IMPACT OF INFLUENZA VIRUS REPLICATION ON THE IFN-γ RESPONSE IN MACROPHAGES

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Keerthana Sekar

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ABSTRACT

IMPACT OF INFLUENZA A VIRUS REPLICATION ON THE IFN-γ RESPONSE IN MACROPHAGES

Macrophages are important for protection against infection with influenza viruses, they may also contribute to disease severity during H5N1 influenza virus infection through the virus induced alteration of important antiviral functions. One reason for these changes might be due to the ability of H5N1 viruses to replicate efficiently in the macrophage. Thus, we hypothesized that H5 virus infection alters the macrophage antiviral response and abrogates the IFN-γ response in RAW264.7 macrophages in a replication dependent manner. Infection of macrophage with live H5 virus decreased the phagocytic activity of IFN-γ activated RAW264.7 cells. Similarly, a reduction in nitric oxide (NO) production was also observed in IFN-γ activated macrophages infected with live H5 virus. Interestingly, infection with UV-inactivated H5 virus restored the phagocytic activity and NO production of IFN-γ activated macrophages. This shows that replication of H5 influenza virus is critical and is required to evade the macrophage antiviral response. H5 virus interferes with these macrophage functions by inhibiting IFN-γ signaling. While tyrosine phosphorylation of STAT1 was found to be intact in IFN-γ activated macrophages infected with H5 virus, expression of the IFN-γ stimulated gene IRF-1 was, blocked. Thus H5 influenza virus alters the antiviral response of

macrophages and antagonize the IFN-γ response in macrophages in a replication dependent manner.

CHAPTER I

INTRODUCTION

Influenza Virus

Widespread epidemics of influenza, acute respiratory disease are reported every year affecting the elderly and children. Influenza virus is the etiological agent of this disease. Influenza virus is a negative-sense, ssRNA virus, belonging to the family *Orthomyxoviridae*. They can be classified into three distinct types A, B and C based on their antigenically distinct proteins [1]. Type A influenza virus (IAV) causes substantial morbidity and mortality in humans. Up to 50% of the population can be infected with flu, and more than 200,000 people are hospitalized each year [2]. Respiratory epithelial cells, alveolar epithelial cells and lung endothelial cells are the targets for the virus infection where they utilize sialic acid receptors to attach to, and enter, target cells [3]. Virus attachment and entry is mediated by the viral hemagglutinin (HA) protein. The HA of human influenza virus isolates preferentially recognizes α2,6- sialic acid linkages which are present on human airway epithelial cells, whereas the HA of avian isolates preferentially recognizes α2,3- sialic acid linkages present in the intestinal epithelium of birds [4].

The RNA genome of influenza virus consists of eight gene segments. Basic polymerase proteins PB2, PB1, and acidic polymerase PA are encoded by segment 1-3. These proteins form the RNA-dependent RNA polymerase complex responsible for

transcription and replication of viral genome. HA and neuraminidase (NA), viral surface proteins are encoded by segment 4 and 6. Segment 5 encodes the nucleocapsid protein that forms ribonucleoprotein complex (RNPs) by binding to viral RNA. Segment 7 codes for matrix proteins M1 and M2. Segment 8 encodes for two different proteins, non-structural protein (NS1) that acts as the antagonist of host innate immune responses and nuclear-export protein (NEP) that helps in nuclear export of newly formed RNPs into the cytoplasm [5].

Based on the antigenic characteristics of the envelope proteins HA and NA, influenza A viruses are further classified into 16 HA and 9 NA serotypes. H1N1 and H3N2 viruses are presently established in humans, causing annual epidemic outbreaks. Novel viruses arise due to antigenic shift, a process where two or more different strains of virus combine to form a new subtype having a mixture of surface antigens of two or more original strains or by the process of antigenic drift, which involves accumulation of mutations within the genes that code for antibody-binding sites [6].

An example of novel IAV emergence through antigenic shift and drift is the emergence in 2003 of highly pathogenic H5N1 viruses in humans in Asia [7]. Patients with H5N1 disease manifest pneumonia leading to acute respiratory syndrome and multiple-organ dysfunction. A 60% mortality rate for confirmed cases of H5N1 is reported. In contrast to the seasonal H1N1 or H3N2 viruses, a sustained human to human transmission of H5N1 by aerosol or by respiratory droplets has not been described yet. Studies have demonstrated mutations in the HA protein and PB 2 protein of H5N1 results

in H5N1 airborne transmission in ferrets [8]. Studies have also shown that few mutations in the HA gene enables the virus to switch from using an avian receptor to human receptor and also enhanced the replication of the virus. Other groups have shown that a minimum of 2-5 amino acid substitutions in PB1, PB2 and HA are required for the emergence of airborne H5N1influenza virus [9, 10]. If H5N1 viruses, through antigenic shift or antigenic drift acquire efficient transmissibility in humans, the impact could be devastating. Thus, a better understanding of the reasons for enhanced disease in H5N1 infected patients is important for pandemic preparedness. The difference in the pathogenesis between H5N1 and seasonal influenza virus is only partially known. Studies show that regions of the HA protein contribute to increased pathogenicity [11, 12]. The host immune system acts against microbial invasion by inducing both innate and adaptive responses [13, 14]. One reason proposed for disease severity during H5N1 infection is an exaggerated inflammatory response relative to H1N1 and H3N2 strains and macrophages are suggested to be central to this response. H5N1 are potent inducers of pro-inflammatory cytokines. Interferon (IFN), Tumor Necrosis Factor-α (TNF-α), IL-6 and other chemokines were found detected in large quantities in H5N1 infection, suggesting that hyper induction of these cytokines have a crucial role in pathogenesis [14, 15,21,22].

Interferon Gamma (IFN-γ)

IFNs are classified into type I and type II IFNs. Type I IFN comprises of IFN-α, IFN-β, IFN-τ and IFN-ω which bind to a common receptor, the IFN receptor. IFN-γ or type II IFN is produced by NK cells and T cells and important for the

activation of other immune cell types [16]. IFN-γ directs the growth, maturation and differentiation of many cell types and is one of the key regulatory cytokines in the host immune response against viral infections [17]. The effects of IFN-γ against viral infection includes induction of various cellular products such as NO upon activation of macrophages [18].

Cellular responses to IFN-γ are mediated by the regulation of gene expression. Most of the signal transduction by IFN-γ are mediated by a Jak-Stat (signal transducer and activator of transcription) signaling pathway (Figure 1). IFN-γ induces cellular responses by binding to a functional IFN-γ receptor (IFNGR) which is comprised of two subunits each of IFNGR1 and IFNGR2. Binding of IFN-γ to its receptor induces receptor oligomerization and activation which allows trans-phosphorylation of receptor-associated Janus kinases (JAKs) Jak1 and Jak2 on the IFNGR. The activated Jaks phosphorylate tyrosine residue 440 (Y440) on IFNGR1 which then serves as a docking site for STAT1. STAT1 undergoes phosphorylation on Y701which induces dissociation of STAT1 from the IFNGR. Phosphorylated STAT1 molecules dimerize and translocate into the nucleus. IFN-γ responses are mediated through the binding of STAT1 homodimers to the genes containing the gamma activated site (GAS) in the promoter region (Figure 1).

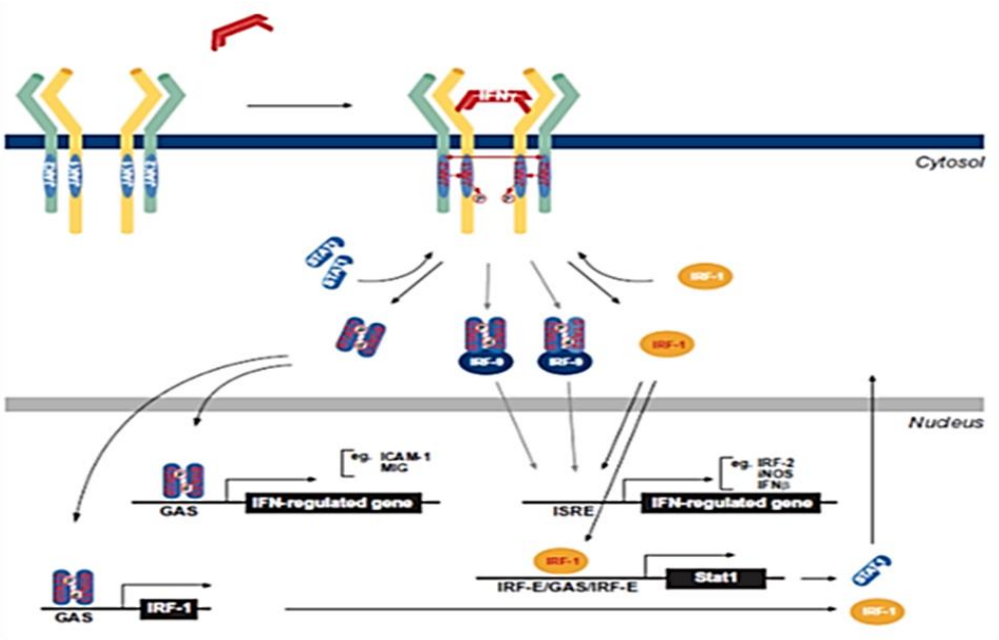

Figure 1: Model of IFN- γ dependent Jak/STAT pathway. Figure adapted from [18]. Upon binding of the ligand to IFNGR1 and IFNGR1, a conformational change takes place that activates the Jak1. The activated Jak1 phosphorylates and forms a docking site. STAT1 undergoes phosphorylation on Y701which induces dissociation of STAT1 from cytoplasm and enter into the nucleus. STAT1 homodimer binds to promoter IFN-γ activation site (GAS) to initiate/suppress transcription of IFN-γ regulated genes.

Some of the IFN-γ induced genes are interferon regulatory factor (IRF-1), a transcription factor that amplifies the antiviral response and ICAM-1, required for recruitment and activation leukocytes. The importance of STAT1 mediated antiviral responses is demonstrated in previous studies showing that STAT1-knock out mice suffer from severe viral and bacterial infections [19, 20]. Interferon regulatory factors (IRF) are involved in mediating IFN signal cascades. Among the IRF family, IRF-1 expression is

upregulated in response to virus infection and various cytokines, including type I and type II IFNs. IRF-1 transcription increases upon IFN-γ induced STAT1 activation [20].

Role of Macrophages and IFN-γ in viral infection

Macrophages play a central role in the antiviral immune response. They are important in the body's defense against various microorganisms [23]. Alveolar macrophages present in the intranasal passages are among the first cells that are exposed to respiratory pathogens such as an influenza virus infection. They modulate the innate and adaptive immune response both at early and later stages of the infection by phagocytosis of pathogenic agents and apoptotic cells and by interacting with cells of the adaptive immune response [24]. *In vivo* studies have shown that macrophages are essential to host defense against influenza viruses as they control severity of the disease by limiting viral replication, initiating various cytokine and chemokine responses and mounting adaptive immune responses [25,26]. Thus, depletion of macrophages resulted in greater replication of influenza virus in lungs and also increased the severity of the disease in animal models of influenza virus infection, indicating how essential macrophage responses are to host defense [27].

Although macrophages prevent severe influenza virus infection, H5N1 influenza viruses are thought to be potential inducers of various cytokines in macrophages. This excessive pro inflammatory cytokine response such as IFN-γ, TNF α

by macrophages, and subsequent tissue damage resulting from a heightened immune response are thought to be one of the causes of death in patients experiencing H5N1 virus infection [20, 22, 28]. Early infiltration of macrophages into the lungs was noticed in H5N1 infected patients, which might be a reason for increased level of expression of pro-inflammatory cytokines when compared to seasonal influenza virus infection [29]. Studies have also shown that genetic factors of H5N1 influenza virus might also contribute to this excessive cytokine response [30].

However, the contribution of macrophages to H5N1 pathogenesis may also result from an ability of these viruses to replicate in macrophages. While the replication of most influenza strains has been shown to be abortive in macrophages, the ability of H5N1 virus to replicate in macrophages is unclear [30, 31]. It has been shown that the primary targets of H5N1 influenza virus infection are the respiratory epithelium and the alveolar macrophages. Studies on replication of H5N1 influenza virus infection in alveolar macrophages has produced inconsistent results. van Riel *et al.,* demonstrated a failure of HPAI H5N1 viruses to productively replicate in alveolar macrophages [32], while Cline *et al.,* demonstrated that H5N1 viruses productively replicate in a mouse cell lie and in primary alveolar macrophages [33]. Finally, Yu *et al.,* showed that productive replication of H5N1 influenza viruses in human alveolar macrophages correlated with increased expression of various proinflammatory cytokines [34]. These findings highlight the impact that replication of H5N1 influenza viruses may have on the course of infection. Previous data demonstrates that H5N1 viruses are unique in their ability to

replicate in macrophages and that the HA protein is sufficient to confer this ability. However in a non-H5 virus infection, macrophages effectively block virus transcription and genome replication [33]. Several cellular factors and cell-intrinsic innate immune pathways responsible for blocking virus replication in macrophages are being studied [35].

Once activated by an external stimulus macrophages ingest and clear the pathogens by a process called phagocytosis. In this antiviral process, the pathogen is internalized and becomes trapped in the phagosome, which then fuses with a lysosome. Enclosed in phagolysosome, enzymes and toxic free radicals digest and destroy the pathogens [36]. As a response to infection, macrophages secrete proinflammatory mediators such as nitric oxide (NO), IL-1 and tumor necrosis factor (TNF). All these factors in turn contribute to eliminating the infection [37, 38]. The importance of macrophage phagocytosis for protection against IAV infection is evident from previous studies demonstrating that inhibition of phagocytosis leads to increased virus replication and associated morbidity. The importance of phagocytosis and its protection against H5N1 infection has not been addressed [39].

NO is generated by the enzyme nitric oxide synthase (NOS) which is induced by IFN-γ and catalyzes the biosynthesis of NO in a range of tissues. The inducible form of NOS, iNOS, serves as a key molecule in combating viral infection. NO acts as a mediator of apoptosis and excessive production has negative effects reacting with other damaging oxidants leading to inflammation [40]. Various studies have demonstrated the

role of NO in the immune response to virus infection. It has also been shown that NO may inhibit the early stage of virus infections thus leading to viral clearance. IFN-γ induced expression of iNOS in macrophages requires IRF-1 as a transcription factor [41]. Priming with IFN-γ along with triggering stimuli with LPS or TNF-α increases the expression of iNOS. IFN-γ along with LPS has a synergistic effect where, LPS induces macrophages to produce TNF and IFN α/β [42, 43]. LPS is also able to promote IFN-γ dependent STAT1 signaling pathway by inducing STAT1. IFN-γ, along with the exposure of LPS, increases the percentage of molecules phosphorylated on serine and tyrosine and subsequent STAT1 DNA binding [44]. In the case of H5N1 infection, studies have shown an increase in viral load along with excessive production of proinflammatory immune response such as iNOS. Increased production of NO promotes excessive inflammation leading to tissue damage, which might also be the cause for the severity of H5N1 influenza infection [45]. The main objective of this project is to determine how influenza A virus replication in macrophages impacts macrophage functions and to determine if influenza A virus alters the IFN-γ signaling response in macrophages leading to altered macrophage function. I hypothesize that H5N1 influenza virus replication in macrophages alters cellular functions in ways that contribute to increased disease severity. The following aims were framed and were carried out to accomplish the above objective.

Aim 1a: Determine if H5 influenza virus replication impairs antiviral responses.

Aim 1b: Determine if H5 influenza viruses antagonize IFN-γ signaling pathways in a replication dependent manner.

CHAPTER II

MATERIALS AND METHODS

Cell Culture

All experiments were conducted using RAW 264.7 murine macrophages. Raw 264.7 cells were cultured in RPMI 1640 (Hyclone) medium supplemented with 4.5g/L glutamine, 10% Fetal Bovine Serum, and 1% Penicillin and Streptomycin. All cells were grown at 37ºC under 5% CO_2.

Viruses

The study uses the following viruses; Influenza A/California/04/2009 H1N1 (CA/09), CA/09 expressing the HA gene from the H5N1 virus A/Vietnam/1203/04 (H5) and Influenza A/WSN/33 (WSN H1N1). All the viruses were propagated in the allantoic cavity of 10-day-old-specific-pathogen-free embryonated chicken eggs at 37ºC. Allantoic fluid was harvested, cleared by centrifugation, and stored at -80ºC as described previously [46]. Viral titers were determined by tissue culture infectious dose 50 ($TCID_{50}$) analysis in Madin-Darby canine kidney (MDCK) cells as described previously [47]. The limit of detection for the $TCID_{50}$ assay was 100 $TCID_{50}$ units/ml.

In vitro infections

RAW264.7 cells were infected at a multiplicity of infection (MOI) of 5 for 1 h at 37ºC. UV inactivated viruses were prepared by exposing virus to UV radiation for 1 minute. Cells were washed with Phosphate Buffered Saline (PBS) three times to remove unbound virus, and infected cells were maintained in RPMI 1640 media containing 0.075% of bovine serum albumin (BSA) and 1% Penicillin and Streptomycin. The cells were infected for 5 h and the cells were treated with IFN-γ (100U) either for 12 h or 1 h based on the experiment.

Determination of NO concentration

The concentration of nitric acid (NO) in culture supernatants was determined by assaying its stable end product Nitrite (NO_2^-), an index of cellular NO production using Griess reagent. RAW264.7 cells were infected with either live or UV treated viruses for 5 h and then stimulated with IFN-γ (100U) and LPS (20ng/ml). After 12 h of incubation, 50µl culture supernatant was transferred to a well in a 96-well microtiter plate, followed by addition of 50 µl of Griess reagent. Absorbance was measured within 5 min at 540nm on an automated microtiter plate reader, and nitrite concentrations were calculated by comparison to a standard curve generated with sequential dilutions of sodium nitrite.

Phagocytotic Activity Assay

RAW264.7 cells were infected with live or UV treated viruses for 5 h and stimulated using IFN-γ (100U). Cells were incubated for 12 h and were tested for their ability to ingest fluorescein isothiocyanate – labelled *Escherichia coli* using Vybrant Phagocytosis assay kit (Molecular probes) according to manufacturer's instructions.

Western Blot

Cell lysates were quantitated using the Pierce BCA protein assay kit. Ten micrograms of total cell lysate was separated on a 10% SDS-PAGE gel under reducing conditions. After a transfer to nitrocellulose, blots were blocked in 3% nonfat dry milk in Tris-buffered saline (TBS) for 1 h at room temperature and then probed for phosphorylated STAT1 (1:1,000;) in TTBS for overnight at 4°C . Blots were washed and incubated with goat anti-rabbit HRP (1:10,000; Jackson Immunoresearch Laboratories) in TTBS. Chemiluminescence substrates (Supersignal west pico chemiluminescent substrate, ThermoFisher Scientific) was added based on the manufacturer's instruction and incubated at room temperature for 2 minutes. Chemiluminescence was detected using digital imagers.

RNA extraction and RT-PCR

RNA was isolated using RNA was eluted in 100µl nuclease free water. RT-PCR was performed using briefly 1µg total RNA for each sample was added to the PCR. For gene expression of IRF-1 after reverse transcription (30 min at 50C; 15 min at 95 C),

25 cycles of PCR were performed for 30 s at 94 C, 1 min at 65C and 1 min at 72 C and the final elongation time was 10 min at 72 C. PCR products were analyzed on ethidium bromide-stained 1.5% agarose gel. The IRF-1 antisense oligonucleotide primers were used based on previous data [19].

CHAPTER III

RESULTS

Phagocytic activity of macrophage is impacted by H5 influenza virus replication

During influenza virus infection, the phagocytic capacity of macrophages is enhanced within the host, contributing to the host's ability to prevent virus replication and clear the infection [39, 46]. This is due, in part, to the phagocytosis of apoptotic cells by activated macrophages [39]. The significance of macrophage phagocytosis on the outcome of infection can be seen in experiments in which mortality and morbidity in infected mice was increased when phagocytosis was inhibited [47]. However, these studies were performed using lab-adapted or seasonal influenza virus strains, and there is no information available on the impact of H5N1 influenza virus replication on the phagocytic capacity of macrophages.

To test the hypothesis that influenza virus replication alters the phagocytic capacity of macrophages, phagocytic activity was deduced using the Vybrant Phagocytosis Assay Kit (Life Technologies) according to manufacturer's protocol. While infection with the CA/09 virus activated macrophage phagocytic capacity to 150% of that of mock-infected cells, macrophages infected with either the H5 or WSN virus only had ~50% of the phagocytic capacity of a mock-infected cell (Figure 2). Thus, infection with an influenza virus strain that is capable of replication in macrophages decreased the phagocytic capacity of the macrophages by ~3-fold relative to infection with a virus that is not capable of replication in macrophages. To test the hypothesis that

the decrease in phagocytic capacity of WSN- or H5-infected macrophages was dependent on virus replication, we UV-inactivated the three virus strains and repeated the phagocytosis assay. Interestingly, inactivation of the H5 or WSN strains by UV-irradiation rescued the phagocytic capacity of the macrophages. There was no significant change in phagocytic capacity of macrophages infected with UV-inactivated CA/09 relative to live CA/09. This data demonstrates that influenza virus replication in macrophages inhibits macrophage phagocytosis in a replication-dependent manner (Figure 2).

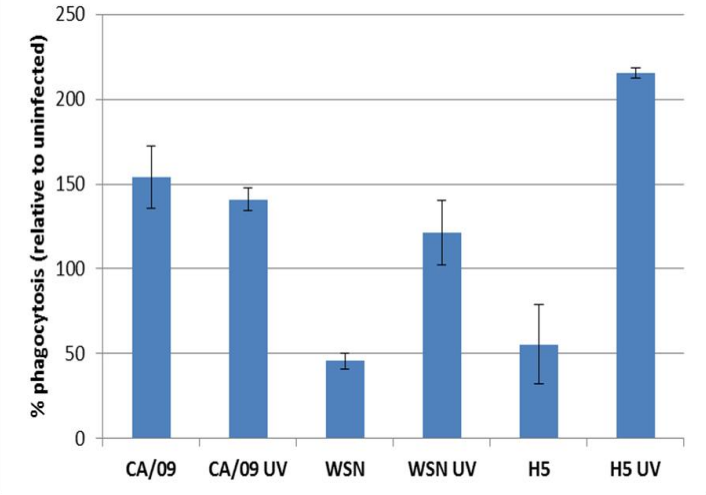

Figure 2 – H5 influenza viruses inhibits macrophage phagocytosis in a replication dependent manner.

RAW264.7 macrophages were mock-infected or infected (MOI=3) with the indicated viruses for 12 hours. Phagocytosis was determined using fluorescein-labeled *E. coli* particles (Vybrant Phagocytosis Assay Kit, Molecular Probes).

H5 influenza virus replication impairs IFN-γ activated macrophage phagocytic activity

Macrophages respond to various cellular products during the antiviral immune response. Of these, IFN-γ plays a major role in activating macrophages to become better at carrying out antiviral responses (ie – phagocytosis) [15, 48].

Having identified that H5- or WSN-infected macrophages have a decreased phagocytic capacity, experiments were conducted to investigate whether IFN-γ stimulation can override this virus-mediated suppression of phagocytosis. RAW264.7 macrophages were infected with live or UV-inactivated CA/09 or with H5 or WSN influenza virus strains (MOI=3) for 5 hours and then treated with IFN-γ (100 units) for 12 hours. The phagocytic capacity of macrophages was again determined with the Vybrant phagocytosis assay kit. The phagocytic capacity of uninfected, IFN-γ-treated cells was set as maximal (ie – 100%) phagocytosis. As shown in Figure 3, IFN-γ treatment of macrophages infected with live CA/09 resulted in a 20% decrease in maximal phagocytic capacity and infection with UV-inactivated CA/09 restored phagocytic capacity to maximum levels, suggesting that infection with CA/09 results in a slight inhibition of IFN-γ-mediated activation of macrophages. However, infection of macrophages with live WSN or H5 virus resulted in a significantly greater inhibition of IFN-γ-mediated phagocytic capacity (50% and 30% maximal capacity, respectively). Again, this inhibition of macrophage responsiveness to IFN-γ was shown to be replication-dependent as the phagocytic capacity of IFN-γ-treated macrophages infected with UV-inactivated WSN or H5 virus approached maximum levels (100% and ~80%, respectively; Figure 3).

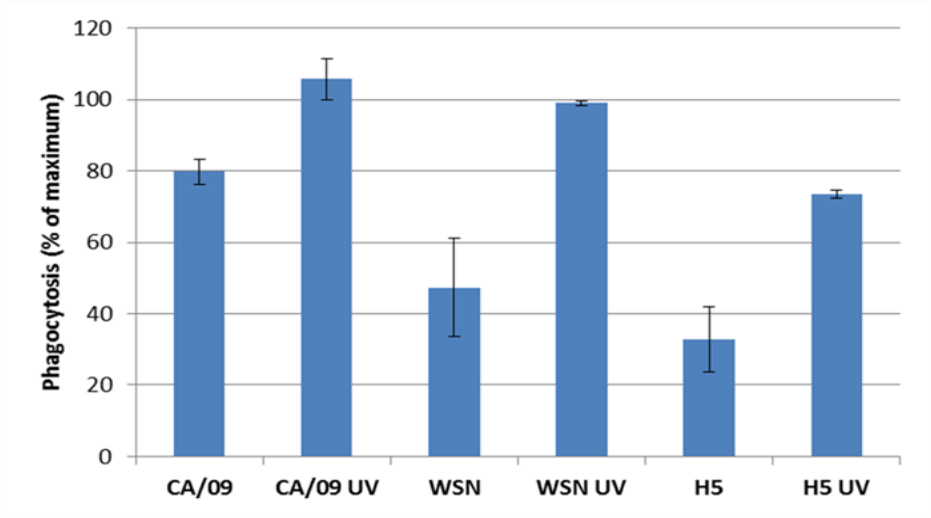

Figure 3 – H5 influenza viruses alter macrophage phagocytosis and IFN-γ responsiveness in a replication-dependent manner. RAW264.7 macrophages were infected (MOI=3) in duplicate with the indicated viruses for 12 hours and treated with IFN-γ (100 units) for 12 hours beginning at 5 hours post-infection. Phagocytosis was determined using fluorescein-labeled *E. coli* particles (Vybrant Phagocytosis Assay Kit, Molecular Probes). Phagocytic activity of mock-infected IFN-γ-treated cells was set as 100% and the phagocytic activity of virus-infected cells is presented as a percentage of that value. Error bars represent the standard deviation from the mean value.

H5 influenza virus inhibits nitric oxide production in a replication dependent manner

IFN-γ activated macrophages, apart from increasing in phagocytic activity, also produce nitric oxide (NO) due to the IFN-γ-mediated induction of the enzyme nitric oxide synthase. Nitric oxide acts as an intracellular messenger molecule in mammalian cells and also plays an important role in protection against viral infections [40,41]. Previous studies have demonstrated in an increased killing by macrophage induced by IFN-γ and LPS due

to augmented production of NO. Higher production of NO was noticed in mice infected with H5 influenza virus. Mice that were deficient of iNOS showed a decrease in NO production [43, 44, 45].

To determine if influenza virus replication alters NO production in activated macrophages, RAW264.7 macrophages were mock-infected or infected (MOI=3) with the indicated viruses for 12 hours, or treated with 20ng LPS and IFN-γ (100 units) for 12 hours, beginning at 5 hours post-infection (hpi) and NO levels were measured using the Griess method. In the absence of IFN-γ treatment (exposure to influenza virus alone), the measured nitrite levels in macrophages were slightly above the nitrite detection limit. Here, influenza virus-infected macrophages did not generate nitrite levels above that which was detected in mock-infected cells. Incubation of RAW264.7 cells with IFN-γ (100U) and LPS (20ng) significantly increased the production of NO (~100μM) when compared to mock-treated cells. Nitrite levels in macrophages infected with live and UV-inactivated CA/09 reached ~60μM and 100μM respectively, indicating that, similar to our results with the phagocytosis assay, live CA/09 virus moderately disrupted the antiviral response of macrophages. However, infection with the live H5 virus resulted in a significantly greater inhibition of IFN-γ-mediated NO production. In fact, NO production was reduced to levels similar to what was observed in uninfected, unstimulated cells. Demonstrating that this inhibition is replication-dependent, NO production increased to ~80μM in macrophages infected with UV-inactivated H5 virus and treated with IFN-γ (Figure 4).

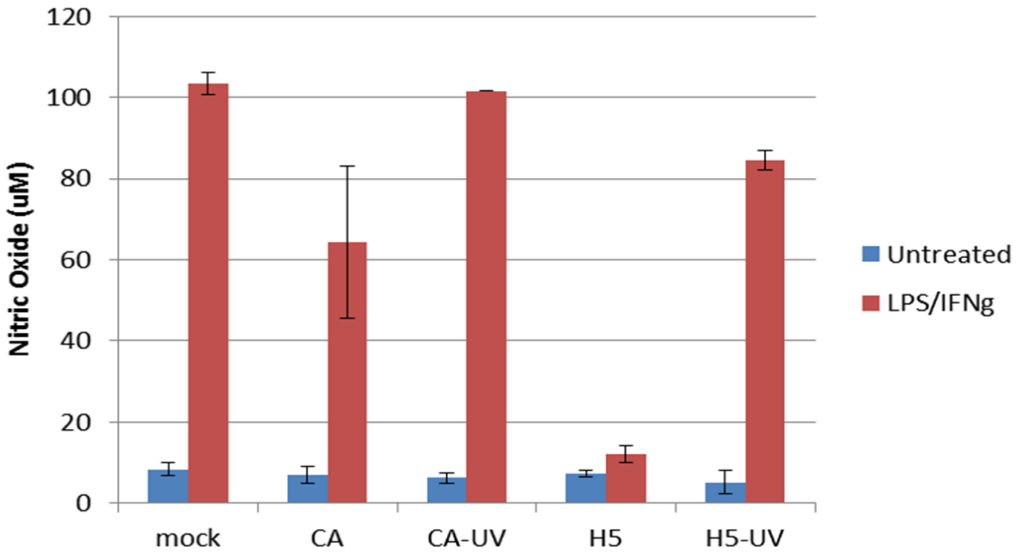

Figure 4 – H5 influenza viruses inhibits nitric oxide production (NO) in a replication-dependent manner. RAW264.7 macrophages were mock-infected or infected (MOI=3) in duplicate with the indicated viruses for 12 hours, or treated with LPS(20ng)/ IFN-γ (100 units) for 12 hours beginning at 5 hours post-infection (hpi) . NO production in the culture supernatants was quantified by the Griess method. Blue bars represent nitric oxide production in untreated cells cells and red bars represent nitric oxide production in LPS/IFN-γ-treated cells.

H5 virus do not inhibit IFN-γ dependent STAT1 phosphorylation

Our previous experiments have demonstrated that macrophages infected with influenza viruses capable of replicating in this cell type are not responsive to IFN-γ. In order to study the mechanism behind the inhibition of the IFN-γ response, we asked whether STAT1, the major transcription factor activated in response to IFN-γ signaling was activated in H5 or WSN-infected cells.

RAW264.7 cells were infected with live CA/09, H5, or WSN virus for 5 hours, followed by IFN-γ treatment for one hour, and whole cell lysates were subject to SDS-PAGE electrophoresis and Western blot for tyrosine phosphorylation (Y701) of STAT1. No phosphorylation of Y701 was seen in mock-infected control cells that were not treated with IFN-γ (Figure 5). Phosphorylated STAT1 was detected following IFN-γ treatment regardless of which virus the cells were infected with (Figure 5).

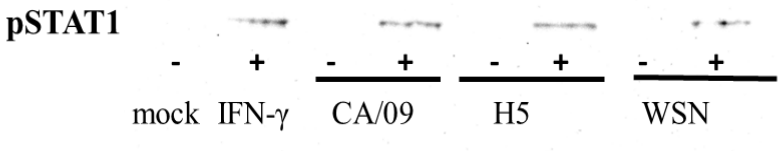

Figure 5. Influenza virus infection does not inhibit STAT1 activation. RAW264.7 macrophages were infected with the indicated influenza virus strains for 5 hours and then treated (+) with IFN-γ (100U) for one hour, or left untreated (-). Tyrosine 701 phosphorylation of STAT1 was detected by Western blot. Uninfected cells with and without IFN-γ treatment were used as controls.

This result shows that inhibition of STAT1 activation does not account for the ability of the H5 and WSN viruses to block the IFN-γ-mediated phagocytosis and NO production in macrophages as shown in Figures 3 and 4.

To better understand the mechanism of IFN-γ signaling inhibition, we asked if H5 and WSN influenza viruses inhibit the expression of the IFN-γ-regulated gene IRF-1 in infected macrophages.

H5 influenza virus inhibits IFN-γ stimulated IRF-1 expression

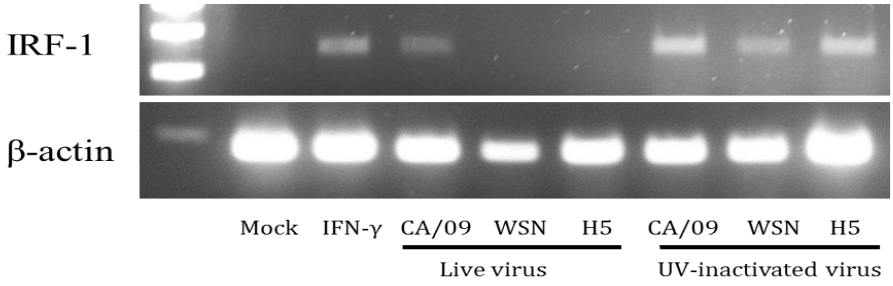

Figure 6: H5 influenza virus inhibits IRF-1 expression in IFN-γ activated RAW264.7 cells. Analysis of IRF-1 and β actin expression in IFN-γ activated RAW264.7 cells infected with live or UV inactivated CA/09, WSN and H5 viruses. RAW264.7 cells were mock-infected or infected with live or UV inactivated viruses at an MOI of 5 for 5 hrs or left uninfected. The infected cells and uninfected control were treated with IFN-γ (100U) for 5 hrs to stimulate IRF-1 transcription. Total RNA was extracted and subjected to RT-PCR analysis with primers specific to IRF-1 and β-actin.

Macrophages were infected with live or UV-inactivated CA/09, H5, or WSN viruses for 5 hours and then treated with IFN-γ for five hours to stimulate IRF-1 expression. As shown in Figure 6, IRF-1 expression was induced following IFN-γ treatment of CA/09 infected cells. However, WSN and H5 infection resulted in a complete block of IRF-1 expression. Consistent with our previous results demonstrating the replication-dependent restriction of IFN-γ mediated macrophage activation, UV-inactivation of the WSN and H5 viruses restored IRF-1 expression in IFN-γ treated macrophages. These results suggest that replication of influenza viruses in macrophages results in general inability of infected cells to respond to IFN-γ. This may be due to a

disruption of the IFN-γ-activated Jak/STAT pathway downstream of STAT1 activation. Further experiments need to be performed to elucidate the exact mechanism of IFN-γ signaling inhibition.

CHAPTER IV

DISCUSSION

The emergence of highly pathogenic H5N1 avian influenza viruses in humans in 2003 was immediately recognized as a major public health concern. Since that time, H5N1 viruses have become endemic in domestic and wild birds on several continents, resulting in occasional epidemic outbreaks in domestic poultry and sporadic infections of humans with a mortality rate of ~60%. Fortunately, efficient person-to-person transmission of H5N1 viruses has not been observed [22, 25]. Accompanying this public health threat has been a strong research effort to understand why these viruses cause such severe disease in humans. A number of studies have focused on molecular or genetic signatures of these viruses that have contributed to their ability to jump the species barrier from birds to humans [31, 32, 33,51] . One aspect of H5N1 pathogenesis that has been less well characterized is the role of the immune response in exacerbating disease symptoms. While it has been known for some time that human H5N1 infection is accompanied with an exaggerated cytokine response and resulting tissue damage, the exact cell types involved in severe H5N1 disease have not been completely studied [35].

It has been shown by others that H5N1 infection results in an excessive infiltration of macrophages into the airways and that these cells, when stimulated ex vivo, secrete higher levels of inflammatory cytokines than macrophages infected with seasonal influenza virus strains. The risk that a highly pathogenic H5N1 influenza virus might

reassert with a currently circulating H1N1 virus to create a virus of increased virulence and efficient human-to-human transmission is of great concern and highlights the need to understand more about the mechanisms of enhanced disease severity [33].

Historically, influenza virus infection was thought to be abortive in macrophages. Thus, serving as a "dead end" cellular target for influenza viruses and contributing to effective host defense [56]. However, previous work in the Cline lab aimed at understanding the role of macrophages in H5N1 influenza disease demonstrated that H5N1 viruses are unique in their ability to replicate in macrophages, a feature that was mapped to the HA gene alone. [33] Following up on that work, we have now proposed the hypothesis that replication of influenza viruses in macrophages contributes to changes in the antiviral functions of the macrophage in a replication-dependent manner and in ways that might contribute to the increased disease severity observed in vivo.

Macrophages produce antiviral and inflammatory cytokines when activated in response to various cytokines produced during the innate and adaptive immune responses [47,48]. IFN-γ is the most potent activator of macrophages, driving important antiviral responses such as nitric oxide induction and phagocytosis [49, 50, 51, 52]. We have shown here that influenza virus strains capable of replicating in macrophages (WSN and H5) decrease the phagocytic capacity of macrophages (Figure 2). Additionally, IFN-γ-mediated enhancement of the phagocytic response (Figure 3) and nitric oxide production (Figure 4) was abrogated in macrophages infected with these viruses. In contrast, infection with the CA/09 virus (which does not replicate in macrophages) enhanced

macrophage phagocytic capacity and did not inhibit cell responsiveness to IFN-γ as measured to phagocytic capacity and nitric oxide production. In support of our hypothesis that changes in macrophage function require virus replication, UV-inactivation of the WSN and H5 viruses restored the phagocytic response and ability of macrophages to respond to IFN-γ.

To further elucidate the mechanism by which H5 viruses antagonize the IFN-γ signaling transduction, IFN-γ dependent gene expression was investigated. The expression of the IFN-γ-induced gene IRF-1 was decreased in macrophages infected with live, but not UV-inactivated, WSN and H5 virus. There was no block in IRF-1 expression in macrophages infected with the CA/09 virus and treated with IFN-γ (Figure 6). Previous studies have demonstrated that the transcription factor IRF-1 is essential in iNOS gene induction. It has also been shown that macrophages with targeted disruption of IRF-1 showed very little nitric oxide production and also increased the severity of the disease in mice [53, 54]. This might be one possible explanation for the decrease in nitric oxide production observed in IFN-γ treated cells infected with H5 in my data. Thus an inhibition in IRF-1 gene is observed in IFN-γ treated cells infected with H5 or WSN virus, might decrease the iNOS gene transcription leading to little production of NO. While the expression of IFN-γ-regulated genes (IRF-1) was inhibited in a virus replication-dependent manner in macrophages infected with the H5 or WSN viruses, we did not observe a block in STAT1 phosphorylation. This suggests that the mechanism by which influenza viruses abrogate the IFN-γ response is downstream of STAT activation.

While further experimentation is required to elucidate the mechanism by which influenza virus replication inhibits IFN-γ signaling, we hypothesize that activated STAT1 homodimers fail to translocate to the nucleus in infected cells.

The data presented here are consistent with a previous study by Uetani *et al.*, [20] in which it is demonstrated that influenza viruses inhibit IFN-γ signaling in epithelial cells. In contrast to our work, Uetani *et al.,* demonstrated that the block does involve a lack of STAT1 phosphorylation. Our work has added to that of Uetani *et al*., by demonstrating that virus replication is required for the inhibition of IFN-γ signaling. Additionally, we have connected the block in IFN-γ signaling to antiviral responses that are important for controlling virus replication in vivo.

Further, numerous studies have investigated the mechanism by which viruses interfere with IFN signal transduction which support our findings here [55, 56]. Studies have shown that influenza A virus inhibited nuclear translocation of STAT1 upon IFN-γ stimulation in influenza A infected respiratory epithelial cells [21,22]. It has been shown that Ebola virus impairs the nuclear translocation of tyrosine phosphorylated STAT1 in IFN signaling. This impairment of nuclear translocation is mediated by the expression of glycoprotein VP24 of Ebola virus. VP24 does not interfere with tyrosine phosphorylation of STAT1 and keeps it intact. However, Ebola virusVP24 was found to specifically interact with karyopherin α1, the nuclear localization signal receptor required for nuclear accumulation of tyrosine phosphorylated STAT1 [57]. A slightly similar mechanism was seen to be adapted by rabies virus to interfere with IFN-γ. It has been shown that the V

protein in rabies virus did not preclude the tyrosine phosphorylation of STAT1 but rather retained the activated STAT1 in the cytoplasm, blocking nuclear import [58]. Yet another study shows that vaccinia virus not only block STAT 1 phosphorylation but also blocks the IFN-γ signal transduction through the dephosphorylating activity of the viral phosphatase VH1[59,60]. All these finding shows the importance for a viruses to advance various mechanisms to target different steps within the host IFN response.

In conclusion, the data presented here demonstrate that influenza virus replication alters the function of macrophages in ways that have been shown to contribute to disease severity in animal models of infection. Further studies on the strategies used by influenza viruses to block IFN-γ responses in macrophage are warranted and may reveal important clues to viral pathogenesis and the development of directed therapies or novel vaccination strategies to control and prevent future H5N1 outbreaks in animals and humans.

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