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Identification of cellular partners of Influenza A virus (H5N1) non-structural protein NS1 by yeast two-hybrid system

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Summary. – Influenza A virus (IAV) subtype H5N1 is associated with the re-emergence of severe human influenza. The virus is highly virulent and viral non-structural protein 1 (NS1) is believed to play a crucial role in the viral pathogenesis. A screening for human proteins interacting with NS1 was performed by a yeast two-hybrid system (Y2H). Two bait plasmids that expressed DNA binding domain (BD) fused to either RNA binding domain or to effector domain of NS1 were constructed and transformed into yeast. The bait yeast was mated with a prey yeast containing human macrophage cDNA library fused to DNA activation domain. Obtained clones were interacting with interleukin-6 receptor (IL-6R), MHC class I HLA-B, cathepsin B, ubiquitin, and adenosine deaminase acting on RNA (ADAR1). These proteins play important role in the immune response, targeting for proteosomal degradation, and RNA editing. Thus, IAV H5N1 may use NS1 to manipulate these host proteins for its own benefit and in that way confer an unusual severity to the infection.

Keywords: Influenza A virus; H5N1 subtype; NS1; protein-protein interaction; yeast two-hybrid system; ADAR1; IL-6 receptor; HLA-B; cathepsin B; ubiquitin

Introduction

IAV (the family *Orthomyxoviridae*) is a negative-stranded RNA virus. The virus genome consists of eight RNA segments encoding 10 proteins. IAVs are divided into different subtypes based on the antigenic differences of the two surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA). Currently, 16 different HA subtypes and 9 NA subtypes of IAV isolated from avian species have been identified.

The "bird flu" outbreak in Hong Kong in 1997 caused by IAV (H5N1/97) was first documented as a solely avian influenza virus causing respiratory disease and death in humans. It was associated with an overall mortality rate of 33%. The clinical symptoms featured viral pneumonia progressing to acute respiratory distress and multiple organ dysfunction syndromes associated with lymphopenia and hemophagocytosis (Abdel-Ghafar *et al*., 2008; Chan *et al*., 2002; Claas *et al*., 1998). Since 2001, the precursor of H5N1 viruses has continued to re-assort and has given rise to novel virus genotypes. One of these H5N1 genotypes was associated with the re-emergence of human influenza in 2003 that eventually led to a widespread outbreak in Asia with transmission to humans in Vietnam and Thailand (Allen *et al*., 2006; Peiris *et al*., 2004). This outbreak posed a significant threat to human health and a potential for the onset of IAV pandemic.

Presently, it is still not clear why H5N1 IAV is so highly virulent. However, the IAV-encoded NS1 is suggested as one of the putative contributing factors (Li *et al*., 2006). The results obtained with recombinant mutant viruses indicated that NS1 protein contributed to the virulence of IAV during infection primarily by allowing the viruses to disarm the interferon (IFN)-based defense system of the host cell

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Abbreviations: ADAR1 = adenosine deaminase acting on RNA; BD = binding domain; ED = effector domain; HA = hemagglutinin; HDV = Hepatitis D virus; IAV = Influenza A virus; IF = immunofluorescence; IFN = interferon; IL-6 = interleukin-6; IL-6R = IL-6 receptor; NA = neuraminidase; NS1 = non-structural protein 1; PKR = protein kinase R; RD = RNA-binding domain; TNF = tumor necrosis factor; Y2H = yeast two-hybrid system

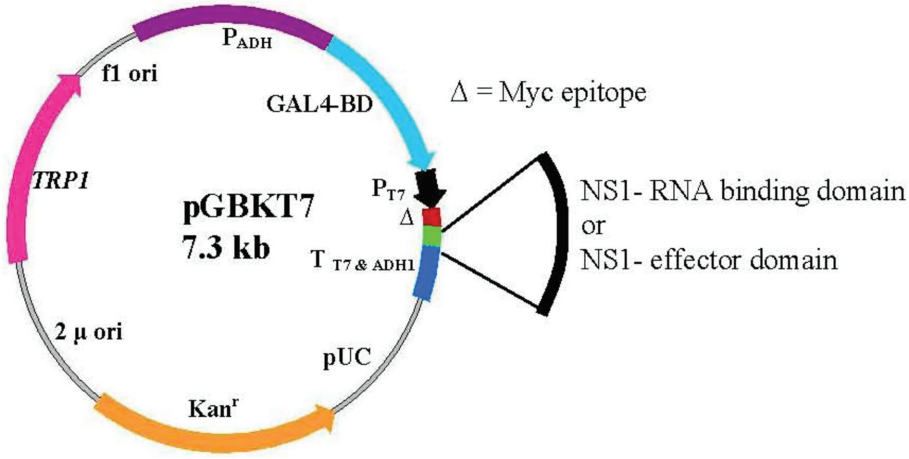

Fig. 1

Schematic diagram of the bait plasmids

GAL4-BD = GAL4-DNA binding domain; T7 = T7 RNA polymerase promoter; P_{ADH} = transacted *Saccharomyces cerevisiae* ADH1 promotor; pUC = plasmid replication origin; Kan^r = kanamycin resistance gene; 2 μ ori = yeast 2μ replication origin; TRP1 = tryptophan coding sequence; f1 ori = f1 bacteriophage origin of replication.

(Lipatov *et al*., 2005; Seo *et al*., 2004). Additional studies reported that the NS1 gene was associated with the virulence of IAV in a mouse model. On the other hand, IAV with the deleted NS1 gene exhibited an attenuated phenotype in the mice and pigs. Amino acid Glu92 of NS1 was shown to confer high virulence to the infecting virus together with a resistance to antiviral cytokines (Solorzano *et al*., 2005; Seo *et al*., 2002). NS1 protein has two functional domains e.g. N-terminal dsRNA-binding (RD) and dimerization domain and C-terminal effector domain (ED).

The multifunctional NS1 protein is widely regarded as a virulence factor that contributes to the viral pathogenesis by modulating many viral and host cellular processes (Cheung *et al*., 2002; Falcon *et al*., 1999). However, detailed mechanism of the NS1 function as well as the influence of cellular factors interacting with NS1 is not completely understood.

The aim of the present study was the search for cellular proteins interacting with IAV (H5N1) NS1 protein using Y2H system, nucleotide sequencing, and sequence identification. Furthermore, the relevant proteins that interacted with NS1 protein were confirmed by co-localization in the infected cells using immunofluorescence (IF) assay.

Materials and Methods

Cells and virus. MDCK (Madin-Darby canine kidney) cells were grown in DMEM supplemented with 10% fetal bovine serum (Gibco). The H5N1 viral isolate was A/Thailand/1(KAN-1)/04. Virus infectivity was assessed by 50% tissue culture infectious dose titration in MDCK cells. The experiments of H5N1 virus were performed in a biosafety level-3 facility**.**

Bait plasmids construction. The NS1 gene of H5N1 IAV was amplified by RT-PCR using forward and reverse primers that contain *Eco*RI and *Bam*HI restriction sites as follows: H5N1NS1-*Eco*RIF (5'-CCGGAATTCATGGATTCCAACACTGTGTC-3') and H5N1NS1-*Bam*HIR (5'-GCGGGATCCTCAAACTTCTGACT CAAT-3'). A bait plasmid was initially constructed by inserting full-length NS1 in-frame into *Eco*RI and *Bam*HI site of pGKBT7 containing GAL4 DNA-binding domain (BD) (Clontech Laboratories). To eliminate the auto-activation of NS1 protein full range, NS1 was separated into RNA binding domain (RD) by H5N1NS1-*Eco*RIF and H5N1NS1-*Bam*HIR 341-357 primers (5'-GCGGGATCCTTA CATTATTGCCTGGTCC-3') and effector domain (ED) by primers H5N1NS1-*Eco*RIF 358-380 (5'-CCGGAATTCGATAAAGTCGT CATATTGAAAGC-3') and H5N1NS1-*Bam*HIR. Each domain was individually cloned into pGKBT7 (Fig. 1). The plasmids were subjected to DNA sequencing and individually transformed into Y187 yeast (Clontech Laboratories).

Construction of macrophages cDNA library. Peripheral blood mononuclear cells were separated by gradient centrifugation through Ficoll-Hypaque (Pharmacia). The cells were washed twice and viable cells were brought to a final concentration of 5×10^5 cells/ml in 100 mm tissue-culture plates (Corning) for 90 mins at 37°C. The monocytes were enriched by adsorption onto tissue-culture plates in RPMI-1640 (Invitrogen). Cultures was washed 10 times with medium to remove unattached lymphocytes, while adherent monocytes were cultured in RPMI-1640 medium supplemented with 10% normal human serum (Sigma) and 20 ng/ml granulocyte-macrophage colony-stimulating factor (GM-CSF) (R&D Systems). The cells were allowed to differentiate for 10 days. Differentiated macrophages were identified by a typical morphol-

ogy. Total RNA was prepared from macrophages by using TRIzol reagent (Invitrogen). From this total RNA, mRNA was purified by NucleoTrap® mRNA purification (BD Biosciences). cDNA was synthesized using the MATCHMAKER Library Construction and Screening Kit (Clontech Laboratories) and co-transformed with the plasmid pGADT7-Rec carrying GAL4 activation domain (Clontech Laboratories) into AH109 yeast. The transformation mixture was spread on 150 mm SD/-Leu agar plates for a total of 40 plates. The plates were incubated at 30°C for 5 days. After colonies appeared, the plates were chilled at 4°C for 4 hrs. Subsequently, 5 ml of a freezing medium (YPD medium with 25% glycerol) was added to each plate. The transformants were swirled and all liquid was combined in a sterile flask, and then stored at -80°C for using as prey plasmid cDNA library.

Screening of NS1-binding protein by Y2H. To screen for NS1-binding proteins from the human macrophage cDNA library, bait yeast (Y187 containing pGBKT7-NS1) was mated with prey yeast (AH109 transformed with human macrophage cDNA library). Bait yeast in log phase growth was mixed with prey yeast carrying human macrophage cDNA library and cultured in 50 ml of YPDA broth contain 50 μg/ml kanamycin with gentle swirling (30–50 rpm) for 24 hrs. After mating, the culture was centrifuged and cell pellet was resuspended in 10 ml of 0.5 x YPDA containing 50 μg/ml kanamycin and spread onto 150 mm on SD/-Trp/-Leu/-His/-Ade plates. The library screening plates were incubated at 30°C until yeast colonies appeared. After 2–3 days, the colonies were visible on plates. The plates were incubated for at least 2 weeks to allow slower growing colonies to appear. During the incubation, 2 mm yeast colonies were harvested and re-streaked on SD/-Trp, -Leu agar and incubated at 30°C for 2 days before keeping at 4°C. To select yeast clones with bait and prey fusion protein interactions, the mated diploid yeast clones were re-cultured on SD/-Trp/-Leu/-Ade/-His agar and SD/-Trp/-Leu/-Ade/-His agar plus X-α-gal at 30°C for 2 days. The positive yeast clones could grow on this SD medium plate and showed blue colonies on the SD medium plate with X-α-gal. Plasmid pGADRecT7-cDNA plasmids were isolated from the positive yeast transformants grown in a Leu-deficient medium using lithium acetate method and transformed into *Escherichia coli* (Gietz *et al*., 2002). cDNA inserts were PCR amplified and the PCR products were digested with *Alu*I restriction enzyme and analyzed by gel-electrophoresis. Plasmids giving similar restriction patterns were considered as repetitive clones.

Specificity of the interactions of positive yeast clones. Plasmid shuffling in yeast was performed to test specific interactions between human and viral protein. Plasmids from positive clones were individually transformed into Y187 yeast. The pGBKT7-NS1-RD or -ED plasmid was transformed into AH109 yeast. Y187 containing pGADRecT7/SV40 T-Ag was mated with AH109 containing pGBKT7/p53 and pGBKT7/laminC as positive and negative controls, respectively. The specific interaction assay was then performed by mating of individual transformed Y187 yeast and AH109 containing pGBKT7-NS1-RD or -ED, pGBKT7, pGBKT7/p53 or pGBKT7/laminC. Specific interaction was indicated by growth of mated yeast on SD/-Trp/-Leu/-Ade/-His dropout medium and appearance of blue colony on X-α-Gal plates. cDNA sequences from the positive clones were identified through BLAST search on the NCBI GenBank and EMBL databases.

IF assay. MDCK cells grown on 22 mm glass base dishes were infected with A/Thailand/1(KAN-1)/04. At 8 hrs post infection, the cells were fixed with 80% cold acetone in PBS for 30 mins and permeabilized with 0.1% Triton X-100 for 10 mins. NS1 was detected by incubation of cells with anti-NS1 antibody for 1 hr and subsequently with Alexa 488-labeled secondary antibody (Invitrogen) for 1 hr. NS1-binding proteins were detected by incubating cells with anti-ADAR1 or anti IL-6R antibodies for 1 hr and with Cy3-labeled secondary antibody (Invitrogen) for 1 hr. Nuclei were visualized with Hoechst 33258 dye (Invitrogen). Cellular localization of NS1 and NS1-binding protein was observed by using a laser scanning confocal Zeiss LSM 510 microscope.

Results

Bait plasmid expression in yeast

Y187 yeast transformed with pGKBT7-NS1 could grow on SD/-Ade/-His/-Leu/-Trp medium showing auto-activation by NS1. Therefore, it could not be used in the screening of NS1-binding protein in the yeast two-hybrid system. In order to eliminate this auto-activation, NS1 was firstly separated into RD and ED. Each domain was individually cloned into pGKBT7. The constructs expressed fusion protein at high level and grew on SD/-Trp medium but not on SD/-Ade/-His/-Leu/-Trp medium (Fig. 2). Thus, these transformed yeasts could be used in Y2H analysis. The fusion proteins were expressed in the bait yeast showing expected M_r of RD and ED at 33.5 K and 31.5 K, respectively.

Y2H screening of NS1-binding proteins

Positive colonies (250) from mating of the pGKBT7-NS1-RD-transformed yeast and positive colonies (60) from mating of the pGKBT7-NS1-ED-transformed yeast with the prey yeast grew on SD/-Ade/-His/-Leu/-Trp plates containing X-α-Gal. In these plates, α-galactosidase enzyme was secreted by the $MEL1^+$ positive colonies, which could catalyze a shift of X-α-Gal to the blue pigment. Positive results showed appearance of strong blue colony from X-α-Gal plate (Fig. 3a). The plasmids in the clones were screened by restriction length analysis and duplicate clones were eliminated. After checking for repetitive clones and specificity by re-transformation and mating, 7 clones remained. Of these, 4 clones interacted with H5N1 NS1-RD and 3 clones interacted with H5N1 NS1-ED.

Specificity of the interaction of positive yeast clones

To test the specificity of interaction between H5N1 NS1-RD or H5N1 NS1-ED (bait plasmids) and H5N1 NS1-binding proteins (prey plasmids), individual prey yeast colony was mated with bait yeast including yeast containing the

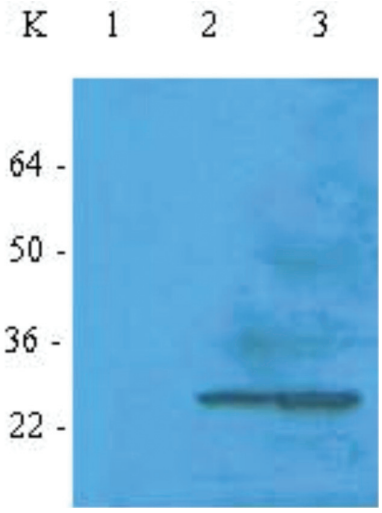

Fig. 2

Expression of NS1-RD and NS1-ED fusion proteins in bait yeast

Y187 cells, negative control (lane 1), Y187 cells containing pGBKT7H5N1-RD (lane 2), Y187 cells containing pGBKT7H5N1-ED (lane 3). M_r standards on the left.

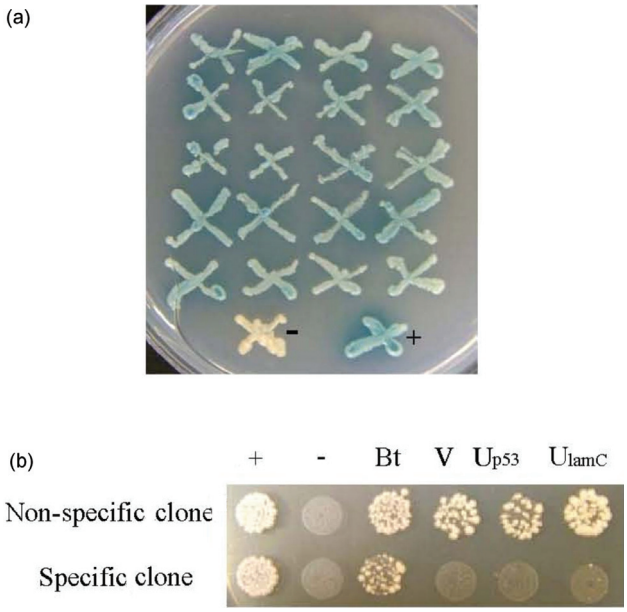

Fig. 3

Screening of yeast clones expressing NS1-binding proteins

(a) Plating of yeast AH109 transformed with pGBKT7NS1 containing macrophage cDNA library on the SD/-Trp/-Leu/-His/-Ade +X-α-Gal medium. (b) Verification of the specificity of interaction. Non-specific and specific prey yeast clone mated with a specific bait clone (Bt) and nonspecific bait yeasts (V, U_{p53}, U_{lamC}). Positive (+) and negative (-) control.

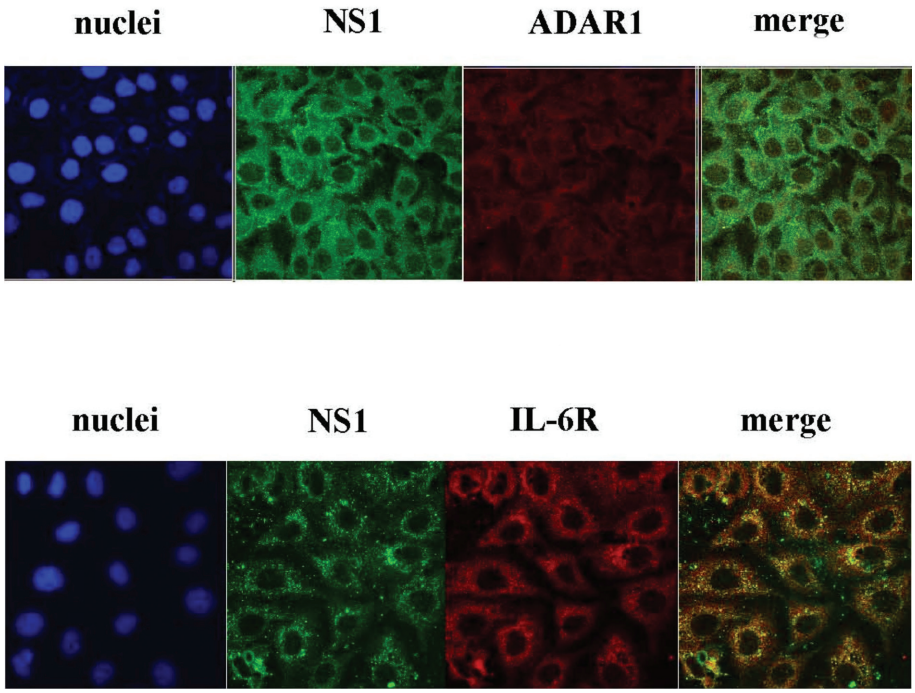

Fig. 4

Cellular co-localization of NS1 with ADAR1 (upper panel) and IL-6R (lower panel) in the infected MDCK cells

Staining for NS1 (green), ADAR1 or IL-6R (red) and cell nuclei (blue).

original bait plasmid, an empty bait vector pGBKT7 and unrelated bait pGBKT7/p53, and pGBKT7/lamin C plasmids. The last two plasmids expressed two unrelated proteins that should not interact with H5N1 NS1-binding proteins. Only bait and prey colony could grow on SD/-Trp/-Leu/-His/-Ade plate, what is considered as a specific clone. For positive control, AH109 yeast containing pGBKT7/p53 was mated with Y187 yeast containing pGADRecT7-SV40-T Ag and a negative control was set up by a mating between AH109 yeast containing pGBKT7/lamin C and Y187 yeast containing pGADRecT7-SV40-T Ag. The specific interaction between H5N1 NS1-RD and H5N1 NS1-binding protein is shown as selective growth of only mated yeast carrying the specific interacting partners (Fig. 3b). In contrast, a non-specific clone allowed the growth of yeast colonies, when mated with specific NS1 bait or non-specific baits pGBKT7, pGBKT7/p53, and pGBKT7/lamin C (Fig. 3b).

Sequencing and identification of NS1-binding proteins

To identify the NS1-binding proteins, cDNA from 7 positive clones were sequenced and subjected to BLAST search in NCBI GenBank <http://www.ncbi.nlm.nih.gov> and EMBL databases <http://www.ebi.ac.uk>. Of 4 clones interacting with NS1-RD, 2 clones contained IL-6R, 1 clone contained ADAR1 and 1 clone cathepsin B. Of 3 clones interacting with NS1-ED, 2 clones contained MHC class I HLA-B and 1 clone contained ubiquitin.

Co-localization of NS1 and NS1-binding proteins in infected MDCK cells

MDCK cells were infected with A/Thailand/1 (KAN-1)/2004 (H5N1). At 8 hrs post infection, the localization of NS1 and binding proteins ADAR1 or IL-6R were analyzed by IF using confocal microscope. The results showed co-localization of NS1 and both ADAR1 and IL-6R in the cytoplasm of infected cells (Fig. 4).

Discussion

The Y2H system provides a way of identification for the interaction between two proteins. In the present study, human macrophage proteins interacting with IAV NS1 RD and IAV NS1 ED were identified. Subsequently, a co-localization of H5N1-NS1 with ADAR1 and IL-6R were confirmed by double IF staining and confocal microscopy. These putative NS1-binding proteins may contribute to the viral pathogenesis. These proteins play an important role in the immune response, protein targeting for proteosomal degradation, and RNA editing. The interaction between NS1 and those proteins may contribute to the unusual severity of human H5N1 influenza. However, a confirmation of these interactions requires further studies.

ADAR1 is an RNA editing enzyme targeting dsRNA that plays an important role in post-transcriptional regulation of cellular RNA (Keegan *et al*., 2004; Bass *et al*., 2002). It edits a Hepatitis D virus (HDV) RNA during viral replication what is required for production of a large delta antigen. Therefore, this enzyme is essential for HDV replication. On the other hand, over-expression of ADAR1 could lead to a hyper-editing of HDV genome and inhibition of the viral replication (Jayan *et al*., 2002). Recently, ADAR1 was shown to up-regulate Human immunodeficiency virus-1 expression by a post-transcriptional mechanism (Phuphuakrat *et al*., 2008). NS1 of IAV can inhibit host cellular mRNA expression post-transcriptionally. Because both ADAR1 and NS1 are dsRNA binding proteins and are involved in the post-transcriptional regulation of RNA, interactions between these two proteins may be involved in the inhibition of host mRNA expression by NS1 (Hale *et al*., 2008; Fernandez-Sesma *et al*., 2007). In addition, ADAR1 was shown to inhibit dsRNA-activated protein kinase (PKR) activity, which is an important component of the interferon-induced antiviral state (Nie *et al*., 2007). NS1 has also been shown to counteract the antiviral function of IFN inducible PKR (Min *et al*., 2007). Recently, ADAR1 has been shown to suppress IFN signaling and to block a premature apoptosis in hematopoietic cells (Hartner *et al*., 2009; Iizasa *et al*., 2009). The ability of ADAR1 to suppress IFN signaling makes it a good partner for NS1 to mediate viral evasion of the host innate antiviral mechanisms.

IL-6 is a pleiotropic cytokine that regulates an immune reaction. IL-6R is the binding component specific to IL-6. IL-6R binds to plasma membrane receptor complexes containing the common signal transducing receptor chain gp130. Signal transduction involves the activation of JAK (Janus kinase) tyrosine kinase family members leading to the activation of transcription factors of the STAT (signal transducers and activators of transcription) family. Another major signaling pathway for IL-6R is the MAPK (mitogen-activated protein kinase) cascade (Heinrich *et al*., 2003; Akira *et al*., 1997). IL-6 is the predominate inducer of the acute-phase response, an innate immune mechanism, which is triggered by the infection and inflammation. IL-6 also plays a multiple role during subsequent development of acquired immunity against incoming pathogens, stimulation of antibody production by B cells, regulation of macrophage and dendritic cell differentiation, and response of regulatory T cells to microbial infection (Rose-John *et al*., 2007). In addition, IL-6 can inhibit TNF (tumor necrosis factor) production *in vitro* and *in vivo* (Benigni *et al*., 1996). Therefore, IL-6 may play an important role in a down-regulation of innate response in transition to the specific immune response. Increased amounts of TNF were detected in the human macrophages infected with highly virulent H5N1 viruses and TNF hyperinduction is believed to be a key virulence factor

(Cheung *et al.*, 2002). Interaction of NS1 to IL-6R may interfere with signaling of IL-6R and the immune regulatory function of IL-6. This may contribute to the hyperactive responses and severe inflammation in H5N1 infection.

Cathepsin B is a lysosomal cysteine protease of the papain family. It is found in lysosomes and also associated with the plasma membrane or secreted, what indicates its role in the digestion of extracellular matrix component (Chwieralski *et al.*, 2006). Cathepsin B has been shown to be involved a number of inflammatory diseases and pathological conditions, such as bronchitis, rheumatoid arthritis, acute pancreatitis, and cancer progression. In addition, cathepsin B has been implicated in an apoptotic pathway that involves the TNF signaling pathway (Chwieralski *et al.*, 2006; Canbay *et al.*, 2003). Finding a solution to the problem of NS1 contribution to airway inflammation and apoptosis through its interaction with cathepsins B requires further studies.

Antigen presenting cells present virus-derived antigenic peptides in association with MHC class I antigens to prime antiviral CTL (cytotoxic T-lymphocyte). Viruses interfere with this antiviral response by down-regulation of the expression of MHC class I molecule on the surface of antigen presenting cells. The virus-specific CTL recognizes virus-derived antigenic peptides in association with MHC class I antigens (Martin *et al.*, 2002; Bodmer *et al.*, 1987). A decreased expression of these antigens on the surface of virus-infected cells prevents their recognition and killing by the CTL. Interaction of NS1 protein with HLA-B may interfere with the antigen presentation by HLA-B providing an immune escape mechanism for the virus.

Ubiquitin is a small protein that is composed of 76 amino acids. Ubiquitin modification and protein degradation by the ubiquitin-proteasome pathway is a mechanism for controlling the function and availability of regulatory proteins in the cell. It also provides for many different viruses to achieve successful viral infection. Many viruses have been reported to involve different strategies to utilize the ubiquitin-proteasome pathway for their own benefits (Gao *et al.*, 2006; Corbin-Lickfett *et al.*, 2003; Dantuma *et al.*, 2003; Eom *et al.*, 2003; Kalejta *et al.*, 2003; Luo *et al.*, 2003; Andrejeva *et al.*, 2002; Strack *et al.*, 2000; Didcock *et al.*, 1999). IAV requires ubiquitin-proteasome activity at an early stage of the infection. It has been recently shown that IAV requires the proteasome for endosomal progression (Ros *et al.*, 2004; Khor *et al.*, 2003). Interaction of NS1 protein with the ubiquitin may direct ubiquitination to certain cellular or viral targets, thereby regulating cellular functions required for the effective viral replication.

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