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### ***Research Fund for the Control of Infectious Diseases***

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# **The role of influenza virus gene constellation and viral morphology on cytokine induction, pathogenesis, and viral virulence**

Submitted to the Grant Review Board (15<sup>th</sup> September 2007)

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## **Acknowledgements**

This research was supported by Research Fund for the Control of Infectious Diseases, Hong Kong Special Administrative region

## **Summary**

### ***Background***

Since the first emergence of highly pathogenic H5N1 avian influenza in 1997 (H5N1/97), the virus has continued to genetically re-assort and evolve to become increasingly pathogenic with an expanding host range. Between late 2003 and mid 2006, the newly emerged Z genotype H5N1 avian influenza has spread across ten Asian countries and beyond Southeast Asia, causing high morbidity and mortality in aquatic birds, poultry and humans. The pathogenesis of these highly virulent H5N1 viruses in humans is still largely unknown. Recent findings have suggested that virus-induced cytokine dysregulation may play a crucial role in H5N1 pathogenesis. Investigation of the viral determinants responsible for such cytokine imbalance is critical to the further understanding of the possible mechanisms underlying the disease, and may help define potential therapeutic interventions in human disease associated with H5N1 for better influenza pandemic preparedness.

### ***Aims and Objectives***

1. To investigate the role of virus morphology on the ability of influenza A H5N1 viruses to induce tumour necrosis factor and other proinflammatory cytokines from macrophages
2. To elucidate the viral gene component(s) involved in above (1) by the use of influenza reverse genetics and naturally available reassortant viruses.

### ***Study Design and Methods***

We have a panel of naturally occurring and recombinant viruses (generated by reverse genetics) which hyper-induce cytokines from macrophage in either a virus replication dependent or independent manner. We propose to correlate these phenotypes with virus replication competence, virus morphology (filamentous versus spherical particles) and virus genotype. In the second phase of the study, we will use reverse genetics to engineer recombinant viruses which contain human or avian influenza matrix or nucleoprotein genes which are known to alter virus morphology.

### ***Results and Conclusions***

Previously it has been demonstrated that H5N1/97 causes hyper-induction of pro-inflammatory cytokines, most notably TNF- $\alpha$ , in primary human macrophages in vitro. Here, we further explored the TNF- $\alpha$  induction potential of the recent Z genotype H5N1 viruses in a human macrophage model and the viral determinants involved in high TNF- $\alpha$  induction. By measuring mRNA levels using quantitative PCR and protein levels using ELISA, the Z genotype viruses were found to be potent inducers of TNF- $\alpha$ , with some strains inducing up to 4 folds greater than that induced by H5N1/97. In the light of these findings, the viral determinants responsible for the TNF- $\alpha$  hyperinduction were further investigated. We hypothesized that TNF- $\alpha$  hyperinduction in primary human macrophages in vitro may be related to differences in viral morphology, or to certain viral genetic determinants. In this study, it was demonstrated that TNF- $\alpha$  hyperinduction was not associated with the morphology of virus particles, but was closely linked to the presence of certain H5N1 viral gene components. By employing reverse genetics, recombinant viruses containing genes from H5N1 (A/Vietnam/1203/2004) and genes from a low TNF- $\alpha$  inducing virus, H1N1 (A/WSN/33), were constructed to further explore the genetic contribution of the 8 viral segments of Z genotype H5N1 viruses towards TNF- $\alpha$  hyper-induction. Here, it was demonstrated that while recombinants containing the H5N1 non-structural, matrix or nucleoprotein gene alone in an H1N1 genetic background did not confer high TNF- $\alpha$  inducing phenotype, high TNF- $\alpha$  induction was likely a polygenic trait and was observed in recombinants that had specific combinations of H5 genes. One of

these combinations involved the H5 replication complex (NP/PA/PB1/PB2), while another combination involved the H5 surface proteins in addition to the H5 matrix proteins (HA/NA/M). Both of these combinations led to significantly increased TNF- $\alpha$  production in primary human macrophages in vitro.

### ***Implications***

This study has determined that a severe inflammatory reaction was triggered when cells were infected with the avian influenza H5N1 virus. The extreme immune response could then aggravate the inflammatory response and increase damage to the lung. This may be a key contributor to what makes the H5N1 viruses more pathogenic than the ordinary influenza viruses. These studies also provide a framework for understanding the disease severity that may ensue if the H5N1 virus becomes pandemic. We hypothesise that if it becomes pandemic by reassortment (as happened in 1957 and 1968) the cytokine induction would be less and the disease may be attenuated. However if the virus directly adapts to human-to-human transmission, as happened in 1918, the severity of human disease may remain very severe.

## Introduction

Severe disease has been caused by the influenza A (H5N1) viruses in 1997 (H5N1/97) and in 2003 (H5N1/03)<sup>1</sup>. Despite its high pathogenicity, it was fortunate that the viruses was not highly transmissible between humans. From working with the H5N1 viruses, it was observed that different strains of H5N1 has different ability to propagate in MDCK cells. The difference in titre can be 3-4 log<sub>10</sub> units.

The reason for this difference is unclear but work by Liu et al. suggested that replication efficiency and the morphology of Influenza A viruses may be linked<sup>2</sup>. They further suggest that the nucleoprotein and matrix protein may together influence the morphology of the virus. Their work was later reinforced by Bournalina and Garcia Sastre who revealed that both the amino and carboxy-terminal parts of the M1 gene (amino acid residues 95 and 204) in influenza A viruses are critical in determining filamentous particle formation<sup>3</sup>.

Further to our published work on the role of pro-inflammatory cytokines in the pathogenicity of human disease caused by the H5N1/97 virus<sup>4</sup>, it was observed that the substitution of the nucleoprotein gene from H5N1/97 virus into a WSN/33 gene background (rWSN-NP-H5) significantly increases the ability of TNF- $\alpha$  induction from human macrophages. The level of TNF- $\alpha$  induction even surpass that induced by the wild type H5N1/97 viruses but unlike the H5N1/97 viruses, ultra-violet light irradiation of the rWSN-NP-H5 virus did not abolish the effect. This suggests that the induction of TNF- $\alpha$  by the rWSN-NP-H5 virus is not a replication dependent event and that some thing associated with the virus structure may be responsible. One potential mechanism is pattern recognition of certain influenza morphology by human macrophages through possibly toll-like receptors. Another observation is the apparent reduction in the efficiency in propagation in MDCK cells. Titre of the rWSN-NP-H5 virus is consistently 2-3 log<sub>10</sub> units lower than the plasmid derived WSN/33 virus.

Although H5N1 viruses with a genetic constellation of H5N1/97 were successfully eradicated by the slaughtering of poultry in 1997, its putative precursors including A/Goose/Guangdong/1/96 (H5N1) (H5N1 Gs/Gd) continued to circulate in Southern China. Continued reassortment of the HA donor H5N1 Gs/Gd with other unknown aquatic viruses created the H5N1 viruses belonging to the Z genotype which re-emerged to cause fatal human H5N1 infection in 2003<sup>1</sup>. This virus is genetically distinct from the H5N1/97 and it is therefore important to focus to work with this genotype in this study.

The role of virus morphology in pathogenesis and cell-to-cell transmission is still very much unclear. Thus, further studies are required to determine the role that virus morphology play in influenza virus life-cycle and virulence.

## Method

### Viruses and cells

Influenza viruses isolated from humans and animals Influenza A viruses isolated from birds and humans in various countries in Asia including China, Hong Kong, Indonesia, Thailand and Vietnam were used in this study (Table 1). Virus stocks for each of these viruses (except for A/Hong Kong/437.6/99 H5N1) were propagated in MDCK cells in minimum essential medium (MEM) supplemented with 1% penicillin and streptomycin and harvested on the third day post-infection for storage and titering. As 437.6/99 (H5N1) did not grow to high titers in MDCK cells, this virus was propagated in allantoic cavities of 10-day-old embryonated chicken eggs followed by incubation at 37°C for 2 days. Allantoic fluid from multiple eggs was pooled, clarified by centrifugation, aliquoted, and stored at -70°C. All experiments that involve the handling of H5N1 viruses were performed in

the BSL-3 biosafety containment facility of the Department of Microbiology at HKU.

### **Influenza viruses generated from reverse genetics**

Recombinant viruses were generated by a recently established reverse genetics system<sup>5</sup>. RT-PCR products of viral RNA segments were cloned into plasmid pHW2000. The newly introduced viral RNA in the recombinant viruses was sequenced for confirmation. All procedures involving live H5N1 viruses and recombinant viruses were carried out in a facility of biosafety level 3.

### **Cell culture**

Madin-Darby canine kidney (MDCK) cells and 293T cells were maintained in minimum essential medium (MEM) (GIBCO BRL, Gaithersburg, MD, USA) supplemented with 10% fetal-calf serum (FCS) and 1% penicillin & streptomycin at 37°C in 5% CO<sub>2</sub>. The cells were passaged when confluent by incubation with trypsin-EDTA at 37°C for 10 min to detach the cells, then a ten-fold dilution of the cells was added to new flasks with fresh medium and further incubated at 37°C in 5% CO<sub>2</sub> until confluent. MDCK cells were passaged up to a maximum of 20 times before they were discarded, while 293T cells were passaged up to a maximum of 15 times.

### **Isolation of human primary monocytes**

Human peripheral blood leucocytes were isolated from buffy coats of anti-coagulated blood obtained from healthy blood donors (the Hong Kong Red Cross Blood Transfusion Service) by centrifugation on a Ficoll-Paque density gradient (Pharmacia Biotech, Uppsala, Sweden) and subsequently purified by adherence according to method as previously described<sup>6</sup>.

### **Electron Microscopy**

#### **Negative staining**

Influenza viruses were propagated in MDCK cells and purified by sucrose gradients as previously described<sup>3</sup>. Briefly, MDCK cells were infected with influenza viruses at a low MOI of 0.001. At day 4 post-infection, the culture supernatants were harvested and preclarified by centrifugation at 4000 rpm for 10 min at 4°C. The viruses were subsequently purified and pelleted through a 25% sucrose cushion in NTE buffer (100 mM NaCl, 10 mM Tris, 1mM EDTA, pH 7.4) by ultracentrifugation at 28 000 rpm for 90 min at 4°C in a SW28 rotor (Beckman). Purified virions were resuspended in 200µl of NTE buffer and were allowed to adhere to carbon/Formvar-coated 400-mesh copper grids (Electron Microscopy Sciences, Fort Washington, PA, USA) for 5 min prior to subsequent staining with 4% phosphotungstic acid pH 7.4 (Electron Microscopy Sciences) for 30 s. Excess stain was blotted away with filter paper and the grids were air-dried for 2 min. Both sides of the grids were then subjected to high energy UV-irradiation for 15 min inside the biosafety cabinet to inactivate the live viruses. Specimens were viewed under Philips EM208S transmission electron microscope (Electron Microscopy Unit, HKU) for examination of viral morphology.

#### **Ultrathin section electron microscopy**

MDCK cells were infected with virus at an MOI of 2 and incubated in MEM with TPCK-trypsin at 37°C. At 8 hours post-infection (p.i.), the MDCK cells were harvested by incubating with EDTA-trypsin for 10 min at 37°C. The cells were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer pH 7.4 (Electron Microscopy Sciences) for 1 hour at 4°C and post-fixed with 1% osmium tetroxide for 30 min at room temperature. The cells were then subsequently embedded in 1% agar and cut into 1 mm cubes. The specimens were dehydrated in a series of ethanol gradients followed by propylene oxide, embedded in epoxy resin, and allowed to polymerase at 60°C for a day.

Ultrathin sections (80 nm) of the embedded specimens were cut with an ultra-microtome, stained with uranyl acetate (Electron Microscopy Sciences) and lead citrate (Electron Microscopy Sciences), and examined with a Philips EM208S transmission electron microscope. (Electron Microscopy Unit, HKU).

### **Influenza virus infection of human macrophages**

Differentiated macrophages (from monocytes seeded at  $1.5 \times 10^5$  cells per well) were infected with influenza viruses at a multiplicity of infection of two unless otherwise stated. After 30 mins of adsorption at  $37^\circ\text{C}$ , the virus inoculum was removed, the macrophages were washed once with PBS and re-fed with 1ml serum-free macrophage medium (GIBCO) supplemented with 1% penicillin & streptomycin and 2 mg/L L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin. The macrophages were incubated at  $37^\circ\text{C}$  and the infection was monitored by collection of total RNA and culture supernatants at different time-points for further analysis of RNA transcription and cytokine secretion, respectively<sup>4</sup>.

### **Results**

#### ***Induction of TNF- $\alpha$ by H5N1 Z genotype influenza viruses in a primary human macrophage model***

Although the exact cause of H5N1 virulence in humans is still unknown, we hypothesize that H5N1-induced cytokine dysregulation is one crucial factor accounting for the unusual disease severity associated with H5N1 infection. Cheung et al. (2002) have previously demonstrated that A/Hong Kong/483/97 (H5N1) caused hyper-induction of a variety of pro-inflammatory cytokines, particularly TNF- $\alpha$ , in primary human macrophages<sup>4</sup>. However, as the genetic constellation of the internal genes of 483/97 differs from that of the newly emerged Z genotype viruses, it would be interesting to compare the cytokine induction profile of the Z genotype viruses with 483/97. It has been shown earlier that high levels of TNF- $\alpha$  could be induced by an early strain of Z genotype virus (A/Ck/Hong Kong/61.9/02) isolated in 2002 from chicken faeces<sup>7</sup>. Here we further investigate the TNF- $\alpha$  induction profile of recent Z genotype viruses (from late 2003 to 2005) isolated from different hosts (avian and human) in several regions in Southeast Asia including China, Indonesia, Thailand and Vietnam.

Human macrophages were infected with viruses from Table 1 at an MOI of 2, and immunofluorescence staining for influenza nucleoprotein at 8 hours post-infection showed the presence of viral nucleoprotein in 95 – 100% of the cells, indicating that the majority of the macrophages were infected. At 3 hours post-infection, 483/97 (H5N1) and all the H5 Z genotype viruses showed significant upregulation of TNF- $\alpha$  expression when compared with human H1N1 viruses 54/98, 178394/04 and 87856/05 (Student's t-test,  $p < 0.05$ ) (Figure 1). A similar trend was observed in TNF- $\alpha$  protein concentrations in the culture supernatant assayed by ELISA at 3, 8 and 24 hours post-infection (Figure 2). Although the HA genes of these H5 viruses were evolutionarily related to 437.6/99 (H5N1), this virus exhibited low levels of TNF- $\alpha$  expression compared to 483/97 (H5N1) and Z genotype viruses. Thus, it is postulated that the hyper-induction effect was not due to the HA gene segment alone and that the remaining 7 genes may be potentially contributing to cytokine hyper-induction. Despite the closely related genomic sequences among the Z genotype viruses, TNF- $\alpha$  hyper-induction levels of Z genotype viruses were variable, ranging from levels similar to 483/97 (H5N1) such as for 3046/04, 33/04, 2A/04, 5/05, QH5/05 up to 4-folds higher in protein concentration at 8 hours post-infection (1194/04).

#### ***Association of viral morphology with TNF- $\alpha$ hyper-induction***

The morphology of the virus particles was studied by examination of negative stains of concentrated

viruses or ultra-thin sections of infected cells under the electron microscope (Philips EM208S). For negative staining of viral particles, viruses were first grown in MDCK cells, purified and concentrated through ultracentrifugation through a 25% sucrose cushion and subsequently stained with 4% phosphotungstic acid. Influenza viruses were recognized by the fringe of spikes formed by the surface proteins haemagglutinin and neuraminidase. Ten random views of the negative stains were examined for each virus under low magnification (28,000x) and their particle diameter measured using Carnoy 2.0 software (K.U. Leuven, Belgium). Particles greater than 300 nm in length were counted as filamentous particles.

For ultrathin section electron microscopy, MDCK cells were infected at MOI of 2, fixed at 8 hours post-infection, processed and sectioned accordingly. Influenza strain Udorn/72 (H3N2), a strain which readily forms filamentous virus particles, was used as a positive control for filamentous particles. The high TNF- $\alpha$  inducing H5N1 viruses 483/97, 1203/04 and 1194/04 were selected for the study, as well as 54/98 (H1N1) which is a low TNF- $\alpha$  inducer. The morphologies of these viruses were determined and compared against their cytokine induction profile. The Udorn/72 (H3N2) was shown to be a low inducer of TNF- $\alpha$  in macrophages comparable to 54/98 (H1N1) (Figure 3). The low TNF- $\alpha$  expression of Udorn/72 observed was not due to poor replication. In terms of viral morphology, 13% of the Udorn/72 (H3N2) viral particles were found to be filamentous (>300 nm) (Figures 4) with filaments reaching up to 3.65  $\mu$ m in length. Viral particles of filamentous forms can also be observed in thin-sections of Udorn/72-infected MDCK cells (Figure 5).

### ***Reverse genetics to define viral genes responsible for TNF- $\alpha$ hyper-induction***

In order to elucidate the viral gene(s) responsible for the hyper-induction of TNF- $\alpha$  in H5N1 infected macrophages, the reverse genetics technique was employed. Through the use of reverse genetics, one can manipulate the viral genome in any desired way, such as shuffling gene segments from different viruses and introduction of any desired mutations into the viral genome, allowing us to study the functions of viral genes and their effects on host interactions. By employing reverse genetics, recombinant viruses, which contain genes from H5N1 and genes from a low TNF- $\alpha$  inducing human influenza virus, A/WSN/33 (H1N1), were constructed to further explore the genetic contribution of the 8 viral segments of H5N1 viruses towards TNF- $\alpha$  hyper-induction.

### ***Contribution of H5N1 Z genotype NS gene to TNF- $\alpha$ hyper-induction***

By constructing recombinant viruses containing the NS gene segment of BL/03, AIV-1/04, 1203/04 and 1194/04 in A/WSN/33 (H1N1) background (Table 2), the role of this gene in TNF- $\alpha$  induction was further investigated.

The concentration of TNF- $\alpha$  protein released at 3, 8 and 24 hours post-infection induced by the WSN(H5N1-NS) recombinants in human macrophages were compared against the A/WSN/33 (WSN) virus and the wild-type H5N1 parent (Figure 6). At 8 hours post-infection, each of the recombinants containing an H5N1 NS gene induced higher levels of TNF- $\alpha$  ranging from 280% to 488% compared to the WSN parent (Table 3; Figure 7). However, the wild-type H5N1 viruses induced even higher levels of TNF- $\alpha$  compared to the WSN(H5N1-NS) recombinants. The increase of TNF- $\alpha$  produced by wild-type H5N1 viruses is therefore only partly contributed by the H5N1 NS gene. For all of the recombinants tested, similar levels of M gene transcription was observed, suggesting that the differential in the TNF- $\alpha$  levels observed was not due to poor replication of the viruses. UV-irradiation of the viruses before infection was able to reduce TNF- $\alpha$  expression to mock levels and abolish viral replication, indicating that the effects seen was not due to other molecules (eg. cytokines) present in the viral culture supernatant.

### ***Genetic determinants of A/Vietnam/1203/04 to TNF- $\alpha$ hyper-induction in human macrophages***

In order to further elucidate the role of other viral genes besides the NS segment of H5N1 Z genotype viruses in cytokine hyperinduction, the remaining 7 viral segments of A/Vietnam/1203/04 were cloned and recombinants carrying different gene segment(s) from the virus were reconstituted in the background of WSN/33 (H1N1). 1203/04 (H5N1) is a Z genotype virus isolated from pharyngeal swabs of a fatal 10-year-old male in Vietnam. It has previously been demonstrated to be a highly pathogenic strain causing high lethality in infected mice<sup>8</sup> and ferrets<sup>8,9</sup>. This strain has also been shown to induce high levels of TNF- $\alpha$ , making the strain a good choice for further study of viral genetic contribution to cytokine hyper-induction.

Comparison of TNF- $\alpha$  induction of plasmid-derived 1203r and wildtype 1203/04 virus using the 8 plasmids cloned from the wildtype strain 1203/04 (1203WT), the plasmid-derived 1203/04 (1203r) was reconstituted and its TNF- $\alpha$  induction profile was compared with its wild-type parent. (Figure 7) shows that the plasmid-derived 1203r induced significantly high levels of TNF- $\alpha$  in human macrophages when compared with WSN (a 10-fold increase). However, when compared to its wild-type parent, the amount of TNF- $\alpha$  induced by 1203r was only 42% of that induced by the 1203WT.

### ***TNF- $\alpha$ hyper-induction is not solely a single-gene effect***

To assess the viral genetic contribution to TNF- $\alpha$  hyperinduction, different combinations of viral segments from 1203/04 (H5N1) and WSN/33 (H1N1) were used to reconstitute a series of recombinants. These recombinants were then used to infect human macrophages at an MOI of 2, and the amount of TNF- $\alpha$  transcribed and released is shown in Figures 8 and 9 respectively. The TNF- $\alpha$  mRNA levels (Figure 8) correlated well with the TNF- $\alpha$  protein levels in the supernatant (Figure 9). Recombinants that contain the H5 surface glycoproteins HA and NA (WSN(1203-HA/NA)) exhibited low levels of TNF- $\alpha$  similar to that of the H1N1 parent WSN. The H5 surface glycoproteins alone thus did not appear to have a contributive role in TNF- $\alpha$  hyperinduction. The introduction of an additional H5 NS segment together with the HA and the NA segments (WSN(1203-HA/NA/NS)) resulted in an increase in induction levels to one that was similar to WSN(1203-NS), further supporting that the Z genotype NS segment was partially involved in cytokine hyper-induction. For recombinants containing single “internal” H5N1 genes, such as WSN(1203-NS), WSN(1203-M) and WSN(1203-NP), TNF- $\alpha$  induced was partially increased compared to their parent virus WSN but remained lower than that of 1203r. With the presence of both M and NP from 1203/04 (WSN(1203-M/NP)), the induction effect was increased to up to 53% of the parental 1203r virus at 8 hours post-infection. When the H5 polymerase complex and NP genes (NP/PA/PB1/PB2) from 1203/04 were incorporated, the virus WSN(1203-NP/PA/PB1/PB2) was able to induce high amounts of TNF- $\alpha$  close to that induced by the parental virus 1203r (98%) (Figure 9). Interestingly, however, this study showed TNF- $\alpha$  can be hyperinduced by another combination of H5 genes in WSN background. This combination involved the H5 surface glycoproteins together with the H5 matrix protein in the background of WSN. As shown in figures 3.13 and 3.14, WSN(1203-HA/NA/M) and WSN(1203-HA/NA/M/NS) were able to induce very high levels of TNF- $\alpha$ , exceeding even that of the parental 1203r virus by 59% and 139% respectively at 8 hours post-infection.

By comparing the M1 protein of avian H5 viruses and that of other human influenza isolates, it was observed that the avian H5 viruses, including 1203/04, contained a different nuclear localization signal, with lysine at position 101. Human influenza viruses, on the other hand, contained arginine at position 101 in the M1 protein, including the WSN/33 virus used in this study. In order to investigate the involvement of this amino acid difference to TNF- $\alpha$  induction in human macrophages, the amino acid at position 101 of the 1203/04 M1 protein was mutated to arginine by site-directed mutagenesis, and a new recombinant, WSN(1203-HA/NA/M(K101R)), which contained the 1203 surface glycoproteins in addition to the mutated 1203 matrix segment, was reconstituted. The mutant, however, when used to infect macrophages at an MOI of 2, did not exhibit any differences in TNF- $\alpha$

induction compared to WSN(1203-HA/NA/M), suggesting that position 101 of the H5 M1 protein was not involved in TNF- $\alpha$  hyperinduction.

## Discussion

Although studies in ferrets showed Z genotype human isolates appeared to be particularly lethal in infected ferret<sup>8</sup>, there was no such correlation in our study in terms of TNF- $\alpha$  hyper-induction, as some of the human isolates from Vietnam such as MK2/04, 3046/04 and the Indonesian human isolate 5/05 exhibited lower levels of induction than avian viruses BL/03 and AIV-1/04. No correlation was also found between the geographical region of isolation and the TNF- $\alpha$  induction potential of the viruses. Both clade 1 Z genotype viruses (eg. those originated from Vietnam and Thailand, such as 3046/04 and AIV-1/04, respectively) and clade 2 Z genotype viruses (eg. Those originated from Indonesia and China, such as 5/05 and QH5/05, respectively) were all high TNF- $\alpha$  inducers in human monocyte-derived macrophages.

Previous studies using reverse genetics have shown that alanine at position 41, arginine at position 95 and glutamate at position 204 of the M1 protein are crucial in filamentous particle formation<sup>3,10</sup> and are present in Udorn/72 (H3N2). Interestingly, for all of the H5N1 tested (which includes 483/97 and Z genotype viruses 1203/04, 1194/04 and 5/05), the particles observed were predominantly spherical (>99%, Figures 3.8 – 3.10) although all of these viruses contained the same amino acids as Udorn/72 at the three positions just mentioned. The majority of the virus particles of 54/98 (H1N1) were also found to be spherical (98%). The spherical morphology observed for H5N1 viruses which exhibit high TNF- $\alpha$  expression in human macrophages, taken together with the filamentous morphology observed for Udorn/72 (H3N2) and spherical morphology observed for 54/98 (H1N1) which both exhibit low TNF- $\alpha$  expression in macrophages, suggest that virus morphology is not related to TNF- $\alpha$  upregulation in human macrophages.

From our study with recombinant viruses, high TNF- $\alpha$  induction appears to be a polygenic trait which was observed in recombinants that had specific combinations of H5 genes. One of these combinations involved the H5 replication complex (NP/PA/PB1/PB2), while another combination involved the H5 surface proteins in addition to the matrix protein (HA/NA/M). Both of these combinations led to significantly increased TNF- $\alpha$  productions in primary human macrophages in vitro.

## Conclusions

- H5N1 viruses that cause severe disease in human are very potent inducers of proinflammatory cytokines when compared to seasonal influenza viruses, and this may play a role in the mechanism of H5N1 pathogenesis
- H5N1 viruses are predominantly spherical in morphology, and morphology of influenza viruses does not influence the ability to induce proinflammatory cytokines
- The NS1 viral protein may play a partial role in the potency of proinflammatory induction
- The H5N1 haemagglutinin and neuraminidase do not appear to transfer the high cytokine phenotype.
- The ability to induce cytokines is a polygenic trait, involving a combination of different viral genes

## **Implications**

The H5N1 Z genotype viruses can cause fatal disease in human and are causing worldwide concern at this current time. In this study we found that the H5N1 Z genotype viruses, just like the H5N1 viruses from 1997, are strong inducers of TNF- $\alpha$  in macrophages. The extreme immune response could then aggravate the inflammatory response and increase damage to the lung. This may be a key contributor to what makes the H5N1 viruses more pathogenic than the ordinary influenza viruses.

Our findings suggest that the genetic determinants for TNF- $\alpha$  hyperinduction do not rest on the presence of the H5 surface glycoproteins, but are likely to be contributed by other H5 internal genes and gene products. Also we demonstrated that TNF- $\alpha$  hyperinduction is closely associated with the viral genetic constellation. Specific combinations of H5 genes were able to confer a high TNF- $\alpha$  inducing phenotype, and that a polygenic effect is implicated in H5N1 virulence. These findings serve as an important basis for further understanding of the molecular mechanisms underlying H5N1-induced cytokine dysregulation and pathogenesis and also provide a framework for understanding the disease severity that may ensue if the H5N1 virus becomes pandemic. We hypothesise that if it becomes pandemic by reassortment (as happened in 1957 and 1968) the cytokine induction would be less and the disease may be attenuated. However if the virus directly adapts to human-to-human transmission, as happened in 1918, the severity of human disease may remain very severe.

## **Dissemination**

The findings and observations are in preparation for further publications and meeting presentation

## **Publications**

Chan MC, Cheung CY, Chui WH, Tsao SW, Nicholls JM, Chan YO, Chan RW, Long HT, Poon LL, Guan Y, Peiris JS. Proinflammatory cytokine responses induced by influenza A (H5N1) viruses in primary human alveolar and bronchial epithelial cells. *Respir Res.* 2005 Nov 11;6:135.

## **Bibliography**

1. Peiris JS, Yu WC, Leung CW, Cheung CY, Ng WF, Nicholls JM, Ng TK, Chan KH, Lai ST, Lim WL, Yuen KY, Guan Y. Re-emergence of fatal human influenza A subtype H5N1 disease. *Lancet.* 2004 Feb 21;363(9409):617-9.
2. Liu et al (2002) Association of influenza virus matrix protein with ribonucleoproteins may control viral growth and morphology *Virology.* 304:89-96.
3. Bournalina and Garcia-Satre (2003) Reverse Genetics studies on the filamentous morphology of influenza A viruses. *J. Gen. Vir.* 84:717-527
4. Cheung CY, Poon LL, Lau AS, Luk W, Lau YL, Shortridge KF, Gordon S, Guan Y, Peiris JS.(2002) Induction of proinflammatory cytokines in human macrophages by influenza A (H5N1) viruses: a mechanism for the unusual severity of human disease? *Lancet* Dec 360:1831-7
5. E Hoffmann, G Neumann, Y Kawaoka, G Hobom and RG Webster, A DNA transfection system for generation of influenza A virus from eight plasmids, *Proc Natl Acad Sci USA* 97 (2000), pp. 6108–6113.

6. LJ Montaner, M Collin and G Herbein, Human monocytes: isolation, cultivation, and its applications. In: LA Herzenberg, Editor (5th edn.), Weir's handbook of experimental immunology vol IV, Blackwell Science, Cambridge, MA (1996), pp. 1–11.

Guan Y, Poon LL, Cheung CY, Ellis TM, Lim W, Lipatov AS, Chan KH, Sturm-Ramirez KM, Cheung CL, Leung YH, Yuen KY, Webster RG, Peiris JS. H5N1 influenza: a protean pandemic threat. *Proc Natl Acad Sci U S A*. 2004 May 25;101(21):8156-61.

Salomon, R., Franks, J., Govorkova, E. A., Ilyushina, N. A., Yen, H. L., Hulse-Post, D. J., Humberd, J., Trichet, M., Rehg, J. E., Webby, R. J., Webster, R. G. and Hoffmann, E. (2006) The polymerase complex genes contribute to the high virulence of the human H5N1 influenza virus isolate A/Vietnam/1203/04. *J Exp Med* 203(3):689-697

Govorkova, E. A., Rehg, J. E., Krauss, S., Yen, H. L., Guan, Y., Peiris, M., Nguyen, T. D., Hanh, T. H., Puthavathana, P., Long, H. T., Buranathai, C., Lim, W., Webster, R. G. and Hoffmann, E. (2005) Lethality to ferrets of H5N1 influenza viruses isolated from humans and poultry in 2004. *J Vir* 79(4):2191-2198

Elleman, C. J. and Barclay, W. S. (2004) The M1 matrix protein controls the filamentous phenotype of influenza A virus. *Virology* 321:144-153

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