to the mock-treated cells, showed significantly higher expression of spliced XBP1 mRNA ($P=0.0344$), there was no significant difference in the expression of spliced XBP1 mRNA between NDVB1-NS1 and mock-treated cells ($P=0.1361$). These results indicate that influenza virus NS1 inhibits the IRE1 pathway of ER stress response by inhibiting the endoribonuclease activity of IRE1, as well as downstream mediator XBP-1.

Effect of NS1 on ATF6 pathway: Expression of chaperone genes such as ERDJ3, GRP94 and ERP72, at least partly, depend on ATF-6 for their full upregulation [345, 346]. Therefore, to investigate ATF6 activation, mRNA expression of chaperone genes was analyzed. Results of this experiment are presented in fig 3.4. DelNS1 virus, as compared

to the PR8 virus, induced significantly higher expression of chaperone genes ERDJ3 (P=0.0029) and GRP94 (P=0.047). However, PR8 virus, as compared to delNS1 virus, induced significantly higher expression of ERP72 (P<0.0029). While expression of ERDJ3 and GRP94, were lower in the PR8 virus infected cells (P=0.0003 and 0.0015, respectively); expression of ERP72 mRNA was significantly higher (P<0.0003) in the PR8 infected cells as compared to the mock-infected cells. There was no significant difference in the expression of chaperone genes between NDVB1 and NDVB1-NS1 viruses (P= 0.1447, 0.1079, 0.1447 for ERDJ3, GRP94 and ERP72, respectively). These data suggest that influenza virus differentially regulate the expression of ATF-6 pathway that may involve NS1 mediated as well as NS1 independent mechanisms.

Effect of NS1 on PERK pathway: Activation of the PERK pathway leads to selective enhancement of CHOP-10 transcription; therefore to analyze the PERK pathway, mRNA expression of CHOP-10 was examined in cells treated as above. Results are presented in Fig 3.5. Cells treated with TG showed a significantly higher expression of CHOP-10 (P<0.0001), as compared to all other treatments including mock and virus infections. While delNS1 virus, as compared to the PR8 virus, induced significantly higher expression of CHOP-10 (P=0.456), there was no significant difference in the expression of CHOP-10 between NDVB1 and NDVB1-NS1 infected cells (P=0.1029). However, expression of CHOP-10 was approximately two-fold lower in the NDVB1-NS1 infected cells, as compared to the NDVB1 infected cells (mean relative expression 6.815 and 3.275, respectively). DelNS1 infected cells showed approximately five-fold higher expression of CHOP -10, as compared to the mock-infected cells (mean relative expression 4.93 and 1, respectively). On the other hand, expression of CHOP-10 was approximately three-fold

lower in the PR8 infected cells as compared to the mock-infected cells (mean relative expression 0.3 and 1, respectively).

Differences in the CHOP-10 expression levels between PR8 and delNS1 virus infected cells were also reflected on the protein levels. Western blotting was performed on total cellular protein from the cells treated as above. Western blotting was performed using anti-CHOP -10 antibody (SC-575, 1:250 dilution, Santacruz Biotechnology) and anti-actin (1:1000 dilution, cell signaling technology) was used as loading control antibody. Results are presented in Fig 3.6. In the TG treated cells as well as delNS1 infected with delNS1 virus, higher levels of CHOP-10 protein were detected. On the other hand, CHOP-10 protein was barely detectable in the PR8 virus or mock infected cells. During the ER stress response, once activated CHOP-10 translocates to the nucleus where it activates transcription of other genes that have CCAT elements. Therefore, translocation of CHOP-10 was examined using double immunofluorescence using anti-CHOP-10 (SC-575) and anti-influenza NP antibodies in the delNS1 virus and PR8 virus infected cells. Mock infected cells were used as a negative control, and TG treated cells were used as a positive control. IFA analysis revealed that there was no detectable level of CHOP-10 in PR8 infected cells (Fig 3.7). On the other hand, delNS1 infected cells did show expression of CHOP-10 that was mainly localized in the nucleus. Taken together these data suggest that influenza virus inhibits the activation of PERK pathway. Furthermore, inhibition of CHOP-10, at least in part, is mediated by NS1.

3.4. Discussion

In the recent years, the ER stress response has emerged as a critical player in the pathogenesis and replication of viral infections and several groups have reported a

differential regulation of ER stress response pathway by viruses including influenza [3, 314, 338]. One of the earliest reports on ER stress linking to influenza showed that overexpression of a mutated, misfolded form of influenza HA protein induces ER stress in the simian cells [347]. Recently, differential activation of UPR pathways by influenza virus infection was investigated in murine tracheal epithelial cells [314] and human tracheobronchial epithelial cells [338]. Macrophage cells have been implicated in the pathogenesis [299] as well as in protection [348] from influenza virus; hence activation of ER stress pathway in these cells may play a critical role in the pathogenesis of influenza. Therefore, the current study was designed to examine the ER stress pathway in influenza virus infected macrophage cells. Furthermore, this study, to best of my knowledge, also appears the first to examine the role of viral NS1 protein in the ER stress response pathway. ER stress-induced transcription factor CHOP-10 has been shown to induce apoptosis as well as regulate the expression of certain cytokines such as IL-23 [4] and thus may have important implications in the replication, pathogenesis and immune response against influenza. Therefore the focus of this study was CHOP-10.

Overall, it was found that in murine macrophage cell line RAW264.7, influenza virus infection resulted in inhibition of the IRE1 as well as PERK pathway. We also observed that PR8 virus resulted in slight activation of ATF6 pathway with the exception of chaperone protein ERP72 that was significantly upregulated by PR8 virus. Preferential activation of the ATF6 pathway was also observed in murine tracheal epithelial cells where influenza infection resulted in activation of ATF6 and an increase in ERp57 but not CHOP-10 [314]. However, in contrast to the present study, in HTBE cells, influenza virus activated the IRE1 pathway with little or no concomitant activation of PERK or ATF-6

pathway [338]. These differences might be explained by the differences between cell systems and time points for ER stress analysis. Macrophage cells are potent APCs and secretory cells thus these might be more adept at handling the protein overload as compared to the epithelial cells; thus macrophages cells may be more resistant to ER stress. Furthermore, Hassan et al., [338] studied the ER stress response at early time point (12 hrs) but present study and study by Roberson et al., [314] analyzed the ER stress marker expression at later time point (20 hrs or later).

Most of the ER chaperone proteins depend on induction of the ATF-6 pathway, therefore it is conceivable that up-regulation of chaperones might be crucial for viral replication by preventing severe ER stress and eventual cell death. ERp57, a member of protein disulfide isomerase (PDI) family of protein, is critical in the folding of influenza virus HA [315]. ERp72 is also a member of PDI family of proteins and closely related to ERp57. Therefore, upregulation of ERP72 might help in viral protein folding. Inhibition of the ER stress response was reported to be critical in influenza viral replication. Tauroursodeoxycholic acid (TUDCA), a naturally occurring bile salt, has been shown to possess molecular chaperone properties, and to alleviate ER stress response in vitro as well as in vivo [332]. Restoration of the ER stress response using treatment of cells with TUDCA, prior to influenza virus infection of HTBE cells resulted in significantly lower titers of influenza virus as compared to the TUDCA non-treated control cells. While viral RNA replication was not affected, there was markedly reduced viral protein synthesis in TUDCA treated cells as compared to the non-treated cells, suggesting that influenza virus required an impaired ER stress response for efficient viral replication [338]. Differential activation of ER stress pathways has also been reported in other viruses. While hepatitis C

virus induces the ATF6 pathway but blocks the IRE1 pathway [349], hepatitis B virus induces ATF6 and IRE1 but not PERK [350]. As African swine fever virus (ASFV) uses ER as a site for the assembly and maturation it is expected to induce ER stress, however, in ASFV-infected cells, it did not induce activation of PERK pathway [351]. Currently, the only known mechanism of activation of PERK, ATF6, and IRE1 is their release from BiP. The mechanism behind differential activation of the different arms of the UPR is unknown [3].

Activation of PERK leads to the phosphorylation of eIF2α that results in host protein shutdown to resolve the ER stress. Phosphorylation of eIF2α, however, results in preferential translation of ATF4 that activates downstream targets, including CHOP-10. Induction of CHOP-10 and the consequent apoptosis might play a critical role in the development of viral cytopathic effects, viral spread and pathogenesis. For example, UPR was associated with induction of apoptosis in virus infected cells in Japanese encephalitis virus [352], bovine viral diarrhea virus [353], tula virus [354], severe acute respiratory syndrome coronavirus (SARS-CoV) [355]), and West Nile virus [356]).

In the present study, we found that influenza virus, although induced ER-associated chaperone, did not induce CHOP-10. Further analysis of delNS1 virus and PR8 virus revealed that NS1 protein was involved in the inhibition of CHOP-10 expression and activation. Similar to our finding, AFSV, an ER tropic virus that was expected to induce CHOP-10, was found not to inhibit induction and activation of CHOP-10 [351]. Although, induction CHOP-10 and consequent host cell death by apoptosis may help in viral pathogenesis, it may also affect the viral replication. Therefore, viruses might have evolved with the mechanism to regulate the induction of ER stress associated apoptosis to facilitate

their replication. In support of our hypothesis, a recent study found that although influenza virus-induced ATF6 pathway it did not induce CHOP-10 [314].

Comparison of expression of ER stress response markers in wild type and mutant virus either lacking NS (PR8 vs. delNS1) or expressing NS1 (NDVB1 vs. NDVB1-NS1) suggests that NS1, at least in part, plays some role in the differential activation of ER stress pathway. Moreover, higher expression of ERP72 was present in the viruses that expressed NS1 protein (PR8 and NDVB1-NS1) suggesting that the NS1 protein might participate in differential upregulation of ERp72. Moreover, influenza virus lacking NS1 (delNS1) have been reported to induce apoptosis earlier as compared to the wild type virus [195]. Therefore, it seems possible that influenza virus NS1 protein might be the viral factor involved in the inhibition of CHOP-10. In conclusion, our results support the hypothesis that influenza virus infection results in a differential activation of UPR pathway that, at least in part, may be dependent on the NS1 protein of influenza virus.

Table 3.1: List of Taqman® assays used in this study

Target	Other name	Taqman ®Assay ID
BiP	Hspa5	Mm00517690_g1
ERP72	Pdia4	Mm00437958_m1
ERdj3	Dnajb11	Mm00518196_m1
GRP94	Hsp90b1	Mm00441926_m1
XBP-1 (U)	XBP1 unspliced	Mm00457357_m1
XBP-1 (S)	XBP1 spliced	Mm03464496_m1
CHOP-10	DDIT3, Gadd153	Mm01135937_g1
GAPDH	GAPD	Mm99999915_g1

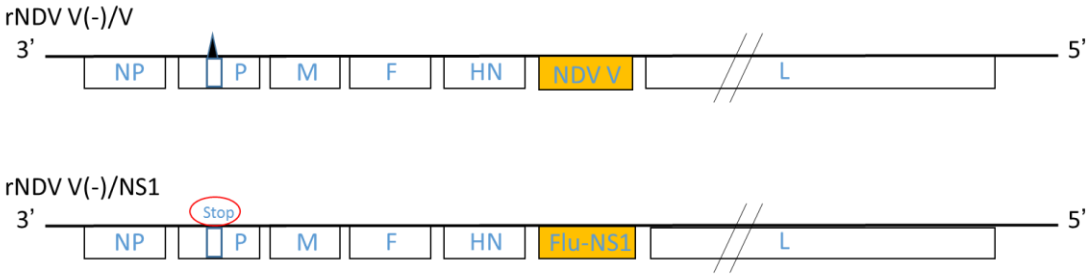


Figure 3.1: Schematic representation of NDVB1 and NDVB1-NS1 constructs

Adapted from: Park M et al. J. Virol. 2003; 77: 9522-9532

Figure 3.2: Effect of influenza virus on BiP/GRp78 mRNA expression

Data are presented as mean ± SD and are representative of at least two independent experiments carried out in duplicate.

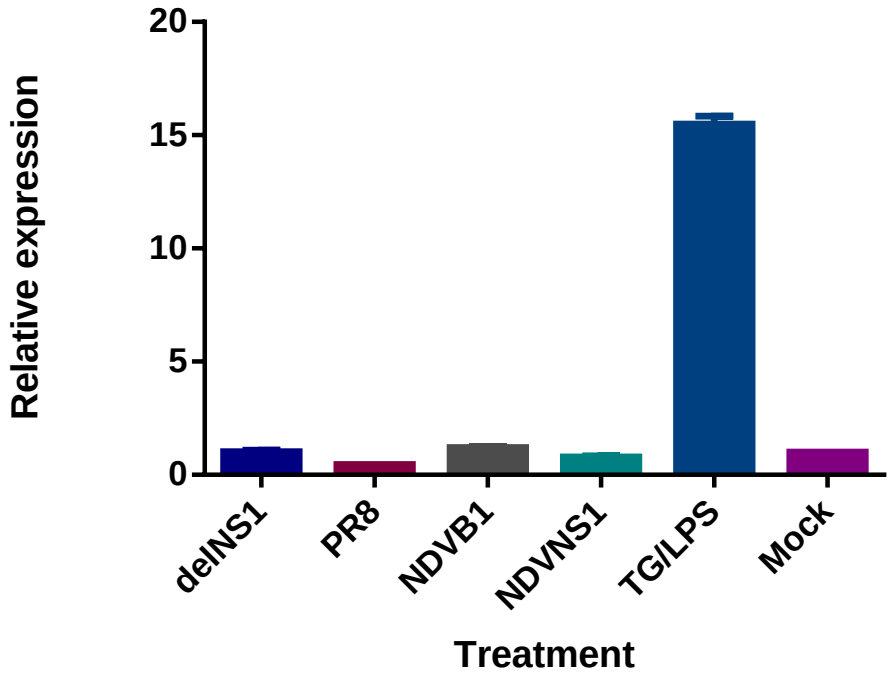


Figure 3.3: Effect of influenza virus on XBP1 mRNA expression

Data are presented as mean ± SD and are representative of at least two independent experiments carried out in duplicate.

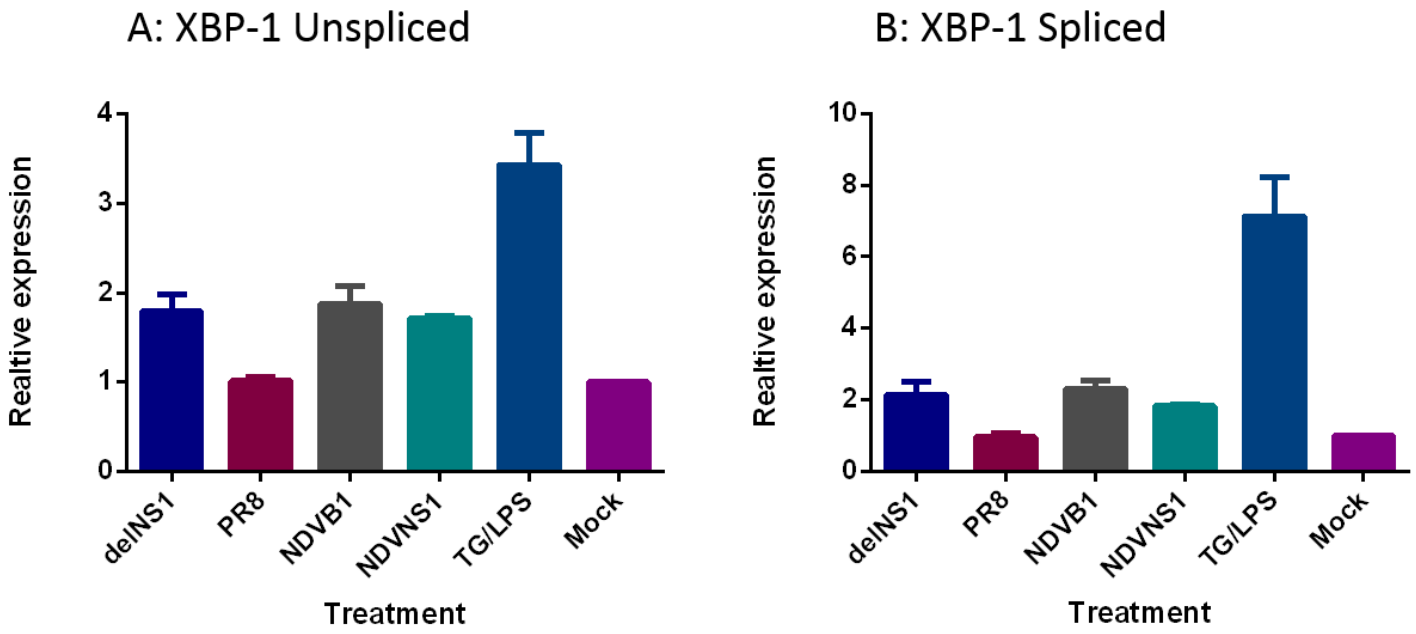


Figure 3.4: Effect of influenza virus on ATF6 pathway

Data are presented as mean ± SD and are representative of at least two independent experiments carried out in duplicate.

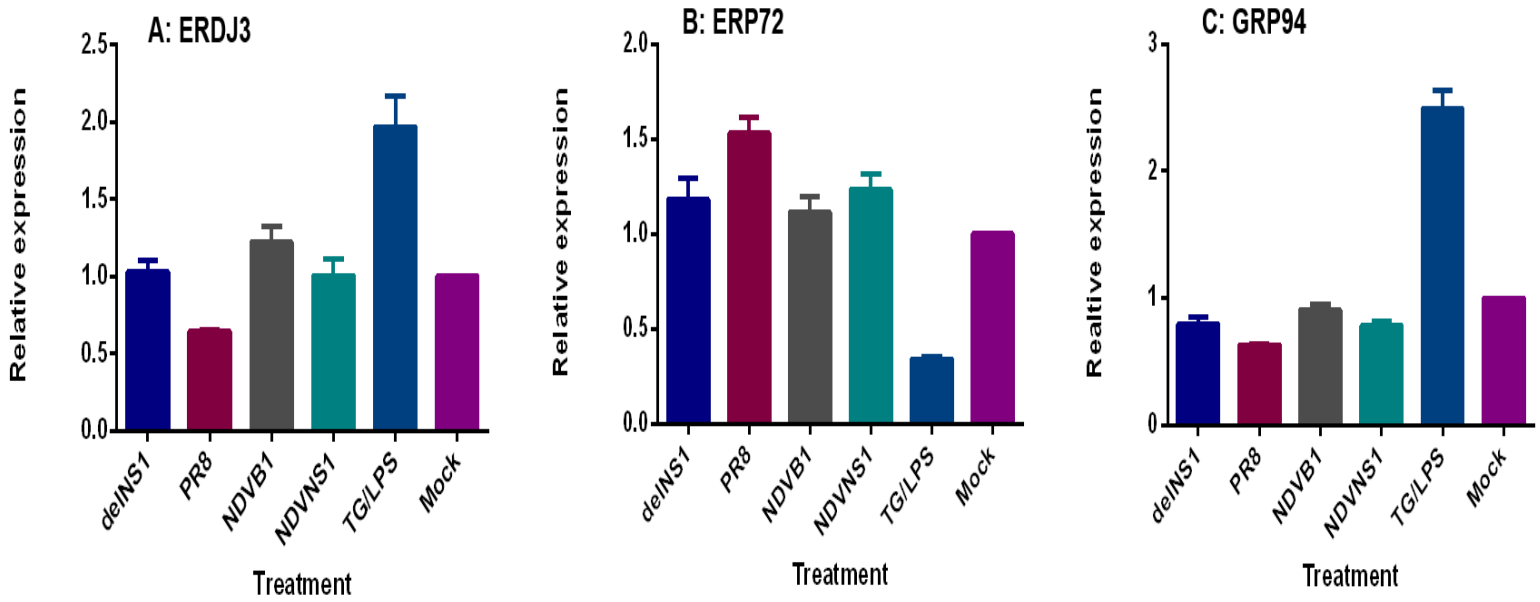


Figure 3.5: Effect of influenza virus on expression of CHOP-10 mRNA

Data are presented as mean ± SD and are representative of at least two independent experiments carried out in duplicate.

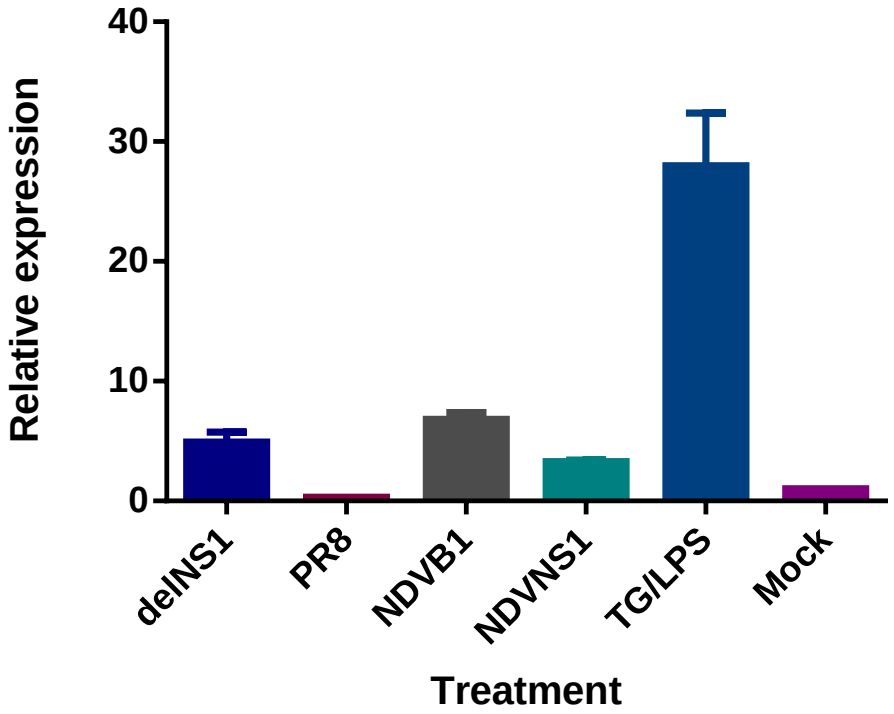


Figure 3.6: Effect of influenza virus on expression of CHOP-10 protein

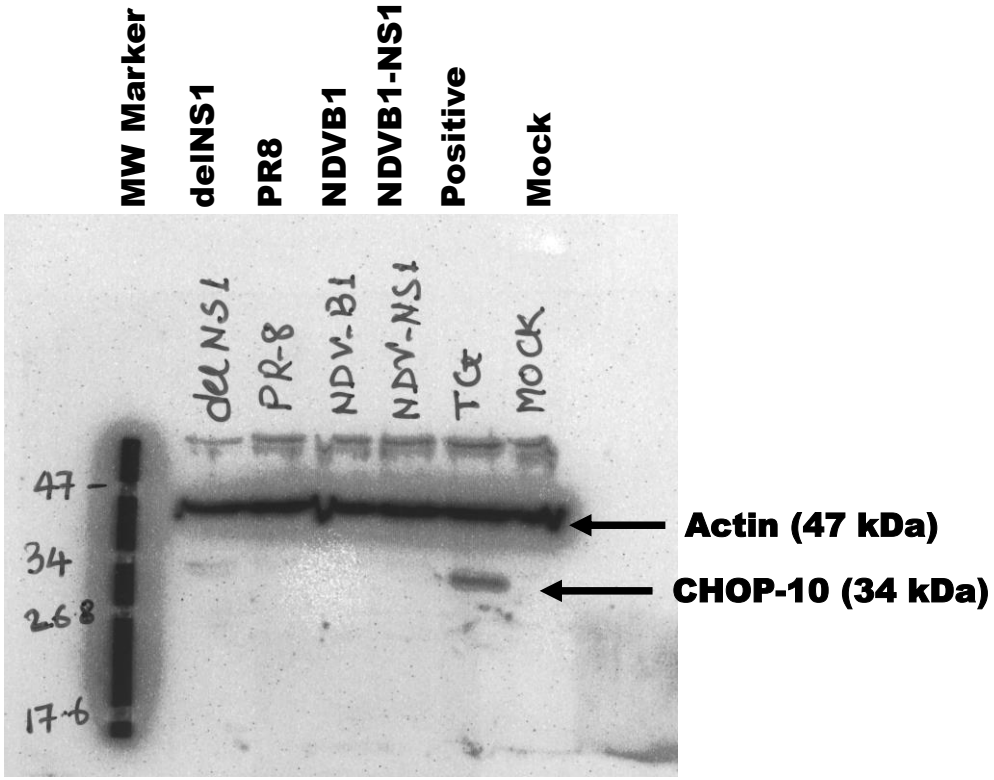
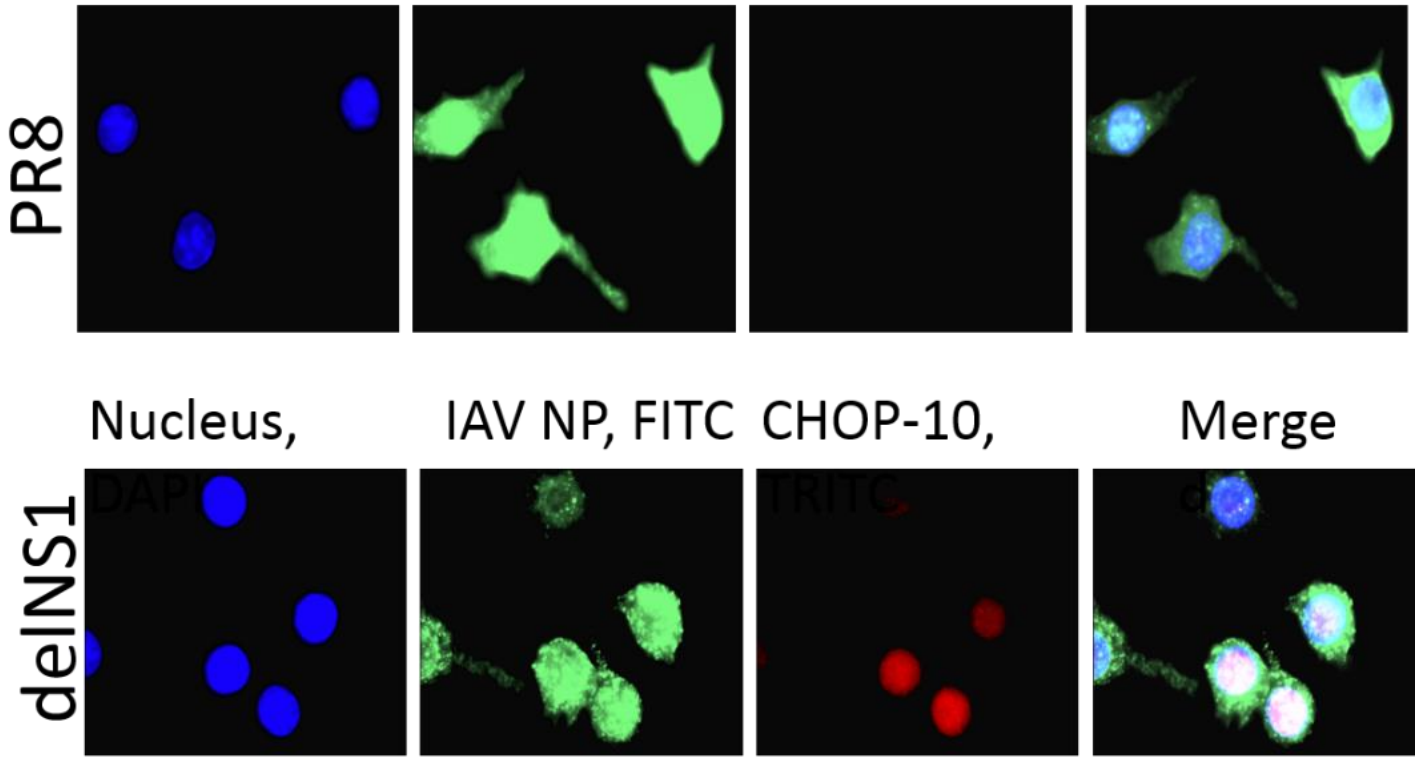


Figure 3.7: Effect of influenza virus on activation of CHOP-10

Nucleus is stained with DAPI (Blue), Influenza virus nucleoprotein detected with FITC conjugated secondary antibody (green) and CHOP-10 detected with Texas red conjugated secondary antibody. 100X magnification.



CHAPTER 4

Influenza A virus NS1-mediated inhibition of CHOP-10 downregulates IL-23

4.1. Introduction

Influenza virus is a negative-sense single-stranded RNA genome-carrying virus that causes one of the major respiratory diseases worldwide. Recent epidemiological and laboratory studies provide a strong evidence that secondary bacterial pneumonia is the major cause of death during influenza [209]. With emerging drug-resistant strains of both influenza virus and bacteria, along with the looming threat of pandemic influenza, alternate therapeutic measures become important. Pathogenesis of influenza virus depends on intricate interaction of viral and host cellular factors, and the host innate immune responses. Thus understanding these interactions would be important in identifying new therapeutic targets.

IL-23 is a recently identified member of IL-12 family of heterodimeric cytokines that is essential for proliferation of IL-17 producing Th17 cells. IL-17 has been shown to induce other cytokines that help recruit other critical cells such as macrophage, monocytes and neutrophils. IL-23/IL-17 mediated immune responses have been implicated in control of a variety of respiratory bacteria [279], however, its role during influenza virus infection, especially in the setting of bacterial co-infection, has just begun to be appreciated. In a recent study, Kudva et al., reported that influenza virus-induced type-I IFN resulted in inhibition of IL-23/IL-17 that caused increased susceptibility of the host to secondary bacterial infection [300]. However, influenza virus is known to inhibit type-I IFN response. Thus, there is a possibility that another mechanism might be involved in the influenza virus mediated inhibition of IL-23.

Multiple cellular transcription factors have been proposed to control the regulation of IL-23, including CHOP-10 [4]. In previous experiments (Chapter III) influenza virus was found not to induce the expression of CHOP-10. Therefore, considering these factors, it was hypothesized that influenza virus NS1 mediated CHOP-10 inhibition results in the downregulation of IL-23/IL17 pathway. In order to test this hypothesis, expression of IL-23 was studied *in vitro* in murine macrophages as well as *in vivo* in a influenza mouse model.

4.2. Materials and methods

Cells: Vero cells and RAW264.7 cells were cultured as described in the previous chapter. Mouse primary alveolar macrophage cells (AM) were isolated and purified by a method described previously [357] with minor modifications. Briefly, 6-8 week old female c57BL/6 mouse (Harlan) was euthanized by intraperitoneal injection of 200 µL Buthanasia-D™. Mouse was then sprayed with 70% ethanol, and the abdominal cavity was carefully opened to bleed the mouse by severing abdominal aorta. Trachea was exposed aseptically, and a small puncture was made with a sterile needle to insert a sterile catheter made in-house using 20G Tygon® tubing. Catheter was attached to a three-way valve connected to two 5cc syringes. One ml warm PBS (37°C) was infused through the trachea with one syringe then the inlet valve was closed. The outlet valve was then opened and while gently massaging the thoracic cavity fluid was aspirated into the second syringe. After this, the valve was switched to the inlet syringe, and the process was repeated for a total of 5 mL PBS. The BAL fluid thus obtained from several mice was pooled and centrifuged at 400g/10 min at 4°C to pellet the cells. If BAL cells were contaminated with red blood cells (RBC), the cell pellet was resuspended in 1ml ACK lysing buffer (Gibco)

for 30 sec to lyse the RBC then washed again with PBS. The Cell pellet was resuspended in RPMI-1640 (Invitrogen) medium supplemented with 10% FBS and 1X PSA solution (AM-growth medium). Cell concentration was determined using a hemacytometer and adjusted to $1x10^6$/mL in AM-growth medium. In order to obtain pure population of alveolar macrophages, BAL cells were plated in 100 mm tissue culture dishes and incubated at 37°C/5% CO_2 for two-four hours with gentle rocking at every hour. After incubation, the plate was gently rocked and tilted to collect aspirate medium containing unattached lymphocytes and other cells. Attached cells are then washed once with growth medium and then scraped, counted and seeded into desired culture vessels as per requirement of the experiment. After adherence step, the cells are usually in ≥90% pure population of macrophage and this method usually yields approximately $3\text{-}5x10^5$ AM cells /mouse [357].

Infectious agents: PR/8/34 strain (PR8) of IAV and an isogenic recombinant virus lacking NS1 (delNS1) as described in the previous chapter (chapter III) were used in the current study.

For the bacterial infection, *Streptococcus zooepidemicus* (*S. ze*) strain 7e was used (kind gift from Dr. John Timoney at Gluck Equine Research Center). The bacteria was isolated from a clinical case of bronchopneumonia in donkey. One day before mouse inoculation, bacteria were freshly streaked on Columbia CNA (Sigma) blood agar containing 5% horse blood and incubated for 24 hrs at 37°C/5% CO_2. A single isolated colony was inoculated in 4 mL Todd-Hewitt broth for 6 hrs to achieve log growth phase. After 6 hrs, bacterial culture was pelleted at 6000g/4ºC for 20 minutes and then washed twice with normal saline. Bacterial pellet was resuspended to $1x10^8$ CFU/mL in normal saline. Bacterial concentration was confirmed retrospectively in the inoculum by plating

serial dilutions on Columbia CNA (Sigma) blood agar containing 5% horse blood, and colonies were counted at 24 hrs after incubation and results were presented as colony forming units per mL (CFU/mL).

Infection of cells with the virus: For the mRNA expression studies in RAW264.7 cells, cells were cultured and infected as described in the previous chapter. For mRNA expression studies in the primary murine alveolar macrophage, $0.5x10^6$ purified cells were seeded in 24 well tissue culture plate and allowed to rest overnight. For infection, cells were washed three times with pre-warmed PBS and inoculated with virus at 2 MOI in 100uL final volume of inoculum prepared in serum free RPMI-1640 (Gibco) supplemented with 0.35% BSA (Lampire Biologicals), 1x PSA (CellGrow) and 1ug/mL trypsin (Sigma). Virus was allowed to adsorb for 1hr at 37°C/5% CO_2. After adsorption, cells were washed three times in pre-warm PBS to remove unabsorbed virus. Cells were then fed with pre-warmed serum free RPMI-1640 supplemented with 0.35% BSA (Lampire Biologicals), 1x PSA (CellGrow) and 1ug/mL trypsin (Sigma). Cells were transferred to the incubator and incubated for 20hr at 37°C/5% CO_2.

RNA extraction and Real-time PCR: Total cellular RNA, cDNA synthesis and real-time PCR using predesigned validated Taqman® assays (see Table 4.1) and relative expression analysis was carried out at described in chapter III.

Stable expression of CHOP-10: An **e**xpression plasmid containing murine CHOP-10 cDNA under CMV promoter and neomycin resistance as a eukaryotic selection marker was purchased from Origene (pCMV-Kan/neo) and used to generate RAW264.7 cells stably expressing murine CHOP-10. RAW264.7 cells were transfected using Targafect-RAW transfection reagent system (Targeting systems) following protocol supplied with the kit.

For the selection of stable clones, 24 hrs after transfection, cell culture medium was replaced with growth medium containing 0.6 mg/mL neomycin (G-418, Sigma) and continued to be grown with replacing fresh selection medium every two days until individual clumps of cells appeared. Individual clumps of the cells were then lifted with cell scraper and transferred to separate cell culture dishes and cultured and passaged in selection media. Stable clones were screened for expression of CHOP-10 using a Taqman® assay as described earlier in this chapter. Stable clones of empty vector were prepared as described above and were used as a negative control.

Lentivirus mediated knockdown of CHOP-10: Commercially available pre-validated lentiviral particles for CHOP-10 shRNA and control shRNA were purchased from Sigma. Lentiviral transduction was performed following manufacturer's protocol. Briefly, the day before transduction cells were seeded to achieve about 70% confluency on the day of transduction. Immediately before transduction the cell culture medium was replaced with growth medium containing hexadimethrine bromide (Sigma) at a final concentration of 8 µg/mL followed by addition of lentiviral particles to achieve MOI of five. Cell culture medium was replaced at 24 hrs after transduction and followed by adding selection media containing 0.5ug/mL puromycin on the next day. Fresh selection medium was added every two days until individual clones of stable cells appeared. Individual clones of the cells were then carefully lifted and transferred to the new culture dishes and cultured in selection medium until ready for passage. Selection was continued for at least two weeks before screening the clones for efficiency of knockdown which was analyzed by real-time PCR using Taqman® assay as described earlier in this chapter.

Animals: Six-eight week old female c57BL/6 mice were purchased from a commercial supplier (Harlan). On arrival, mice were housed in microisolater cages with *ad libitum* supply of food and water. Mice were acclimatized for one week before infection and examined daily for any signs of stress or illness following the guidelines of Institutional Animal Care and Use Committee (IACUC) at University of Kentucky. All the animal work was approved by IACUC at University of Kentucky.

Inoculation of mice: Mice were sedated with ketamine/xylazine administered intraperitoneally. Once sedated mice were held upright by holding from the loose scruff at the neck. A small drop of inoculum was placed near each nostril so that mice involuntarily inhaled the inoculum. Inoculum was instilled from alternating the nostrils so that whole inoculum (60uL) was divided between two nostrils. After inoculation mice were transferred back to the microisolater cages with *ad libitum* supply of fresh food and water and observed for recovery. Mice were divided into six independent experimental groups with six mice in each group. Experimentally treated groups were housed separately as three mice/cage. Details of experimental groups are as following:

- **Control:** intranasally inoculated with 60µL normal saline
- **delNS1:** intranasally inoculated with 1000 $TCID_{50}$ units of delNS1 virus in 60µL normal saline
- **PR8:** intranasally inoculated with 1000 $TCID_{50}$ units of PR8 virus in 60µL of normal saline
- **Zoo:** intranasally inoculated with approximately $1x10^6$CFU of *S. zooepidemicus* (strain 7e) in 60µL of normal saline

- **Zoo+delNS1:** intranasally inoculated with 1000$TCID_{50}$ units of delNS1 virus+ approximately 1x10^6 CFU of *S. zooepidemicus* (strain 7e) in combined volume of 60µL of normal saline
- **Zoo+PR8:** inoculated with 1000$TCID_{50}$ units of PR8 virus+ approximately 1x10^6 CFU of *S. zooepidemicus* (strain 7e) in combined volume of 60µL of normal saline

Bronchoalveolar lavage (BAL) fluid collection: At 12 hrs post infection mice were euthanized by intraperitoneal injection of 200µL ButhanasiaD™, and BAL fluid was collected aseptically using a sterile intra-tracheal catheter. A total of one mL sterile normal saline was instilled in the lungs and aspirated. The aspirated fluid was re-infused and aspirated for a total of three times. BAL fluid was centrifuged at 600g/5min, and cell-free BAL fluid was transferred to sterile microcentrifuge tube as single use aliquots and frozen at -80°C.

Data Analysis: Results in the RAW cells are presented as mean ±SEM and are representative of at least three independent experiments. Results of primary alveolar macrophage cells are from a single experiment done in duplicate. Data was analyzed by using Prism6 (GraphPad Software).

4.3. Results

Effect of influenza virus NS1 on type-I IFN expression: Recently, induction of type-I IFN was implicated in the influenza virus mediated inhibition of IL-23. However, influenza virus NS1 is known to inhibit the type-I IFN response [172].Thus, before testing my primary hypothesis, effect of NS1 on relative mRNA expression of IFN-β was tested in the RAW cells as well as in primary alveolar macrophages. Cells were infected either with PR8 virus or delNS1 virus at MOI of 2TCID50/cell and incubated at 37 C/5% CO2 for 20

hrs after infection. Two sets of cells were mock infected to serve as controls. One set of mock-infected cells was treated with TG (0.025uM) and LPS (100ng/mL, Sigma) for last 7 hrs of incubation to serve as a positive control while mock-infected cells were used as negative control and calibrator for relative expression analysis. Murine GAPDH was used as internal control for relative expression. Results are presented in Fig 4.1. In RAW cell, delNS1 virus, as compared to the PR8 virus induced significantly higher expression of IFN- β ($P<0.0477$). Similarly, in primary AM cells, expression of IFN-β was approximately 10 fold higher in the delNS1 virus-infected cells as compared to the PR8 virus-infected cells. These results indicate that influenza virus NS1 inhibited the expression of type-I IFN, as expected.

Effect of influenza virus NS1 on IL-23p19: IL-23 is a heterodimeric cytokine composed of a p40 subunit that is shared with IL-12 and an IL-23p19 subunit that is unique to IL-23 [279]. Thus, in order to determine the effect of NS1 on IL-23, we analyzed the relative expression of IL23p19 in the RAW cells and primary AM cells using total cellular RNA from the experiment described above. The underlying hypothesis was that influenza virus NS1 inhibits the expression of IL-23p19. Results of relative mRNA expression are presented in fig 4.2. Results in the RAW cells are presented as mean ±SEM and are representative of at least three independent experiments performed in duplicate. Results of primary alveolar macrophage cells are from a single experiment done in duplicate. Expression of IL-23p19mRNA, in the RAW cells, was significantly higher in the delNS1 virus-infected cells as compared to the PR8 virus infected cells ($P<0.0446$). Also, delNS1 virus infected AM showed higher relative expression of IL-23p19 as compared to the PR8

virus infected cells. These results suggest that influenza virus NS1 inhibits the expression of IL-23.

Effect of Influenza virus NS1 on expression of CHOP-10: CHOP-10 is an ER stress-induced factor that, in human dendritic cells, has been shown to be critical in the expression of IL-23. The hypothesis for this experiment was that Influenza virus NS1 protein inhibits the expression of CHOP-10. In order to test this hypothesis, expression of CHOP-10 was analyzed in primary AM of mouse and RAW cells were used for the purpose of comparison. Relative expression of CHOP-10 was compared between delNS1 virus and PR8 virus infected cells and results are presented in Fig 4.3. Results from the RAW cells are presented as mean ±SEM and are representative of at least three independent experiments performed in duplicate. Results of primary alveolar macrophage cells are from a single experiment done in duplicate. As expected, RAW cells infected with delNS1 virus infected cells showed significantly higher expression of CHOP-10 mRNA, as compared to the PR8 virus infected cells ($P<0.0286$). Also, in the AM cells infected with the delNS1 virus CHOP-10 expression was approximately eight fold higher than the PR8 virus-infected cells. These results suggest that influenza virus NS1 inhibits the expression of CHOP-10.

Effect of CHOP-10 overexpression on IL-23p19 expression: The hypothesis for this experiment was that CHOP-10 overexpression will rescue the inhibition of IL-23 in PR8 virus infected cells. To test this CHOP-10 was overexpressed in the RAW cells and cells were then infected with PR8 virus and expression of IL-23p19 was analyzed. Results of this experiment are presented in Fig 4.4. Overexpression of CHOP-10 was confirmed by real-time PCR. Expression of CHOP-10 was significantly higher in the expression vector

(pCHOP-10) transfected cells as compared to the empty vector (pCMV) transfected cells ($p<0.0001$). Although lower than the mock-infected cells, expression of CHOP-10 was significantly higher ($P=0.0027$) in the PR8 virus infected cells transfected with overexpression vector (pCHOP-10+PR8) as compared to the cells transfected with empty vector (pCMV+PR8), suggesting that expression of CHOP-10 in the PR8 infected cells was restored, at least partially. IL-23p19 mRNA expression was highest in the mock-infected CHOP-10 overexpressing cells (pCHOP10+Mock). Also, expression of IL23p19 mRNA was significantly higher ($P<0.0001$) in the PR8 infected cells overexpressing CHOP-10 (pCHOP-10+PR8) as compared to the PR8 infected cells transfected with empty vector (pCMV+PR8). These results suggest that restoration of CHOP-10 by plasmid-mediated overexpression overcomes the influenza virus mediated inhibition of IL-23p19.

Effect of CHOP-10 knockdown on IL-23: In previous experiments, it was observed that delNS1 virus infection of RAW cells and AM led to the induction of CHOP-10 and IL-23p19 mRNA. Hence, this part of study was designed to investigate whether CHOP-10 inhibits the induction of IL-23p19 in the delNS1 virus infected cells. To test this, CHOP-10 was knocked down in the RAW cells by lentiviral-mediated delivery of CHOP-10 siRNA. Cells were then infected with delNS1 virus and relative expression of CHOP-10, and IL-23p19 mRNA was analyzed. Results in the Fig 4.5A confirm the siRNA mediated knockdown of CHOP-10. As expected, cells transduced with CHOP-10 siRNA lentivirus resulted in significantly lower expression of IL-23p19 as compared to control siRNA transduced cells after infection with delNS1 virus ($P=0.0019$). These results suggest that delNS1 virus-induced expression of IL-23p19 is mediated by CHOP-10.

Effect of influenza virus NS1 on IL-23/IL-17 pathway *in vivo*: The delNS1 virus infected cell induced significantly higher expression of both IFN-β and IL-23p19 *in vitro* suggesting that IL-23 expression is not inhibited by type-I IFN. Therefore, it was hypothesized that the influenza virus infection will inhibit the IL-23 pathway *in vivo* by a mechanism that does is not mediated by type-I IFN. It was also, hypothesized that this inhibition will result in downregulation of IL-17 that is secreted by Th17 cells. In order to test these hypotheses, respiratory IL-23 and IL-17 induction was determined by analyzing the expression of IL23p19 and IL-17A mRNA in the BAL cells of mice from different treatment groups as described in the materials and methods section of this chapter. Expression of IL23p19 was highest in the mice co-infected with *S. ze* and delNS1 and it differed significantly from the mice co-infected *S. ze* and PR8 virus (Fig 4.6, $P<0.0001$). Likewise, IL-17A expression was also highest in the *S. ze* and delNS1 virus-infected mice, and it was significantly different as compared to the *S. ze* and PR8 virus co-infected group (Fig 4.7, $P<0.0001$). However, contrary to *in vitro* results, there was no significant difference in the expression of IFN-β between the S. ze and delNS1 virus co-infected group and S. ze and PR8 virus infected groups (Fig 4.8). Moreover, induction of IFN-β was minimal in all the treatment groups, and it was lowest in the delNS1 virus alone-infected group.

4.4. Discussion

The IL-23/IL-17 pathway has been shown to play a critical role in the clearance of secondary bacterial infection during influenza. However, viral and host factors involved in the modulation of the IL23/IL-17 pathway during influenza are poorly understood. In a recent study Kudva et al., suggested that influenza virus mediated induction of type-I IFN,

especially IFN-β, is responsible for the downregulation of IL-23/IL-17 pathway [300]. However, influenza viruses are known to suppress type-I interferons in infected cells. Therefore, presence of an interferon independent mechanism to downregulate the IL-23/IL-17 pathway was speculated. CHOP-10, an ER stress-induced transcription factor, was found to be critical in the regulation of IL-23 [4], and current study suggested that NS1 inhibits the expression of CHOP-10 (Chapter III). Thus, it was hypothesized that influenza virus NS1 protein-mediated inhibition of an ER stress-induced transcription factor CHOP-10 causes downregulation of IL23/IL17 pathway. The hypothesis was tested *in vitro* using murine macrophage cell line (RAW cells) as well as in murine primary AM cells and subsequently confirmed *in vivo* using a mouse model.

Relative mRNA expression of IFN-β, IL-23p19 and CHOP-10 in the delNS1 virus-infected and PR8 virus-infected RAW cells as well as in primary macrophages was analyzed. As expected, in both cell types delNS1 virus resulted in significantly higher expression of IFN-β suggesting that influenza virus NS1 inhibits the expression of type–I IFN. This finding is in corroboration with previously reported inhibitory effects of NS1 on the induction of type-I IFN [172, 175, 273, 358]. Next, in order to test if IFN-β has any inhibitory effect on IL-23, mRNA expression of IL-23p19 was investigated in the experimentally treated cells as above. As expected, delNS1 virus infection, as compared to the PR8 virus infection, resulted in significant upregulation of IL23p19 in both the macrophage types tested. While studying the effect of influenza virus on DC maturation, significantly higher induction of both type-I interferon and IL-23p19 was also observed in delNS1 virus infected cells as compared to the PR8 virus infected cells [274]. In the previous study (Chapter III), it was observed that NS1, at least in part, was responsible for

the inhibition of CHOP-10 in RAW cells. Those observations held true in the present study as well. DelNS1 virus, as compared to the PR8 virus, induced significantly higher expression of CHOP-10 in the RAW cells as well as in the primary alveolar macrophages. Taken together these results support the possibility of the existence of a type-I interferon independent mechanism that may involve CHOP-10.

Next, the effect of siRNA-mediated knock-down of CHOP-10 was investigated in cells infected with delNS1 virus. The underlying hypothesis was that the CHOP-10 knockdown will lead to downregulation of delNS1 virus-induced IL-23p19 expression. Results of this experiment supported the hypothesis that a CHOP-10 knock down caused downregulation of IL-23p19 expression. My findings are in agreement with the previous reports on the effect of CHOP-10 knockdown on IL23p19 expression. CHOP-10 knockdown in U937 cells, a human monocytic cell line, resulted in a substantial reduction in the TG/LPS induced IL-23p19 expression as well as in response to *Chlamydia trachomatis* infection [4]. Further, overexpression of CHOP-10 rescued, at least partially, the IL23p19 expression in the PR8 virus infected cells. These results implied that CHOP-10, at least in part, is responsible for influenza virus NS1 mediated down-regulation of IL-23p19.

Results of *in vivo* experiments in mice further confirmed the primary hypothesis and co-infection of bacteria and PR8 virus resulted in significant lower IL-23 and IL-17 as compared to the delNS1virus infected cells. Similar inhibition of bacteria-induced IL-17 was observed when influenza virus-infected mice were superinfected with *Streptococcus pneumoniae* [359]. Unexpectedly, although lower than the S. Ze alone infected animals, mice infected with PR8 virus showed higher expression of IL23p19 and IL-17A as

compared to the delNS1 alone infected mice. A possible explanation could be that delNS1 virus replicate poorly in the type-I interferon competent cells [276] and bacterial co-infection results in the increased replication of the virus. Increased replication of the virus was observed in the combined *S. aureus* and influenza infection possibly due to enhanced cleavage of viral HA1 by streptococcal proteases [360]. Proteases derived from S. aureus and concentrated in vitro can cleave the HA, supporting the hypothesis [361].

Unlike *in vitro* observations, IFN-β expression was lower in the delNS1 virus infected mice. Also, while mice co-infected with delNS1 and bacteria showed a significant difference in the induction of IFN-β, the induction was minimal in the all the treatment groups relative to the control group. These results are intriguing and do not exclude the role of type-I interferon in the influenza virus mediated subversion of IL23/IL17 pathway as reported previously by Kudva et al., [300]. A possible explanation for the minimal induction in virus infected mice could be that viral replication in the lung was not sufficient to induce substantial IFN-β. It is also possible that IFN-β induction is time and dose dependent. In the mouse model, it has been observed that while mutant viruses lacking NS1 induce IFN-β early that peaks around 24 hrs, wild type virus does not induce substantial levels of IFN-β during the first 24 hrs [362, 363]. Additionally, the difference in the source of type-I IFN in lungs of mice infected with PR8 virus and delNS1 could also contribute to our observation. Kallfass et al., reported that, while the primary source of IFN-β in IAV lacking NS1 was infected epithelial cells and CD11c- macrophage cells, in the lungs of mice infected with wild type virus , IFN-β was mainly produced from CD11c+ cells and the contribution of epithelial cells was minimal [363]. Since mice were infected under anesthesia, it is possible that most of the inoculum was deposited in the lungs and

hence there was not much contribution from the epithelial cells. However, studying the effect of time and dose of viruses on the kinetics of IFN-β induction *in vivo* could provide better insight and needs to be investigated further.

Although, there are certain limitations and, some areas need further investigation, results from this study support the hypothesis that influenza virus NS1 mediated inhibition of CHOP-10 leads to the inhibition of respiratory IL-23/IL17 axis of innate immune response. However, the present study does not exclude the possible role of type-I IFN in the regulation of IL-23/IL17 pathway during influenza infection.

Table 4.1: List of Taqman® assays used in this study

Target	**Gene aliases**	**Taqman ®Assay ID**
CHOP-10	DDIT3, Gadd153	Mm01135937_g1
IFN-β	IFNb1	Mm00439552_s1
IL-23p19	IL-23a	Mm01160011_g1
IL-17	IL-17A	Mm00439618_m1
GAPDH	GAPD	Mm99999915_g1

Figure 4.1: Relative expression of type-I interferon mRNA in macrophage cells
Mock: cell culture supernatant from non-infected Vero cells; **Positive:** Treated with Thapsigargin (0.025µM) and LPS (100ng/mL) for last 7 hrs of the culture; **PR8:** Influenza A virus strain PR/8/34; **delNS1:** Influenza A virus isogenic to PR8 but lacks NS1

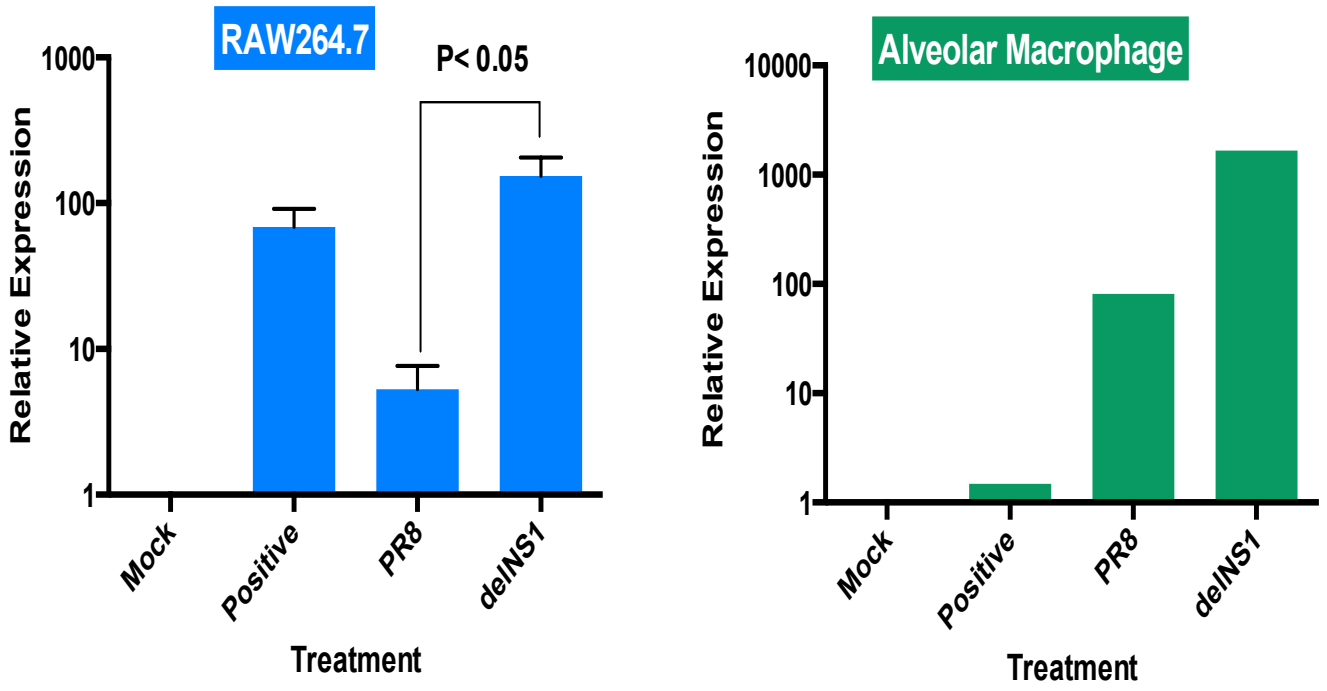


Figure 4. 2: Relative expression of IL-23p19 mRNA in macrophage cells
Mock: cell culture supernatant from non-infected Vero cells; **Positive:** Treated with Thapsigargin (0.025µM) and LPS (100ng/mL) for last 7 hrs of the culture; **PR8:** Influenza A virus strain PR/8/34; **delNS1:** Influenza A virus isogenic to PR8 but lacks NS1

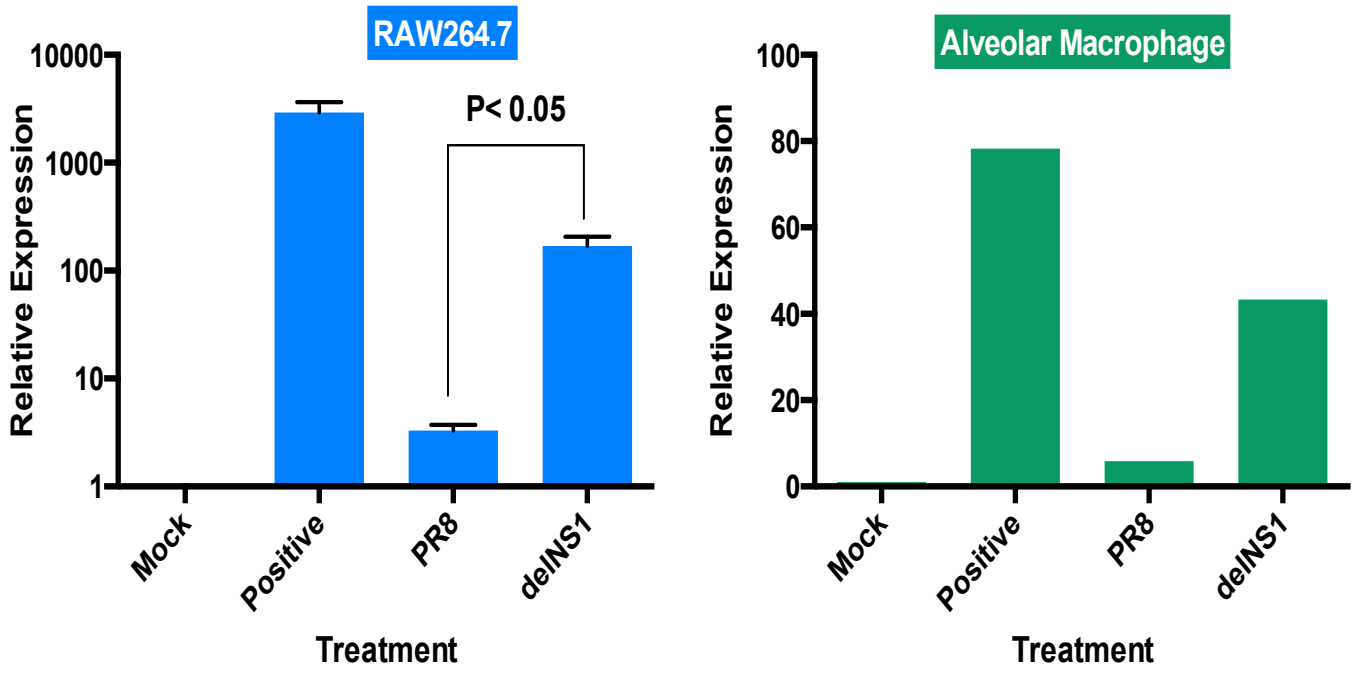


Figure 4.3: Relative expression of CHOP-10 mRNA in macrophage cells
Mock: cell culture supernatant from non-infected Vero cells; **Positive:** Treated with Thapsigargin (0.025µM) and LPS (100ng/mL) for last 7 hrs of the culture; **PR8:** Influenza A virus strain PR/8/34; **delNS1:** Influenza A virus isogenic to PR8 but lacks NS1

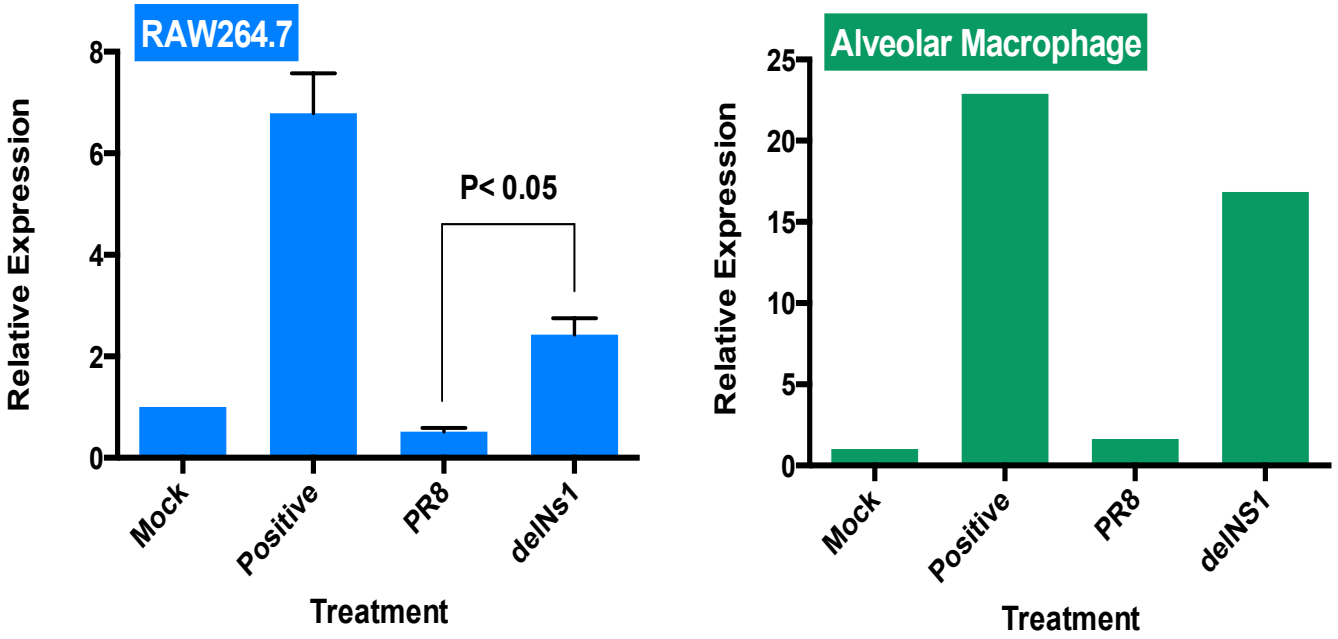


Figure 4.4: Effect of CHOP-10 overexpression on IL-23p19 mRNA expression

pCHOP-10+Mock: cells transfected with CHOP-10 overexpression plasmid then mock infected
pCHOP-10+PR8: cells transfected with CHOP-10 overexpression plasmid then infected with PR8 virus
pCMV+PR8: cells transfected with empty control vector then infected with PR8 virus
pCMV+Mock: cells transfected with empty control vector then mock infected
**= p<0.001; ***= P<0.0001

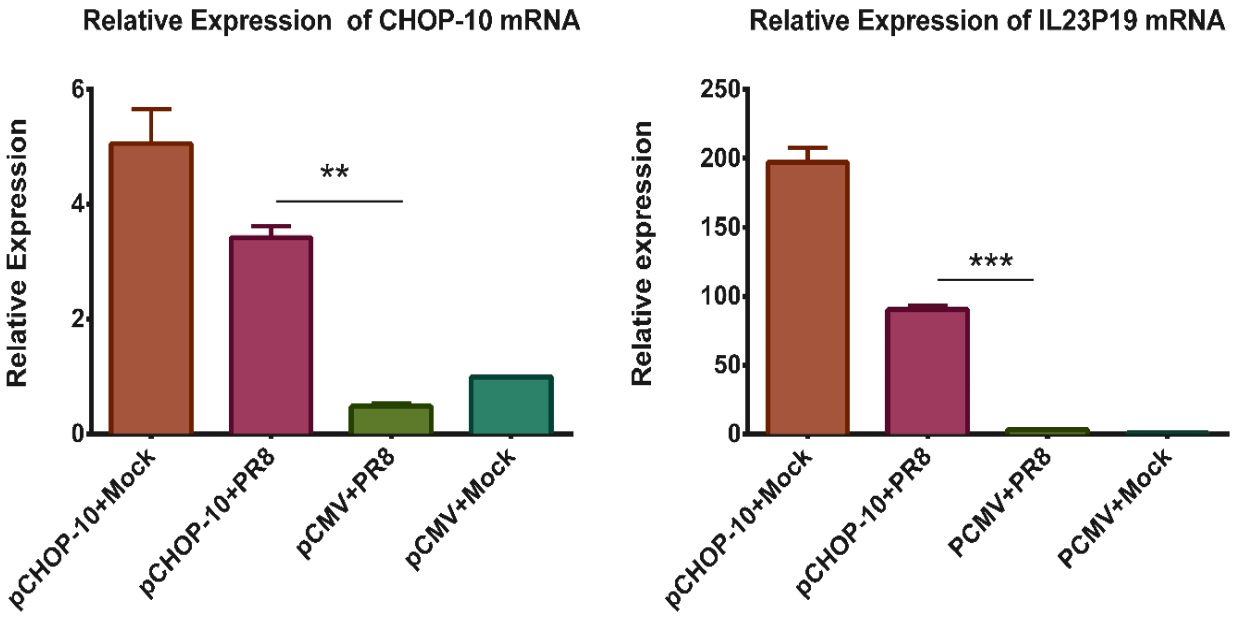


Figure 4.5: Effect of CHOP-10 knockdown on IL-23p19 mRNA expression

RAW+delNS1: Non-transduced RAW cells infected with delNS1
CHOP-10-siRNA+delNS1: cells transduced with CHOP-10 siRNA lentivirus then infected with delNS1 virus
Sic+delNS1 cells transduced with non-target control siRNA lentivirus then infected with delNS1 virus
*=P<0.05; **=P<0.001

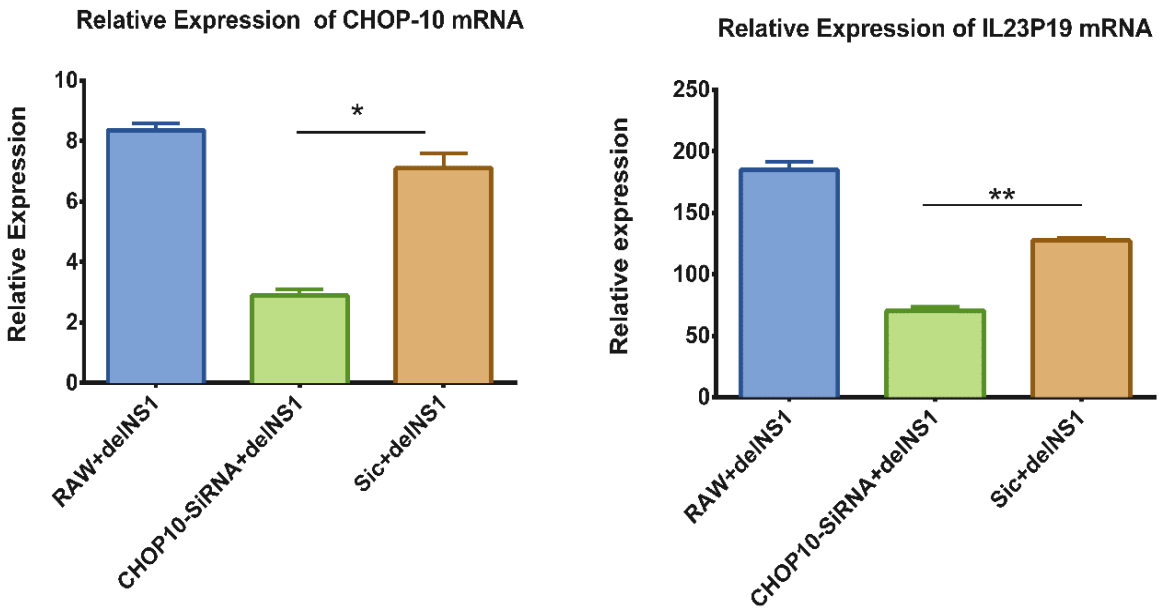


Figure 4.6: Relative expression of IL23p19 mRNA in mouse lung
Mice were inoculated intranasally with Saline (Control), virus diluted in saline (delns1 or PR8), or *S. Ze* in combination with either delNS1 or PR8 virus (Zoo+delNS1 and Zoo+PR8). cDNA was prepared from RNA extracted from BAL cells and Real-time PCR was performed. Relative quantitation was done Δ ΔCT method.

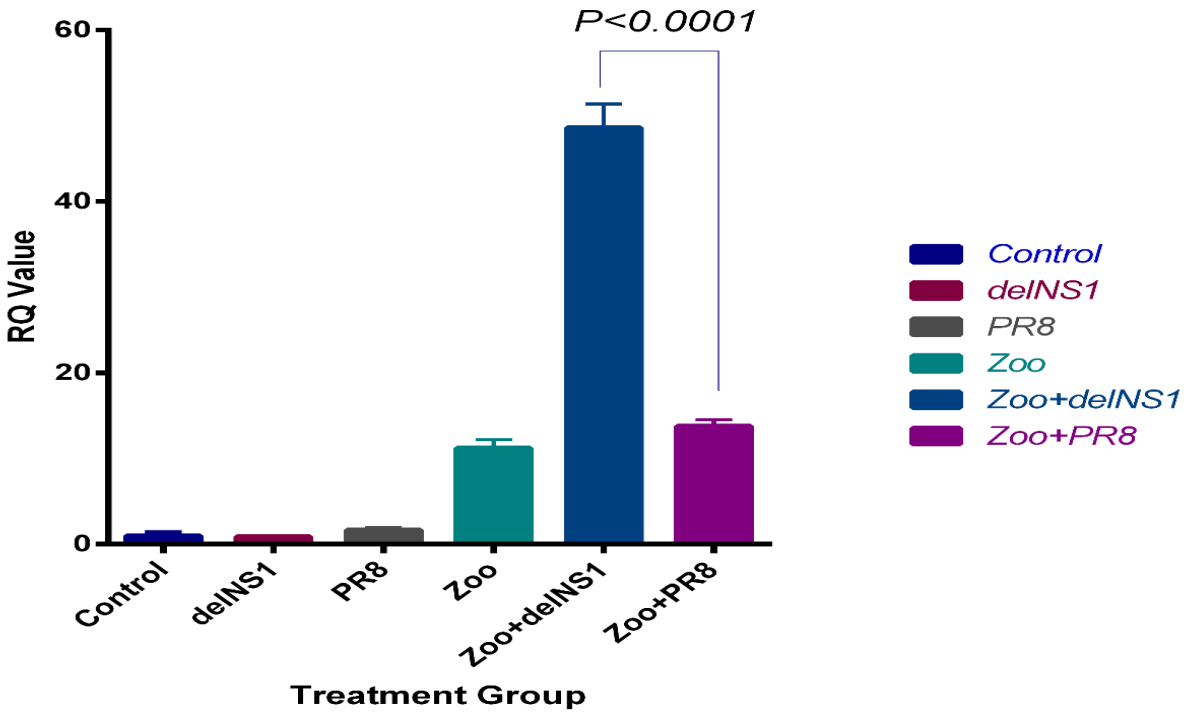


Figure 4.7: Relative expression of IL-17A mRNA in mouse lung
Mice were inoculated intranasally with Saline (Control), virus diluted in saline (delns1 or PR8), or *S. Ze* in combination with either delNS1 or PR8 virus (Zoo+delNS1 and Zoo+PR8). cDNA was prepared from RNA extracted from BAL cells and Real-time PCR was performed. Relative quantitation was done Δ ΔCT method.

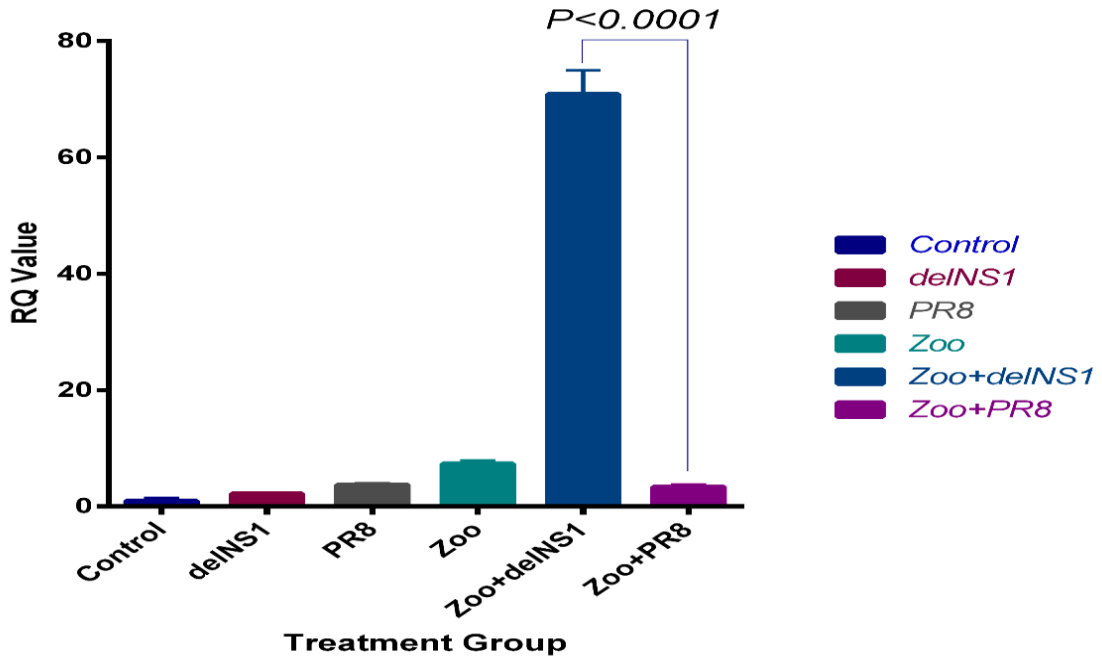
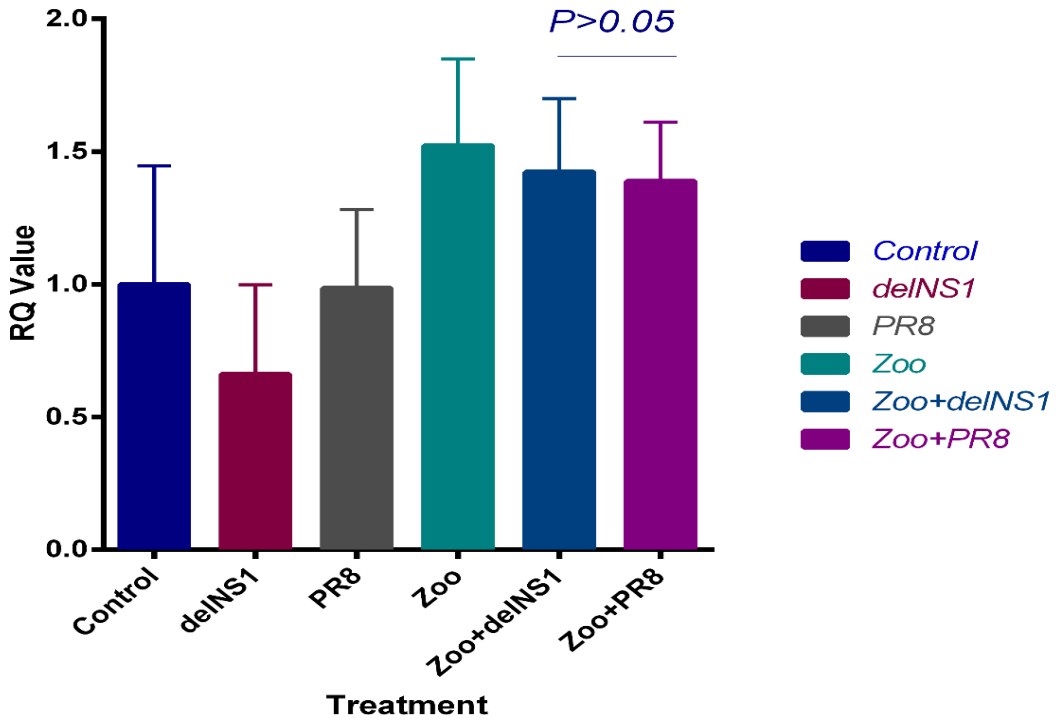


Figure 4.8: Relative expression of IFN-β mRNA in mouse lung
Mice were inoculated intranasally with Saline (Control), virus diluted in saline (delns1 or PR8), or *S. Ze* in combination with either delNS1 or PR8 virus (Zoo+delNS1 and Zoo+PR8). cDNA was prepared from RNA extracted from BAL cells and Real-time PCR was performed. Relative quantitation was done Δ ΔCT method.



CHAPTER 5

Intranasal administration of recombinant IL-23 protects mice from lethal co-infection of influenza A and *Streptococcus zooepidemicus* co-infection

5.1. Introduction

Influenza A virus (IAV) infection is one of the major respiratory disease worldwide. Although, some IAV are sufficiently virulent to cause mortality in the host, secondary bacterial infections appear to be major contributors in the influenza associated deaths. During the influenza pandemic of 1918 that caused the death of about 40 to 50 million persons [214] while viral pneumonia killed many healthy young persons, most of the deaths were due to secondary bacterial pneumonia [225]. Bacterial infection during influenza virus infection can be either concurrent with the virus infection or subsequent to influenza virus infection. Concurrent bacterial and influenza pneumonia, although less frequent, have a worse outcome than sequential infection [364]. Concurrent influenza virus/bacterial infection is multifactorial and involves interaction between the host, bacteria and virus, whereas post-influenza infection involves interaction between host and bacteria only. Therefore, in post-influenza pneumonia the host response will be against bacterial pathogens only, but in concurrent infection the host will respond to the virus as well as the bacterial pathogen. These differences could affect the outcome and are important in deciding the therapeutic regimen for treating influenza pneumonia [207].

Vaccination against seasonal influenza has been shown to reduce influenza incidences greatly. Epidemics of seasonal influenza have been attributed to random single point mutations. Current advances in surveillance and epidemiological modeling could help in predicting these vaccine strains and allow satisfactory vaccine design in advance.

However, pandemic strains of IAV arise due to genetic shifts and therefore difficult to predict. Hence, effective therapeutic interventions remain important especially to prevent secondary bacterial complication, which is a major contributor in pandemic deaths. Antibiotics, as well as antiviral, have been used with limited success. Despite the availability of antibiotics, bacterial pneumonia was involved in almost two-thirds of fatal cases during the influenza pandemic of 1957. Inhibitors of viral replication have also been investigated. Neuraminidase inhibitors significantly improved the survival of mouse form pneumonia following influenza [365]. There is a relatively small window, however, in which neuraminidase inhibitors can reduce viral replication and missing that time-window will result in failure to prevent mortality in mice with influenza complicated by bacterial pneumonia [366]. Survival of mice with post-influenza bacterial pneumonia did not improve after treatment with Rimantadine [256]. Moreover, efficacy of these inhibitors in concurrent influenza and bacterial infection has not been investigated. Development of antibiotic resistant bacteria and antiviral-resistant influenza viruses further limit the use of these therapeutics.

A classical explanation for increased secondary bacterial infection during influenza has been the mechanical damage to the respiratory tract. However, altered host respiratory innate immune response has begun to be appreciated. Cytokines are an integral part of the host innate immune system, a broadly reactive defense mechanism of the host, which acts as the first line of defense against a variety of invading pathogens. IL-23 is a recently identified cytokine that has been shown to be important in the respiratory innate immune response. IL-23, in concert with IL-17, constitutes a newly identified innate immune pathway. The host IL-23/IL-17 pathway has been shown to play a critical role in the

clearance of many respiratory bacterial pathogens. However, its role during influenza and bacterial co-infection has not been investigated much. Thus, it was hypothesized that inhibition of respiratory IL23/IL17 pathway by influenza virus predisposes the host for secondary bacterial infection and restoring the respiratory IL-23 by rIL-23 administration will increase the clearance of bacteria and reduce the pathogenesis of influenza. In order to test this hypothesis, effect of restoration of respiratory IL-23 by intranasal administration was studied in a mouse model of concurrent influenza and bacterial infection. To study the effect of IL-23 restoration on clearance of virus and bacteria, weight loss of the animals and survival analysis was performed.

5.2. Materials and methods

Infectious agents: PR/8/34 strain (PR8) of IAV and an isogenic recombinant virus lacking NS1 (delNS1) as described in the previous chapters (chapter III) were used in the current study. For bacterial co-infection *S.ze* strain 7e was used. Bacteria were cultured and inoculum was prepared as described in chapter IV.

Animals: Six-eight week old female CD-1 mice were purchased from a commercial supplier (Harlan). On arrival mice were housed in microisolater cages with an *ad libitum* supply of food and water. Mice were acclimatized for one week before infection and examined daily for any signs of stress or illness following the guidelines of Institutional Animal Care and Use Committee (IACUC) at University of Kentucky (fig 5.4, table 5.1). All the animal work was approved by IACUC at University of Kentucky.

Inoculation of mice and rIL-23 administration: Mice were sedated with ketamine/xylazine administered by intraperitoneal injection. Once sedated mice were held upright by holding from the loose scruff at the neck. Inoculum was prepared to contain

approximately $1x10^6$ CFU of bacteria and 1000 TCID50 units of PR8 virus in a final volume of 60µL normal saline. A small drop of inoculum was placed near each nostril so that mice involuntarily inhaled the inoculum. The inoculum was instilled from alternating the nostrils so that whole inoculum (60µL) was divided equally between two nostrils. Mice (n=18/group) were then assigned randomly to either rIL-23 or vehicle group that received PBS that was used to resuspended IL-23. Immediately after the viral/bacterial inoculation, a total of 3 µg rIL-23 in 20µl PBS was administered intranasally to the rIL-23 treatment group and 20µL PBS was administered intranasally to the vehicle only group as described for the inoculum. Investigator was blinded for which group received which treatment until data were collected and analysis was completed.

BAL Fluid collection: Mice were euthanized 72 hrs post infection and BAL fluid was collected aseptically with sterile intra-tracheal catheterization as described in chapter IV. A total of 1 mL sterile normal saline was instilled in the lungs and aspirated. The aspirated fluid was re-infused and aspirated for a total of three times. BAL fluid was centrifuged at 600g/5min and cell-free BAL was transferred to sterile microcentrifuge tubes as single use aliquots and frozen at -80°C.

Determination of bacterial and viral load in the lungs: Lung bacterial and viral burden was determined from the BAL fluid of co-infected mice. In order to determine the bacterial burden, 10 fold serial dilutions of cell free BAL fluid were plated in duplicate onto Columbia CNA agar supplemented with 5% horse blood. Plates were incubated at 37°C/5% CO_2 for 24 hrs before counting the number of colonies and recording the results as CFU/mL.

Lung virus titer was determined by IAV matrix gene-based quantitative real-time PCR. Viral RNA was extracted from 50ul cell-free BAL fluid using the magmax™ 96 viral nucleic acid extraction kit (Ambion AM-1836) following manufacturers instruction. Quantitative real-time PCR was performed using 1-step RT-PCR kit on ABI 7500 platform using the primer probes described previously (CDC REF #I-007-05). Sequence of primer and probes is provided in Table 5.2.

Data analysis: Pathogen burden and weight loss data was analyzed using students T-test with Welch's correction using GraphPad (Prism6) statistical software. Survival fractions of the two groups were estimated using Kaplan-Meier method and survival curves were compared by log-Rank (Mentel-Cox) test and Gehan-Breslaw-Wilcoxon test using GraphPad statistical software (Prism6).

5.3. Results

Effect of IL-23 administration on bacterial and viral burden in the lungs of ci-infected mice: Mice were observed daily for clinical signs and scored following the guidelines provided by IACUC (Table 5.1 and Fig. 5.4). Pronounced clinical signs of disease characterized by ruffled fur, lethargy, hunched posture and labored breathing were displayed by both the treatment groups of mice as early as day 2. However, subsequent clinical signs were more pronounced in the vehicle only group and some of the mice in rIL-23 treatment group started to recover by day 6 onwards.

Since IL-23 treated mice showed less severe clinical signs as compared to the vehicle only treated mice, it was hypothesized that treated mice would have improved clearance of bacteria and virus from the lungs. As mortality started on day three PI, we chose 72 hrs PI time point to assess the bacterial and viral burden in the two groups. Results

of bacterial load in the BAL fluid are presented in Fig 5.1. Although statistically non-significant (P=0.5490), bacterial count in the IL-23 treated mice trended towards lower side as compared to the non-treated mice suggesting that IL-23 might have a role in the clearance of bacteria.

Viral burden was determined in the cell free BAL fluid by using quantitative real-time PCR. Results of the quantitative real-time PCR (Fig 5.1B) are presented as threshold cycle (CT) value. There was no statistically significant difference in viral load between two groups (P=0.3066), suggesting that IL-23 had no effect on viral clearance.

Effect of intranasal rIL-23 treatment on weight loss in co-infected mice: In order to determine whether IL-23 could affect the clinical outcome of the co-infection, we compared the weight loss of the treated and non-treated mice. Weight of each mouse was recorded every 24 hrs and percent weight loss was calculated respective to weight on day zero. Mice with a weight loss of $\geq 25\%$ of day zero weight were euthanized and considered dead for data analysis purposes. Mean percent weight loss of mice from each group are presented in Fig 5.2. As expected, mice treated with PBS (vehicle), as compared to the rIL-23 treated mice, showed higher weight loss. Weight loss was statistically significant as early as day two (p=0.0005), and by day six all the mice were dead in the non-treated vehicle group.

Effect IL-23 treatment on survival of co-infected mice: Next we wanted to determine whether intranasal rIL-23 treatment affects the survival of mice. Age and sex matched 6-8 week old mice were randomly allotted to either rIL-23 treated group or a control group treated with vehicle only (PBS). Each mouse of both the group was observed daily and clinical scores were recorded as per the IACUC guidelines (Table 5.1). Any mouse

showing a cumulative score of ≥6 or a score of three on a single category was euthanized (IACUC guideline, Fig 5.4) and considered dead for data analysis purposes. Also, any mouse showing ≥ 25% of weight loss of day zero weight was euthanized and considered dead for data analysis purpose. Results of mortality are presented in Fig 5.3. First death was observed in the rIL-23 treated group on day 3. However, later on mortality was more pronounced in non-treated group and by day 6, all the mice in non-treated group were succumbed to death. On the other hand, 50% (9/18) of the rIL-23 treated mice were surviving on day 6. Most of the surviving mice either did not show any further weight loss or showed a minimal weight loss suggesting recovery. Survival curve analysis showed a highly significant difference with a p-value of 0.0015 and 0.0069 in Log-rank (Mentel-cox) and Gehan-Breslaw-Wilcoxon tests, respectively, and the mean death time in the treated group was prolonged by 1 day.

5.4. Discussion

Influenza infection is a leading cause of death worldwide. However, most of the deaths associated with influenza have been ascribed to the secondary bacterial infection. Secondary bacterial infection associated with influenza may either be due to concurrent infection or follow a preceding influenza infection. As bacterial pneumonia often ensues 3-7 day post-influenza infection, most of the studies have used a sequential model of secondary bacterial infection where bacterial inoculations were performed up to seven days after preceding influenza challenge. However, although less frequent, concurrent influenza and bacterial infections involve more complex interactions of host, virus, and bacteria than the sequential infection and often result in more serious outcome than sequential infection [364]. Furthermore, some of the secondary bacterial invaders during influenza infection

are opportunistic pathogens commonly present in the upper respiratory tract of humans as well as animals which could lead to secondary infection during influenza. Therefore, in the current study a concurrent infection model was used to investigate the therapeutic potential of rIL-23 on influenza-associated secondary bacterial infections. Since *S. zoo* is a commonly associated secondary invader during equine influenza, the overreaching goal of the study was to investigate the effect of rIL-23 supplementation on outcome secondary *S. zoo* infection during equine influenza infection. However, recombinant equine IL-23 is not available yet, therefore, we tested our hypothesis in the mouse model. Mouse has been successfully used as a model for *S. zoo* by Dr. P Timoney at Gluck equine research center. Furthermore, the PR8 strain of influenza virus is well adapted to the mouse model and has been used extensively to study the pathogenesis and immune response against influenza.

IL-23 is a recently identified member of IL-6 family of cytokines that is important in the induction and maintenance of Th17 cells that secrete IL-17. IL-17 is an early proinflammatory cytokine that mediates host defense against several respiratory bacterial pathogens. Although there are reports on using intranasal rIL-23 as a therapeutic intervention against respiratory bacterial infections, it has not been investigated much during influenza and bacterial co-infection. There is only one study that investigated the effect of restoration of IL23 in the lungs of influenza and bacterial co-infection in which Adenovirus expressing rIL-23 was found to improve the clearance of bacteria in *S. pneumoniae* and influenza co-infected mice [300]. Thus, current study appears to be the first to utilize intranasal rIL-23 as therapeutic intervention during concurrent influenza and bacterial co-infection.

The hypothesis in the current study was that rIL-23 treatment will improve the clearance of virus and bacteria. Unexpectedly, there was no significant difference between respiratory viral load between rIL-23 and control animals. Likewise, although bacterial counts were lower in the treated mice, they were statistically not significant. In contrast to the present study, Kudva et al., observed that overexpression of IL-23 in the lungs of mice resulted in improved clearance of virus as well as bacteria [300]. It should be noted, however, that authors in that study used a sequential model of infection where bacterial infection was performed at day six post-influenza infection. It is possible that during this time there was already some repair of the tissue damage caused by influenza virus resulting in decreased adherence of bacteria. Additionally, since adenovirus was used to express the IL-23, it would be difficult to determine the exact level of IL-23 in the lungs. Furthermore, timing and duration of the IL-23 administration may have important implications on the outcome. One of the limitations of the present study is that only a single intranasal dose of rIL-23 concurrent with the infection was used. In the case of adenoviral overexpression levels of IL-23 could be maintained during the infection and may have a significant effect on the clearance of virus as well as bacteria. It is possible that a subsequent dose may help in maintaining the levels of IL-23 in the lungs and further improve the clearance of virus and bacteria and needs to be investigated.

As expected, rIL-23 treated mice, as compared to the vehicle only treated mice, showed less severe signs of disease, less weight loss and improved survival suggesting that intranasal rIL-23 could limit the pathology of co-infection. One possible mechanism could be the suppression of the early strong inflammatory response (cytokine storm) that plays an important role in the pathogenesis of influenza. It was recently found that adenovirus-

mediated overexpression of IL-23 resulted in the decreased lung inflammation in influenza and S. pneumoniae co-infected mice [300]. Additionally, improved survival of rIL-23 treated mice could also be due to increased tolerance of the host for infection due to accelerated tissue repair and homeostasis. Recently, it was observed that, despite normal anti-bacterial immune response, hosts were susceptible to lethal secondary bacterial infection in influenza infected mice due to impaired ability to tolerate respiratory tissue damage [102]. Studies with amphiregulin, a member of the family of epithelial growth factors, revealed an important role in lung tissue homeostasis in the survival of influenza and bacteria co-infected mice. While amphiregulin treatment did not affect the bacterial and viral burden in co-infected mice, it resulted in decreased lung tissue damage and significantly improved weight loss and the survival of co-infected mice [102]. IL-23 could help in the repair of damaged respiratory tissue mediated by IL-22. IL-22 is important for the modulation of tissue responses during inflammation, and it induces proliferative and anti-apoptotic pathways, as well as anti-microbial molecules that help prevent tissue damage and aid in its repair [367]. IL-23 has been shown to be critical in the differentiation and proliferation of Th17 cells [368]. Also, IL-22 has been reported to be robustly secreted by Th17 cells in IL-23 dependent fashion [369]. Although the extent of tissue damage was not evaluated between IL-23 treated and control groups and needs to be investigated, it seems plausible that the difference in tissue repair could have resulted in improved tolerance in the rIL-23 treated mice.

In the present study we observed that while IL-23, to some degree, helped in clearance of bacteria in co-infected mice, it did not affect the viral clearance. Although, we did not establish the underlying mechanism, we did observe that intranasal rIL-23

administration, significantly improved the survival of co-infected mice. From our study, we conclude that intranasal administration of rIL-23 has a beneficial effect during concurrent bacterial and influenza virus co-infection and in the wake of emerging drug resistant influenza viruses and bacteria, IL-23 could potentially be included as alternative therapeutic intervention.

Figure 5.1: Bacterial and viral burden in the lungs of influenza and *S. ze* co-infected mice

Mice were co-infected with PR8 virus and *S. ze* by intranasal inoculation. Immediately after inoculation mice were intranasllly given recombinant IL-23 (rIL-23) or PBS (Vehicle). At 72 hr PI BAL fluid was collected.Cell free BAL fluid was plated on CAN agar supplemented with 5% horse blood and colonies were counted after 24 hr and bacterial burden (CFU/mL of BAL fluid) was determined (A). viral RNA was extracted from cell free BAL fluid and Real-Time PCR CT values were used to compare the viral burden.

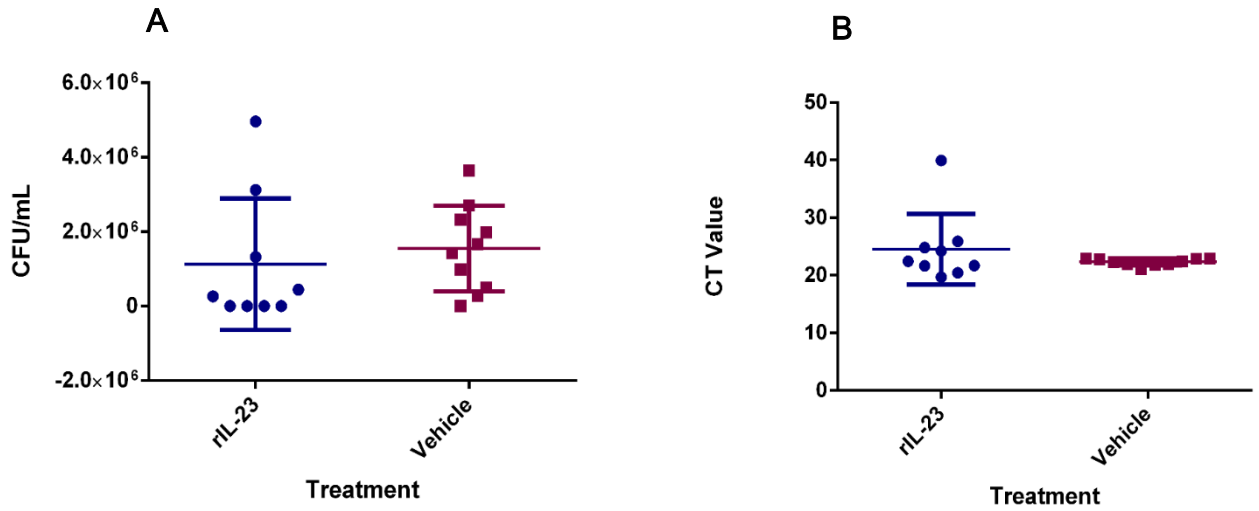


Figure 5.2: Effect of intranasal rIL-23 administration on weight loss in influenza and S. ze co-infected mice

Mice were co-infected with PR8 virus and *S. ze* by intranasal inoculation. Immediately after inoculation mice were intranasllly given recombinant IL-23 (rIL-23) or PBS (Vehicle). Mice were weighed daily and weight loss was calculated to as percent of day 0 weight. Data was analyzed by using t-test with Welch's correction using GraphPad Prism 6 software. (*= P<0.05; **= P<0.01; ***=P<0.0001)

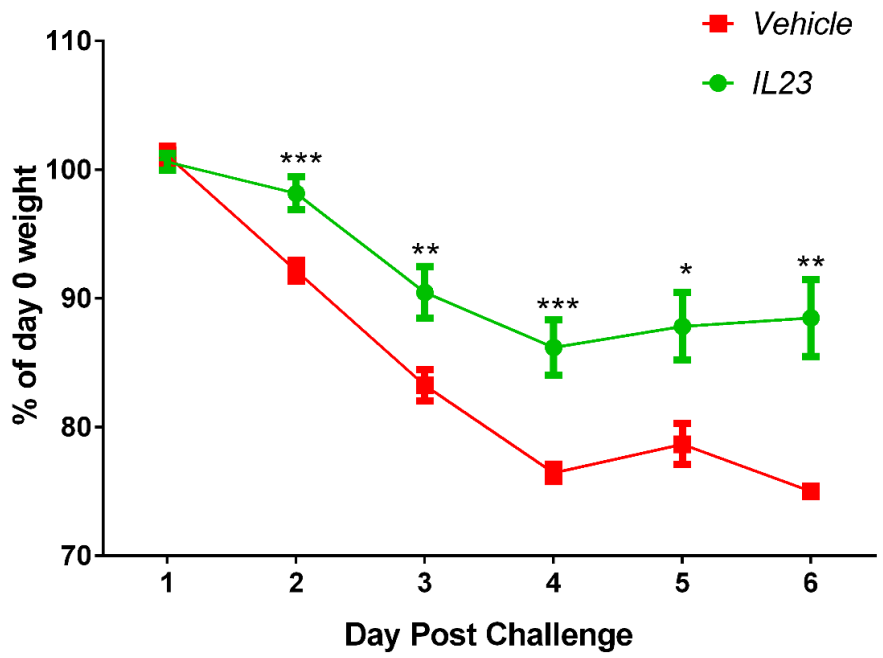


Figure 5.3: Effect of intranasal rIL-23 administration on survival of influenza and *S. ze* co-infected mice

Mice were co-infected with PR8 virus and *S. ze* by intranasal inoculation. Immediately after inoculation mice were intranasllly given recombinant IL-23 (rIL-23) or PBS (Vehicle). Mortality was recorded andsurvival fractions were calculated. Survival curve comparison was performed using Prism 6.0 (GraphPad software) that uses Kaplan-Meier method to generate survival fractions and survival curves are compared by log-Rank (Mentel-Cox) test and Gehan-Breslaw-Wilcoxon test. Two curves were significantly different with **P value 0.0015 and 0.0069** in Log-rank (Mentel-cox) and Gehan-Breslaw-Wilcoxon tests, respectively.

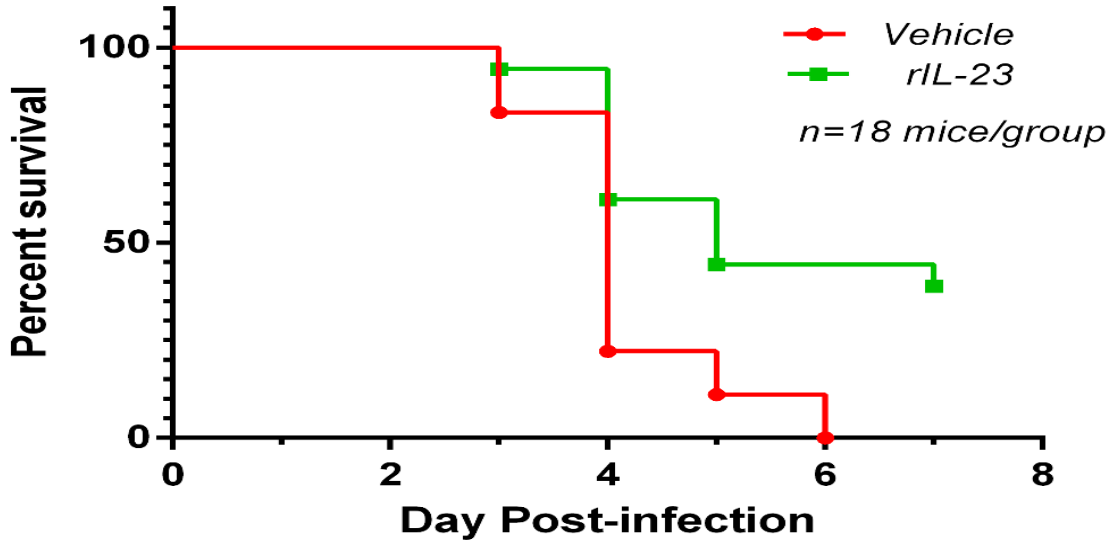


Figure 5.4: Treatment flow chart for mice with respiratory infections

From (IACUC, University of Kentucky)

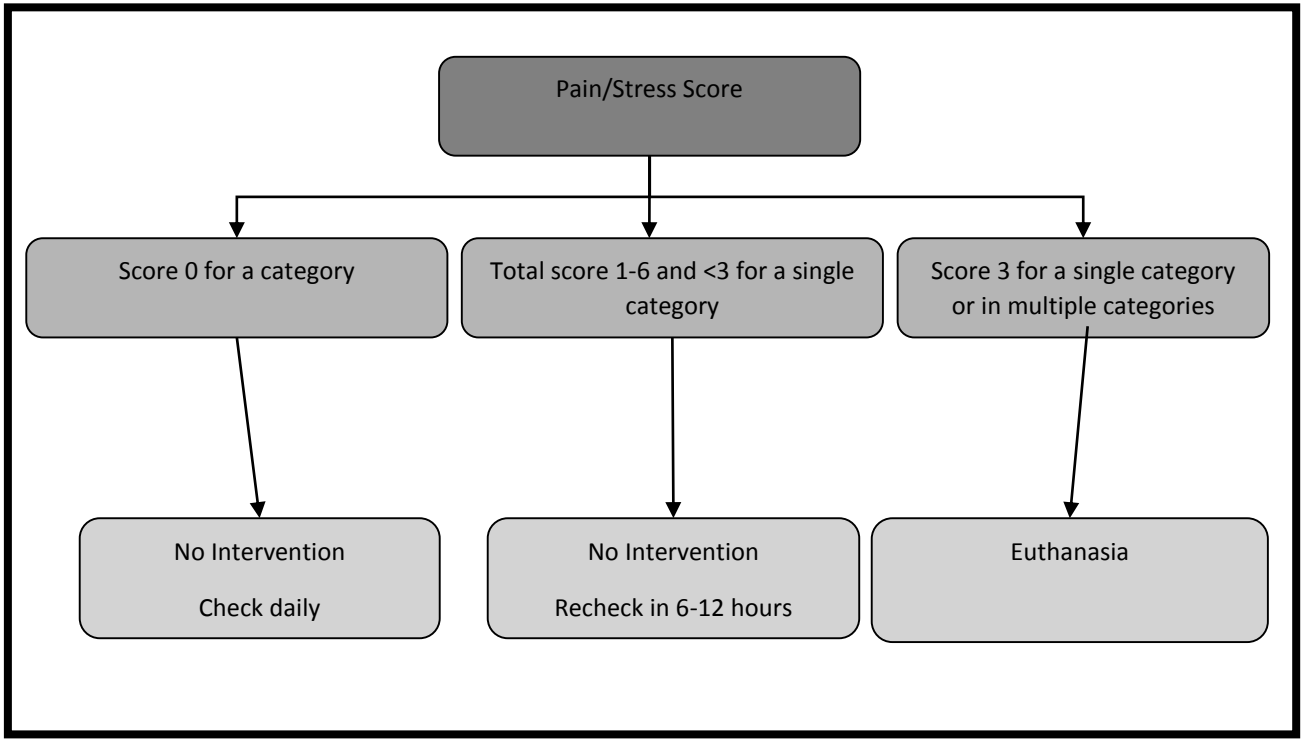


Table 5.1: Clinical scoring criteria for mice

Criteria/Score	0	1	2	3	Total
Locomotion	Moving normally around cage, not hugging the sides of the cage.	Stumbling , falling, or hugging the sides of the cage.	Writhing, stumbling and/or falling. OR Movement only when stimulated.	No movement.	
Respiration	Normal rate with no audible respiratory sounds to naked ear	Mild “chattering” or “snoring”	Moderate “chattering” or “snoring”	Labored breathing , increased respiratory rate	
Behavior	Normal cage exploration, normal food and water consumption, animal calm in cage. Previously social animal still social.	Minimal exploration, increased or decreased food and/or water consumption. Previously social animal has become withdrawn or aggressive.	No cage exploration, hunched posture, anorexic for 24 hrs.	No cage exploration, hunched posture, piloerection, anorexic, or moribund	

Table 5.2: Primer and probe sequence for influenza virus real-time PCR

Primer/probe	Sequence (5'>3')	Target IAV gene	Final Concentration
InfA Forward	GAC CRA TCC TGT CAC CTC TGA C	Matrix (M1)	800nM
InfA Reverse	AGG GCA TTY TGG ACA AAK CGT CTA	Matrix (M1)	800nM
InfA Probe	FAM-TGC AGT CCT CGC TCA CTG GGC ACG-BHQ1	Matrix (M1)	200nM

CHAPTER 6

General discussion and conclusions

The goal of the present study was to identify the possible viral and host factors that predispose the host to secondary bacterial infection during influenza virus infection. The major hypothesis was that influenza virus NS1 protein-mediated inhibition of an ER stress transcription factor mediates the inhibition of the respiratory IL-23/IL17 axis of innate immune response. I tested my hypothesis in vitro using mouse macrophage cell line as well as primary alveolar macrophage cells and in vivo using mouse model. Although, there are certain areas that need to be further investigated, results from the study support our hypothesis.

In the first part of the study, effect of NS1 protein on ER stress response of mouse macrophages was studied *in vitro*. Results of this study support the hypothesis that influenza virus NS1 mediates differential activation of the ER stress response. While influenza virus induced activation of chaperones, it inhibited the induction of CHOP-10. Since influenza viruses rely heavily on the host cellular machinery for their protein synthesis, it appears counterintuitive that virus would not induce ER stress.

Why would influenza virus inhibit ER stress? ER stress response is an evolutionary conserved host response that is used to resolve the excessive protein load on the ER. By activating three arms of UPR, the host cell tries to restore the ER homeostasis. It seems that influenza virus cleverly inhibits the PERK and IRE1 branches, but does not inhibit the ATF6 branch. While activation of PERK leads to the EIF2α phosphorylation that shuts down the global protein synthesis, activation of IRE1 branch leads to induction of transcription factors involved in the ER-associated degradation. Thus, activation of these

branches would limit the viral protein synthesis. Additionally, EIF2α phosphorylation leads to selective transcription of ATF4 that induces CHOP-10, a pro-apoptotic factor induced during the ER stress. While apoptosis may help in the viral spread and pathogenesis, premature cellular death would be detrimental for virus replication.The ATF6 branch activates transcription of ER chaperones that could promote viral replication by increasing the folding capacity of the ER. Indeed, ER chaperone protein Erp57 is reported to be crucial in the folding of influenza virus HA [315]. Therefore, the differential activation of the UPR pathway by influenza virus in the present study would support influenza viral replication.

Recently, a type-I IFN mediated mechanism for IL-23 inhibition has been reported, but influenza virus is known to inhibit type-I IFN. So, an interferon independent mechanism was speculated. The hypothesis was that influenza virus NS1 protein-mediated inhibition of CHOP-10 results in the inhibition of IL-23 expression. The hypothesis was tested in vitro in mouse macrophage cells and *in vivo* using a mouse model. Although *in vivo* results in the present study could not exclude the possibility of type-I IFN dependent mechanism, results from the *in vitro* study do suggest the presence of type-I interferon independent mechanism mediated by CHOP-10. Recent findings of the critical role of CHOP-10 in IL-23 regulation in human dendritic cells [4], further support our hypothesis.

Innate immune responses are mainly triggered by the PRRs. Then why would ER stress influence the innate immune response? PRR act as sentinels to sense the environmental danger signals such as extracellular microbial products and products of stressed or damaged tissues such as PGE2, ATP and urate [370]. Similar to the PRR, activation of UPR may serve as a counterpart to the external danger signal detection system

especially in the case of intracellular pathogens such as virus and intracellular bacteria. By activating UPR, cell may sense the presence of intracellular pathogens. Thus, integrating the PRR signaling with UPR activation would favor a failsafe mechanism for the host to detect danger signals especially in the case of intracellular pathogens.

Why would ER stress signal induce IL-23? In a review of IL-23 and IL-17 immune pathway, Mckenzie et al., [370] suggested that IL-23 is critical in driving the early immune response to infection. IL-23 rapidly induces IL-17 from Th17 cells that allow early recruitment of phagocytic cells such as neutrophils and macrophages to provide early clearance of pathogens [370]. Therefore, inhibition of the respiratory IL-23/IL-17 response could be important for the replication and pathogenesis of influenza.

Having found that influenza virus inhibits the IL23/IL17 pathway, it was speculated that restoration of IL-23 in the lungs would reduce the pathogenesis of influenza virus infection. Secondary bacterial infections are common during influenza, and IL-23 has been shown to provide protection from a variety of respiratory bacterial pathogens. Therefore, the hypothesis was that the restoration of respiratory IL-23/IL-17 innate immune pathway in the lungs would help in clearance of the pathogens and protect the host from lethal co-infection of influenza and bacteria. In order to test this hypothesis, intranasal rIL-23 administration was used to restore the respiratory IL-23/IL-17 pathway in a mouse model of concurrent influenza and bacterial infection. Although, rIL-23 administration had no effect on viral clearance, it did help in the clearance of bacteria, albeit to a limited extent. However, a notable finding was that a single dose of intranasal IL-23 significantly improved the survival of co-infected infected mice.

How could rIL-23 administration protect the host without significantly affecting the pathogen clearance? Protection of the host can be achieved in three ways: avoidance, resistance, and tolerance [371]. While avoidance reduces the risk of exposure to infectious agents, resistance reduces pathogen burden once the infection is established [371]. On the other hand, tolerance increases the fitness of the host by reducing the negative impact of an infection without directly affecting the pathogen load [372-374]. It is possible that IL-23, by inducing an additional mediator such as IL-22, induces a rapid tissue repair and could increase host tolerance. Indeed, recent findings by Jamieson et al., support this idea. They found that influenza virus infection enhanced susceptibility to secondary bacterial infection, even when bacterial infection was controlled by the immune system [102]. However, it should be noted that we have used a single intranasal dose of rIL-23, and we could not completely determine whether the protective effect of IL-23 was due to increased immune response or the tolerance.

Based on findings from current study and previously published reports, a working model for how influenza virus NS1-mediates inhibition of CHOP-10 and how this leads to inhibition of IL-23 and increases the susceptibility of the host to secondary bacterial infection has been presented in Fig 6.1.

In conclusion, this dissertation demonstrates for the first time that influenza virus NS1 protein is the viral factor that is involved in differential activation of cellular ER stress response. Additionally, this study also provides the evidence for the first time that influenza virus mediated inhibition of IL-23 is mediated by ER stress-induced transcription factor CHOP-10.The study also provides valuable insights on the increased susceptibility of bacterial infection during influenza that could be utilized for future therapeutic targets

against influenza virus. This study also shows that IL-23 could be used as potential therapeutic agent to reduce influenza-associated mortality.

Limitations and future directions: This dissertation provide evidence to support the primary hypothesis that influenza virus NS1-mediated inhibition of CHOP-10 mediates the inhibition of IL-23/IL-17 pathway of respiratory innate immune response which results in enhanced bacterial susceptibility during influenza. However, the study did not identify the exact mechanism of inhibition and proposed mechanism is substantially built upon findings in different cell type. Therefore, future research needs to be done to confirm the proposed model in alveolar macrophages. In the current study, effect of single intranasal dose rIL-23 administration on pathogen clearance was investigated in an immunocompetent host. To further delineate the role of IL-23 in protective immune response, future research using IL23 knockout host could further confirm the protective roles of IL-23. Also, characterization of innate immune cells, cytokines and tissue repair after IL-23 administration would be useful in understanding the IL-23/IL-17 immunity against secondary bacterial infections during influenza infection.

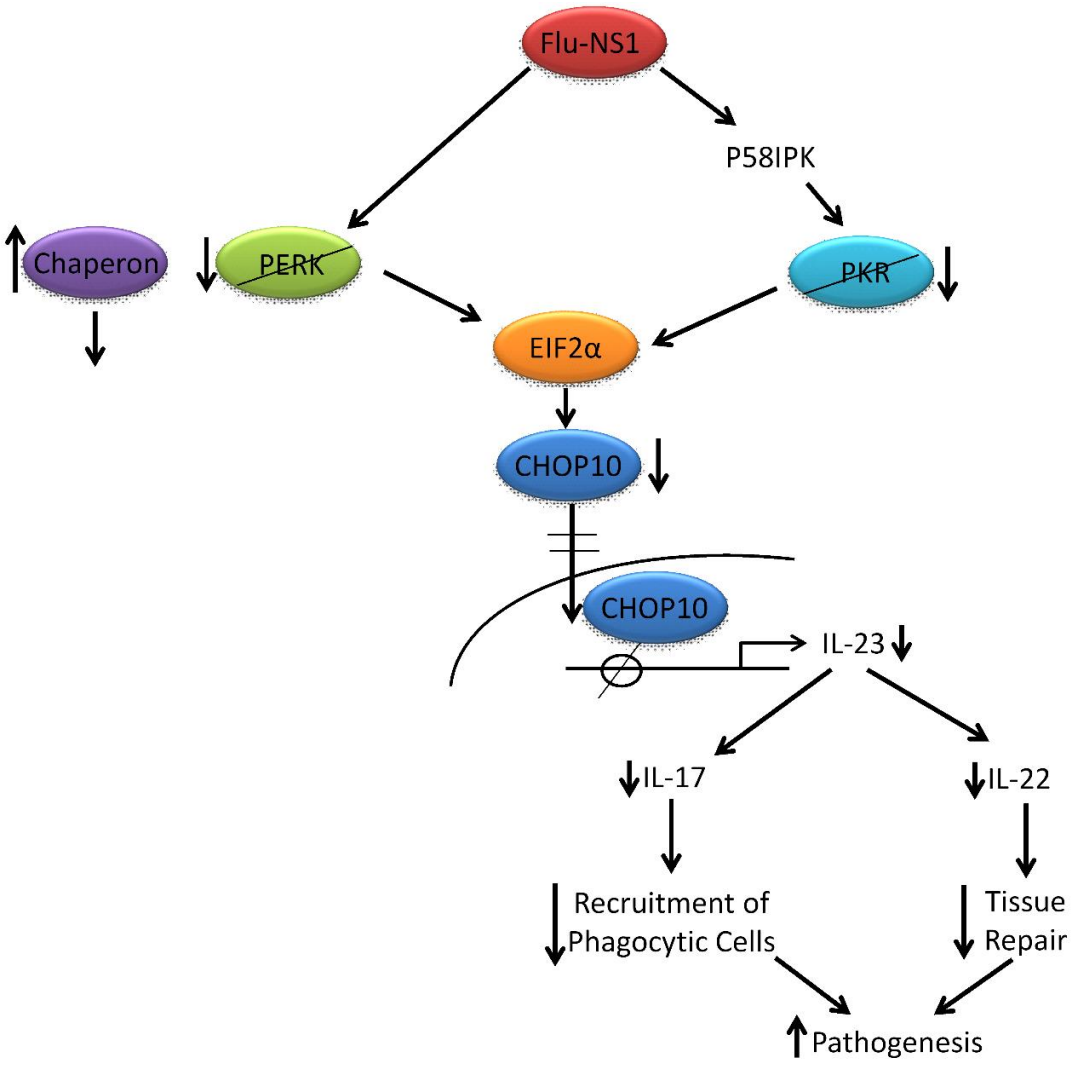


Figure 6.1: Model for influenza virus NS1-mediated inhibition of IL-23

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- First Position in Master's Program in Veterinary Microbiology Division
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Publications

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