INVESTIGATION OF INHALED NITRIC OXIDE AND MESENCHYMAL STROMAL CELLS AS NOVEL THERAPEUTIC STRATEGIES TO IMPROVE CLINICAL OUTCOME IN EXPERIMENTAL SEVERE INFLUENZA

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science

Institute of Medical Science
University of Toronto

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Investigation of Inhaled Nitric Oxide and Mesenchymal Stromal Cells as Novel Therapeutic Strategies to Improve Clinical Outcome in Experimental Severe influenza

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Abstract

Severe influenza, recognized as a clinical syndrome characterized by hyper-induction of pro-inflammatory cytokine production, results in approximately 250–500 thousand deaths annually worldwide. Current influenza research is focused on therapeutics to target the influenza virus or modulate influenza virus-induced inflammation as potential treatment options to improve clinical outcome in experimental influenza A (H1N1) virus infection. The goals of this work were: (1) to evaluate the utility of inhaled nitric oxide (iNO) for decreasing influenza virus production in the lungs, and (2) investigate the use of mesenchymal stromal (stem) cells (MSCs) for mitigating deleterious host responses to influenza infection. Here, we report that MSCs and iNO, administered alone either prophylactically or post-influenza virus infection, fail to modulate host inflammation, fail to improve acute lung injury, fail to dampen lung viral load, and fail to improve survival of infected mice.
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List of Abbreviations

A/Mex/4108: A/Mexico/4108/09
A/PR/8: A/PuertoRico/8/34
A/WSN/33: A/Wisconsin/33
AICAR: aminoimidazole carboxamide ribonucleotide
ALI: acute lung injury
AMPK: AMP-activated protein kinase
Ang-1: angiopoietin-1
APC: antigen presenting cell
ARDS: acute respiratory distress syndrome
BAL: bronchoalveolar lavage
CFU: colony forming unit
COX: cyclooxygenase
CV-N: cyanovirin-N
DC: dendritic cell
eNOS: endothelial nitric oxide synthase
FDA: Food and Drug Administration
GFP: green fluorescent protein
GvHD: graft-vs-host disease
HA: hemagglutinin
HLA: human leukocyte antigen
HMGB-1: high mobility group box 1
HMG-CoA: 3-hydroxy-3-methylglutaryl coenzyme A
HPAIV: high pathogenic avian influenza
IDO: indoleamine 2,3-dioxygenase
IFN: interferon
IL: interleukin
ILI: influenza-like-illness
IL-1R: interleukin-1 receptor
iNO: inhaled nitric oxide
iNOS: inducible nitric oxide synthase
KGF: keratinocyte growth factor
LPAIV: low pathogenic avian influenza
LPS: lipopolysaccharide
MDCK cell: Madin-Darby canine kidney cell
MHC: major histocompatibility complex
M1: matrix 1
M2: matrix 2
M2 macrophage: alternatively activated macrophage
NA: neuraminidase
NAI: neuraminidase inhibitor
NEP: nuclear export protein
NF-κB: nuclear factor kappa B
NK cell: natural killer cell
NO: nitric oxide
NOS: nitric oxide synthase
NP: nucleoprotein
NSAID: non-steroidal anti-inflammatory
NS1: non-structural protein 1
NS2: non-structural protein 2
PA: polymerase acidic protein
PB1: polymerase basic protein 1
PB2: polymerase basic protein 2
PB1-F2: polymerase basic protein 1 – F2
PFU: plaque forming unit
PGE2: prostaglandin E2
PPAR: peroxisome proliferator activated receptor
ROS: reactive oxygen species
SARS: severe acute respiratory syndrome
siRNA: short interfering RNA
SNAP: S-nitroso-N-acetylpenicillamine
sTNFR: soluble TNF receptor
TGF-β: transforming growth factor beta
TNF: tumour necrosis factor
TSG-6: TNF inducible protein 6
VCAM-1: vascular cell adhesion molecule-1
vRNP: viral ribonucleoprotein
WHO: world health organization
Chapter 1: Literature Review

1.1 Global burden of severe influenza

Influenza viruses A, B, and C belong to the Orthomyxoviridae family of single stranded negative-sense RNA viruses. Influenza B viruses are known to infect only humans and seals, while influenza A viruses can infect a wide range of avian and mammalian hosts. Influenza B viruses can cause mild to severe respiratory tract infections and can account for a significant proportion of laboratory confirmed cases of influenza; however, they do not cause pandemics [299]. This is likely due to their host range, which limits the generation of new strains by reassortment. Influenza C viruses are known to infect both humans and pigs and may cause mild upper respiratory tract illness in humans which can rarely lead to infection of the lower respiratory tract; nearly all adults have been infected with these viruses [300].

While wild aquatic birds are the primary reservoir for influenza A viruses, periodic reassortment between avian and mammalian influenza viruses or adaptation to novel host species can give rise to influenza epidemics or pandemics [1]. During annual influenza epidemics in humans, approximately 5–15% of the population will be infected with influenza A virus [2]. Of those infected, an estimated 3–5 million of these cases are classified as severe illness, leading to 250–500 thousand deaths each year [2]. In the United States alone, seasonal influenza accounts for more than 226,000 hospital admissions annually [3]. The spectre of an influenza pandemic, however, poses a drastically worse scenario.
Up to 50% of the population can be infected with influenza A virus during a pandemic year [4].
The most devasting pandemic to date, the 1918 Spanish influenza, was estimated to have killed
50 to 100 million people [5], with an estimated case fatality rate of 2–3% [6]. Case fatality rates
for the 1957/1958 Asian flu and the 1968 Hong Kong flu pandemics were estimated to be
approximately 0.2% [6], while the most recent pandemic, the 2009 swine origin influenza virus
(H1N1) pandemic, had a relatively low case fatality rate of 0.026% [7].

Most recently, 602 cases of avian influenza virus (H5N1) have been reported to the World
Health Organization (WHO) between 2003 and April 2012, of which 355 cases resulted in
fatalities [8]. However, the WHO employs stringent criteria to ascertain H5N1 disease
excluding asymptomatic individuals. A recent meta-analysis which calls the WHO H5N1
disease criteria into question reveals that 1–2% of study participants from 20 studies were
seropositive for H5N1 [10]. Therefore, the existence of mild or subclinical H5N1 infection
means the true case fatality rate is likely to be less than 60% as reported by WHO.
Furthermore, individuals with limited access to healthcare may not receive a laboratory-
confirmed diagnosis of H5N1 influenza virus infection [9].

1.2 Structure and life cycle of the influenza virion

The influenza virus genome is comprised of eight single-stranded negative-sense viral RNA
segments which encode the ten following gene products: hemagglutinin (HA), neuraminidase
(NA), matrix 2 (M2), polymerase basic protein 1 (PB1), polymerase basic protein 2 (PB2),
polymerase acidic protein (PA), nucleoprotein (NP), matrix 1 (M1), non-structural protein 1
(NS1) and non-structural protein 2 (NS2). An alternate reading frame in the PB1 polymerase gene segment which encodes the protein PB1-F2 may also be generated in select influenza A viruses [33].

The eight influenza viral segments are encapsulated by a host-derived lipid bilayer envelope in which viral encoded glycoproteins HA and NA, as well as M2 are embedded [11]. Currently there are 17 known subtypes of HA and 10 known subtypes of NA, which can be found in a number of combinations on the influenza A virus surface. Subtypes H1N1, H2N2, and H3N2 are known to circulate in humans while other subtypes are commonly found in other species including 9 different subtypes of H5, H7, and H9 found mainly in wild birds and poultry. H7N7 influenza A viruses can infect horses and H3N8 viruses can infect both horses and dogs. Other subtypes have been isolated from bats, pigs, marine mammals and guinea pigs [301,302].

Each influenza A viral RNA segment is structurally bound to NP to form viral ribonucleoprotein (vRNPs), which are associated with the 3 viral polymerase proteins that form the polymerase complex (PB1, PB2, and PA) located at the ends of the nucleocapsids. Finally, M1 forms a shell surrounding the virion nucleocapsids, underneath the virion envelope [1].
The process by which influenza A virus infects host cells begins with the binding of HA to sialic acid on host epithelial cells. The HA$_0$ precursor protein undergoes post-translational cleavage into the HA$_1$-HA$_2$ complex, a process critical to influenza virulence [13]. The virus is then internalized to form a host cell endosome containing each RNA segment. Hydrogen ions in the endosome are pumped through the opening of a proton pore, the M2 ion channel, which results in endosomal acidification and subsequent release of vRNPs into the cytoplasm. Via facilitated translocation with the aid of at least two nuclear localization sequences (NLS) contained by the NP known as NLS1 and NLS2, the vRNPs are then imported into the nucleus of the host cell where RNA replication, transcription and assembly of progeny vRNPs occurs [291]. Newly-made vRNPs assemble in the nucleus and are exported into the cytoplasm. HA, NA and M2
migrate via the golgi apparatus and anchor in the plasma membrane forming the new virion membrane. Viral segments are then arranged so that each of the eight segments is included in a newly formed virion. The process by which this occurs is not well understood however it is thought to involve several protein-protein interactions between the cytoplasmic tails of the viral integral membrane proteins, the matrix protein and the vRNPs [297]. Finally, sialylated progeny virions gather at the host cell membrane where their sialic acid is cleaved by NA which frees the newly assembled infectious influenza progeny virions from the host cell [11]. This process of influenza respiratory tract virus replication peaks within 24–72 hours after illness onset [88].

1.3 Uncomplicated and severe influenza

Most influenza virus infections are uncomplicated and resolve within 7 – 10 days. Symptoms of acute mild infection are sudden onset of high fever, coryza, cough, headache, malaise and transient tracheo-bronchitis [4]. Individuals who are more susceptible to developing severe disease include the elderly, children, pregnant women, immunocompromised individuals and individuals with known underlying health conditions including asthma, chronic obstructive airway disease, cardiovascular disease, obesity, and diabetes [4,14]. Clinically, severe influenza is characterized by dyspnea, cyanosis, bloody (or coloured) sputum, chest pain, persistent fever (duration > 3 days), altered mental status, and/or hypotension [15]. Severe influenza can lead to diffuse alveolar damage with histopathologic features of acute lung injury (ALI) and the acute respiratory distress syndrome (ARDS) [16–20], which directly contributes to influenza-associated morbidity and mortality. ARDS is characterized by increased permeability of the microvascular endothelium and disruption of the alveolar-capillary barrier, leading to a proteinaceous exudate in the alveolar airspaces, accompanied by neutrophil, macrophage, and
erythrocyte infiltration [21,22]. Post-mortem examination of the lungs of patients who succumbed to infection with the 2009 pandemic H1N1 influenza virus revealed diffuse alveolar damage with or without a hemorrhagic component or necrotizing bronchiolitis; severe neutrophilic inflammation was noted in a substantial number of cases [23]. Severe infection may also result in renal dysfunction and multi-organ failure, and secondary bacterial pneumonia may also cause substantial illness and death, as was observed with the 1918 influenza pandemic.

### 1.4 Viral determinants of influenza pathogenesis

#### 1.4.1 Hemagglutinin

High and low pathogenic avian influenza viruses (HPAIV and LPAIV, respectively) differ in their HA protein cleavage sequence. HPAIV possesses a polybasic cleavage site (QRERRRKRR/G) while LPAIV possesses one to two basic amino acids at this site [24–26]. The multibasic cleavage motif found in HPAIV is recognized by subtilisin-like endoproteases, such as furin, which are present in all tissues, while the LPAIV lacking this motif is recognized by trypsin-like enzymes preferentially expressed at the surface of respiratory and gastrointestinal epithelia [23]. Therefore, LPAIV, although infectious, replicates in a limited number of cell types and is therefore restricted in its ability to spread in the host, while HPAIV is able to replicate in all tissues leading to widespread lethal systemic infection [23,26]. Hatta et al. also found that high cleavability of HA was essential for lethal murine H5N1 infection as conversion of the multibasic amino acid HA site to the sequence typical of avirulent avian influenza viruses highly attenuated an H5N1 modified virus [27].

The importance of the avian HA glycoprotein in the induction of pro-inflammatory cytokines has been demonstrated in vitro [28]. The importance of influenza virus HA in immunomodulation is
also illustrated by the 1918 influenza pandemic, in which virus containing the 1918 HA/NA was associated with enhanced pulmonary infiltrates and production of IFN-γ, TNF, MIP-2, and CCL3 [29].

1.4.2 Polymerase proteins including PB1-F2 peptide

PB1-F2 is a critical influenza virulence determinant for some influenza virus strains in certain hosts [30]. The PB1-F2 peptide, encoded in the +1 reading frame of the PB1 segment of the trimeric viral RNA-dependant RNA polymerase complex, is expressed in the highly virulent 1918 pandemic influenza virus, highly virulent murine-adapted influenza A/Puerto Rico/8/34 (A/PR/8; H1N1) virus and HPAIV [31]. It is pro-apoptotic [32] and has been associated with increased pathogenicity and mortality in a mouse model [33]. PB1-F2 has also been implicated in copathogenesis with bacterial pathogens such as *Streptococcus pneumoniae*; the C-terminal portion of the peptide contributed to acute lung injury, even in the absence of viral replication [34]. The PB1-F2 peptide of the 2009 pandemic H1N1 influenza virus is truncated due to the presence of several stop codons in the PB1 gene [35,36]. This may partially account for the relatively low virulence associated with the 2009 pandemic virus. However, 2009 pandemic H1N1 influenza virus genetically modified to express PB1-F2 had similar virulence compared to wild-type 2009 pandemic H1N1 influenza virus in mouse and ferret models, despite elevated levels of IFN-γ, CCL2, CCL4, and CCL5 [36]. Since the initial viral strains were sequenced, several isolates circulating during the pandemic were found to express a 57 amino acid PB1-F2 due to a stop-to-leucine substitution at position 12. This appeared to increase virus replication in cell culture but not pathogenicity in a mouse model [37].
Expression of PB1-F2 was also shown to confer increased pathogenicity without altering virus replication [38] when a serine amino acid is present at position 66 [39]. Influenza viruses with the N66S mutation in PB1-F2 have been shown to cause enhanced production of cytokines and associated cellular infiltrates [40]. Thus, the presence of PB1-F2 influenza virus expression is an important contributor to influenza A virulence and is involved in immunopathology. PB2 mutations have also been noted to promote virulence and transmission, including D701N and E627K [27,41,42]. More recently, the PB2-E158G mutation was associated with increased viral replication and mortality in 2009 pandemic H1N1 influenza virus-infected mice as well as H5N1 virus-infected chickens [43].

1.4.3 Other viral determinants

Other virulence determinants, including influenza virus NS1 and NA, have been associated with increased virus replication as well as inflammation [44,45]. NS1 in particular has been well-studied, and an NS1-truncated live attenuated virus has been proposed as a vaccine candidate [46–48]. NS1 is a multifunctional protein and several reviews outlining its role in immune evasion, viral replication, and host cell modulation have been published [49–52]. Interferon antagonism is a primary function of the NS1 protein, acting at both the pre- and post-transcriptional levels of type I interferon production [53–55]. NS1 has also been shown to directly inhibit the actions of antiviral proteins [56,57]. NS1 activity and virulence varies depending on viral strain. For example, the NS1 in a highly pathogenic H5N1 virus from an outbreak involving humans was shown to play a central role to the high mortality associated with this virus [58]. Finally, NS1 has been implicated in host cell signaling, whereby the C-terminal
domain of avian influenza viruses contains a PDZ ligand capable of binding host cell proteins and leading to decreased survival and increased lung damage in a mouse model [59,60].

1.5 Antiviral therapy

1.5.1 Neuraminidase inhibitors, M2 ion channel inhibitors and other antivirals

While influenza vaccines may only provide moderate protection against influenza [292], annual immunization remains the primary means of protection against influenza. However, in the event of a pandemic, strain-specific vaccine production can take up to six months. During this time, entire unvaccinated populations remain susceptible to a novel pandemic strain. In the interim, antiviral drugs are important for patient management and the abrogation of transmission.

The United States Food and Drug Administration (FDA) has approved two classes of antiviral drugs for the prevention and treatment of influenza. These include the M2 ion channel inhibitors, also known as the adamantanes (amantadine and rimantadine) and the neuraminidase inhibitors (NAIs) (oseltamivir and zanamivir). The first class of drugs, the adamantanes, block the influx of H+ ions through the M2 ion channel, preventing the acid-triggered fusion reaction and thus interfering with viral endosomal release inside the cell [61]. The second class of drugs, the NAIs, competitively bind to the highly conserved enzyme NA active site by mimicking sialic acid, the natural substrate of NA. This inhibits the enzyme’s key function by destroying neuraminic acid-containing receptors, which prevents newly assembled virion cleavage and release of progeny virions from infected cells [61].
Because the licensure of NAIs was obtained based on studies in healthy adults with uncomplicated seasonal influenza, it is not clear that these drugs are effective for treatment of severe disease. A major Cochrane review which amalgamated two large reviews of oseltamivir use for influenza in healthy adults and children, respectively, found that oseltamivir shortens the duration of symptoms by less than a day in people with influenza-like illness (ILI) but there is no evidence of an effect on hospitalizations [62]. It is uncertain whether oseltamivir has an effect on complications. However, treatment with oseltamivir has been associated with a reduction in mortality in a number of studies [91,294,295].

Because influenza A virus possesses an error-prone RNA polymerase, a high incidence of mutation occurs and variants resistant to antiviral drugs may be selected; permissive mutations restoring viral fitness ensure ongoing transmission and infection [65,66]. With the development of viral resistance, the utility of adamantanes has been all but eliminated. Since 2005, nearly all influenza A (H3N2) viruses isolated globally were resistant to adamantanes [67,68]. All 2009 pandemic influenza A (H1N1) viruses isolated globally from September 2010 to January 2011 were also resistant to adamantanes [68]. As the continued use of adamantanes further exerts selective pressure on the development of drug resistance, the use of these drugs as mono-therapy should be eliminated from current clinical practice.

Unlike adamantanes, the frequency of resistance to NAIs has been minimal, until recently. Since 2007, a significant increase in seasonal influenza A (H1N1) virus mutations conferring resistance to oseltamivir have been observed [69]. The majority of these mutations have been characterized by a histidine to tyrosine mutation at residue 274 of the NA; the glutamine at position 276 is pushed away by the bulkier tyrosine substitute, thus disrupting the hydrophobic pocket which
otherwise accommodates the pentyloxy portion of the oseltamivir molecule [69,70]. During the 2008–2009 influenza season, nearly 100% of tested seasonal influenza A/H1N1 virus strains in the United States and in over a dozen other countries were found to be oseltamivir-resistant [71,72]. As of October 2010, 313 cases of oseltamivir-resistant 2009 pandemic H1N1 influenza virus have been identified, and these cases were mostly linked to prophylactic or therapeutic use of oseltamivir [68,73]. Oseltamivir resistance has also been found in H5N1 virus-infected and oseltamivir-treated patients [74,75]. However, the majority of 2009 pandemic H1N1 influenza viruses tested from September 2010 to January 2011 remained susceptible to oseltamivir [68].

Oseltamivir is restricted to oral administration, though a phase I trial is planned by Hoffmann La Roche for an intravenous (IV) formulation [76]. Clinical trials have also demonstrated the safety of peramivir, an NAI developed for IV administration. Japan has recently licensed peramivir under the name Rapiacta [77,78].

Another licensed NAI, zanamivir, has a low incidence of resistance and is currently an alternative treatment strategy for oseltamivir-resistant strains [79]. Zanamivir’s low resistant rate may be attributable to a low prescription rate as well as differences in the way it binds to NA [61]. However, zanamivir is delivered to the lungs by a dry powder inhaler which may pose difficulties for patients on mechanical ventilation [80], individuals with severe cough or dyspnea, small children and the elderly [81]. There is also concern of sub-optimal delivery to sites of infection in influenza virus-infected patients with pneumonia or pulmonary disease [16,80,82]. Therefore, H5N1 virus-infected patients who are viremic may not respond to NAIs as the antiviral is unlikely to reach the necessary end-target sites, as has been observed where patients
shed virus for many days despite combined NAI treatment [293]. Nebulization of zanamivir is not recommended due to the formulation’s potential to cause mechanical ventilator obstruction, which may result in death [83]. Zanamivir is currently undergoing phase II trials for parenteral I.V. use [84,85]. CS-8958 (laninamivir prodrug), an NAI similar to zanamivir but with a prolonged half-life, is also being studied for use by inhalation and is currently commercially available as Inavir in Japan [86,87].

In addition to the development of influenza virus drug resistance, another important caveat to consider with respect to antivirals includes limitations in the timing of administration [88–92]. Initiation of oseltamivir treatment within the first 48–72 hours after symptom onset is associated with lower mortality and greater antiviral efficacy compared with treatment initiated beyond the first 48 hours of symptom onset for H1N1, H3N2 and H5N1 infected patients [89,90]. This may be attributed to the peak in influenza respiratory tract viral replication at 24–72 hours after illness onset [88]. Furthermore, influenza patients in the intensive care unit or those who died from severe influenza were less likely to have received antiviral treatment within 48 hours after the onset of symptoms [91]. Late antiviral treatment may also be unable to prevent the development of ARDS; 2009 pandemic H1N1 influenza virus-infected patients with ARDS who died began oseltamivir treatment an average of 5 days after the onset of symptoms compared to infected patients who survived without ARDS and those with mild-disease, where treatment was initiated an average of 4 and 2 days post symptom onset, respectively [92]. However, late antiviral treatment is still associated with a significant reduction in mortality compared to no treatment [294].
Other caveats include limited efficacy in cases of H5N1 virus infection [75] and limited access to stockpiles during a pandemic [93,94]. More effective, affordable and accessible drugs to treat severe influenza are urgently required. Other antiviral agents which may target different stages of the viral life cycle are currently being investigated, including the polymerase inhibitor favipiravir (T-705), HA inhibitor prokaryotic cyanovirin-N (CV-N), short interfering RNA (siRNA), and a sialic acid receptor inhibitor, fludase (DAS-181) [95].

1.6 Investigational antiviral therapy - inhaled nitric oxide

Nitric oxide (NO) is an important cellular signaling molecule synthesized from L-arginine by NO synthase (NOS). In the airways, NOS is present in a variety of cells, including macrophages, vascular endothelial cells, airway epithelial cells and neurons, where NOS activity is known to mediate neurotransmission, smooth muscle contraction and mucin secretions. There are three types of NOS: constituent and calcium-dependant isoforms (both principally present in endothelial cells and neuronal cells), and the inducible or calcium-independent isoform (iNOS) [96]. NO is also a well known biological mediator in the host response to infection [96,97]. Various inflammatory stimuli such as lipopolysaccharide (LPS) and cytokines including IFN-γ and TNF can cause high and sustained NO production by iNOS. Depending on the species, strain, infection dose and pathogen entry route, iNOS activity can result in pro- or anti-inflammatory responses, cytotoxicity, or cytoprotection [reviewed in 96].

In vitro, NO antimicrobial activity has been demonstrated against a variety of viruses including ectromelia virus, vaccinia virus, herpes simplex type 1 viruses, coronavirus, and influenza A and B viruses [98–102]. In these studies, administration of the NO donor S-nitroso-N-
acetylpenicillamine (SNAP) to virus-infected cells significantly reduced viral burden. Lin et al. reported that SNAP did not have any direct anti-viral capability since pre-treatment of viral stocks with SNAP for 1 hour failed to affect virus infectivity, but rather biochemical analysis revealed that NO exerts its antiviral effects by profound inhibition of viral RNA synthesis, viral protein accumulation, and viral release from infected cells [103].

Severe cases of influenza are often associated with multisystem organ failure and hypoxemic respiratory failure, including ALI/ARDS requiring ventilatory support [104,105]. Affected individuals may receive ‘rescue’ therapies, including inhaled nitric oxide (iNO), in an attempt to improve outcome [105]. However, iNO administration for ARDS secondary to viral pneumonia has not been specifically reported to improve clinical outcome [104,105].

iNO therapy is currently FDA approved for the treatment of term and near-term neonates with hypoxemic respiratory failure associated with clinical or echocardiographic evidence of pulmonary arterial hypertension [106,107]. Variable findings have been reported for iNO efficacy when administered at 1 ppm and up to 80 ppm. For its indicated use, iNO has been found to increase vasodilation, transiently improve arterial oxygenation, reduce length of mechanical ventilation, reduce oxygen requirement, and decrease length of stay in the intensive care unit [107–109]. One study found iNO, at 30 ppm or less, decreased the spread and intensity of lung infiltrates and improved arterial oxygen saturation in patients with severe acute respiratory syndrome (SARS) [110]. However, systematic reviews and meta-analysis of randomized controlled trials have shown that iNO, when used therapeutically in the management of ARDS, does not reduce mortality [111–115]. Moreover, iNO therapy for ARDS may increase
the risk of iNO treated patients developing renal dysfunction [114,115]. Despite this, 39% of critical care specialists surveyed reported using iNO for the management of patients with ARDS in Ontario, Canada [116].

### 1.7 Host determinants of pathogenesis

#### 1.7.1 Cytokine dysregulation

Various host factors can influence susceptibility to influenza and progression to severe disease including immune status, underlying health conditions and age. Recently, it has become clear that one of the most important factors contributing to influenza-related morbidity and mortality is inflammation. Severe influenza can be characterized as a disease which induces hyper-production of inflammatory cytokines during host inflammation. In physiologic conditions, anti-inflammatory cytokines are immunoregulatory molecules that control pro-inflammatory responses. In pathological conditions, insufficient control of anti-inflammatory responses over pro-inflammatory responses may occur and the net response of this imbalance determines individual patient outcome. Specifically, the extent of the host inflammatory response triggered by various influenza virus strains is positively correlated with clinical severity of influenza including HPAIV infection and infection with reconstructed 1918 pandemic H1N1 influenza virus in animal models. Importantly, the degree or magnitude of dysregulated cytokine production fails to correlate with viral titers in both animal models [117–123] and in humans [123,124].
Cytokine dysregulation has repeatedly been observed in human influenza virus infection from the earliest H5N1 outbreaks in 1997 onward [13,123,124]. H5N1-induced hypercytokinemia has also been observed in mouse models [120] and in cell culture [125]. In these H5N1 studies, levels of cytokines, including CXCL10, CCL5, CXCL9, CCL2, IL-8, IL-10, IL-6, IFN-γ, TNF, CCL3 and soluble IL-2, were elevated compared to other influenza virus strains.

Influenza caused by seasonal H1N1 and H3N2 virus strains and the recent 2009 pandemic H1N1 influenza virus have not been associated with cytokine dysregulation, which may partially account for the low case fatality rate of 2009 pandemic H1N1 influenza virus [7,126–128]. In vitro experiments with seasonal H3N2 influenza virus and other non-pandemic H1N1 influenza viruses showed no evidence of hypercytokinemia [126,127]. In vitro [128] and ex vivo [127] experiments did not reveal cytokine dysregulation induced by 2009 pandemic H1N1 influenza virus infection. However, To et al. found higher levels of pro-inflammatory cytokines in patients with 2009 pandemic H1N1 influenza virus infection and ARDS [92]. This often resulted in death compared to patients who survived without ARDS or those who suffered mild forms of the disease, independent of viral load amongst the three groups. Taken together, these studies suggest that 2009 pandemic H1N1 influenza virus may be associated with cytokine dysregulation in the setting of ARDS.

The significance of each elevated cytokine in influenza virus-induced cytokine dysregulation is not well defined. For example, Peiris et al. found significantly elevated levels of CXCL10 and CXCL9 but no significant differences in levels of CCL2, CCL5 and IL-8 in patients with H5N1 virus infection compared to patients infected with other influenza viruses [123], while Perrone et
al. did not observe significantly elevated levels of CXCL10 or CXCL9, but rather significantly elevated levels of CCL3, KC (mouse IL-8), IL-1α, IFN-γ, and IL-6, in HPAIV infected mice [120].

In order to elucidate which cytokines are most important in mediating lung pathology, investigators have taken advantage of mice with targeted deletions of specific cytokine or cytokine receptor genes (i.e., cytokine ‘knock-out’ mice). Research involving cytokine knock-out mice has revealed protective or deleterious roles of certain cytokines in the host response to influenza, which seems to vary depending on the specific strain or subtype of influenza virus studied. CCR5−/− mice (receptor for CCL3, CCL4 and CCL5) developed massive inflammation and severe pulmonary damage associated with decreased survival when infected with influenza A/PR/8 virus [117]. Similarly, mortality was significantly increased or accelerated in IL-1R1−/− mice (receptor for IL-1α and IL-1β) compared to wild-type mice when infected with influenza A/PR/8 virus or reconstructed 1918 virus, respectively [129,130]. This suggests signaling through IL-1R1 is protective and these observations indicate that a proper balance in immune-mediated inflammation is necessary to achieve eradication of influenza virus while avoiding deleterious host-mediated inflammatory tissue injury. Other research has also found similar morbidity and mortality in CCL3−/−, IL-1R−/−, IL-6−/−, TNFR1−/−, IL-6−/−, TNF−/− or CCL2−/− mice inoculated with avian influenza virus H5N1 compared to wild-type mice [131,132].

To the contrary, other studies have shown improved outcome in cytokine or cytokine receptor knock-out mice. CCR2−/− mice infected with influenza A/PR/8 virus had reduced lung injury and inflammation and mortality compared to wild-type controls [117]. The chemokine receptor
CCR2 binds its ligand CCL2 to cause the migration of monocytes and the development of monocyte-derived inflammatory cells in the lungs which has been associated with influenza virus induced lung injury and subsequent mortality [117,122,133]. This finding is in contrast to that observed with $CCL2^{-/-}$ mice reported by Salomon et al. [132]. A further study by Lin et al. showed reduced lung pathology and an 89% survival increase of $CCR2^{-/-}$ mice compared to influenza virus-infected wild-type controls [133]. However, Aldridge et al. showed $CCR2^{-/-}$ mice infected with A/PR/8 had comparable morbidity and mortality to infected wild-type controls [134]. Subsequent studies using a CCR2 inhibitor, PF-04178903, administered prophylactically and twice daily post-infection resulted in 100% survival of influenza A/PR/8 virus-infected mice compared to 25% survival and decreased pulmonary immune pathology in untreated mice [122]. Therefore, CCR2 inhibition may be effective in reducing influenza virus-induced lung pathology and overall mortality.

It is important to bear in mind that conclusions based on experiments employing mice with specific targeted genetic deletions (‘knock-out’) may be misleading as other downstream factors may be modified and ‘hidden from view’. Functional redundancy amongst signaling pathways may result in host compensation which could mask the immunomodulatory benefits of single cytokine depletion. To account for such host responses, Perrone et al. examined $TNF-R1$, $TNF-R2$, and $IL-1RI$ triple knock-out mice [121]. When infected with a lethal dose of H5N1, these mice exhibited decreased inflammatory cell infiltrates and cytokine levels, reduced morbidity, and a significant delay in mortality compared to wild-type controls [121]. Taken together, this research suggests that reducing overall inflammation rather than targeting individual cytokines may prove to be a superior strategy for decreasing influenza-related morbidity and mortality.
1.8 Immunomodulatory therapy

The outcome of influenza virus infection is determined by both viral and host factors; therefore, it would be logical to investigate the efficacy of therapeutic strategies which target the host in combination with antiviral therapy. Such therapeutic strategies could potentially target either signaling pathways to reduce viral replication or host-mediated inflammation in an effort to dampen deleterious tissue/organ dysfunction and injury. Importantly, several experimental studies in mice show that mortality is reduced by immunomodulatory agents without concomitant antiviral treatment and without any decrease in virus replication, implying modulation of host inflammation alone may be sufficient to improve influenza virus-infected patient outcome or provide additional benefit when used in combination with antiviral agents.

A number of FDA-approved drugs have received investigational interest as immunomodulatory strategies for the treatment of influenza (described in sections 1.8.1-1.8.8). All of these drugs, with the exception of CCR-2 antagonist PF-04178903, are produced as generic agents and, thus, would be relatively inexpensive and available for deployment in the event of a pandemic. In addition, these agents could potentially circumvent waning efficacy in the face of increasing antiviral resistance. Chapter 1.8 will focus on immunomodulatory agents for the treatment of severe influenza which target dysregulated host inflammatory responses (summarized in Table 1.1).
Table 1.1. Summary of investigational immunomodulatory agents for treatment of influenza (reprinted with permission from © Expert Reviews Ltd, ref. [135].)

<table>
<thead>
<tr>
<th>Mechanism of action</th>
<th>In vivo effects</th>
<th>Overall potential</th>
</tr>
</thead>
<tbody>
<tr>
<td>COX-2 inhibitor: celecoxib</td>
<td>Decreases host proinflammatory cytokine and chemokine production</td>
<td>Ineffective as monotherapy in mouse models of influenza [119,120]. Combined with zanamivir and amantadine, celecoxib improved survival of H5N1-infected mice from 13.3% to 53.3% compared to zanamivir alone [114].</td>
</tr>
<tr>
<td>CCR2 inhibitor: PF-04178903</td>
<td>Inhibits binding of the chemokine, MCP-1</td>
<td>Increased survival of A/PR/8-infected mice from 25% to 100% [90]</td>
</tr>
<tr>
<td>Macrolides: azithromycin, clarithromycin, erythromycin</td>
<td>Lowers host proinflammatory cytokine production</td>
<td>Erythromycin increased survival of H2N2-infected mice from 14% to 57% [103].</td>
</tr>
<tr>
<td>Anti-TNF agents</td>
<td>Mediator of pulmonary inflammation during influenza A viral pneumonia</td>
<td>Decreased pulmonary inflammation and prolonged survival of A/PR/8-infected mice [113]. Reduced pulmonary inflammatory cell infiltrates, T-cell cytokine production, weight loss and illness severity [114].</td>
</tr>
<tr>
<td>Statins</td>
<td>Inhibits activation of immune effector cells</td>
<td>Associated with 16% reduction in 30-day pneumonia mortality in a cohort study over ten influenza seasons [112].</td>
</tr>
<tr>
<td>Glucocorticoids</td>
<td>Suppresses proinflammatory cytokine production via activation of the GC receptor</td>
<td>H3N2-infected rats treated with tamoxifen showed decreased macrophage activation and reduced pulmonary inflammation [96].</td>
</tr>
<tr>
<td>PPAR-α agonist: gemfibrozil</td>
<td>Reduces proinflammatory cytokine production</td>
<td>Daily gemfibrozil increased survival of H2N2-infected mice from 26% to 52% [104].</td>
</tr>
<tr>
<td>PPAR-γ agonist: pioglitazone, rosiglitazone</td>
<td>Reduces proinflammatory cytokine production</td>
<td>Pioglitazone increased survival of A/PR/8-infected mice by 20–40% [92,155]. Rosiglitazone increased survival of A/PR/8-infected mice from 30% to 100% [155].</td>
</tr>
<tr>
<td>AMPK agonist: AICAR</td>
<td>Activates AMPK</td>
<td>AICAR increased survival of A/PR/8-infected mice by 40%, while combination therapy including AICAR and pioglitazone improved survival by 60% [155].</td>
</tr>
</tbody>
</table>

A/PR/8: Influenza A/PuertoRico/8/34; AICAR: Aminomimidazole carboxamide ribonucleotide; AMPK: AMP-activated protein kinase; ARDS: Acute respiratory distress syndrome; CCR2: Chemokine receptor 2; COX-2: Cyclooxygenase 2; GC: Glucocorticoid; MCP-1: Monocyte chemotactic protein-1; PPAR: Peroxisome proliferator activated receptor; TNF: Tumor necrosis factor.
1.8.1 Glucocorticoids

Glucocorticoids are a group of corticosteroids which exert anti-inflammatory activity via binding to cytoplasmic receptors that subsequently interact with glucocorticoid response elements to regulate transcription of a number of genes [136]. Clinical trials using prolonged low-to-moderate dose glucocorticoid therapy for ARDS, a prominent feature of severe influenza, have demonstrated that glucocorticoid therapy enhances the anti-inflammatory activity of the glucocorticoid-activated glucocorticoid receptor; a major regulator of inflammation in ARDS [reviewed in 137]. Consistent with these clinical observations, glucocorticoids suppress cytokine levels [138] and IFN-γ induced pro-inflammatory gene expression including cyclooxygenase-2 (COX-2) expression in human bronchial epithelial cells in vitro [136].

Evidence to date supporting the use of glucocorticoids for the treatment of severe influenza is inconclusive. In a rat model of experimental H3N2 influenza, daily doses of the glucocorticoid triamcinolone acetonide combined with an NAI suppressed IFN-γ induction, resulting in decreased macrophage activation and pulmonary inflammation [139]. However, survival benefits were not reported in this study, and a study of experimental H5N1 influenza in mice found no difference in mortality between daily corticosterone-treated mice and controls [132]. In humans, glucocorticoid therapy has yielded equivocal H5N1 infection with uncertain effects. In a randomized trial in Vietnam, all 4 H5N1 virus-infected patients who received glucocorticoids died [15]. Moreover, in a clinical series reported from Thailand, only 2 of 8 H5N1-infected patients treated with methylprednisolone survived [140]. In contrast, a recent study, while lacking an ‘influenza infected with ARDS’ control group, reported that glucocorticoid treatment improved ALI in individuals with ARDS secondary to severe 2009 pandemic H1N1 influenza.
In view of these mixed results, the use of glucocorticoids as adjunctive therapy to antiviral drugs for severe influenza may warrant further study in the form of a randomized clinical trial, but there is currently insufficient evidence to endorse its routine use.

### 1.8.2 Macrolides

Macrolides, such as erythromycin, azithromycin, and clarithromycin, are bacteriostatic protein synthesis inhibitors that exert broad immunomodulatory activities including downregulation and upregulation of pro- and anti-inflammatory cytokines, respectively, modulation of leukocyte recruitment, and improvement of macrophage phagocytic function, but without causing global immunosuppression [142–144]. *In vitro*, clarithromycin reduces cytokine content in culture supernatant of seasonal H3N2 influenza virus-infected tracheal epithelial cells [145]. Erythromycin treatment of H2N2 influenza virus-infected mice increased survival from 14% to 57%, and reduced inflammatory cell counts [146].

Secondary bacterial pneumonia is frequently observed during influenza pandemics, resulting in increased influenza viral titers and lung inflammation [147,148]. As non-lytic antibiotics, macrolides may be important in clearing influenza bacterial co-infection in addition to decreasing host inflammation. Ampicillin, a β-lactam antibiotic targeting the bacterial cell wall has been shown to clear bacterial infection from influenza virus-infected lungs, however ampicillin use did not alter mortality rate in a mouse model of severe pneumococcal pneumonia with bacteremia following influenza [149]. This prompted investigation into the use of azithromycin, which significantly improved outcome of a milder bacteria-influenza co-infection compared to ampicillin by dampening inflammatory responses caused by ampicillin-induced
bacterial lysis, due to their non-lytic antimicrobial activity [150]. Therefore, macrolides may be advantageous when used in secondary bacterial pneumonia following influenza, and clinical trials are required to demonstrate a benefit from macrolide use in severe influenza. However, a note of caution is warranted because of the risk that widespread routine use of antibiotics for treatment of individuals with influenza may promote the development of problematic antimicrobial resistance in the long-term [151]. Also, safety concerns including increased risk of cardiovascular death have been associated with macrolides [296].

### 1.8.3 TNF inhibitors

Researchers have explored the use of therapeutic agents to lower host inflammation by directly targeting specific cytokines. TNF-neutralizing monoclonal antibodies and soluble TNF-receptor fusion proteins are important in managing inflammation caused by immune-mediated disorders, such as inflammatory bowel disease and rheumatoid arthritis [152,153] and increasing survival of LPS injected mice [154]. In contrast, anti-TNF strategies have not been beneficial in the treatment of other inflammatory conditions such as sepsis [155].

Experimental use of anti-TNF therapy in mouse models of influenza has shown inconsistent results. Few studies have reported that TNF neutralizing strategies for severe influenza in mouse models reduce lung lesion severity, pulmonary inflammatory cell infiltrates, and T-cell cytokine production, however with no survival benefit reported [131,132,156,157]. $TNFR-1^{-/-}$ mice survived longer than wild-type mice when infected with reconstructed 1918 influenza virus, suggesting that anti-TNF therapy might provide some therapeutic benefit in severe influenza. However, it should be noted that all mice died in both groups [130].
Anti-TNF therapy for severe influenza in humans has not been studied in a controlled clinical trial, and its failure for the treatment of sepsis casts doubt on its potential benefit in severe clinical influenza. Additionally, increased risk of bacterial infection and infections with rare intracellular pathogens are well-described complications of anti-TNF therapy [158]. Thus, overall experimental evidence to support the clinical use of anti-TNF therapy for severe influenza is currently lacking, especially when considering the attendant risks.

1.8.4 Cyclooxygenase-2 inhibitors

Cyclooxygenase (COX) enzymes, which catalyze the conversion of arachidonic acid into prostaglandins, play important roles in modulating immune responses and inflammation [118,159,160]. Widely used clinically for their analgesic, anti-inflammatory and antipyretic properties, the COX inhibitors consist of the well known non-selective non-steroidal anti-inflammatory drugs (NSAIDs), such as ibuprofen, which inhibit both COX-1 and COX-2, and the selective COX-2 inhibitors, such as celecoxib, which target COX-2 specifically [118].

In vitro but not necessarily in vivo data support the use of COX-2 inhibitors for severe influenza. Pronounced COX-2 upregulation has been reported following in vitro infection of macrophages with H5N1 virus and in lung tissue at autopsy from patients who died of H5N1 virus-associated disease [161]. In vitro, COX-2 inhibitors, including celecoxib, have been shown to suppress influenza virus-induced inflammatory cytokine production [128,161]. In mouse models of H3N2 influenza, inflammation was reduced and survival increased in COX-2⁻/⁻ compared to wild-type mice [118], suggesting a benefit from COX-2 deficiency. However, experiments in knock-out mice may yield misleading results secondary to effects on unidentified downstream pathways. In
murine models of influenza, the use of the pharmacological COX-2 inhibitor celecoxib did not confer any survival advantage [162,163]. These studies suggest that COX-2 inhibitors alone may not be effective agents for severe influenza therapy; however, retrospective studies to determine whether COX-2 inhibitor use was associated with improved clinical outcome may still be warranted.

Research examining the use of COX-2 inhibitors in combination with anti-viral drugs has shown promising results. Combination therapy including zanamivir, celecoxib and the anti-inflammatory drug mesalazine significantly improved survival of mice infected with H5N1 influenza virus compared to treatment with zanamivir alone [162]. Importantly, the triple combination therapy reduced levels of IL-6, IFN-γ, TNF and MIP-1 in infected mice compared to zanamivir treatment alone. Single daily use of either immunomodulator or the mesalazine and celecoxib combination failed to confer any survival advantage. It should also be noted that combination therapy of mesalazine and celecoxib was not investigated further in this study. The experimental data suggests that COX-2 inhibitors may provide additional benefit when used adjunctively to existing antiviral strategies. Further research is necessary to strengthen the evidence in support of combined celecoxib, mesalazine, and antiviral therapy use for severe influenza.

Animal models of ALI have shown COX-2-derived mediators to exert a protective role against inflammation-induced lung injury, in part via enhanced lipoxin signaling [164]. Lipoxins are a unique class of arachidonic acid-derived lipid pro-inflammatory mediators of inflammation which display in vivo inhibition of polymorphonuclear cell activation, cytokine release and
angiogenesis [reviewed in 165]. Inhibition of lipoxin signaling has been associated with enhanced lethality of H5N1 virus infection in mice [166]. This finding, when considering the unconvincing *in vivo* evidence to support the use of COX-2 inhibitors as monotherapy, provides indication of the potential use for lipoxin agonists to reduce inflammation in severe influenza and improve patient outcome.

### 1.8.5 HMG-CoA reductase inhibitors

A group of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, commonly known as statins [167], were proposed as a potential therapeutic strategy to reduce influenza virus-induced inflammation in 2005 [93]. Statins have been shown to modulate host immunity by inhibiting activation of immune effector cells, such as macrophages, and antigen presentation by macrophages to T cells, as well as affecting endothelial function through the inhibition of reactive oxygen species (ROS) production and upregulation of endothelial nitric oxide synthase (eNOS) [168, reviewed in 169]. Statins also antagonize high mobility group box 1 protein (HMGB-1), a potent pro-inflammatory cytokine, and suppress CCR2 gene expression [170–173]. Advantages of this class of drug include its widespread availability, relatively inexpensive cost, and long shelf life [174].

No study has reported statin reduced mortality in murine models of influenza. In humans, a cohort study conducted during the 1996–2006 influenza seasons revealed a 16% reduction in 30 day pneumonia mortality with the use of statins, although interpretation of these results may have been complicated by confounding factors [94,174]. Among patients admitted with laboratory-confirmed influenza, those who used statins before or during hospital stay had lower
odds of dying within 30 days and after adjusting for other variables they reported a 41% reduction in mortality [175]. It is important to note that adverse drug reactions, such as autoimmune hepatitis and myopathy, are associated with statin therapy. Taken together, there is currently insufficient evidence to recommend routine clinical use of statins for the management of influenza. A randomized clinical trial of acute statin therapy in hospitalized statin-naïve, influenza virus-infected patients may be warranted [176].

1.8.6 High mobility group box 1 protein antagonists

High mobility group box 1 (HMGB-1) is a 215 amino acid protein that functions intracellularly as a transcription factor and is highly conserved among species [177]. When released either passively from dying, necrotic cells or actively from macrophages, HMGB-1 functions in the extracellular milieu as a signal of tissue injury/damage [178]. Implicated in the pathogenesis of severe sepsis [179,180] and arthritis [181], HMGB-1 is a potent mediator of pro-inflammatory activity in the presence of inflammatory cytokines such as IL-1β, IFN-γ or TNF [171,172]. Given these properties, HMGB-1 antagonists could potentially prove useful in the treatment of ‘the cytokine storm’ in severe influenza.

However, HMGB-1 is characterized as a relatively late mediator of inflammation in sepsis [171,180,182] and, thus, its inhibition may not impact clinical outcome in severe influenza. For example, plasma levels of HMGB-1 in H2N2 influenza virus-infected mice peaked at day 9 post-infection (P.I.), a full 2 days after peak mortality was reached [183]. Furthermore, treatment with the HMGB-1 antagonist ethyl pyruvate failed to reduce mortality [183]. Further studies of
other HMGB-1 antagonists will provide a better understanding of the role of HMGB-1 in severe influenza.

Glycyrrhizin, a compound extracted from liquorice root, has been reported to bind and inhibit HMGB-1 [184, 185], increase T-cell IFN-γ production, and reduce endocytic viral uptake [186–188]. Glycyrrhizin treatment increased survival in murine models of influenza when administered prophylactically 1 day prior to infection and continued on days 1 and 4 P.I. [187]. However, protection against LPS induced lung injury in mice receiving glycyrrhizin pre-treatment has been attributed in part to suppression of COX-2 and iNOS expression [189]. Therefore, additional study is needed to determine whether inhibition of HMGB-1 is in fact the mechanism by which glycyrrhizin increases survival in experimental influenza. Metformin and statins also antagonize HMGB-1 [170, 190]. Molecular modeling of the HMGB-1-glycyrrhizin interaction may aid in the development of potent anti-HMGB-1 specific drugs which could potentially be useful as adjunctive therapy to antiviral drugs in the treatment of severe influenza.

1.8.7 Peroxisome proliferator activated receptor agonists

Peroxisome proliferator activated receptors (PPARs) are a family of lipid activated transcription factors, consisting of PPAR-α, PPAR-δ, and PPAR-γ, which are key regulators of inflammation and lipid metabolism [reviewed in 191]. PPAR activation antagonizes crucial inflammatory pathways such as NF-κB, AP1 and STAT [reviewed in 192], which results in a reduction of inflammatory cytokines such as IL-6 and IFN-γ [193].
Gemfibrozil, a PPAR-α agonist belonging to the fibrate family of drugs, was discovered over 40 years ago to reduce plasma lipid and cholesterol level and is widely used as treatment for hypertriglyceridemia [194,195]. Gemfibrozil reduces the release of inflammatory cytokines including TNF, IL-6 and IFN-γ [196,197] and has been proposed for the treatment of severe influenza [194,196]. Daily injections of gemfibrozil administered in a treatment strategy following the onset of experimental H2N2 influenza in mice increased survival from 26% to 52% [194]. In contrast, gemfibrozil failed to increase survival of H5N1 influenza virus-infected mice when administered 48 hours post-inoculation [162]. These studies suggest the need to conduct further research on the use of gemfibrozil for severe influenza.

Thiazolidinediones, also known as glitazones, are a class of PPAR-γ agonists, including pioglitazone and rosiglitazone. Pioglitazone has been shown to suppress inflammation in sepsis [198] and improve insulin sensitivity in patients with impaired glucose tolerance [199] by reducing CCL2 expression, which is the primary ligand for CCR2 and an important mediator of host inflammation [199,200]. In experimental murine models, prophylactic administration of pioglitazone to A/PR/8 influenza virus-infected mice has been reported to increase survival by 20–40% [134,201]. Aldridge et al. found the benefits of pioglitazone administration pre- and post-influenza virus inoculation in mice to be independent of pulmonary viral load [134]. Therefore, the benefit of pioglitazone therapy can be attributed to modulation of host inflammatory responses.

Rosiglitazone has also recently been shown to increase survival in experimental murine influenza. Specifically, Moseley et al. reported 100% survival of A/PR/8 influenza virus-
infected mice treated with rosiglitazone compared to 30% survival of influenza virus-infected controls [201]. Moseley et al. also reported 20% survival of influenza A/California/04/09 (a 2009 pandemic H1N1 strain) virus-infected mice treated with rosiglitazone compared to 100% fatality of infected controls. These results are by far the most promising reported to date in the search for an effective immunomodulatory strategy to improve clinical outcome in severe influenza. Because of the encouraging results observed with PPAR agonists in the treatment of influenza, biochanin A, a component of red clover with both PPAR-\(\alpha\) and PPAR-\(\gamma\) agonistic properties, has been proposed as a potentially effective immunomodulatory agent for the treatment of influenza [195].

1.8.8 AMP-activated protein kinase agonists

Increases in the intracellular AMP-to-ATP ratio trigger the activation of AMP activated protein kinase (AMPK), an enzyme which exerts anti-inflammatory effects upon activation [202]. Aminoimidazole carboxamide ribonucleotide (AICAR) is an AMP-activated protein kinase activator. In a murine model of LPS-induced ALI, administration of AICAR resulted in AMPK activation in the lungs which diminished the severity of lung injury as shown by decreased pulmonary edema, and reduced bronchoalveolar lavage fluid levels of IL-6 and TNF [202]. Moseley et al. reported a 40% increase in survival of A/PR/8 influenza virus-infected mice treated with AICAR while combination therapy including AICAR and pioglitazone improved survival by 60% [201].

Metformin, another AMPK agonist and known HMGB-1 antagonist, is routinely used in combination with pioglitazone for patients with diabetes mellitus and has been shown to improve
survival in murine endotoxemia [190]. Metformin and pioglitazone have been shown to have synergistic effects in type 2 diabetes [203]. This compound is also commercially available. Therefore, metformin and AICAR might prove effective in the treatment of severe influenza when used either alone, or in combination with PPAR-\(\gamma\) agonists and/or antiviral drugs.

### 1.9 Investigational immunomodulatory therapy – mesenchymal stromal cells (MSCs)

In 1968, Friedenstein and colleagues described a murine bone marrow cell population with the ability to form colonies and differentiate into several cell types [204]. These cells, now referred to as mesenchymal stromal (stem) cells (MSCs), represent a heterogenous subset of non-hematopoietic pluripotent stromal cells with multilineage potential. MSCs can be isolated from embryonic tissue, adipose tissue, liver, muscle and dental pulp; however, adult bone marrow remains the most common source of MSCs for pre-clinical and clinical studies [205,206]. A position paper released by the International Society for Cellular Therapy [207], defines MSCs by the following 3 minimum criteria:

1. MSC’s must adhere to plastic when maintained in standard culture conditions.

2. MSCs must possess the ability to differentiate into osteoblasts, chondroblasts and adipocytes *in vitro*.

3. MSCs must lack several lineage markers including haematopoietic and endothelial markers (CD34, CD45, CD11b, CD14, CD79\(\alpha\), CD19, and HLA-DR), and must possess several mature stromal cell markers (CD105, CD73, CD90).
While the first two criteria are mostly universally accepted, the third criteria is complicated by varying MSC markers identified between species and strains of species; therefore, the ability to identify a unique MSC cell surface marker remains unresolved [208].

Initial clinical interest in MSCs focused on their ability to differentiate into injured cell types as a means to improve outcome in pre-clinical models of disease. However, more recent pre-clinical studies demonstrate that while MSCs can reduce injury, engraftment rates are low [209-211]. Rather, MSCs are now thought to exert their effects by controlling inflammatory and immunological reactions, and secreting multiple paracrine factors.

1.9.1 MSCs and the immune system

*In vitro*, MSCs have been shown to suppress both innate and adaptive immune responses including an ability to suppress effector and cytotoxic T-cell functions, impair cytolytic potential of natural killer (NK) cell functions, induce regulatory T-cells, and modulate dendritic cell (DC) and macrophage function. A number of studies have reported that the addition of MSCs to primary mixed lymphocyte co-cultures can suppress T-cell proliferation [212-214]. MSCs may also inhibit T-cell proliferation indirectly by inhibiting monocytes from maturing into DCs [215]. DCs play a fundamental role in antigen presentation to naïve T-cells following DC maturation, which can be induced by pro-inflammatory cytokines or pathogen associated molecules [216]. In a study by Jiang *et al.*, the allostimulatory ability of MSC-treated mature DCs on allogeneic T-cells was impaired [215]. Here, MSCs were shown to induce an immunosuppressive DC phenotype by inhibiting their production of pro-inflammatory cytokines TNF, IFN-γ, and IL-12 and inducing DC production of anti-inflammatory cytokine IL-10 [215].
MSCs may also affect NK cells of the innate immune system. NK cells play an important role in antiviral and anti-tumor immune responses due to their cytolytic properties and pro-inflammatory cytokine production. Under low NK-to-MSC ratios, MSCs alter the phenotype of NK cells and suppress proliferation, cytokine secretion, and cytotoxicity against major histocompatibility complex (MHC) class I expressing targets [217]. MSC-mediated inhibition of NK cells is related to the downregulation of NK cell-activating surface receptors, while conversely, cytokine-activated NK cells can kill MSCs in vitro [218]. Some of these effects require cell-to-cell contact, whereas others are mediated by soluble factors, including transforming growth factor-β (TGF-β) and prostaglandin E2 (PGE2). This suggests diverse mechanisms may be responsible for MSC-mediated NK-cell suppression. Neutrophils are also important cells in innate immunity which are rapidly mobilized and activated to kill microorganisms during infection. After binding to bacterial products MSCs have been shown to delay neutrophil apoptosis through an IL-6 dependant mechanism [219].

Finally, MSCs have been shown to alter macrophage phenotype, in vitro [220] and in vivo [221]. Monocytes preferentially differentiate into IL-10 secreting alternatively activated (immunosuppressive) macrophages (M2 macrophages) in the presence of MSCs in vitro [220]. Macrophages isolated from MSC-treated septic mice produced significantly higher amounts of anti-inflammatory cytokine IL-10 than those from non-MSC treated mice, suggesting a temporary reprogramming of monocyte and therefore macrophage function [221].
The clinical use of MSC therapy is likely to rely heavily on the use of allogeneic and not autologous MSCs. This is because autologous cells may require several weeks to expand in culture before they could be used in the clinic; by contrast, allogeneic cells would be readily available “off-the-shelf” as they could be manufactured in advance for immediate use in the treatment of acute disease. Also, allogeneic transplantation would ensure that the cells are derived from healthy immunocompetent individuals and thus improving their likelihood of functionality.
However, the use of allogeneic MSCs would require “universality” – that is, a low immunogenic profile so that the cells could evade the recipient’s immune responses. Expression of human leukocyte antigens (HLAs) – the major histocompatibility complex (MHC) molecules I and II – present antigenic peptides generated in the cytosol to CD8⁺ T-cells or in intracellular vesicles to CD4⁺ T-cells, respectively. Low human MSC expression of MHC Class I and lack of MHC class II and classic co-stimulatory molecules such as B7-1, B7-2, CD80, CD86, CD40 and CD40L allow human MSCs to evade host immune responses [223]. Therefore, human MSCs could potentially be administered to patients without the need for HLA matching. Indeed, patients who underwent MSC allotransplants experienced no anti-allogeneic MSC antibody formation or T-cell sensitization [224].

However, under certain stimuli including low level IFN-γ co-culture, MSCs may upregulate expression of MHC class II to the cell surface (can detect MHC Class II intracellularly in basal conditions) and therefore act as antigen-presenting cells (APCs) to activate antigen specific immune responses [225,226]. Studies have also demonstrated that infusion of allogeneic mouse MSC can elicit a host immune response and lead to MSC rejection [227,228]. Therefore, MSCs may not be intrinsically immunopriviliged and may be unable to serve as a “universal donor” in MHC mismatched recipients, but this may be specific to mouse rather than human-derived cells. Further investigation is necessary to understand the complex interaction between MSCs and the immune system.

The use of MSCs for therapeutic applications has also attracted attention for their ability to “home” to sites of inflammation upon IV injection. Homing has been described as the process by
which cells migrate to and engraft in the tissue which they will exert their effects. Since MSCs may localize to the injured tissue, concerns of MSCs inducing systemic immunosuppression - which could lead to major complications - may be lessened. In a mouse model of multiple organ failure, green fluorescent protein (GFP)-tagged MSCs homed to sites of injury within numerous tissues, with MSCs proportionally homing to regions of most severe disease [229]. Akin to leukocyte trafficking, a key indicator of MSC homing is the increase in inflammatory chemokines at the site of injury.

In basal conditions, MSCs express several chemokine receptors located on their surface, including CCR2, CXCR3, CXCR4, CXCR5, and CX3CR1 as well as RNA encoding the following chemokines – CCL2, CXCL8, SDF-1(CXCL12), and cytokines TGF-β and IL-6 [230-232]. Studies have shown that MSCs respond to chemoattractant factors including CCL2, CXCL12, CX3CL1, CCL3, and IL-8 leading to MSC chemotaxis [233,234]. However chemokine / cytokine and receptor expression varies in the literature, possibly due to different culture conditions, including passage and the initial heterogeneity of the isolated cells obtained from bone marrow [232,235,236]. It is not known which chemokines and their receptors are most critical for MSC migration.

1.9.2 MSC duality – immunostimulant or immunosuppressant?

MSCs are located at the interface of the bone marrow cavity and on the peripheral surface of the main sinusoidal vessels [237]. This position gives MSCs a unique ability to function as “gatekeepers” to the marrow by interacting with all cells transiting in and out of the marrow and by regulating immune responses as the first line of defense. The role of MSCs in immune
regulation has been shown to be homeostatic, namely, they possess an ability to serve as either immunostimulatory or immunosuppressive, depending on the environment.

The presence of IFN-γ and other cytokines in the MSC microenvironment play an important role in induction of immunosuppression and MSC homing. Ren et al. have demonstrated that the immunosuppressive effect of human MSCs depends on IFN-γ in the co-presence of either TNF, IL-1α or IL-1β [238]. Under co-stimulation with these cytokines in vitro, human MSCs secreted large amounts of indoleamine 2,3-dioxygenase (IDO) and chemokines which in turn drove T-cell migration in proximity with MSCs, whereas high level IDO production suppressed T-cell function. Aggarwal and Pittenger demonstrated that the level of PGE2 - an important MSC-secreted protein - is significantly upregulated by MSCs in the presence of TNF [213]. IFN-γ mediated MSC activation has also been demonstrated in vivo in a model of graft-vs-host disease (GvHD) where recipients of IFN-γ⁻/⁻ T-cells did not respond to MSC treatment [239]. Mechanistically, IFN-γ may act directly on MSCs by upregulating B7-H1, an inhibitory molecule on the surface of MSCs [240].

On the contrary, low levels of IFN-γ or the absence of specific proteins in the MSC microenvironment may cause MSCs to behave as APCs [241]. Human MSCs may also act as immune-enhancers when IDO is knocked-down. Therefore, a clinical scenario where MSCs may encounter insufficient pro-inflammatory cytokines in vivo could potentially lead to MSC-mediated immunostimulation. It may then be difficult to determine the point in the disease progression at which administering MSCs could achieve an immunosuppressive response.
**1.9.3 Effects of MSCs in pre-clinical models**

MSC mediated immunosuppression after infusion *in vivo* has been documented in a diverse array of pre-clinical animal models of disease. MSC-based prophylactic and treatment strategies have yielded significant therapeutic benefits in pre-clinical models of a variety of inflammatory diseases, including rheumatoid arthritis [243], sepsis [221,244] and ALI [209,210,245–248]. However, several studies report no MSC-mediated immunosuppression or therapeutic benefit in pre-clinical models of GvHD, organ transplantation and rheumatoid arthritis [249-251]. Furthermore, MSCs used in combination with the universal immunosuppressant cyclosporine A for the treatment of GvHD resulted in accelerated graft rejection in mice [251]. The clinical trial registry at the National Institute of Health indicates over 200 registered clinical trials currently investigating the use of MSCs in humans as a therapy for diverse diseases including Crohn’s disease, GvHD, osteogenesis imperfecta and multiple sclerosis [252].

Most importantly, MSCs have been shown to reduce dysregulated inflammatory responses, to improve alveolar fluid clearance, and to maintain lung epithelial and endothelial integrity in the lung during pulmonary inflammation and injury in murine models [209,210,245–248, reviewed in 253]. These models consist of lung injury induced by either: (1) bleomycin - an antitumour agent known to cause lung injury by inducing oxidative stress and elevated inflammation, or (2) endotoxin / LPS – a major constituent of the outer cell wall of gram-negative bacteria. In these murine models of ALI, the beneficial effects of MSC therapy have been attributed to the secretion of growth factors, cytokines and lipid mediators [reviewed in 253 and discussed in section 1.9.3].
1.9.4 MSC derived paracrine mediators

While the initial driving force behind therapeutic interest in MSCs was for their regenerative capacity, it is their paracrine properties and ability to modulate host inflammation that markedly increased the known range of MSC therapeutic applications and attracted even greater scientific interest in MSC research. MSCs secrete a vast array of soluble mediators and growth factors which can modulate the host response. Importantly, these findings have been observed independent of direct MSC-to-cell contact. For example, concentrated MSC-conditioned media reversed multi-organ dysfunction syndrome in a rat model [254]. While many immunosuppressive mediators are suspected to be important for treating lung injury, the proteins under greatest investigation include IL-1 receptor antagonist (IL-1ra) [255], soluble TNF receptor (sTNFR) [256], IDO [214], IL-10 [221], angiopoietin-1 (Ang-1)[298], TNF stimulated gene 6 protein (TSG-6) [258,259] TGF-β [260], PGE$_2$ [213,221], and keratinocyte growth factor (KGF) [248]. These proteins may exert their positive effects by immunomodulation, improving alveolar fluid clearance, or reducing lung endothelial permeability.

IL-1ra, sTNFR-1, IL-10, IDO, PGE$_2$ and TSG-6 may be important in MSC-mediated immunomodulation. In a bleomycin-induced animal model of lung injury, injected MSCs decreased inflammatory responses and prevented the development of lung fibrosis. These effects were attributed to MSC secretion of IL-1ra [255]. In another pre-clinical mouse model of disease, human MSCs administered to LPS induced sepsis in mice attenuated systemic inflammation by secreting sTNFR1 [256]. sTNFR1 binds to TNF to neutralize its inflammatory activity. MSC secretion of IL-10 is also important. MSCs can mediate downregulation of pro-inflammatory cytokines TNF and MIP-2 and upregulation of anti-inflammatory cytokine IL-10.
in BAL (BAL) fluid of endotoxin induced lung injury in mice [247]. MSC secretion of PGE$_2$ may be implicated in reprogramming macrophages to increase IL-10 production resulting in reduced mortality and improved organ function in pre-clinical sepsis [221]. Human MSC released IDO has been attributed to the immunosuppressive properties of MSCs as well [214]. In vitro, MSC released IDO – an important protein in tryptophan metabolism - inhibits T-cell proliferation directly and indirectly via IDO induced monocyte differentiation into M2–like immunosuppressive cells [220]. Improved myocardial infarction and lung injury in MSC treated mice has also been attributed to MSC expression of TSG-6 [258,259]. TSG-6 is a TNF induced anti-inflammatory protein.

In addition to MSC secreted immunomodulatory proteins, the effects of MSCs may be mediated by endothelial/epithelial specific growth factors including Ang-1 and KGF which are potentially important in maintaining lung-vascular endothelial integrity. The integrity of the lung microvascular endothelium is essential to prevent the influx of protein-rich fluid from the plasma which may further aggravate the ability of the lung epithelium to reduce alveolar edema. Ang-1, a ligand for the endothelial-specific receptor tyrosine kinase Tie2 [261], is a potent mediator of angiogenesis that functions to prevent leakage and promote blood vasculature quiescence via strengthening of endothelial cell junctions and downregulation of surface adhesion molecules, such as vascular cell adhesion molecule-1 (VCAM-1) and E-selectin [262–265]. In vitro, the effects of MSCs have been shown to be mediated by Ang-1 which resulted in reduced epithelial protein permeability in cultured human alveolar type II cells [298]. KGF, another important MSC soluble mediator, has also been shown to reduce lung injury in animal models of pulmonary edema [266]. KGF may stimulate the proliferation and differentiation of alveolar type
II cells, which in turn may promote lung repair during injury. In an ex vivo perfused human lung, intra-bronchial instillation of human MSCs 1 hour following endotoxin-induced lung injury restored alveolar fluid clearance, in part by the secretion of KGF [248].

It is important to note that inhibition of most MSC secreted molecules does not result in complete loss of MSC immunosuppressive function. For example, upon MSC knockdown of TSG-6, Danchuk et al. reported a decrease but not complete loss of MSC mediated immunosuppression in vivo [259]. Also the relative importance of each MSC secreted protein is dramatically varied between studies. Lastly, administering isolated proteins implicated as the most important MSC soluble factor does not usually fully recapitulate the immunosuppressive function of MSC administration. For example, MSCs were more effective in mouse model of bleomycin induced lung injury, than recombinant or virally delivered IL-1ra [255]. Therefore, it is likely that no single molecule exerts an exclusive role and MSC mediated effects are likely the result of concerted actions mediated by many important soluble mediators in combination.

1.10 Mouse models of influenza

To date, mouse models of influenza have demonstrated utility in the guidance of novel therapeutic investigation and intervention for the treatment of severe influenza in humans. As in humans, influenza virus virulence in mice is dependent on the virus subtype and strain. In addition, the lethal dose, replication kinetics, and inflammatory response are affected by a complex and multigenic host component [303]. Symptoms of severe disease are comparable in mice and humans as both may feature reduced blood oxygen saturation, lung edema, increased
lung viral load, pulmonary immune cell infiltrates and elevated cytokine production and hemorrhage [267]. Also, onset of symptoms positively correlates with viral titers [268]. However, experimental mice may not show signs of fever, and both cyanosis and dyspnea are not easily detectable in many models aside from H5N1 influenza [267]. The most widely accepted indicator of disease progression in influenza murine models is weight loss [269,270].

Antiviral compounds as well as several investigational immunomodulatory agents have shown efficacy against influenza and host inflammation in mice [271, previously discussed]. Advantages of mouse models include well characterized immune responses and a wide availability of murine immune reagents. Additionally, mice are inbred so experimental results are usually reproducible; however, results may be misleading due to genetic variability in the human population. For example, while zanamivir has demonstrated efficacy against H5N1 in mice [271], antivirals have shown limited efficacy against H5N1 in humans [75] and the efficacy of oseltamivir is not entirely clear [62]. Conversely, statins have been shown to reduce disease severity in humans when their use was evaluated in retrospective studies, while statins are ineffective at improving outcome in murine models (previously discussed). Therefore, while mouse models are important in guiding the development of novel therapies for use in humans, results should be interpreted with caution.

Historically, influenza A/PR/8 virus (H1N1) and A/WSN/33 virus (H1N1) are the most commonly used strains in experimental influenza [272]. While both A/PR/8 and A/WSN/33 are known to cause severe inflammation, A/WSN/33 is better known for its neurotropism and its capacity to cause systemic infection [273]. Both influenza A/PR/8 virus and A/WSN/33 virus have been serially passaged and adapted to mice in order to cause severe disease. Therefore, it is important to bear in mind that conclusions based on results obtained using these strains may be
misleading due to molecular changes acquired during adaptation. Nonetheless, A/PR/8 has been employed in the evaluation of many immunomodulatory agents (e.g. PPARs) for the treatment of severe influenza [134,201].
1.11 Research aims and objectives

While the vast majority of influenza virus infections resolve without complications, millions of individuals develop severe disease each year and are at risk of death. Given the importance of the innate host response in severe influenza – particularly inflammation - adjunctive therapies to complement anti-viral treatment strategies are in need. In the face of rising antiviral drug resistance, novel anti-influenza drugs are also sorely in need. The aim of this work was to investigate two novel therapeutic treatment strategies for severe influenza. This included an assessment of iNO therapy as an antiviral agent and exploration of mesenchymal stem cell therapy as an immunomodulatory strategy.

In Chapter 2, we examined whether iNO administration could reduce viral load and improve survival in a murine model of severe influenza. Our specific aims were as follows:

1. To determine if iNO delivered either continuously or intermittently could improve survival in an experimental murine model of severe influenza;

2. To establish whether iNO therapy could reduce the influenza viral burden in an experimental murine model of severe influenza.

We found that iNO therapy failed to improve survival or decrease viral load in experimental influenza.

In Chapter 3 we examined whether MSC therapy could modulate the host response to infection in an in vivo mouse model of severe influenza and therefore improve outcome. The specific aims of this work were as follows:
1. To establish and characterize the influenza A/PR/8 virus mouse model of severe influenza which develops inflammation and acute lung injury;

2. To determine whether MSC therapy can improve survival and/or dampen host inflammation and lung injury in a mouse model of severe influenza;

3. To assess the ability of MSC prophylaxis, with or without MSCs pre-incubated with cytokines, to improve survival in experimental influenza model;

4. To test whether combining oseltamivir with MSCs is effective in reducing mortality and/or dampening host inflammation and lung injury in experimental influenza model.

We found that MSCs were not effective in dampening host inflammation and acute lung injury in experimental influenza. Thus, MSCs were unable to decrease morbidity and mortality in our experimental model of influenza.
Chapter 2: Inhaled Nitric Oxide Antiviral Therapy for Severe Influenza

2.1 INTRODUCTION

Further investigation of antiviral strategies which have shown promising results against influenza A viruses \textit{in vitro} but have not been investigated \textit{in vivo} are warranted. One of these agents which may be effective when used against influenza virus is NO. \textit{In vitro}, NO has antimicrobial activity against a wide range of viruses, including influenza A virus; therefore, we hypothesized that inhaled NO (iNO) would increase survival \textit{in vivo} by reducing the viral load in C57Bl/6 mice infected with a lethal dose of influenza A/WSN/33 virus (H1N1). iNO delivery would provide a safer and easier delivery method than administration of NO donors such as SNAP, as iNO is currently approved for treating term and near-term neonates with hypoxemic respiratory failure [106,107].

iNO was delivered to influenza virus-infected mice either continuously or intermittently at 80 or 160 ppm, respectively, using both prophylactic and post-infection treatment strategies. These dosing regimens were chosen for the following reasons:

1. iNO delivery in hypoxemic respiratory failure is already approved up to a dose of 80 parts per million (ppm) [106,107].

2. Gaseous NO at a high dose of no less than 160 ppm and with five hours of continuous exposure, can elicit a non-specific antimicrobial response against a broad range of microorganisms \textit{in vitro} [274].
In vivo, 160 ppm iNO treatment would result in NO binding to hemoglobin to form methemoglobin, resulting in reduced oxygen transport and hypoxemia, as well as the potential for elevated levels of the harmful NO metabolite NO$_2$. However, Miller et al. have shown that iNO in an intermittent delivery regimen of 160 ppm for 30 min every 3.5 hours can prevent methemoglobinemia and reduce the potential of host cell toxicity in vivo [Miller C, personal communication]. Therefore, we chose to deliver 160 ppm iNO in a similar manner.

We chose to investigate the use of iNO over other antiviral agents for several reasons. Firstly, it will be available off patent as of 2013 and therefore will be relatively cheap to administer as treatment for severe influenza. Secondly, it is relatively easy to administer with a breathing mask in resource poor areas. Lastly, iNO would likely evade the development of influenza drug resistance that is often associated with antiviral therapy, due to its non-specific antiviral effects including enzyme nitrosylation, such as nitrosylation of proteases (e.g. reverse transcriptases and ribonucleotide reductase) containing cysteine residues and viral-encoded transcription factors that are involved in replication [97, 98].
2.2 MATERIALS AND METHODS

Murine influenza model

Animal use protocols were reviewed and approved by the University Health Network Ontario Cancer Institute Animal Care Committee, and all experiments were conducted in accordance with institutional guidelines in an animal biosafety level 2 facility. Female C57Bl/6 mice, 9–11 weeks old, were obtained from Jackson Laboratories (Bar Harbor, ME, USA) and maintained under pathogen-free conditions with a 12-hour light cycle. On day 0, while under light isofluorane anesthesia, experimental mice were infected via nasal instillation with 1000 plaque forming units (PFU) of influenza A/WSN/33 virus (H1N1) (stock kindly provided by Dr. Eleanor Fish, University Health Network/University of Toronto) in 50 µl PBS. Weight was recorded daily for a maximum of twelve days P.I., and mice were sacrificed when euthanasia criteria was met (greater than 20% weight loss). Lung tissue was harvested for analysis on day 5 P.I..

In Vivo NO Delivery

Prophylactic or post-infection iNO therapy was initiated either 1 hour prior to or 4 hours P.I., respectively. Mice were placed in flow-through chambers with free access to food and water and received either compressed room air, continuous NO at 80 ppm +/-5ppm mixed with compressed room air, or intermittent NO for 30 min every 3.5 hours at 160 ppm +/-5ppm mixed with compressed room air. Soda lime (200 g) was supplied to each chamber, and gas flow was maintained at 10–12 L/min to scavenge and minimize NO₂ levels, respectively. NO₂ levels were
limited to <2 ppm for continuous iNO therapy and <8 ppm for intermittent iNO therapy. NO and NO₂ levels were measured using an AeroNOX machine (Pulmonox Medical, AB, CA).

**Lung influenza viral load analysis**

Lungs were harvested and frozen at -80°C. Lungs were thawed, weighed, and homogenized in 1 ml PBS for 30 sec using a Tissue Miser homogenizer (Fisher Scientific, ON, CA). Lung homogenates were spun at 10,000xg for 10 min, aliquoted, and stored at -80°C for viral yield titration. Influenza A/WSN/33 viral yield in lung homogenates was quantified by plaque assay in Madin-Darby canine kidney (MDCK) cells (ATCC, VA, USA). MDCK cells (ATCC, Manassas, VA, USA) were maintained in Eagle’s MEM (ATCC) supplemented with 10% FBS and 0.25% gentamicin. All cell lines were cultured at 37°C with 5% CO₂. MDCK cells were plated at a concentration of 8x10⁶ cells/plate in 6 well culture plates. 12–24 hours later, medium was removed and MDCK cells were washed twice with PBS. 10-fold dilutions of lung homogenates were added to MDCK cells in 500 µL Eagle’s MEM, in duplicate, and incubated at 37°C with 5% CO₂ for 1 hour with plates rocked every 15 min. After incubation, 1 mL of serum-free 2X Eagle’s MEM supplemented with 8 µl/ml trypsin, 60 µl/ml of 7.5% sodium bicarbonate and 20 µl/ml antibiotics, combined with 1 ml of 1.2% agarose, was added to each well. Once the agarose set, plates were incubated at 37°C for 42–72 hours until syncytia were observed. Plates were fixed with Carnoy’s fixative (3:1, methanol:glacial acetic acid) for 30 min then stained with 0.1% crystal violet in 20% ethanol to visualize plaques. Viral load was expressed as plaque forming units per gram of lung tissue (PFU/g).
Statistical Analysis

Logrank tests were performed on Kaplan-Meier survival curves. Significant differences in weight loss between groups were assessed by two-way analysis of variance (ANOVA) and Bonferroni post-tests were performed. Student’s t-tests were carried out on viral yield data to assess significant differences ($p \leq 0.05$) between experimental groups.
2.3 RESULTS

2.3.1 Continuous iNO at 80 ppm decreased survival and intermittent high dose iNO at 160 ppm did not increase survival of influenza A virus-infected mice

We evaluated the ability of iNO to improve survival of influenza A/WSN/33 virus-infected mice. Experimental C57Bl/6 mice were inoculated intranasally with an 80–100% lethal dose of A/WSN/33 virus (1000 PFU). At 5 days P.I., the majority of mice in all experimental groups experienced weight loss (Figure 2.1A and 2.2A). At 7 days P.I., mice began to reach euthanasia criteria (≤80% of day 0 weight), and by day 10 P.I., most mice were euthanized (Figure 2.1B and 2.2B). If 20% weight loss was not met by day 10 P.I., the infection typically resolved, and surviving mice gained weight.

Figure 2.1. Prophylactic iNO therapy increased weight loss and decreased survival of C57Bl/6 mice infected with influenza A/WSN/33 virus. C57Bl/6 male mice were infected with 1000 PFU A/WSN/33 virus and administered continuous NO at 80 ppm or compressed room air starting 1 hour prior to infection (n=17–18/group, 2 independent pooled experiments). (A) Shown are the weight loss data up to the day of first death. Mice receiving iNO displayed a significant reduction in weight compared to infected controls (Two-way ANOVA p < 0.001 with Bonferroni post-tests; **, p < 0.01 on day 6 and 7 P.I.). Error bars represent standard deviations. (B) Survival curve. iNO significantly reduced survival of treated mice compared to infected controls as shown by Kaplan-Meir survival curves; **, logrank test: P < 0.01).
Weight loss over the course of infection was accelerated in mice administered continuous iNO at 80 ppm starting 1 hour prior to inoculation compared to infected control mice administered compressed room air (P < 0.001) (Figure 2.1A). Continuous iNO administered at 80 ppm starting 1 hour prior to inoculation significantly decreased survival of A/WSN/33 virus-infected mice compared to infected control mice administered compressed room air (P < 0.01). During the course of infection, 100% of continuous iNO treated mice were euthanized compared to 80% of infected control mice (Figure 2.1B). Intermittent iNO administrated at 160 ppm for 30 min intervals every 3.5 hours starting either 1 hour prior to or 4 hours P.I. resulted in similar weight loss kinetics (Figure 2.2A) and consequent survival kinetics (Figure 2.2B) of infected mice compared to infected control mice administered compressed room air.
Figure 2.2. Prophylactic and post-infection intermittent iNO does not alter weight loss kinetics or survival of C57Bl/6 mice infected with influenza A/WSN/33 virus. C57Bl/6 male mice were infected with 1000 PFU A/WSN/33 virus and administered NO intermittently at 160 ppm for 30 min intervals every 3.5 hours starting either 1 hour prior to infection or 4 hours P.I. Infected control mice were administered compressed room air (n=9–10/group). (A) Shown are the weight loss data up to the day of first death. No difference was observed (Two-way ANOVA). Error bars represent standard deviations. (B) Survival curve. No difference was observed (logrank test).

2.3.2 Continuous or intermittent iNO administration does not reduce lung viral load

As NO at high concentrations has been shown to decrease the viral load of infected cells in vitro (Miller C, personal communication), we examined whether iNO could reduce the viral load of influenza virus-infected mice. iNO was administered starting 1 hour prior to influenza A/WSN/33 virus infection and continued either continuously at 80 ppm or intermittently at 160 ppm for 30 min every 3.5 hours until mouse lungs were harvested at peak influenza viral load in the lungs (determined to be day 5 P.I. based on preliminary studies, data not shown). Since iNO was administered both prior to and for 5 days P.I., we were able to test whether iNO at intermediate (80 ppm) or high concentration (160 ppm) could prevent either viral entry or viral
replication in vivo, and thereby reduce viral load. Continuous iNO administered at 80 ppm, intermittent iNO administered at 160 ppm, and administered compressed room air yielded similar lung viral loads of infected mice on day 5 P.I. (Figure 2.3A and B, respectively). Therefore, iNO administered both continuously and intermittently failed to reduce lung viral load of infected mice, compared to infected control mice administered compressed room air.

Figure 2.3. Intermittent high dose iNO prophylactic therapy failed to decrease viral load of C57Bl/6 mice infected with influenza A/WSN/33 virus. Lungs were collected 5 days post-influenza A/WSN/33 virus infection (1000 PFU) from mice treated with (A) continuous iNO at 80 ppm or compressed room air starting 1 hour prior to infection (n=5/group) or (B) intermittent iNO at 160 ppm or compressed room air for 30 min intervals every 3.5 hours starting 1 hour prior to infection (n=5/group). Error bars represent standard deviations. Lung viral load was quantified for all experimental groups by plaque assay on MDCK cells.
2.4 DISCUSSION

In this report, we investigated the application of iNO therapy as an antiviral strategy for prophylaxis and treatment of severe influenza. We found that iNO was unable to reduce the viral burden in the lungs of influenza virus infected mice when administered continuously or intermittently throughout the course of infection. We also showed that iNO, administered either intermittently or continuously at varying concentration, was unable to reduce morbidity and mortality in experimental severe influenza.

Typically, iNO is administered at initial doses of 5–20 ppm in randomized controlled trials and observational studies for neonatal hypoxic respiratory failure [107]. Although FDA-approved at concentrations up to 80 ppm, no specific dose of iNO has been proven more advantageous than another [107,113]. Rather, methemoglobinemia, defined as 7% methemoglobin by Davidson et al. was more likely to occur at higher concentrations [276]. We speculated that methemoglobinemia may account for the decrease in survival observed in our study with continuous iNO administration at 80 ppm. However, we could not draw this conclusion as methemoglobin levels were not measured. NO₂ concentrations were measured daily over the course of infection and kept below 2 ppm as is acceptable in humans, however, lung toxicity may still explain these results as the toxic threshold in mice may be lower. On the other hand, given previous in vitro findings by McMullen et al. [274], 80 ppm may also have been too low of a concentration to provide an antiviral effect.

A high dose of NO at 160 ppm was administered intermittently, not to target the airway vessels specifically, but rather to induce an antimicrobial effect while avoiding the harmful effects of high dose continuous iNO delivery. iNO administered to influenza virus-infected mice in this
manner, either prophylactically or therapeutically, failed to improve survival of infected mice, change the course of weight loss, or decrease the lung viral load, compared to control mice administered compressed air. Therefore, although administration of high dose intermittent iNO may have reduced the harmful side-effects of NO, antimicrobial activity was not observed in vivo. However, the variation in weight loss over the course of infection within either the iNO treated or untreated groups, as indicated by large and overlapping standard deviations, may suggest that perhaps too few animals were studied to identify a true effect of iNO therapy.

In conclusion, despite the demonstrated antimicrobial activity of NO against influenza A virus in vitro, the results of this study do not support the use of iNO as a prophylactic or treatment strategy to reduce viral burden or improve clinical outcome in severe influenza in vivo. Furthermore, it may be difficult to achieve virucidal concentrations of NO in the airways using iNO at concentrations that are safe in the living host.
Chapter 3: Mesenchymal Stromal Cell Immunomodulatory Therapy for Severe Influenza

3.1 INTRODUCTION

MSCs offer considerable promise as a novel influenza treatment strategy. While previous strategies to inhibit a specific component of the immune response in influenza have met with all but failure, a more complex overall immunomodulatory effect demonstrated by MSCs may be more effective in decreasing influenza associated morbidity and mortality. In addition, MSCs may be able to reprogramme the immune response to reduce destructive host inflammation while maintaining a sufficient inflammatory response to control virus replication. Also, MSCs may enhance the repair of lung injury and restore epithelial and endothelial integrity, either by direct MSC cell contact or secretion of various paracrine mediators including KGF and Ang-1.

Indeed, several reports have suggested that mesenchymal stem cells (MSCs) could exert a potent immunosuppressive effect in pre-clinical models of acute lung injury, and thus may have a therapeutic potential for other inflammatory / lung injury dependent pathologies [209,210,245-248]. Therefore, we aimed to establish whether MSCs could be used to control overzealous host inflammatory responses and decrease acute lung injury in experimental severe influenza, a major cause of morbidity and mortality in severe influenza.

Most experimental evidence to date regarding the use of MSC treatment for ALI comes from studies that employed mouse MSCs (mMSCs) [209,210,245,247]. However, it is critical that the
efficacy and mechanism of human MSCs (hMSCs) be studied in depth before proceeding to clinical trials so that the human physiological response could be accurately predicted. Evidence suggests that the mechanism by which hMSCs exert their effects varies from that of mMSCs [238]. Therefore, MSC investigators have more recently employed hMSCs in pre-clinical study [256,258,259]. Likewise, we chose to evaluate the use of hMSCs rather than mMSCs in our experimental model of severe influenza.

As basal levels of MSC chemokines / cytokine expression can be modified by pre-stimulation with IFN-γ and TNF [231,238], we sought to assess whether cytokine pre-incubated hMSCs could improve survival in our experimental influenza model. We also examined the effect of hMSCs in combination with antiviral therapy, oseltamivir, for the two following reasons. Firstly, any future MSC therapy for severe influenza patients will be administered in combination with antiviral therapy, as is standard treatment. Secondly, oseltamivir might have important effects on the host inflammatory environment and therefore effect MSC functionality and migration/homing. Because inflammation and ALI/ARDS are major determinants of morbidity and mortality in severe influenza, MSC therapy represents a promising adjunctive immunomodulatory strategy to improve outcome in severe influenza and warrants investigation.
3.2 MATERIALS AND METHODS

Murine influenza model

Animal use protocols were reviewed and approved by the University Health Network Ontario Cancer Institute Animal Care Committee, and all experiments were conducted in accordance with institutional guidelines in an animal biosafety level 2 facility. Male C57Bl/6 mice, 8–10 weeks old, were obtained from Jackson Laboratories (Bar Harbor, ME, USA) and maintained under pathogen-free conditions with a 12-hour light cycle. On day 0, while under light isofluorane anesthesia, experimental mice were infected by nasal instillation with 425 EID$_{50}$ of influenza A/PR/8 virus (H1N1) (stock kindly provided by Dr. David Kelvin, University Health Network/University of Toronto) in 50 ul PBS. Weight was recorded daily for a maximum of twelve days P.I., and mice were sacrificed when euthanasia criteria was met (greater than 20% weight loss). BAL fluid, lung tissue, and blood were harvested for analysis.

Cells

Human mesenchymal stromal cells (hMSCs) were provided by the Texas A&M Health Science Center College of Medicine Institute for Regenerative Medicine at Scott & White. hMSCs (isolated from a 24 year old male donor) were quickly thawed to 37°C and plated for 24 hours in α-MEM with 2–4 mM L-glutamine supplemented with 100 U/ml penicillin, 100 ug/ml streptomycin and 16.5% Fetal Bovine Serum (FBS). After 24 hrs, plates were washed with PBS and cells were lifted by incubation with trypsin/EDTA for 2 min at 37°C and re-plated at 60 cells/cm$^2$. Cells were incubated for 7–10 days for each subsequent passage (P). P3–P4 hMSCs were lifted as described above and spun at 300xg for 5 min at 4°C with 10 ml of media. Media
was then discarded and cells were spun twice at 300xg, 4°C with 10 ml of fresh Dulbecco’s Phosphate Buffered Saline (PBS; Gibco/Invitrogen, Burlington ON, CA). After last wash, cells were re-suspended in 1 ml fresh PBS and total cell count was determined via hemocytometry.

hMSCs were re-suspended in PBS at 2.5x 10^6 cells/ml and 100 ul was injected via the tail vein into experimental mice on day -2, 0, 2, and 5 P.I.. Injections were performed using 26 1/2 gauge needles and a typical mouse restrainer.

MDCK cells (ATCC, Manassas, VA, USA) were maintained in Eagle’s MEM (ATCC) supplemented with 10% FBS and 0.25% gentamycin. All cell lines were cultured at 37°C with 5% CO₂.

Reagents

Oseltamivir phosphate was extracted from Tamiflu capsules (Roche, Switzerland) and administered to mice via oral gavage. Experimental mice were administered 5 mg/kg in 100 ul ddH₂O, twice daily, every 12 hours, beginning either 2 or 5 days P.I. for a maximum of 5 days [as described in 277]. Recombinant human IFN-γ and TNF were purchased from R&D Systems (Minneapolis, MN, USA) and hMSCs were pre-incubated for 24 hours prior to cell harvest at 10 and 3 ng/ml, respectively [as described in 220].

BAL fluid analysis

BAL fluid of both lungs was obtained by instillation and aspiration of three consecutive 500 ul aliquots of PBS. BAL fluid was spun at 800xg at 4°C for 5 min. Supernatant from the first lavage was removed and stored at -80°C for further analysis. Red blood cells in BAL fluid cell pellet were lysed with 250 ul red blood cell lysis buffer for 5 min then spun again at 800xg for 5
min at 4°C. Combined cell pellets from all washes per mouse were re-suspended in 200 μl PBS. Total cell numbers were determined using a hemocytometer. BAL fluid concentrations of CCL3, CCL2, CXCL10, CCL5, mouse keratinocyte-derived cytokine (KC) and IFN-γ were determined by sandwich ELISA (R&D Systems, Minneapolis, MN, USA) as per manufacturer’s instructions. BAL fluid total protein concentration was measured using a BCA protein assay, and BAL fluid IgM and albumin concentration was determined by sandwich ELISA (Bethyl Laboratories, Montgomery, TX, USA) as per manufacturer’s instructions.

**Lung homogenate analysis**

Lungs were harvested and frozen at -80°C. Lungs were thawed, weighed, and homogenized in 1 ml PBS for 30 sec using a Tissue Miser homogenizer (Fisher Scientific, ON, CA). Lung homogenates were spun at 10,000xg for 10 min, aliquoted, and stored at -80°C for viral yield titration. Influenza A/PR/8 viral yield in lung homogenates was quantified by plaque assay in MDCK cells (ATCC, VA, USA) as described in section 2.2.

**Blood Analysis**

Infected mice and uninfected controls were anesthetized with intraperitoneal (IP) injection of ketamine (100 mg/kg) and xylazine (5 mg/kg) on day 7 post infection. Cardiac puncture was performed where approximately 600 μL of whole blood was isolated from each mouse. Samples were kept at room temperature for 30 min then stored on ice until centrifugation at 13,000 rpm, 4°C, for 10 minutes. Serum was aliquoted and stored at -80°C for further cytokine analysis. Cytokine levels were determined as described above.
Statistical Analysis

Analysis was performed using GraphPad Prism software. Kaplan-Meier survival curves were compared using the logrank test. Differences between groups were assessed by one- or two-way analysis of variance (ANOVA) with Bonferroni post-tests.
3.3 RESULTS

3.3.1 Influenza A/PR/8 virus infection is characterized by a high mortality rate, elevated inflammation and acute lung injury

To establish a lethal model of severe influenza, experimental C57Bl/6 mice were infected intranasally with 425 EID$_{50}$ influenza A/PR/8 virus. This dose resulted in a mortality rate of 80%, where death was established according to standard euthanasia criteria (≤80% of day 0 weight) (Figure 3.1B). At 6 days P.I., the majority of mice in all experimental groups experienced weight loss while at 7 days P.I., mice began to reach euthanasia criteria (Figure 3.1A and B). By 10 days P.I., most mice were euthanized. If 20% weight loss was not met by day 10 P.I., the infection typically resolved, and surviving mice gained weight.

Figure 3.1. Male C57BL/6 mice infected with 425 EID$_{50}$ influenza A/PR/8 virus develop increased morbidity and mortality. Eight week-old male C57Bl/6 mice were infected with 425 EID$_{50}$ influenza A/PR/8 virus. (A) Weight loss is expressed in percentage of original weight over the course of infection (n=10). Error bars represent standard deviation. (B) Kaplan-Meir survival curve (n=10).
In order to characterize influenza A/PR/8 virus infection as severe influenza which develops elevated host inflammation, BAL fluid, lung homogenate, and serum were collected from C57BL/6 mice on days 0, 2, 4, 5, and 6 over the course of infection and examined for total number of inflammatory cells, as well as cytokine and chemokine protein content. Specifically, CCL2, CCL5, KC, CXCL10, and IFN-γ levels were quantified, as these proteins have been shown to be significantly elevated in and most commonly associated with severe influenza compared to mild or uncomplicated influenza [135]. BAL fluid inflammatory cells began to increase on day 4 P.I. until 6 days P.I., one day prior to the first death (Figure 3.2A). BAL fluid levels of CCL2, CCL5, KC and CXCL10 began to rise at approximately 4 days P.I. and continued to increase until 6 days P.I. (Figure 3.2B). BAL fluid IFN-γ began to increase 6 days P.I., 2 days following the increase in CCL2, CCL5, KC and CXCL10. Lung homogenate levels of CCL2, CCL5, KC, CXCL10, and IFN-γ followed a similar trend as is observed in BAL fluid, while serum levels remained low or below the limit of detection throughout the course of infection.

In order to characterize influenza A/PR/8 virus infection as severe influenza which develops acute lung injury (ALI), BAL was performed on C57BL/6 mice on days 0, 2, 4, 5, and 6 P.I. Total protein, and more specifically, IgM, are indicative of alveolar-capillary membrane barrier leak and, therefore, were quantified in BAL fluid [18]. Levels of total protein and IgM were elevated starting 4 days P.I., and continued to rise until the first day when euthanasia criteria was met (Figure 3.2C).
Figure 3.2. Male C57BL/6 mice infected with 425 EID$_{50}$ influenza A/PR/8 virus develop lung inflammation and acute lung injury. (A) Total inflammatory cell count was measured by haemocytometry in BAL fluid of influenza virus-infected mice on days 0, 2, 4, 5, and 6 P.I. (n=5-7/group). (B) A panel of cytokines and chemokines was examined by ELISA in the serum, lung homogenate and BAL fluid of influenza virus-infected mice on days 0, 2, 4, 5, and 6 P.I. (n=5-7/group). (C) Total protein and IgM, standard markers of acute lung injury, were measured by BCA assay and ELISA, respectively, on days 0, 2, 4, 5, and 6 P.I. (n=5-7/group). Protein levels and cell count are presented as median with interquartile range (IQR).
3.3.2 Mesenchymal stem cell therapy fails to improve survival and/or dampen host inflammation and lung injury in experimental severe influenza

2.5x10^5 hMSCs were administered either 4 hours prior to infection (day 0), early (day 2), or late (day 5) in the course of infection. A dose of 2.5x10^5 hMSCs was chosen since this dose was effective in a report by Mei et al. [210]. MSCs were obtained from the Texas A&M Health Science Center College of Medicine Institute for Regenerative Medicine at Scott & White and were reported by the Centre as meeting the MSC defining criteria proposed by the International Society for Cellular Therapy (207, data not shown).

In the lethal influenza A/PR/8 virus mouse model, hMSCs administered prior to, early, or late in the course of infection, resulted in similar weight loss kinetics (Figure 3.3A) and consequent survival kinetics (Figure 3.3B) of infected mice compared to infected control mice administered PBS. No difference in lung viral titer was observed between experimental groups (Figure 3.3C).
Figure 3.3. hMSCs fail to improve survival, alter weight loss kinetics or affect lung viral load in experimental severe influenza. Eight week-old male C57Bl/6 mice were administered $2.5 \times 10^5$ hMSCs (passage 3), via the tail vein, either 4 hours prior to influenza A/PR/8 virus infection ($425 \text{ EID}_{50}$), 2 days post infection (P.I.), or 5 days P.I. Weight was recorded daily. No significant differences in (A) weight loss kinetics (Two-way ANOVA, $n=18-20$/group, 2 pooled experiments) or (B) survival (logrank test, $n=18-20$/group, 2 pooled experiments) were observed. Error bars represent standard deviation. (C) lungs were collected 7 days P.I and quantified via plaque assay on MDCK cells. No significant differences were observed (One-way ANOVA, $n=5$/group). Error bars represent standard deviation.
To assess whether hMSCs could dampen lung inflammation, experimental mice administered hMSCs or PBS were sacrificed on day 7 P.I. and BAL fluid levels of CCL2, CXCL10, KC, CCL5, and IFN-\(\gamma\) were quantified. All BAL fluid cytokine and chemokine levels were similar for hMSC treated mice compared to control mice (Figure 3.4). Cytokine and chemokine levels were below the limit of detection in BAL fluid of uninfected mice. To assess whether hMSCs could decrease acute lung injury in experimental influenza, we tested the BAL fluid for markers of acute lung injury. Similarly, there was no difference in BAL fluid total protein, IgM, and albumin levels on day 7 P.I. between experimental groups (Figure 3.5).
Figure 3.4. hMSCs fail to alter lung inflammation in experimental severe influenza. Eight week-old male C57Bl/6 mice infected with 425 EID$_{50}$ influenza A/PR/8 virus and treated with 2.5x10$^5$ hMSCs (passage 3) were sacrificed on day 7 P.I. BAL was performed. No significant difference in BAL fluid level of selected cytokines / chemokines was observed between mice treated early (day 2 P.I.) or late (day 5 P.I.) in the course of infection, compared to infected control mice (One-way ANOVA, n=5/group). Error bars represent standard deviation. ND = nondetectable.
Figure 3.5. hMSCs fail to alter acute lung injury in experimental severe influenza. Eight week-old male C57Bl/6 mice infected with 425 EID₅₀ influenza A/PR/8 virus and treated with 2.5x10⁵ hMSCs (passage 3) were sacrificed on day 7 P.I. BAL was performed. No significant difference in total protein, IgM, or albumin, was observed between mice treated early (day 2 P.I.) or late (day 5 P.I.) in the course of infection, compared to infected control mice (One-way ANOVA, n=5/group). Error bars represent standard deviation. ND = nondetectable.

Next we tested whether hMSCs could decrease morbidity in a non-lethal model of A/PR/8 influenza virus infection. Since a dose of 425 EID₅₀ A/PR/8 virus resulted in 80% mortality, we hypothesized that administering hMSCs in a milder infection model would allow greater opportunity for hMSCs to provide a therapeutic effect. Mice were administered a sub-lethal dose of A/PR/8 virus (150 EID₅₀) and hMSCs were administered to infected mice in both prophylactic (2 days prior to infection) and therapeutic (2 and 5 days P.I.) experimental groups. Weight loss over the course of infection was recorded (Figure 3.6). There was no difference in weight loss between experimental groups.
Figure 3.6. hMSCs fail to alter weight loss kinetics in experimental sub-lethal influenza.
Eight week-old male C57Bl/6 mice were administered $2.5 \times 10^5$ hMSCs (passage 3), via the tail vein, 2 days prior to a sub-lethal influenza A/PR/8 virus infection (150 EID$_{50}$), 2 days post infection (P.I.), or 5 days P.I. Weight was recorded daily. No significant difference in weight loss over the course of infection was observed (Two-way ANOVA, n=11/group). Error bars represent standard deviation.
3.3.3 Mesenchymal stem cell prophylaxis, with or without MSC cytokine pre-incubation, fails to improve survival in experimental influenza

Several important findings in MSC biology have centered around the micro-immunoenvironment for which hMSCs persist. One key observation is the requirement of IFN-γ in combination with TNF, IL-1α or IL-1β, to induce human MSC-mediated immunosuppression [238]. Therefore, we tested whether pre-incubating hMSCs with both IFN-γ and TNF [as described in 220], could enhance the immunosuppressive properties of hMSCs and therefore improve survival when administered to infected mice in our experimental model of severe influenza. 2.5x10⁵ cytokine stimulated or non-stimulated hMSCs (passage 3) were administered to influenza virus-infected mice as prophylaxis (2 days prior to infection) or therapy (5 days P.I.). Weight loss kinetics (Figure 3.7A) and consequent survival kinetics (Figure 3.7B) were unchanged with administration of cytokine stimulated or non-stimulated hMSCs 2 days prior to infection, compared to infected control mice administered PBS. Similarly, no improvement in weight loss or survival was observed with cytokine stimulated hMSCs administered on day 5 P.I., compared to infected control mice administered PBS (data not shown).
Figure 3.7. Cytokine stimulated hMSC prophylaxis fails to alter weight loss kinetics or improve survival in experimental severe influenza. Eight week-old male C57Bl/6 mice were administered $2.5 \times 10^5$ hMSCs, pre-incubated for 24 hours with or without 10 ng/ml IFN-$\gamma$ and 3 ng/ml TNF, 2 days prior to infection with 425 EID$_{50}$ influenza A/PR/8 virus. Weight was recorded daily. No significant difference in (A) weight loss kinetics (Two-way ANOVA, n=10/group) or (B) survival (logrank test, n=10/group) were observed. Error bars represent standard deviation.

3.3.4 Mesenchymal stem cell therapy fails to reduce mortality and/or dampen host inflammation and lung injury when used as an adjunctive therapy in two models of experimental influenza

Influenza virus-infected individuals seeking clinical care will likely be treated with antiviral drugs upon presentation. Therefore, we tested the hypothesis that the combination of a neuraminidase inhibitor, oseltamivir, together with hMSCs, could be effective in reducing mortality. To reflect a common clinical scenario, we delayed combination therapy for 5 days after challenging C57Bl/6 with 550 EID$_{50}$ influenza A/PR/8 virus. Mice receiving oseltamivir and control mice receiving PBS had a survival rate of 18% and 17%, respectively. With the addition of hMSC therapy to oseltamivir treatment, the survival rate increased to 35% (Figure
3.8A right). However, this result was not statistically significant (logrank test; p=0.47). Weight loss over the course of infection was similar between experimental groups (Figure 3.8A left).

It is important to employ a variety of influenza virus strains to ascertain that the experimental effects can be applicable to human infection by various strains. Therefore, a similar experiment to assess hMSCs as an adjunctive treatment strategy was performed with a lethal dose (10⁴ EID₅₀) of influenza A/Mexico/4108/09 (A/Mex/4108) virus, a 2009 pandemic H1N1 human influenza strain that can directly infect mice without undergoing serial passage (Figure 3.8B). As the onset of weight loss over the course of infection occurs more rapidly in our influenza A/Mex/4108 virus model compared to our influenza A/PR/8 virus model, oseltamivir and hMSC treatment was initiated on day 2 P.I. No difference in weight loss (Figure 3.8B left) or survival (Figure 3.8B right) between experimental groups was observed.
Figure 3.8. hMSC adjunctive therapy may improve survival in experimental influenza A/PR/8 virus infection but not influenza A/Mex/4108 virus infection and hMSC adjunctive therapy does not alter weight loss kinetics in both models of experimental severe influenza. (A) Eight-week old male C57Bl/6 mice infected with 550 EID$_{50}$ influenza A/PR/8 virus were administered 5 mg/kg of oseltamivir in 100 ul ddH2O via gavage, twice daily for 5 days, either with or without hMSC treatment (2.5x10$^5$ cells), starting 5 days P.I. Left Weight loss kinetics over the course of infection were measured to assess morbidity in mice. Shown are the weight loss data up to day 8 P.I., when most mice reach euthanasia criteria. No difference between experimental groups was observed (Two-way ANOVA, n=29-32/group; 3 pooled experiments). Error bars represent standard deviation. Right Survival curve. Survival of mice treated with hMSCs and oseltamivir in combination was increased compared to control group; however, this was not significant. (logrank test p=0.47, n=29-32/group; 3 pooled experiments). (B) Eight-week old male C57Bl/6 mice infected with 10$^4$ EID$_{50}$ influenza A/Mex/4108 virus were administered 5 mg/kg of oseltamivir in 100 ul ddH2O via gavage, twice daily for 5 days, either with or without hMSC treatment (2.5x10$^5$ cells), starting 2 days P.I. Left Weight loss kinetics over the course of infection were measured to assess morbidity in mice. Shown are the weight loss data up until day 7 P.I., when most mice reach euthanasia criteria. No difference between experimental groups was observed (Two-way ANOVA, n=9-12/group). Error bars represent standard deviation. Right Survival curve. No difference was observed (logrank test, n=9-12/group).
In the A/PR/8 experimental model, mice from each experimental group as described above were sacrificed on day 7 P.I. BAL fluid and lung homogenate levels of cytokines and chemokines CCL2, CXCL10, KC, CCL5, and IFN-γ were quantified. BAL fluid and lung homogenate cytokine and chemokine levels were similar for combination therapy treated mice compared to control mice that received oseltamivir alone or PBS (Figure 3.9). CXCL10 was significantly decreased in the lung parenchyma of mice that received oseltamivir, in combination with or without hMSCs (One-way ANOVA, p<0.05). No difference in BAL fluid markers of acute lung injury (total protein, IgM, and albumin) was observed on day 7 P.I. for hMSC treated mice compared to control mice that received oseltamivir or PBS alone (Figure 3.10).

In our influenza A/Mex/4108 virus experimental model, mice from each experimental group as described above were sacrificed on day 5 P.I. BAL fluid level of cytokines and chemokines CCL2, CXCL10, KC, CCL5, and IFN-γ were quantified and no difference was observed on day 5 P.I. (Figure 3.11). No difference in BAL fluid markers of acute lung injury (total protein, IgM, and albumin) was observed on day 5 P.I. for hMSC treated mice compared to control mice that received oseltamivir or PBS alone (Figure 3.12).
Figure 3.9. hMSCs do not decrease BAL fluid or lung parenchymal inflammation when used as adjunctive therapy in mice infected with influenza A/PR/8 virus. Eight week-old male C57Bl/6 mice infected with 550 EID$_{50}$ influenza A/PR/8 virus were sacrificed on day 7 P.I. Mice treated with antivirals were administered 5 mg/kg oseltamivir in 100 ul ddH2O once every 12 hours via gavage on day 5 P.I. for a maximum of 5 days. For the combined treatment group, 2.5x10$^5$ hMSCs were also administered via the tail vein, 5 days P.I. BAL was performed and lungs were excised. No significant difference in BAL fluid or lung parenchymal level of selected cytokines / chemokines were observed between hMSC treated mice in combination with oseltamivir treatment, compared to oseltamivir alone (one-way ANOVA, n=7/group). Error bars represent standard deviation. ND = nondetectable.
Figure 3.10. hMSCs fail to decrease acute lung injury when used as adjunctive therapy in mice infected with influenza A/PR/8 virus. Eight week-old male C57Bl/6 mice infected with 550 EID<sub>50</sub> influenza A/PR/8 virus were sacrificed on day 7 P.I. Mice treated with antivirals were administered 5 mg/kg oseltamivir in 100 ul ddH<sub>2</sub>O once every 12 hours via gavage on day 5 P.I. for a maximum of 5 days. For the combined treatment group, 2.5x10<sup>5</sup> hMSCs were also administered via the tail vein, 5 days P.I. BAL was performed. No significant difference in BAL fluid total protein, IgM or albumin was observed between hMSC treated mice in combination with oseltamivir treatment, compared to oseltamivir alone (one-way ANOVA, n=7/group). Error bars represent standard deviation. ND = nondetectable.
Figure 3.11. hMSCs do not decrease BAL fluid or lung parenchymal inflammation when used as adjunctive therapy in mice infected with influenza A/Mex/4108 virus. Eight week-old male C57Bl/6 mice infected with $10^4$ EID$_{50}$ A/Mex/4108 virus were sacrificed on day 5 P.I. Mice treated with antivirals were administered 5 mg/kg oseltamivir in 100 ul ddH2O once every 12 hours via gavage on day 2 P.I. for a maximum of 4 days. For the combined treatment group, 2.5x10$^5$ hMSCs were also administered, via the tail vein, 2 days P.I. BAL was performed. No significant difference in BAL fluid or lung parenchymal level of selected cytokines / chemokines was observed between hMSC treated mice in combination with oseltamivir treatment, compared to oseltamivir alone (one-way ANOVA, n=5/group). Error bars represent standard deviation. ND = nondetectable.
Figure 3.12. hMSCs fail to decrease acute lung injury when used as adjunctive therapy in mice infected with influenza A/Mex/4108 virus. Eight week-old male C57Bl/6 mice infected with $10^4$ EID$_{50}$ influenza A/Mex/4108 virus were sacrificed on day 5 P.I. Mice treated with antivirals were administered 5 mg/kg oseltamivir in 100 ul ddH$_2$O once every 12 hours via gavage on day 2 P.I. for a maximum of 4 days. For the combined treatment group, $2.5\times10^5$ hMSCs were also administered, via the tail vein, 2 days P.I. BAL was performed. No significant difference in BAL fluid total protein, IgM or albumin was observed between hMSC treated mice in combination with oseltamivir treatment, compared to oseltamivir alone (one-way ANOVA, n=5/group). Error bars represent standard deviation. ND = nondetectable.
3.4 DISCUSSION

MSC-based prophylactic and treatment strategies have yielded significant therapeutic benefits in various pre-clinical models of acute lung injury [209,210,245-248]. These encouraging findings in which MSCs were efficacious led us to hypothesize that MSCs might dampen host inflammation, decrease acute lung injury, and improve survival in an experimental model of severe influenza, characterized by heightened inflammation and acute lung injury. Despite these positive pre-clinical findings, our study demonstrated that hMSCs were unable to affect inflammation, lung injury and survival in experimental influenza. This finding is in accordance with few published reports where MSCs were unable to dampen host inflammation and improve outcome in vivo [249-251].

Clinical management of severe influenza complicated by ARDS is often guided by the ALI/ARDS literature. For example, it is recommended that lung-protective ventilations strategies such as low tidal volume ventilation be used in managing patients with severe influenza complicated by ARDS, based on recommendations of the ARDSNet trial [278]. Given the demonstrated utility of drawing from ALI/ARDS literature to inform potential treatment strategies for influenza, it was reasonable to evaluate the therapeutic potential of MSCs in the context of severe influenza. Despite similarities in clinical presentation and pathophysiology of ALI and severe influenza, our negative findings reinforce that viral-induced ALI is not wholly comparable with other forms and etiologies of ALI, such as bacterial derived ALI.

We first established the influenza A/PR/8 virus mouse model of severe influenza, characterized by the development of inflammation and acute lung injury, and evaluated the use of hMSCs for the prevention or treatment of severe disease (Fig. 3.1,3.2). While influenza A/PR/8 virus
infection induced a host response and lung injury in vivo, we do not know whether the host response was specifically dysregulated, as we did not compare host responses between various influenza virus infection models. However, in our model, approximately 80% of mice reached euthanasia criteria - defined by 20% weight loss - where such weight loss was associated with elevated inflammation.

The failure of hMSCs to suppress inflammation and decrease lung injury could be attributed to myriad factors. Firstly, the hMSCs may have rapidly differentiated in culture which rendered them ineffective as immunosuppressive agents. In this study, hMSCs were cultured per the “manufacturer’s guidelines”, in terms of low passage number, medium and FBS stocks and concentrations, and cell harvesting at log phase of growth, as these conditions have been deemed optimal [279]. Despite these attempts to create optimal conditions, it is possible that the cultures spontaneously differentiated in vitro rendering them ineffective in vivo. Also, the structure and function of hMSCs tend to vary between donors. For example, Francois et al. found that the amount of IDO produced by each hMSC donor varied, thereby influencing their immunosuppressive potential [220]. Nevertheless, culture-expanded MSCs tested in our experimental model always exhibited a spindle-shaped fibroblastic morphology and were deemed to be of high quality as indicated by measuring the number of colony forming units (a generally accepted indicator of MSC quality [279]) (data not shown).

Our results may also be explained if the hMSCs were unable to home to the lungs in our experimental model. Varied protocols to isolate and expand MSC populations have been reported to influence MSC homing capacity. For example, protocols which employ progressive subculturing are associated with a loss of chemokine receptors and decreased chemotactic
responses [232,235,236]. MSCs delivered intravenously would reach the pulmonary microcirculation first; therefore, homing might not be of concern when lungs are the end target organ. For example, MSCs may be trapped in the lung as microemboli after IV infusion [258]. Mei et al. also found an average 47% of injected MSCs in the lungs 15 minutes after MSC delivery into LPS-challenged mice [210]. However, we did not test for the presence of MSCs in the lung after IV delivery in our model, therefore, we can not definitively conclude that the cells did in fact reach the lungs.

If the cells were indeed delivered to the lungs when administered intravenously in our model of influenza, must they have remained in the lungs in order to elicit a beneficial effect? Since we employed allogeneic MSCs, HLA mismatching may have led to rapid rejection after injection. This result would be consistent with other reports where allogeneic murine MSCs were rejected when administered in MHC mismatched mice compared to autologous cell transplant [227,228]. Additionally our cells were derived from human donors, rather than extracted from mice, which could have exacerbated MSC rejection \textit{in vivo}. This strategy was employed because human clinical trials involving MSCs employ MSCs derived from human donors and a similar cross-species strategy was employed by others in an attempt to elucidate the mechanism of human MSC mediated effects [258,259]. They found that hMSCs attenuated lung inflammation and injury (similar to mouse MSCs) which suggests that the beneficial effects are not restricted to mouse MSCs in mice. Furthermore, MHC class II antigens are not expressed on human MSCs [223] but are constitutively expressed on murine MSCs [227]. Therefore, human MSC rejection may be less likely than murine MSC rejection. Indeed, patients who underwent MSC allotransplant experienced no anti-allogeneic MSC antibody formation or T-cell sensitization.
[224], suggesting human MSCs may not be rejected by the recipient and are indeed immunoprivileged.

Even if the cells were not rejected, their in vivo life span may nonetheless have been too short to mediate outcome. The majority of MSCs were lost from the lung at 3 and 14 days, respectively, after delivery to mice with LPS induced lung injury [209-211]. These findings could suggest that in our experimental model of severe influenza, MSCs did not persist long enough to induce a beneficial response. However, these studies found MSCs to improve outcome in experimental lung injury despite low levels of MSC retention in the lungs. Therefore, the reported benefit in response to cell-based therapy in lung injury models does not necessarily require a high level of long-term MSC persistence in the lung. Furthermore, MSCs may need not reach the lungs to exert their effects. For example, intraperitoneal administration of hMSCs attenuated LPS-induced lung inflammation, despite being less effective than IV delivery or oropharyngeal aspiration [259].

Alternatively, our ascribed cell dose could have been too low to exert an immunosuppressive effect, regardless of where the cells localize and/or persist in vivo. We consistently administered a 2.5x10⁵ dose of hMSCs, via the tail vein, in our experimental influenza model as this dose was effective in a report by Mei et al. [210]. However, in that report cells were administered via the jugular vein and not the tail vein, which could potentially account for the observed difference; cells introduced via the jugular vein have less distance to travel and therefore less potential to be destroyed before reaching the lungs, compared to cells administered via the tail vein. Indeed, Kim et al. found that a dose of 1x10⁶ cells resulted in a significantly prolonged life span and a significantly higher number of motor neurons in mice with amyotrophic lateral sclerosis compared to untreated mice or mice treated with 1x10⁴ cells [280]. Additionally, several positive
findings regarding the use of MSC therapy in lung injury models reported administration of $5 \times 10^5$ cells and $7.5 \times 10^5$ cells [245,247]. Therefore, it is possible that, as opposed to having too few cells persist in the lung, the problem was created at the outset with an insufficient dosage of MSCs delivered via the tail vein. This issue could be circumvented by injecting more cells. However, rats administered 5 million cells at one time point or 2 million cells at two time points in a transplantation model did not suppress immunity but rather accelerated graft rejection [251].

As alluded to earlier, the route of MSC delivery may have affected our findings. MSCs delivered directly into the lung airspaces have been shown to decrease cytokines in BAL fluid and improve survival after endotoxin induced lung injury [247]. However, MSCs delivered intravenously in a similar model did not reduce BAL inflammatory cytokines [246]. Therefore, it is possible that direct rather than IV MSC delivery to the lungs of influenza virus-infected mice might have improved MSC mediated immunosuppression and outcome in our experimental model. However, MSCs administered by various methods in addition to intrapulmonary delivery - including tail vein, jugular vein, and intraperitoneal delivery - were effective in improving outcome in experimental lung injury [209,210,245,246,259]. Therefore, while route of delivery may be important, it is likely insufficient to explain our negative results.

Given the vast amounts of literature describing the immunosuppressive functions of MSCs, it is reasonable to question whether MSC immunosuppression could diminish the ability of the host to control virus replication and mediate viral clearance. However, we did not observe any increase in viral load with hMSC therapy (Fig. 3.3C). To the contrary, recent in vivo studies have provided evidence that MSCs may enhance antimicrobial immune effector cell function and reduce microbial burden during infectious challenge [221,244]. For example, Mei et al. reported
decreased total bacterial counts in septic mice, which was hypothesized to be attributed to increased splenic monocyte and macrophage phagocytosis [244]. In our model, influenza viral titer might have been too overwhelming for any anti-microbial effects to have occurred, or the hMSCs may have been unable to exert anti-viral compared to anti-bacterial effects.

Alternatively, it is possible that the influenza virus in the lung itself interacted with the MSCs in a manner which prevented the MSCs from exerting their immunosuppressive activity. Temporary inactivation of MSC mediated immunosuppression induced by viral activation of toll-like receptors (TLRs) 3 and 4 on MSCs has been described [281]. This finding may represent a mechanism that allows the immune system to function effectively in the presence of virus or bacteria. This finding could account for reduced MSC functionality in the course of infection since influenza RNA can serve as a ligand for TLR3.

As the induction of MSC immunosuppression highly depends on the microenvironment into which the cells are introduced, when is the ideal time to administer MSCs in severe influenza? hMSCs were delivered early (day 2 P.I.) or late (day 5 P.I.) in the course of infection when lung inflammation was minimal or elevated, respectively. In both regimens, MSCs failed to dampen host lung inflammation, decrease acute lung injury, or improve morbidity and mortality (Fig. 3.3-3.5). Despite many in vitro findings of MSC mediated immunosuppression, the microenvironment in vivo is much more variable and unpredictable. When applying MSCs to an animal model of disease, MSC immunosuppression might be easily turned off in vivo, or rather immunostimulatory, as has been reported in other models of disease [250]. This may account for the inability of MSCs to dampen host inflammation in our animal model of severe influenza.
Unfortunately, there is no validated method of measuring MSC bioactivity \textit{in vivo}, which generates further challenges [282].

Another discrepancy which may account for observed differences between the effects of MSCs in our model of lung injury and those with reported MSC therapeutic benefit is the length of time between introducing the injury-causing agent, and the inflammatory response / onset of disease. Most models of acute lung injury result in acute lung inflammation which peaks within 24-48 hours then resolves shortly thereafter, such as in the endotoxin lung injury model. In our model of severe influenza virus-induced lung injury, the host response did not ensue until approximately 4 days post-influenza virus infection - and mice could remain alive for up to 9 days P.I. before they succumbed to death. This may have important implications for our findings. MSCs introduced to the lung on day 5 post-influenza virus infection may not have exerted effects which lasted long enough to affect the outcome of influenza virus-infected mice.

Additionally, MSCs administered prophylactically or early in the course of infection may not have been sufficiently stimulated by the host microenvironment, as the host inflammatory response was only detected beginning around 4 days post-influenza virus infection (Fig. 3.2). Contrary to our hypothesis, we found that MSCs administered prophylactically increased morbidity of influenza virus-infected mice; however, this finding was not significant (Fig. 3.7). An explanation for these effects could be that insufficient \textit{in vivo} levels of pro-inflammatory cytokines rendered MSCs immune-enhancing, despite MSC \textit{ex-vivo} pre-stimulation for one group of MSC treated mice in an attempt to counter this effect. Nevertheless, MSCs administered concurrently with lung injury or before lung injury in experimental models have been effective [209,245,246]. In fact, Ortiz \textit{et al.} reported that MSCs were only effective when administered at
the same time as bleomycin [245]. Therefore, MSCs can exert their beneficial effects in the initial stages of injury, albeit in a model of bleomycin induced injury and not viral induced injury. Although timing of MSC administration is likely to be an important factor, it is difficult to conclude that the non- or minimally inflammatory environment into which the MSCs were introduced when administered prophylactically or early in the course of influenza virus infection is the reason for which the cells lacked efficacy in improving morbidity and mortality.

MSCs administered in combination with oseltamivir also had no effect (Fig. 3.8 – 3.12). Since the mice treated with oseltamivir only did not show improved survival in our influenza A/PR/8 virus or influenza A/Mex/4108 virus experimental models, we postulated that the infection may simply have been too severe for any agent to improve outcome. In particular, the absence of clinical effect could have been because the high levels of inflammation present in severe influenza may have overcome the immunosuppressive properties of MSCs in vivo. This is consistent with the absence of clinical effect in experimental GvHD and rheumatoid arthritis, both diseases characterized by overwhelming inflammation [249,250]. However, this may not necessarily be the case in our study since MSCs have proven effective in pre-clinical sepsis, a disease characterized by an extreme systemic inflammatory response [221].

While antiviral treatment was initiated late in the course of infection to re-capitulate the clinical scenario, the timing of administration was likely too late to inhibit viral replication, since viral load has been shown to peak in the lungs 24-72 hours after infection [88]. Oseltamivir is also known to have limited efficacy when administered beyond 48 hours post-onset of symptoms – mice begin to lose weight around day 5 P.I. which could be deemed the onset of influenza
symptoms, however, it is difficult to correlate symptom onset in humans with our experimental animal model.

Despite a lack of efficacy with oseltamivir treatment, we observed decreased levels of IFN-\(\gamma\), CCL2, CCL5 and KC in BAL fluid and significantly decreased CXCL10 in lung interstitium of oseltamivir-treated mice, independent of MSC treatment (Fig. 3.9). Wong et al. noted oseltamivir could dampen host inflammation independent of an antiviral effect, which may explain our findings [283]. Similarly, others have also hypothesized that a dampened immune response, including decreased cytokine production, may be the mechanism of action of oseltamivir, rather than influenza virus replication inhibition [284]. However, it is difficult to determine whether our finding was attributable to oseltamivir immunomodulatory effects or to an indirect effect of oseltamivir on viral replication, as lung viral load was not quantified in this set of experiments.

Noteworthy, decreased lung inflammation (albeit insignificant) with oseltamivir treatment did not correlate with improved survival in our experimental model. This may indicate that our experimental model was too severe, as not even a modest ability to dampen host inflammation was able to translate into decreased morbidity and mortality. Related, neither oseltamivir or oseltamivir/MSC combination treatment administered starting day 2 post infection in the 2009 pandemic H1N1 experimental influenza (A/Mex/4108) virus model had any effect. Thus, although influenza A/Mex/4108 virus was a human strain which is not murine-adapted (thereby having possibly greater clinical relevance to human influenza virus infection than influenza A/PR/8 virus) our dose of \(10^4\) EID\(_{50}\) influenza A/Mex/4108 virus was probably too
overwhelming to observe any therapeutic effects of either oseltamivir or MSCs as the influenza virus dose proved to be 100% lethal regardless of the treatments applied.

Finally, although MSC-secreted proteins have been attributed to MSC-mediated improved outcome in pre-clinical models of lung injury, they may not have been useful in the treatment of severe influenza, despite underlying pathophysiology common to lung injury derived from viral or bacterial derived pneumonia, sepsis, or trauma. For example, although it may be beneficial to administer recombinant TSG-6 as treatment for LPS-induced lung injury in mice [259], TSG-6 treatment for severe influenza may be detrimental due to its ability to upregulate COX-2 [285], as COX-2 upregulation may contribute to increased morbidity and mortality in severe influenza (discussed in chapter 1). The same reasoning is applicable to MSC upregulation of PGE₂, which is generated by conversion of arachidonic acid via COX-2. Furthermore, IFN-γ and TNF-induced upregulation of MSC secreted factors may have exacerbated disease severity; this may explain why MSCs pre-incubated with these cytokines resulted in increased morbidity and mortality (Fig. 3.7). Further evaluation of individual MSC-secreted proteins for treating severe influenza may be warranted.

In conclusion, our study was not able to demonstrate the therapeutic potential of MSCs in experimental severe influenza in mice. Given the overwhelming number of publications identifying MSCs as an effective treatment in a variety of disease models, we believe that our findings are valuable to the field of MSC research as they provide a noteworthy contrast to some of the existing literature in this field. Ongoing clinical trials should reveal the true potential of these cells and provide insight as to whether further investigation of MSCs for severe influenza treatment is warranted.
Chapter 4: Summary and Future Directions

4.1 Summary

Antiviral drugs currently approved for the treatment of influenza have inherent therapeutic limitations, including unknown efficacy in treating severe disease or complications, timing of administration (i.e. limited clinical impact when administered beyond the first 48 hours of symptom onset) and rising antiviral resistance. Therefore, we investigated the use of iNO as a new antiviral treatment for severe influenza (Chapter 2). Unfortunately, we found that iNO was unable to dampen the viral load and thus improve outcome in experimental severe influenza. This may have been due to the instability and short half-life of NO and the toxic effects of NO metabolites. In future, there may be merit to exploring another vehicle of NO delivery which could perhaps stabilize nitric oxide without generating toxic effects. Overall, our findings emphasize the limitations of drawing conclusions from in vitro experimental findings, as the host response in vivo is much more complex; in vitro findings may not reflect what will be correspondingly observed in vivo where myriad pathways are activated during infection.

Severe influenza is associated with an overexuberant host-mediated innate immune response, leading to excessive inflammation, tissue injury and acute lung injury. It is clear that this deleterious inflammatory host response is a major contributor to influenza-related morbidity and mortality. Development of novel and innovative adjunctive treatment strategies that target the host response to the influenza virus may be critically important for clinical outcome in the case of severe disease. It is clear that severe influenza with acute lung injury is a highly complex disease process involving many pathophysiologic manifestations. Given this, it is not surprising
that strategies targeting one aspect of the disease process have yet to prove efficacious. This suggests that a new multifaceted therapeutic approach, aimed at reducing lung injury while maintaining a balanced immune response and facilitating lung repair, could be of greatest value. In this regard, mesenchymal stem cells were an obvious choice warranting investigation as a new treatment approach to lung injury and enhanced inflammation developed in severe influenza due to their previous demonstrated effects in pre-clinical models of lung injury (Chapter 3). However, contrary to our hypothesis, we found that MSCs did not reduce cytokine/chemokine responses or improve ALI in our murine influenza model. Our experimental findings reinforce that, despite similarities in clinical presentation and pathobiology of acute lung injury caused by various sources, beneficial effects in other models of ALI/ARDS may not necessarily be translated to influenza-virus induced ALI.
4.2 Future directions

Our main focus was to test whether MSCs, given alone or as adjunctive therapy, could reduce host inflammation, specifically, inflammatory cytokines correlated with severe disease and protein level in the lungs - a hallmark of ALI. We were unable to identify any beneficial effect; therefore, a number of plausible explanations merit further investigation. For example, were MSCs in fact mediating the host response? We did not provide any indication for MSC biological activity \textit{in vivo} and host interaction. To determine this, we could investigate biomarkers of MSC activity \textit{in vivo}. Specifically, BAL fluid levels of important MSC-secreted proteins, including KGF, Ang-1 and PGE$_2$, could be measured following MSC delivery.

In light of the fact that oseltamivir did not decrease morbidity and mortality in our experimental model, we could not conclude that MSCs were ineffective as an adjunctive therapeutic agent. Therefore, studies to optimize oseltamivir dosing were recently completed (data not shown). Further examination of an MSC and oseltamivir combination treatment strategy - with a dose of oseltamivir that improves survival by $\sim$30% when used alone - is underway.

Further studies could address the hypothesis that insufficient MSC dosing led to sub-optimal \textit{in vivo} effects, as the optimal MSC dose necessary to obtain a therapeutic benefit remains undefined. A report by Kim \textit{et al.} found MSC mediated effects \textit{in vivo} to positively correlate with increasing cell dose [280]. Therefore, we could determine whether provision of 1 million MSCs to influenza virus-infected mice improves outcome. We could also try intratracheal or intrapulmonary delivery of cells to influenza virus-infected mice, as there is no standardized method of MSC delivery that achieves greatest clinical efficacy (as discussed in Chapter 3).
While it would be favourable to develop a reliable protocol to culture MSCs, it may also be valuable to assess other in vitro culturing systems. Specifically, MSCs could be grown as 3D spheroids under the “hanging drop” method as this form of culture has been shown to enhance MSC mediated immunosuppression, compared to monolayer culture, in a co-culture system with LPS-activated macrophages and in a mouse model of zymosan induced peritonitis [286]. It may also be valuable to assess MSC delivery in a more stable form. To protect against MSC degradation, delivery of hydrogel-encapsulated MSCs as described by Karoubi et al. could provide enhanced cell survival in the lungs [287]. Alternatively, MSC cultures could potentially be modified to improve their immunosuppressive properties.

Lastly, if these investigations reveal a role for MSC-mediated improvement in severe disease, therapeutic strategies based on enhancement of MSC-secreted proteins could be considered. We have not found any literature that reports a therapeutic benefit with the use of MSC-secreted mediators in severe influenza. However, single MSC-released mediators have been effective in improving outcome in pre-clinical models of lung injury. For example, intrapulmonary delivery of recombinant human TSG-6, a known MSC mediator, reduced LPS-induced inflammation in the lung [259]. In particular, proteins which could affect the endothelial barrier may be of interest as inhibition of endothelial activation/dysfunction and associated capillary leak has recently been shown to increase survival of H5N1 virus-infected mice, without altering cytokine levels or viral load [288]. Binding of the ligand Slit to its endothelial receptor Robo4 inhibits hyperpermeability induced by VEGF and promotes cell-surface expression of adherens junction protein VE cadherin [288,289]. London et al. significantly improved survival in experimental H5N1 influenza murine models by administering the active fragment of Slit, Slit2N, which is released via proteolytic cleavage upon binding to Robo4 [288]. This study suggests that
strengthening the vascular-endothelial barrier may be an important therapeutic target for severe influenza treatment.

With this in mind, we could evaluate the role of MSC-secreted protein Ang-1, a potent mediator of angiogenesis that functions to prevent leakage and promote blood vasculature quiescence, in the course of severe influenza virus infection. Should Ang-1 be dysregulated, we could assess Ang-1 rescue strategies in restoring endothelial integrity in the lungs, such as administration of Ang-1 via overexpression in a viral vector. Increasing Ang-1 levels to preserve endothelial integrity may prove to be an effective strategy to prevent or ameliorate ALI caused by severe influenza.
4.3 Final considerations

Antiviral resistance against influenza, including dual (adamantane and oseltamivir) resistance [290], is likely to continue to increase, posing further public health concerns. Therapeutic strategies employing antiviral agents alone are unlikely to significantly improve overall clinical outcome of individuals with severe influenza. Therefore, interest in adjunctive immunomodulatory strategies for the treatment of influenza needs to grow, and innovative approaches targeting the host response to influenza need to emerge.

The current, limited experimental evidence to support the use of immunomodulatory therapy must be strengthened. Promising approaches to combined antiviral and immunomodulatory therapy for influenza have been demonstrated by Zheng et al. using celecoxib, mesalazine and zanamivir in pre-clinical models of severe influenza [162]. Further study of such approaches is clearly warranted. Based on the promising results in pre-clinical models of severe influenza, combination therapy employing PPAR agonists and/or COX-2 inhibitors with conventional antiviral agents should be studied in randomized clinical trials of severe influenza.

Infection and subsequent death due to avian influenza A (H5N1) is likely to continue. In the event that the H5N1 virus develops the ability to transmit from human-to-human, a pandemic may occur. Furthermore, as evidenced by 2009 pandemic H1N1 influenza, it is not currently possible to predict which influenza virus strain will give rise to the next pandemic, and how virulent that strain will be. New adjunctive treatment strategies, in addition to new antiviral therapy, will likely be required to significantly improve clinical outcome.
Finally, immunomodulatory strategies that have yielded positive results in pre-clinical and clinical studies of ALI/ARDS may have therapeutic implications for the management of severe influenza. Targeted strategies to preserve vascular-endothelial integrity in an effort to prevent influenza-related end-organ dysfunction/injury, including ALI/ARDS, should emerge as an innovative approach to improve clinical outcome in severe influenza.
Chapter 5: References


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