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Establishiment of Canine RNA polymerase I-Promoter driven Reverse Genetics for Influenza A virus

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a mio padre, mia madre e mia sorella le colonne della mia vita.

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RIASSUNTO

Reverse Genetics

I vaccini per l'influenza, vivi e uccisi, sono efficaci nel prevenire e tenere sotto controllo il diffondersi della malattia, ma le variazioni antigeniche che caraterizzano le glicoproteine emmaglutinina (HA) e neuraminidasi (NA) del virus dell'influenza A, richiedono frequenti cambi nella loro formulazione. Le nuove tecnologie come la Reverse Genetics (RG), attraverso la quale è possible recuperare il virus infettivo da cellule trasfettate con DNA plasmidico che codifica gli 8 segmenti del genoma virale, potrebbero essere utili per accorciare i tempi di ottenimento del "seme virale" per la preparazione dei vaccini. Infatti, la RG può permettere di ottenere rapidamente riassortanti con ottime caratteristiche di crescita in uova o cellule MDCK (cellule renali di cane) attraverso la combinazione dei sei geni interni da virus ad alta produttività e HA e NA dai ceppi circolanti.
Lo scopo della mia tesi è stato quello di esplorare il possibile uso di questa nuova tecnica per generare e produrre vaccini influenzali.
Per fare questo, abbiamo messo a punto un sistema di Reverse Genetics in cui l'RNA polimerasi (polI) canina, permette al virus di svilupparsi in MDCK, una linea cellulare approvata dall'FDA per la produzione di vaccini influenzali e che supporta un'efficiente crescita del virus stesso. Le nostre scoperte dimostrano che il sistema di RG a 8 plasmidi consente una rapida e riproducibile generazione di riassortanti dell'influenza A da poter utlizzare nella preparazione dei vaccini. Grazie all'aiuto di questa nuova tecnologia, potremmo sviluppare rapidamente vaccini sicuri, cross protettivi tra i vari ceppi virali e che richiedono una quantità minore di virus rispetto a quelli tradizionali.

Fluinnate

Il virus dell'influenza A è un notevole patogeno sia per gli uomini che per diverse specie animali. Questi virus sono stati isolati sia da uccelli che da mammiferi sebbene nei primi siano in una fase di stasi dal punta di vista evolutivo. I virus che infettano gli uccelli acquatici non hanno potere contaminate sull'uomo in quanto hanno necessità di riassortarsi e adattarsi ad un

ospite intermedio prima di emergere nel genere umano. Si pensa che l'impedimento della replicazione negli uomini da parte del virus aviario sia dovuto ad una non ottimale specificità di recettore di legame. Durante il singolo ciclo infettivo, il virus umano preferenzialmente infetta le cellule non ciliate, mentre quello aviario, così come la variante della forma umana adattata a crescere nelle uova che ritorna ad avere una specificità di recettore aviaria, preferenzialmente infetta le cellule ciliate. Questo presupposto è in accordo con la predominante localizzazione dei recettori per la forma umana (acidi sialici legati con un legame di tipo α2-6) sulle cellule non ciliate e dei recettori per la forma aviaria (acidi sialici legati con un legame di tipo α2-3) su quelle ciliate. Queste scoperte rafforzano l'idea che sebbene il virus dell'influenza aviaria può infettare l'epitelio del tratto respiratorio umano, la sua capacità di replicazione rimane limitata ad un tropismo cellulare non ottimale.

Attraverso la RG abbiamo generato diversi virus ricombinanti che defferiscono unicamente nella loro specificità di recettore per l'HA e li abbiamo utilizzati per esaminare se quest'ultima unita all'attività sialidasica del virus può influenzare la risposta immunitaria determinandone la patogenicità. Abbiamo testato le risposte immunitarie e cellulari su colture differenziate di epitelio respiratorio umano e su cellule umane immunocompetenti isolate, scoprendo che l'iniziale risposta immunitaria innata scaturita dall'infezione da parte del virus nell'epitelio respiratorio, può essere determinata in maniera significativa dalle proprietà di legame della molecola di emmaglutinina virale.

ABSTRACT

Reverse Genetics

Killed and live influenza virus vaccines are effective in preventing and curbing the spread of disease but the antigenic variation of influenza A virus hemagglutinin (HA) and neuraminidase (NA) glycoproteins requires frequent changes in vaccine formulation. New technologies such as Reverse Genetics (RG), in which viable infectious virus is rescued from cells transfected with plasmid DNAs encoding the 8 influenza virus genome segments, could be used to shorten the lengthy process of preparing vaccine seed viruses. In fact, RG could allow to quickly generate reassortant seed viruses with optimal growth characteristics in eggs or MDCK cells by combining six internal genes from high-yield viruses and HA and NA genes from the circulating strains.

The aim of my thesis was to explore the use of this new technology for the generation and production of Influenza vaccines.

We have established a canine RNA polymerase I (polI)-driven influenza virus RG system that could work in Madin-Darby canine kidney cells, a cell line that is been approved by the FDA for human vaccine production and can support the efficient growth of influenza virus. Our findings demonstrate that the eight-plasmid system RG allows the rapid and reproducible generation of reassortant influenza A viruses for use in the manufacture of vaccines. By taking advantage of these new technologies, we could quickly develop vaccines that would be safe, cross-protective against variant strains, and require less virus per dose than conventional vaccines.

Fluinnate

Influenza A viruses are important worldwide pathogens for humans and different animal species. These viruses have been isolated from avian and mammalian hosts, although the primary reservoirs are in evolutionary stasis. The aquatic bird viruses do not replicate well in humans, and these viruses need to reassort or adapt in an intermediate host before they emerge in human populations. A non-optimal receptor-binding specificity of avian influenza viruses is

believed to hamper their replication in humans. During the course of a single-cycle infection, human viruses preferentially infected non-ciliated cells, whereas avian viruses as well as the egg-adapted human virus variant with avian virus-like receptor specificity mainly infected ciliated cells. This pattern correlated with the predominant localization of receptors for human viruses (α2–6-linked sialic acids) on non-ciliated cells and of receptors for avian viruses (α2–3-linked sialic acids) on ciliated cells. These findings corroborate the idea that although avian influenza viruses can infect human airway epithelium, their replication may be limited by a non-optimal cellular tropism.

By RG we have generated a panel of recombinant viruses that differs solely in their HA receptor specificity and we have used these viruses to investigate if receptor specificity and sialidase activity of influenza virus can affect innate immune responses to the virus and thus determine its pathogenicity. We have tested immune and cellular responses in differentiated cultures of human airway epithelium and in isolated human immuno-competent cells finding that the first innate immune responses to influenza virus infection in airway epithelium can be significantly determined by the binding properties of the virus hemagglutinin molecules.

1. INTRODUCTION

1.1 The Influenza Virus

Influenza viruses belong to the family *Orthomyxoviridae* that contains five different genera: *the Influenza viruses A, B and C, Thogotovirus and Isavirus* (Kawaoka *et al.*, 2005). The *Orthomyxoviridae* are enveloped viruses with a segmented single-stranded RNA genome of negative orientation. All A and B type influenza viruses possess eight RNA segments, whereas influenza C viruses only have seven RNAs. The genomes of Thogoto viruses consists of only six single-stranded RNA segments of negative polarity, with a total coding capacity for seven proteins. The genome of infectious Isavirus consists of eight negative sense, single-stranded RNA segments (Palese and Shaw, 2006).

Different influenza virus strains are named according to their genus (type, A, B, C), the species from which the virus was isolated (omitted if human), location of isolate, the number of the isolate, the year of isolation, and in the case of the influenza A viruses, the hemagglutinin (HA) and neuraminidase (NA) subtypes. For example, the 220th isolate of an H5N1 subtype virus isolated from chickens in Hong Kong in 1997 is designated: influenza A/chicken/Hong Kong/220/97 (H5N1) virus. So far, 16 different hemagglutinin (H1 to H16) subtypes and 9 different neuraminidase (N1 to N9) subtypes for influenza A viruses have been isolated (Webster *et al.*, 1992; Fouchier *et al.*, 2005).

On initial isolation, influenza A viruses are small (80 to 120 nm in diameter), pleomorphic particles that later become generally spherical. These particles consist of a host-derived lipid bilayer envelope in which the virus-encoded glycoproteins HA and NA and M2 are embedded, an inner shell of matrix protein and, at the center, the nucleocapsids of the viral genome. The genome of influenza A viruses consists of eight unique segments of single-stranded RNA, which are of negative polarity (i.e., complementary to the mRNA sense). The RNA is loosely encapsidated by multiple NP molecules. Complexes containing the three components of the viral polymerase protein (PB1, PB2, and PA) are situated at the ends of the nucleocapsids (Fig. 1).

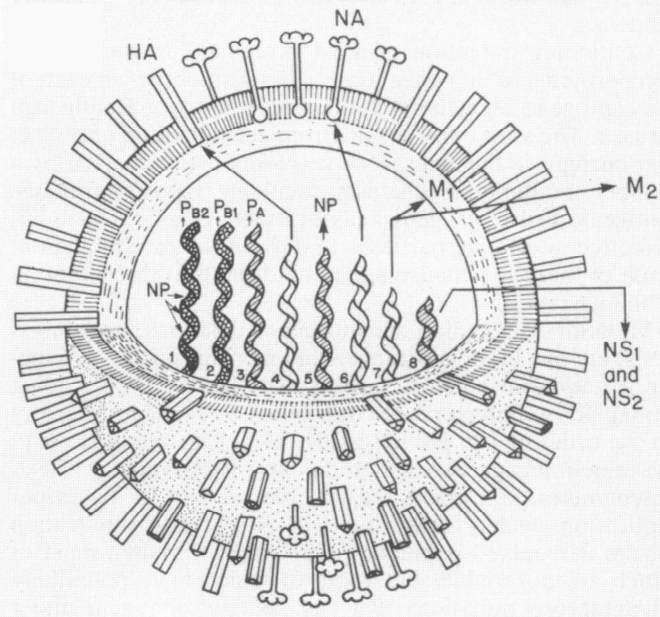


Figure 1. Diagram of the virion. The glycoprotein spikes are formed by two different types of protein: HA predominates and the NA occurs in patches. Inside the lipid bilayer there are eight segments of single-stranded RNA, each of which encodes for one or two proteins (adapted from Webster *et al*., 1992).

To be infectious, a single virus particle must contain each of the eight unique RNA segments. Available evidence suggests that incorporation of RNAs into virions is at least partly random. A random mechanism of incorporation would generate a maximum of one infectious particle for every 400 assembled ($8!/8^8$), a number compatible with the ratio of noninfectious to infectious particles found in viral preparations. The random incorporation of RNA segments allows the generation of progeny viruses containing novel combinations of genes (i.e., genetic reassortment) when cells are doubly infected with two different parental viruses.

The eight influenza A viral RNA segments encode 10 recognized gene products. These are the PB1, PB2, and PA polymerases, NP, NS1 and NS2, Ml and M2, NA and HA proteins (Webster *et al*., 1992).

The viral ribonucleoprotein complexes (vRNP)

Each viral RNA segment exists as a ribonucleoprotein (RNP) complex in which the RNA is coated with NP and forms a helical hairpin that is bound on one end by the heterotrimeric

polymerase complex. The RNA-dependent RNA polymerase is a complex of three proteins: polymerase basic protein 1 (PB1), polymerase basic protein 2 (PB2) and polymerase acidic protein (PA).

- *PB2 (*polymerase basic protein 2): PB2 polymerase is encoded by RNA segment 1 and plays a critical role in the initiation of transcription since it is responsible for binding the cap on host pre-mRNA molecules (Blass *et al.*, 1982; Ulmanen *et al.*, 1981). Endonucleolytic cleavage of these cap structures from host mRNAs is also at least in part a function of PB2. The role of PB2 in the other virus-directed RNA synthetic processes, i.e., synthesis of full-length template cRNA and new negative-sense viral RNA (vRNA), is not known since these processes do not require host cap priming. Newly synthesized PB2 proteins migrate to the nucleus of infected cells (Webser *et al.*, 1992), where viral genome replication occurs.

- *PB1 (*polymerase basic protein 1): PB1 polymerase is encoded by RNA segment 2. PB1 is responsible for binding to the terminal ends of both vRNA (Gonzalez and Ortin, 1999(A); Li *et al.*, 1998) and cRNA (Gonzalez and Ortin, 1999(B)) for initiation of transcription and replication. The interaction with the 3' end of the vRNA activates the endonuclease activity of PB1 (Cianci *et al.*, 1995; Hagen *et al.*, 1994; Li *et al.*, 2001), which generates the capped primer required for mRNA synthesis.

- *PA (*polymerase acidic protein): PA polymerase is encoded by RNA segment 3. No specific function has been ascribed to the PA protein but mutations affecting both transcription and replication have been described, indicating its role in both processes (Fodor *et al.*, 2002; Fodor *et al.*, 2003, Huarte *et al.*, 2003). There is evidence for possible roles as a protein kinase or as a helix-unwinding protein (Sanz-Ezquerro *et al.*, 1998).

- *NP* (nucleocapsid protein): NP nucleocapsid protein is encoded by RNA segment 5. It is an arginine-rich protein and has a net positive charge (at pH 6.5), which reflects its RNA binding activity and its role in encapsidation. After synthesis, it is transported

into the infected cell nucleus, where it binds to viral RNA. In addition to its structural role, NP is believed to play a role in the switching of viral RNA polymerase activity from mRNA synthesis to cRNA and vRNA synthesis. NP is abundantly synthesized in infected cells and is the second most abundant protein in the influenza virus virion. It is phosphorylated and its pattern of phosphorylation is host cell dependent and may be related to viral host range restriction. NP is also a major target of the host cytotoxic T-cell immune response (Webster *et al.*, 1992).

The non-structural proteins:

- *NS1 and NS2*: RNA segment 8 harbors the sequence information for nonstructural NS1 protein and nuclear exporting protein NS2/NEP. NS1 mRNA is colinear with the vRNA, whereas NS2 mRNA is derived by splicing. These proteins, particularly NS1, are abundant in the infected cell where acts as a regulatory factor (NS1 primarily in the nucleus, NS2 primarily in the cytoplasm) but are not incorporated into progeny virions. Both proteins play roles in virus replication, but those roles have not been fully defined (Webster *et al.*, 1992). NS2 is also a minor component of the viron and is found in association with the M1 protein (Ludwig *et al.*, 1999).

The matrix proteins:

- *M1 and M2*: Influenza virus RNA segment 7 is bicistronic, encoding both Ml and M2 proteins and yields mRNA for the matrix protein.

  M1 is the most abundant protein in the influenza virus virion. It associates with lipid membranes (Nayak *et al.*, 2004; Schmitt and Lamb, 2005). Matrix protein forms a shell surrounding the virion nucleocapsids, underneath the virion envelope. M1 has also been shown to be necessary and sufficient for the formation of virus-like particles, providing evidence for its essential role in the budding process (Gomez-Puertas *et al.*, 2000; Lathamet and Galarza, 2001). In the infected cell it is present in both cytoplasm and nucleus.

  M2 is derived from the colinear (Ml) transcript by splicing. It is a tetrameric type III (lacking a signal peptide sequence) integral membrane protein. It has been shown to possess ion channel activity and its major role is thought to be that of conducting

protons from the acidified endosomes into the interior of the virus to allow dissociation of the RNP complex from the rest of the viral components, thus completing the uncoating process (Hay, 1992). Its ion channel activity has also been implicated in stabilizing HAs from premature low pH transitions in the trans-Golgi network (Ciampor *et al.*, 1992). This second function could only be important for viruses carrying highly acid-sensitive HAs, as H5 and H7 hemagglutinin molecules which have a multibasic cleavage site that can be cleaved by ubiquitous proteases and they are therefore more susceptible to a premature low pH-induced conformational change. Finally, there appears to be a role for M2 in assembly and budding (Hughey *et al.*, 1995; Schroeder *et al.*, 2005).

The surface glycoproteins:

- *NA* (Neuraminidase): NA, encoded by RNA segment 6, is a type II integral membrane glycoprotein and the second major surface antigen of the virion. The nine subtypes of the A virus NA fall into two major groups (N1, N4, N5, N8 and N2, N3, N6, N7, N9) based on sequence comparisons (Fouchier *et al.*, 2005). The influenza A virus NAs have a highly conserved short cytoplasmic tail and a hydrophobic transmembrane region which provides the anchor for the stalk and the head domains. Sugar residues are attached to four of the five potential glycosylation sites in the head. The enzyme was found to cleave (at position 2 of neuraminic acid) ketosidically bound sugars of alcohols (Bucher and Palese, 1975). Transition state inhibitors such as 2-deoxy-2,3-dehydro-N-trifluoroacetylneuraminic acid, which mimic the enzymatic substrate, were shown early on to inhibit influenza virus replication (Palese *et al.*, 1974 (A)) and compounds with the same mechanism of action were later developed for use as highly effective antivirals in humans (Garman and Laver**,** 2004; McKimm-Breschkin, 2005). Experiments on cells infected with mutants in the NA portion shown the viral NA must remove the sialic/neuraminic acid receptor from the surface of the cell as well as from the virus particles to prevent recognition by the HA of the virus. Thus, it functions to free virus particles from host cell receptors, to permit progeny virions to escape from the cell in which they arose, and so facilitate virus spread (Palese *et al.*, 1974 (B); Palese and Compans, 1976). In addition, it has been shown that the viral NA may also

play a role early in infection, possibly facilitating entry of the virus (Matrosovich *et al.*, 2004) and/or enhancing late endosome/lysosome trafficking (Suzuki *et al.*, 2005).

- *HA* (Hemagglutinin): HA is encoded by RNA segment 4. The HA protein is a type I integral membrane protein and the major surface antigen of the influenza virion. There are sixteen subtypes of the A virus HA based on sequence comparisons (Fouchier *et al.*, 2005). Remarkably, even though the overall amino acid sequence identity can be less than 50%, the structure and functions of these HAs are highly conserved. The HA is a trimeric rod-shaped molecule with the carboxy terminus inserted into the viral membrane and the hydrophilic end projecting as a spike away from the viral surface. To be fully functional, the HA molecule needs posttranslational modifications (glycosylation and palmitoylation), cleavage of the signal peptide in the ER and cleavage of the HA0 precursor into HA1 and HA2 subunits. The major features of the structure are a long fibrous stem which is made up of a triple-stranded coiled coil of α-helices derived from the three HA2 parts of the molecule and the globular head, which is also made up of three identical domains whose sequences are derived from the HA1 portions of the three monomers (Palese and Shaw, 2006). Both HA1 and HA2 are connected by disulfide linkages and are subunits of the uncleaved HA0. The first major function of HA is binding to receptors and the receptor-binding site lies within the globular head of the molecule. The second major function of the HA is acid pH-triggered fusion, which is required for the uncoating process. Low pH treatment changes the structure of the HA dramatically allowing the fusion peptide to align anti-parallel to the membrane anchor of the HA2. In this way, the fusion peptide brings the endosomal membrane into juxtaposition with the viral membrane, leading to fusion. The presence of more than one hemagglutinin molecuole allows the formation of a fusion pore through which the RNP can exit from the virion and enter the cytoplasm.

1.2 The viral replication cycle

Influenza viruses bind to neuraminic acids (sialic acids) on the surface of cells to initiate infection and replication . The interaction of influenza viruses with a ubiquitous molecule such

as sialic acid is constrained by the fact that the HAs of viruses which replicate in different species show specificity toward sialic acids with different linkages. While some viruses (e.g. paramyxoviruses and herpes viruses) can enter cells directly through the plasma membrane by a pH-independent fusion process, influenza viruses require a low pH to initiate fusion and are therefore internalized by the endocytic compartment. There are four major internalization mechanisms: via clathrin-coated pits, the classical model for influenza virus entry (Matlin *et al.*, 1981), via caveolae, through the non-clathrin, non-caveolae pathways, and through macropinocytosis (Sieczkarski and Whittaker, 2005).

HA binding to cellular surface is followed by the endocytosis of the virus (Klenk *et al.*, 1975; Lazarowitz and Choppin, 1975; Porter *et al.*, 1979). Virus disassembly occurs in the acidic environment of late endosomal vesicles and involves two crucial events. First, the conformation of the hemagglutinin is changed to a low-pH form which results in exposure of a fusion active protein sequence within the HA. This fusion peptide contacts the endosomal membrane and initiates fusion with the viral envelope (Stegmann, 2000). Second, the low pH in the endosomes activates the viral M2 ion channel protein resulting in a flow of protons into the interior of the virion. Acidification further facilitates dissociation of the viral NS2/NEP from the Ml matrix protein (Matlin *et al.*, 1981; Zhirnov and Grigoriev, 1994). The RNPs are subsequently released into the cytoplasm and are rapidly imported into the nucleus through the nuclear pore complexes (NPC).

The polymerase has also to be imported into the nucleus: it has been shown that PB1 binds to both PA and PB2, through its N- and C-terminal domains respectively (Gonzales *et al.*, 1996; Ohtsu *et al.*, 2002) and that PB1 and PA enter the nucleus as a dimer and then bind to PB2, which is imported independently (Fodor and Smith, 2004). The viral nucleoprotein NP which associates with the genomic and antigenomic viral RNAs is a fourth essential cofactor of the viral replicative complex (Huang *et al.*, 1990). Genomic influenza virus RNAs carry at their 5'- and 3'-ends conserved nucleotide sequences of 13 and 12 bases, respectively. These sequences are in part complementary and thus, the ends of viral RNAs can engage in base-pairing interactions resulting in a partially double-stranded promoter structure (Luo and Palese, 1992). The viral RNP binds to and initiates RNA synthesis at these promoter structures of the viral RNA segments. The RNP does not synthesize the 5'-cap structures that are needed for efficient transport and translation of the viral mRNAs. Instead, the 5'-cap structures from

cellular polymerase II transcripts are transferred to viral mRNAs (Krug *et al.*, 1979). The requirement for capped mRNA 5'-ends to function as primers in viral transcription most likely explains the dependency of viral replication on RNA polymerase II. Transcription commences with binding of the 5' end of the vRNA to the PB1 subunit. This induces an allosteric change in the polymerase, which allows the PB2 protein to recognize and bind the cap structure on cellular pre-mRNAs (Fechter and Brownlee, 2005). The change in the polymerase also increases its affinity for the 3' vRNA end, which is bound by PB1. Binding of the 3' terminus stabilizes the polymerase complex (Brownlee and Sharps, 2002) and also serves to activate the endonuclease function of PB1 (Hagen *et al.*, 1994; Li *et al.*, 1998). Endonuclease activation leads to cleavage of the bound pre-mRNAs. This occurs approximately 10-13 nucleotides from their 5' caps, usually after a purine residue (Beaton and Krug, 1981; Plotch *et al.*, 1981). Transcription is then initiated by the addition of a "G" residue to the primer, directed by the penultimate "C" nucleotide at the 3' end of the vRNA template (Beaton and Krug, 1981). RNA elongation is catalyzed by the polymerase function of PB1 and continues until a stretch of uridine residues is encountered approximately 16 nucleotides before the 5' end of the vRNA (Robertson *et al.*, 1981). This is the signal for polyadenylation. The polyadenylation signal is vital for gene expression as replacement of the uridines with adenosines has been shown to result in transcripts with poly(U) tails, which fail to be exported from the nucleus (Poon *et al.*, 2000) (Fig. 2).

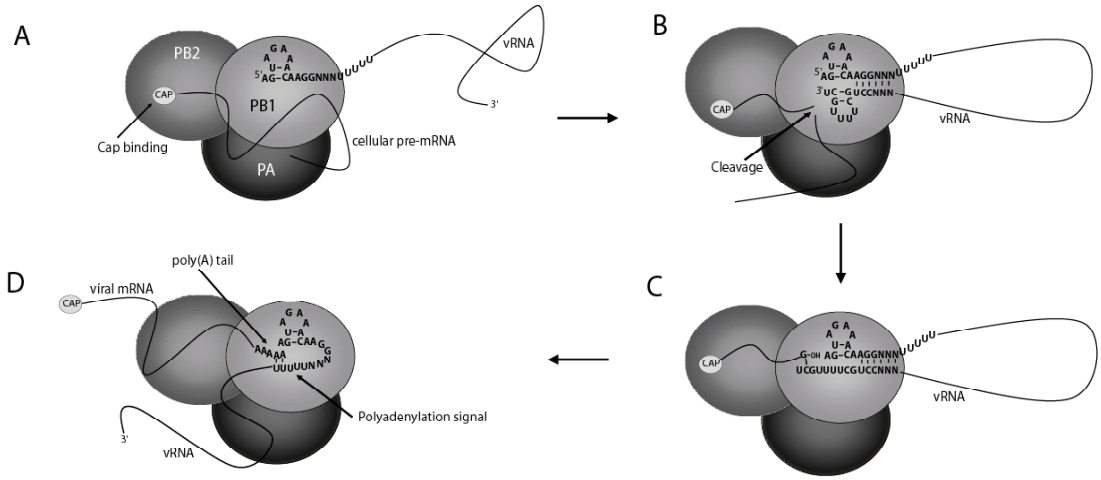


Figure 2 Proposed model for transcription initiation, elongation and polyadenylation of influenza virus mRNA. (A) The 5' end of the vRNA is shown in the corkscrew configuration bound to the PB1 subunit of the polymerase complex. This activates the cap-binding activity of the PB2 subunit. (B) The 3' end of the vRNA binds to PB1 and forms a duplex with the 5' end. The endonuclease activity of the PB1 then cleaves the pre-mRNA 10-13 nucleotides downstream of the cap structure. (C) A guanosine residue is added to the 3' end of the capped primer and base pairs with the penultimate C residue at the 3' end of the vRNA. This initiates transcription and chain elongation is catalyzed by the polymerase function of the PB1 subunit. (D) During elongation the cap detaches from the polymerase. However, the 5' end of the vRNA remains bound while the template vRNA is read in a 3'-5' direction and consequently the polymerase is unable to read beyond the poly-uridine stretch due to steric hindrance. This causes it to stutter and a poly(A) tail is added to the 3' end of the nascent mRNA (adapted from Palese and Shaw, 2006).

The vRNA serves as a template for both mRNA and cRNA synthesis; however, the initiation and termination processes for the generation of these two molecules are quite different. In contrast to the primer-dependent mechanism of initiation of mRNA synthesis, initiation of cRNA synthesis occurs without a capped primer and cRNA molecules are full-length copies of the vRNA and thus are not prematurely terminated and polyadenylated as is mRNAs. In contrast to mRNAs, newly synthesized cRNAs and vRNAs are encapsidated and it has been proposed that the availability of soluble NP, not associated with RNPs, controls the switch between mRNA and cRNA synthesis.

In the second stage of replication, the positive sense cRNA serves as a template for the synthesis of negative sense genomic vRNA. As with cRNA synthesis, this reaction occurs via a primer-independent mechanism and generates full-length products (Palese and Shaw, 2006).

Following virus replication, newly formed RNP complexes are assembled in the nucleus from where they are exported into the cytoplasm. M1 associates with RNPs in the nucleus and promotes the formation of RNP complexes (Huang *et al.*, 2001). NEP/NS2 also associates with M1 (Akarsu *et al.*, 2003; Yasuda *et al.*, 1993), and is responsible for recruiting the export machinery and directing export of the complex (Yasuda *et al.*, 1993). The late expression of M1 determines that export takes place only after a full round of replication has occurred therefore preventing premature exit of RNPs from the nucleus. Similarly, control mechanisms must also exist to stop the re-entry of RNPs into the nucleus following export.

After synthesis, the NA, M2, and the precursor HA (HA0) proteins follow the exocytotic transport pathway from the ER via the Golgi complex. The mature HA and NA glycoproteins and the nonglycosylated M2 are finally integrated into the plasma membrane as trimers (HA) or tetramers (NA, M2), respectively. Ml assembles in patches at the cell membrane. It is thought to associate with the glycoproteins (HA and NA) and to recruit the RNPs to the plasma membrane in the late phase of the replication cycle. Finally the viral RNPs become enveloped by a cellular bilipid layer carrying the HA, NA and M2 proteins and the new virus particles bud from the apical cell surface (Webster *et al.*, 1992). Influenza virus particles have to be actively released after the viral envelope separates from the cell membrane during the completion of budding. This is because the HA anchors the virus to the cell by binding to sialic acid-containing receptors on the cell surface. The enzymatic activity of the neuraminidase protein is required to remove the sialic acid and thereby releases the virus from its host cell. NA activity is also required to remove sialic acid from the carbohydrates present on the viral glycoproteins themselves so that the individual virus particles do not aggregate (Palese and Shaw, 2006). Due to the fact that both HA and NA recognize the same molecule (sialic acid) but have opposing effects (receptor-binding or receptor-destroying), a delicate balance exists between the HA and NA functions (Wagner *et al.*, 2002) (Fig. 3).

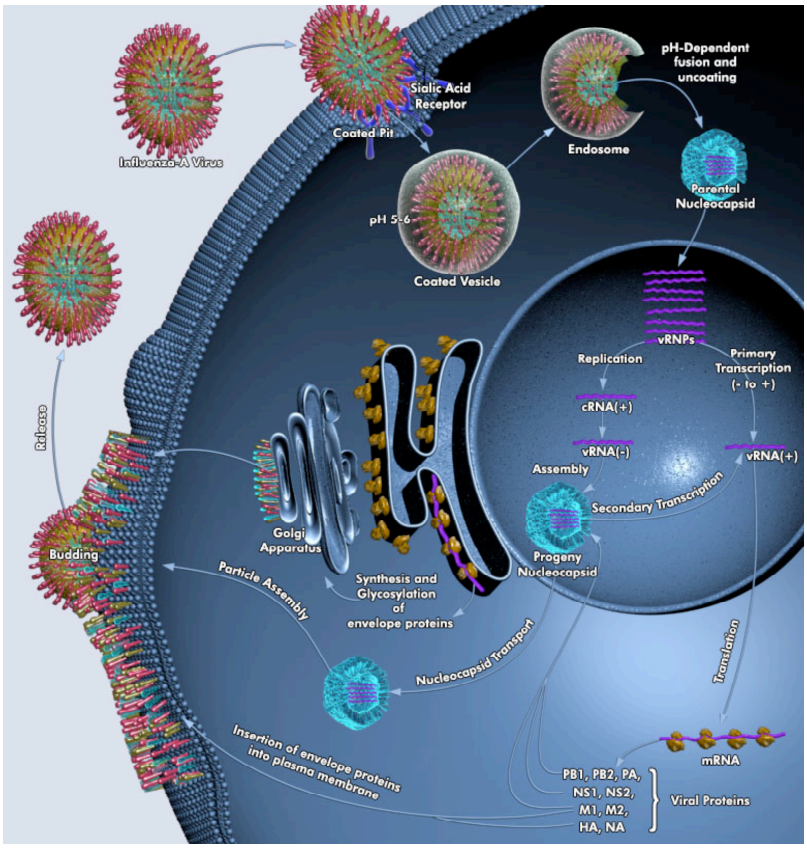


Figure 3 The replication cycle of influenza vimses. The virion attaches to the cellular receptor determinant. The receptor bound particle enters the cell via endocytosis. After fusion of the viral and the endosomal membrane the viral genome is released into the cytoplasm. The RNPs are transported into the nucleus where replication and transcription of the viral RNA segments occurs. The mRNAs are exported into the cytoplasm and are translated into viral proteins. The viral glycoproteins HA, NA and M2 enter the exocytotic transport pathway to the cell surface. Replicative viral proteins enter the nucleus to amplify the viral genome. In the late stage of the infection cycle newly synthesized RNPs are exported from the nucleus and are assembled into progeny virions that bud from the cell surface (image download from https://www1.qiagen.com/GeneGlobe/PathwayView.aspx?pathwayID=247).

The attachment of Influenza virus to the cells results in Influenza disease

Influenza is historically an ancient illness that causes annual epidemics and, at irregular intervals, pandemics. Influenza A viruses have been isolated from a limited number of different animal hosts including humans, birds, horses, whales, seals, mink and swine, some of which are only transiently infect or harbour only a small number of antigenic subtypes. Evidences suggest that the primary reservoir of Influenza A viruses are aquatic birds. Centanni

e Savunozzi demonstrated in 1901, that a filterable agent was responsible for flow plaque, a disease of chickens, but only 50 years later this filterable agent was shown to be an influenza virus (Schafer, 1955). In 1972 antibodies to human influenza NA were identified in Australian pelagic birds (Laver and Webster 1972), demonstrating that aquatic bird species could be hosts for influenza, a finding later confirmed by the isolation of influenza virus from shearwaters (Downi *et al.* 1973) and healthy wild ducks (Slemons *et al.* 1974).

The sites of viral replication in ducks and the illness symptoms contrast with those in humans. Influenza in wild ducks is asymptomatics with the virus replicating preferentially in the cells lining the gastro-intestinal tract (Webster *et al.*, 1978). In humans, influenza often is associated with clinical symptoms, and the virus replicates primarily in the respiratory tract. The asymptomatic nature of infection and the apparent evolutionary status of virus in the acquatic bird reservoir are consistent with a stable host-parasite relationship developed during a prolonged period of coevolution. Upon interspecies transfer, the resultant infection often causes extensive disorder, and an increased viral mutation rate is observed as the virus adapts to new host (Suarez, 2000). Fortunately, both initial human infections by avian viruses and the emergence of pandemic viruses are restricted by a limited fitness of avian viruses in humans. In the case of virus genes other than the HA gene, this restriction can be overcome by gene reassortment between avian and human viruses similar to that which occurred in the 1957 and 1968 pandemic viruses (Webster *et al.*, 1992; Horimoto and Kawaoka, 2001). However, any pandemic virus must carry the HA from a nonhuman virus, for which the human population in mostly naïve. Therefore, one of the key themes in the study of pandemic influenza is to understand the mechanisms of HA-mediated host restriction and how avian viruses manage to breach it.

Avian influenza viruses bind to cell-surface glycoproteins or glycolipids containing terminal sialyl-galactosyl residues linked by 2–3-linkage [Neu5Ac(α2–3)Gal], whereas human viruses, including the earliest available isolates from the 1957 and 1968 pandemics, bind to receptors that contain terminal 2–6-linked sialyl-galactosyl moieties [Neu5Ac(α2–6)Gal] (Paulson, 1985; Nobusawa *et al.*, 1991; Connor *et al.*, 1994; Matrosovich *et al.*, 1997). The 1918 influenza pandemic viruses presumably also had a human-virus like receptor specificity (Matrosovich *et al.*, 2000). By contrast, H5N1 chicken viruses that caused the influenza outbreak in humans in Hong Kong in 1997 (De Jong *et al.*, 1997) had an avian virus-like

receptor specificity (Matrosovich *et al.*, 1999) and were unable to transmit efficiently from human to human (Mounts *et al.*, 1999). It is generally believed, therefore, that alteration of the receptor specificity is a prerequisite for the highly effective replication and human to-human transmission which characterize pandemic virus strains (Cox and Subbarao, 2000; Horimoto and Kawaoka, 2001; Baigent and McCauley, 2003; Matrosovich *et al.*, 2006). However, this theory has never been formally proven, and neither receptor-dependent restriction of avian virus replication in humans, nor restriction mechanisms have been defined. In humans, influenza viruses replicate in the ciliated epithelium of conducting airways (Hers, 1966), which consists of several distinct cell types with different functions (Jeffery and Li, 1997). Observations made late in the infectious process in humans and monkeys show that influenza viruses infect many different types of airway epithelial cells (Tateno *et al.*, 1966; Ebisawa *et al.*, 1969; Rimmelzwaan *et al.*, 2001). However, neither initial targets of the virus attack nor specific cell types that are essential for virus replication have been defined. Our knowledge on the receptor equipment of human airway epithelium is also limited. Predominant expression of 2–6-linked sialic acids on the apical surface of human tracheal epithelial cells has been reported (Baum and Paulson, 1990; Couceiro *et al.*, 1993), but little is known about the presence of 2–3-linked sialic acids on such cells or about variations in sialic acid expression on different cell types.

A better understanding of the ecology of influenza viruses in the reservoir of aquatic birds is therefore required to find ways of intervention to reduce or prevent the occasional catastrophic pandemics.

Influenza viruses are continuously changing. This variability results from accumulation of molecular changes in the eight RNA segments that can occur by a number of different mechanisms including accumulation of point mutations (antigenic drift) due to lack of proof-reading activity of the viral polymerase, gene reassortment (genetic shift), defective interfering particles, and RNA recombination. Each of these mechanisms contributes to the rapid evolution of influenza viruses (Webster *et al.*, 1992) and represent the main causes that hampers a final solution in the treatment of Influenza disease.

1.3 Antivirals and Vaccines

Several drugs have been proposed in the past years and four of these are approved from FDA for use in humas.

Amantadine and Rimantadine block the ion channel activity of the M2 protein (Wang *et al.*, 1993), while Zanamavir and Oseltamivir are inhibitors of the NA protein. However, for all of them, the appearance of resistant variants has been described as a consequence of extensive use.

While no drugs are successfully developed that interfere with the HA/sialic acid interaction, the most important prevention to the Influenza disease remains the vaccination that aims to the generation of anti-HA specific neutralizing antibodies.

There are different types of vaccines:

- *Live, Attenuated Vaccines* (e.g. oral Polio and Varicella vaccine, Flu): these vaccines contain a version of the living microbe that has been weakened in the lab so that it does not cause disease. Because a live, attenuated vaccine is the closest thing to a natural infection, these vaccines are good "teachers" of the immune system; they elicit strong cellular and antibody responses and often confer lifelong immunity with only one or two doses. Viruses often are attenuated through a method of growing generations of them in cells in which they do not reproduce very well. As they evolve to adapt to the new environment, they become weaker with respect to their natural host, human beings. Despite the advantages, this type of approach cannot exclude the remote possibility that an attenuated microbe in the vaccine could revert to a virulent form and cause disease.

- *Inactivated vaccines* (e.g. Polio and Flu vaccine): inactivated vaccines have been produced by killing the disease-causing microbe with chemicals, heat, or radiation. Such vaccines are more stable and safer than live vaccines but stimulate a weaker immune system response than do live vaccines. So it would likely take several additional doses, or booster shots, to maintain immunity.

- *Subunit vaccines* (e.g. Influenza A and B, Hepatitis A and B vaccine): can contain anywhere from 1 to 20 or more antigens. Subunit vaccines can be made in different ways; the microbe can be grown in the laboratory and then broken apart by chemicals in order to gather the important antigens or the antigen molecules can be manufactured using recombinant DNA technology. Vaccines produced in this way are called "recombinant subunit vaccines". In the case of the Influenza A vaccine, the major antigens are the two viral glycoproteins HA and NA. Of course, identifying which antigens best stimulate the immune system is a tricky, time-consuming process.

- *Toxoid Vaccines* (e.g. Diphtheria and Tetanus vaccine): these vaccines are used when a bacterial toxin is the main cause of illness. Scientists have found that they can inactivate toxins by treating them with formalin. When the immune system receives a vaccine produces antibodies that lock onto and block the toxin.

- *Conjugate Vaccines* (e.g. Haemophilus influenzae type B vaccine): if a bacterium possesses an outer coating of polysaccharides, as many harmful bacteria do, it is possible to make a conjugate vaccine for it. Polysaccharide coatings disguise a bacterium's antigens so that the immature immune systems of infants and younger children can't recognize or respond to them. When making a conjugate vaccine, scientists link antigens or toxoids from a microbe that an infant's immune system can recognize to the polysaccharides. The linkage helps the immature immune system react to polysaccharide coatings and defend against the disease-causing bacterium.

- *DNA Vaccines* (e.g. influenza and herpes vaccine): DNA vaccines take immunization to a new technological level. Researchers have found that when the genes for a microbe's antigens are introduced into the body, some cells will take up that DNA. The DNA then instructs those cells to make the antigen molecules. The cells secrete the antigens and display them on their surfaces. In other words, the body's own cells become vaccine-making factories, creating the antigens necessary to stimulate the immune system. A DNA vaccine against a microbe would evoke a strong antibody response to the free-floating antigen secreted by cells, and the vaccine also would

stimulate a strong cellular response against the microbial antigens displayed on cell surfaces. The DNA vaccine could not cause the disease because it would not contain the microbe, just copies of a few of its genes. In addition, DNA vaccines are relatively easy and inexpensive to design and produce. So-called naked DNA vaccines consist of DNA that is administered directly into the body. These vaccines can be administered with a needle and syringe or with a needle-less device that uses high-pressure gas to shoot microscopic gold particles coated with DNA directly into cells. Sometimes, the DNA is mixed with molecules that facilitate its uptake by cells.

- *Recombinant vector vaccines*: are experimental vaccines similar to DNA vaccines, but they use an attenuated virus or bacterium to introduce microbial DNA to cells of the body. "Vector" refers to the virus or bacterium used as the carrier. In nature, viruses latch on to cells and inject their genetic material into them. In the lab, scientists have taken advantage of this process. They have figured out how to take the genomes of certain harmless or attenuated viruses and insert portions of the genetic material from other microbes into them. The carrier viruses then ferry that microbial DNA to cells. Recombinant vector vaccines closely mimic a natural infection and therefore do a good job of stimulating the immune system. Researchers are working for HIV, rabies, and measles.

- *Reverse Vaccinology*: the advent of whole-genome sequencing of bacteria (Fleischmann *et al*., 1995) and advances in bioinformatics have revolutionized the study of bacterial pathogenesis, enabling the targeting of possible vaccine candidates starting from genomic information. A new approach, developed at the end of the years 90 by Rino Rappuoli, Global Head of Vaccines Research in Novartis, and his team, called "reverse vaccinology" has been applied for first time to tackle *N. meningitides* group B (Men B) vaccine development (Pizza *et al*., 2000). Chiron's (at the beginning, Novartis later) scientists used the genome sequence determined in collaboration with Craig Venter and the Institute for Genomic research (TIGR, Waschington, DC) to predict 600 putative surface antigens. Three hundred and fifty of these were expressed in *Escherichia coli*, purified and analysed for their capacity of eliciting bactericidal

antibodies in the mouse model. Very recently, the concept of reverse vaccinology has been applied to virus as well, and all encoded proteins are being considered as potential antigens.

The licensed influenza vaccines in current use are inactivated virus vaccines created by growing virus in embryonated chicken eggs and subsequently purifying and inactivating them by chemical means. Each year, the World Health Organization (WHO) selects subtypes that are representative of strains currently circulating in humans. The efficacy of vaccines requires that the selected vaccine strains is sufficiently closely related to the circulating strains to ensure the induction of effective neutralizing antibodies.

The rapid evolution of Influenza A viruses complicates the effective use of vaccines, as any seasonal vaccine strains becomes quickly outdated. Moreover, large-scale production of vaccines in eggs is a lengthy, labor-intensive process that can take up to 6-9 months and that is heavily dependent on the availability of embryonated eggs, whose supply might be compromised in the case of pandemic viruses that cause widespread mortality in poultry. Therefore, alternative substrates, including mammalian cell lines, must be developed for the production of virus for use in vaccines.

Reverse genetics (RG) technology. The advances of reverse genetics techniques have been of great benefit to the development of alternative vaccine production systems. This technology has allowed the rescue of infectious influenza particles from tissue culture cells transfected with plasmids encoding each of the eight gene segments of the virus.

The first step in making a vaccine-seed virus by RG is converting pathogenic viral RNA to cDNA by reverse transcription. Then, cDNA is amplified by PCR and cloned into a rescue plasmid. In addition, for particular virulent strains, the "virulence associated" HA cleavage site can be changed to an a-virulent one, resulting in a virus that is attenuated for embryonated eggs (Horimototo *et al*., 2006).

In principle, this technology permits the construction of high yields 6:2 seed viruses by mixing 6 plasmid DNAs from a well growing laboratory strain with the HA and NA DNAs obtained by cloning the corresponding genes from currently circulating viruses. The great advantages of this approach is that the backbone of the 6:2 recombinant virus can be generated, tested and

distributed in advance. Moreover, RG allows the introduction into the viral genome of any modification that might improve the yield and the safety of the manufacture process.

2. AIMS

Reverse genetics (RG) technology makes it possible to generate influenza virus entirely from cloned plasmid DNA by co-transfection of appropriate cells with plasmids encoding the eight influenza genome segments. One commonly used system employs eight bi-directional transcription constructs, each of which uses an RNA polymerase I (polI) promoter to synthesize negative-sense viral RNA and an RNA pol II promoter to transcribe positive-sense mRNA from one viral cDNA template. It has been demonstrated that the eight-plasmid RG system efficiently generates influenza A virus in human embryonic kidney 293T cells and, to a limited extent, in African green monkey kidney (Vero) cells. 293T cells, however, are not currently approved for human vaccine production and, although Vero cells can be used to manufacture human vaccines, efficient rescue in these cells is hampered by their low plasmid transfection efficiency and their low productivity for many influenza strains. Therefore, it would be of great interest for human vaccine production to apply the RG technology to rescue influenza viruses in an approved cell substrate that can supports efficient influenza virus production.

The aim of this thesis is to set up a Reverse Genetics system for Influenza virus production in Madin-Darby canine kidney (MDCK) cells, a cell line that support the efficient growth of influenza viruses, already approved by the FDA for the production of seasonal and pandemic influenza vaccines.

In the second part of this thesis, we have used RG to generate recombinant viruses that differs solely in their HA receptor specificities.

Limited data suggest that receptor specificity and sialidase activity of influenza virus can affect innate immune responses to the virus and thus determine its pathogenicity. To test this hypothesis, we have studied how otherwise identical viruses with different receptor specificities can affect expression of genes and cellular responses in differentiated cultures of human airway epithelium and in isolated human immuno-competent cells.

3. MATERIALS AND METHODS

3.1 Cloning of Plasmids

The plasmid pKS10 was made on the basis of the published pHW2000 (Hoffman *et al.*, 2000), using the pUC19 vector as backbone. We introduced 225 bp of the human pol I promoter and 33 bp of the murine terminator sequence separated by two *Bsm*BI sites. The pol I promoter and terminator elements are flanked by a truncated immediate–early promoter of the human cytomegalovirus (CMV), Multiple Cloning Site (MCS), and by the polyadenylation signal of bovine growth hormone (BGH-polyA) (from pcDNA 3, Invitrogen) (Fig. 8 in results).

PKS10 vector was linearized using BsmBI restriction enzyme that cuts in asymmetric way outside of the recognition site allowing the possibility of the insertion of the fragments in the right orientation.

PKS 11 (Fig. 4) was obtained from pKS10 by substitution of human polI promoter with canine polI promoter (synthesized by GeneArt).

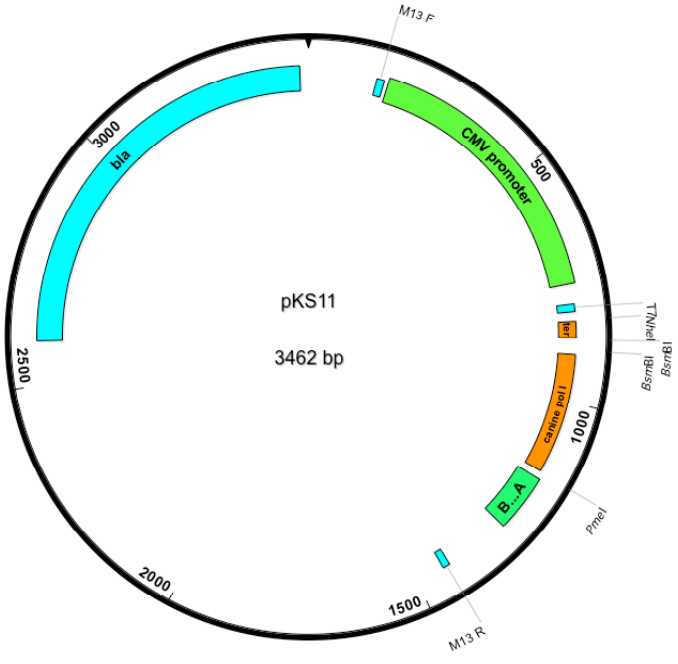


Figure 4 New engineered vector.

3.2 Viral RNA isolation and RT-PCR

RNA was isolated from the following Influenza virus strains:

- A/WSN/33 (H1N1),
- A/PR/8/34 (H1N1),
- A/New Caledonia/20/1999 (H1N1),
- A/Panama/2007/1999 (H3N2),
- A/New York/193/2003 (H3N2)
- A/VietNam/1194/2004 (H5N1),
- A/Wisconsin/67/2005 (H3N2)

by the QiaAmp Viral RNA Mini Kit (Qiagen), according to the manufactures's instructions. The isolated RNA was then reverse trancribed to generate cDNAs by ThermoScript RT-PCR system Kit (Invitrogen), according to manufactures's protocol, using the following oligos:
FLU 07: **AGCAAAAGCAGG,** a universal primer complementary to 3' end of all flu segments.
The cDNA was then amplified by PCR, using different segment specific primers, all of which with the consensus sequence of BsmBI enzyme at the 5' for the cloning into the RG plasmids. For cloning into pKS10, the following couples of oligos were used:

PB1

FLU 89F TTATTCGTCTCAGGGAGCGAAAGCAGGCAAACCATTT (Fwd 5')
FLU 90R TATATCGTCTCGTATTAGTAGAAACAAGGCATTTTTT (Rev 3')

PB2

FLU 95 TATTGCGTCTCAGGGAGCGAAAGCAGGTCAATTATATTC (Fwd 5')
FLU 96 ATATGCGTCTCGTATTAGTAGAAACAAGGTCGTTTTT (Rev 3')

PA

FLU 87 TATTCGTCTCAGGGAGCGAAAGCAGGTACTGATCC (Fwd 5')
FLU 88 ATATCGTCTCGTATTAGTAGAAACAAGGTACTTTTT (Rev 3')

NP

FLU 93F ATATTGCGTCTCAGGGAGCAAAAGCAGGGTAGA (Fwd 5')

FLU 94R TATATGCGTCTCGTATTAGTAGAAACAAGGGTATTTT (Rev 3')

M

FLU 79 TATTCGTCTCAGGGAGCAAAAGCAGGTAG (Fwd 5')

FLU 80 ATATCGTCTCGTATTAGTAGAAACAAGGTAGTTTTTT (Rev 3')

NS

FLU 77 TATTCGTCTCAGGGAGCAAAAGCAGGGTG (Fwd 5')

FLU 78 ATATCGTCTCGTATTAGTAGAAACAAGGGTGTTT (Rev 3')

HA

FLU 83F TTATTCGTCTCAGGGAGCAAAAGCAGGGG (Fwd 5')

FLU 84R AATATCGTCTCGTATTAGTAGAAACAAGGGTGTTTT (Rev 3')

NA

FLU 81F TAATTCGTCTCAGGGAGCAAAAGCAGGAGT (Fwd 5')

FLU 82 ATATCGTCTCGTATTAGTAGAAACAAGGAGTTTTT (Rev 3')

For cloning into pKS11:

PB1: FLU 89F and

FLU 206 TATATCGTCTCGAGGTAGTAGAAACAAGGCATTTTTT (Rev 3')

PB2: FLU 95 and

FLU 209 ATATGCGTCTCGAGGTAGTAGAAACAAGGTCGTTTTT (Rev 3')

PA: FLU 87 and

FLU 204 AATATCGTCTCGAGGTAGTAGAAACAAGGTACTTTTT (Rev 3')

NP: FLU 93F and

FLU 208 TATATCGTCTCGAGGTAGTAGAAACAAGGGTATTTT (Rev 3')

M: FLU 79 and

FLU 202 AATATCGTCTCGAGGTAGTAGAAACAAGGTAGTTTTTT (Rev 3')

NS: FLU 77 and

FLU 201 AATATCGTCTCGAGGTAGTAGAAACAAGGGTGTTTT (Rev 3')

HA: FLU 83F and

FLU 200 AATATCGTCTCGAGGTAGTAGAAACAAGGGTGTTT (Rev 3')

NA: FLU 81F and

FLU 203 AATATCGTCTCGAGGTAGTAGAAACAAGGAGTTTTT (Rev 3')

In red is indicated the sequence corresponding to BsmBI site, in green the one complementary to the specific segment. Once the fragments were obtained, they were isolated by gel electrophoresis, digested using BsmBI and cloned into the vector pKS10 or pKS11. In many case it was necessary to facilitate the cloning, to use a supporting vector, pGEMT-easy. This plasmid contains a poly(T) end that ligates the poly(A) end of the fragments, generate by the polymerase used for the PCR. The cloned segment was then cut-out by BsmBI digestion and sub-cloned into the final vector.

To ensure that the viral fragments derived by RT-PCR amplification and cloned into the RG plasmids were the corrected ones, they were tested by restriction analysis and then sequenced to exclude the presence of mutations.

3.3 Generation and rescue of recombinant viruses

All cell lines were grown at 37°C in 5% CO_2-air and were periodically passaged as soon as they reach 80-90% of confluence. Only low passage cells were used for our experiments.

Recombinant viruses were generated by DNA transfection. The day before 10^6 Embryonic human kidney cells (293T) and $0{,}2x10^6$ Madin Darby canine kidney (MDCK) cells per well, were plated in 6 well-plates (previously treated for 5 min at 37°C with PBS/gelatin 0,1% to promote adhesion) in complete medium (DMEM/10% FCS/1% PSG).
Cells medium was renewed after 24. For transfection assay 1μg each of the eight FLU plasmid were incubated with Lipofectamine 2000 Reagent (Invitrogen) following the manufacture's indications and put drop by drop on plated cells. After 18 hours medium was replaced with DMEM/0,2% BSA/TPCK-trypsin (1:4000) and at 24 and 48 hours post transfection supernatant was collected and titrated.

3.4 Titration assay

15.000 MDCK cells/well were seeded in 96-well flat plates in MEM/3% FCS/1% PSG, 24 h before titration.
The medium was replaced with MEM/1% PSG/ TPCK-trypsin (1:4000) and 10x of viral suspension stocks were inoculate for eight replicas, to be passaged in 10 fold serial dilutions. Cells were grown at 37°C. After 72 hours we evaluated at the microscope the number of wells in which there are lysed MDCK cells and calculated the $TCID_{50}$ (tissue culture infectious dose 50) according to Spearman-Kärber method (Finney, 1978).

3.5 Growth of viruses

To expand the virus, MDCK cells were grown to 80% confluence (about $7x10^6$) on 75 cm^2 flasks. At the moment of infection, growth medium was replaced with MEM/1% PSG/ TPCK-trypsin (1:4000) plus virus at multiplicity of infection (m.o.i.) of 10^{-4}. At 24, 48 and 72 hours supernatant was collected and clarified of cellular debris by low speed centrifugation. Virus was then concentrated by ultracentrifugation (Beckman centrifuge, SW40 tubes) at 28.000 rpm for 4 hours at 4°C through a cushion of 20%(w/v) sucrose solution prepared in TNE (0,02M Tris-HCl pH8, 150mM NaCl, 2mM EDTA). The pellet was resuspended in 0,5 ml of PBS and titrated.

3.6 GFP assay

To ensure the functionality of the cloned canine polI promoter and of the viral genes PB1, PB2, PA and NP cloned from human isolates, we performed a green fluorescent protein (GFP) assay.

$0{,}8x10^6$ /well MDCK cells were plated in complete medium in 6 well plate. The assay was performed as the transfection assay. We co-transfected the plasmids as indicated in the different experiment with one that contains the polI promoter and GFP cloned in the negative orientation flanked by 5' and 3' non coding regions from Influenza A. As positive control we used pMaxGFP (Amaxa), a plasmid that contain the GFP gene under the control of a CMV promoter. The fluorescence was detected under a fluorescence microscope (ZEISS instrument, FITC filter, objectivies 20x, AxioVision 4.6 software).

3.7 Natural Killer (NK) cell Preparation and Cultures

Peripheral blood mononuclear cells (PBMC) were separated from buffy coats of healthy donors over a Ficoll-Paque™ PLUS gradient (GE HEALTHCARE), followed by 1h incubation in plastic flask to remove adherent monocytes and incubated with irradiated EBV^+ RPMI 8866 lymphoblastoid cell line as described previously (Perussia *et al*., 1987). Cultures were collected at days 10. NK cells (>98%, stained $CD56^+/CD3^-$ (BD Biosciences)) were purified by depletion of the magnetically labeled $CD3^+$ cells according to protocol. Beads and Separation Columns are MACS® (Miltenyi Biotec) products. NK cells were maintained in culture in complete medium (RPMI 1640/10% FCS/1%PSG) plus IL12 10^2U/ml.

3.8 Virus infection of P815 cells

P815 (a mouse lymphoblast-like mastocytoma cell line) cells were infected with R1 (HA from original A/Hong Kong/1/68 (H3N2) (HK/68)) or R2 (HA mutated at positions 226 and 228 (L226Q and S228G)) viruses in medium without serum (RPMI 1640/1% PSG/TPCK-trypsin 1:4000) at multiplicity of infection (m.o.i.) of 0,5 at 37°C for 30 min. After this step P815 cells were washed in complete medium (RPMI 1640/10% FCS/1%PSG) and plated in 48 well

plate at 37°C for 16 h.

Staining of cells:

P815 R1, R2 infected or non infected cells were fixed with PBS/4% PFA at 4°C for 15 min.

Abs 1° (20 min RT) anti M/NP (Imagen™ Influenza Virus A and B, OXOID) FICT-labeled antibody on permealized cell (PBS/ 1%BSA/ 0,5% Saponin)(1:100 final concentration), or
goat H3N2 antibody on permealized cell (PBS/ 1%BSA/ 0,5% Saponin)(1:100 final concentration), or
goat H3N2 antibody on not permealized cells.

Abs 2° (20 min RT) α goat FITC (1:10 final concentration) only on goat H3N2 treated cells.

Cells were then analyzed by FACSCalibur™ (Becton Dickinson) cytometer (Cell Quest 3.3 software).

3.9 Neuroaminidase treatment for NK cells

NK cells were treated with a mixture of Neuroaminidase from *Vibrio Cholare* (0,03U/ml final conentration) and *Arthrobacter ureafaciens* (0,003U/ml final concentration) (Roche) for 1 hour at 37°C, then washed in complete medium (RPMI 1640/10% FCS/1%PSG).

Staining of cells:

NK neuroaminidase treated or not, were incubated with:

Lectin (1h at 4°C) digoxigenin-labeled lectins *Sambucus nigra* agglutinin (SNA; 0,6 μg/ml) wich recognize SA α2,6 Gal moieties, or
digoxigenin-labeled lectins *Maackia amurensis* agglutinin (MAA; 1 μg/ml) wich recognize SA α2,3 Gal moieties (Roche) or
PBS alone.

Abs (1h at 4°C) HPR labeled anti digoxigenin Abs (1:200 final concentration, Roche).

Cells were then analyzed by FACSCalibur™ (Becton Dickinson) cytometer (Cell Quest 3.3 software).

3.10 Cell-mediated Cytotoxicity Assays

NK cell killing of P815 (a mouse lymphoblast-like mastocytoma cell line) was determined using a flow cytometric assay for NK cell killing developed by McGinnes *et al.*, 1986 with slight modifications. Briefly, P815 cells (infected or not infected) were loaded with 5 μM CFDA SE in complete medium (RPMI 1640/10% FCS/1%PSG) for 30 min at 37°C. Then a fixed number of target cells were incubated in 96-well U-bottom plates with NK cells at the indicated NK/target ratios for 4 h at 37°C. Using a FACSCalibur™ cytometer (Becton Dickinson) fluorescent P815 cells were acquired for a fixed length of time and the percentage of killing was calculated using the following equation:
(number of CFDA SE^+ cells acquired in the control without NK cells)−(number of CFDA SE^+cells in the sample)/(number of CFDA SE^+cells in the control) x100.

3.11 Infection and BPL inactivation of HTBE cells

Matrosovich's lab provided supernatant of infected human tracheobronchial epithelial cells (HTBE) make as described in Matrosovich *et al.*, 2004.
To obtain β propiolactone (BPL) inactivated virus supernatants, pre-diluted BPL (Ferrak Chemie) in H_2O (final dilution 1:100) was added to virus preparation at a final concentration of 0.05%. Mixed samples was keeped at the inactivation temperature (4°C) for 16-24 h. Hydrolysis of any residual BPL was reached by elevating the temperature to 37°C for 3 h.

3.12 Multiplex cytokines assay

Cytokine's concentrations in the supernatant of HTBE cells rHK-R1, rHK-R2, rHK-5aa, rHK-7aa infected cells have been determined using the Bio-Plex® Cytokine Assay (human 27-Plex and human 23-Plex, Bio-Rad). Bio-Plex Cytokine assay are multiplex bead-based assay designed to quantify multiple cytokines in diverse matrices in a single microplate well. Bio-Plex assays contain dyed beads conjugated with monoclonal antibodies specific for a target cytokine. Each of the 100 spectrally addresses bead sets can contain a capture antibody specific for a unique target cytokine. The antibody-conjugated beads are allowed to react with

sample and a secondary, or detection, antibody in a microplate well to form a capture sandwich immunoassay. The constituents of each well are drown up into the flow-based Bio-Plex array reader, wich illuminates and reads the sample. When a read diode classification laser (635nm) in the Bio-Plex array reader illuminates a dyed bead, the beat's fluorescent signature identifies it as a member of one of the 100 possible sets. Bio-Plex Manager 4.1 software correlates each bead set to the assay reagent that has been coupled to it. In this way the Bio-Plex system can distinguish between the different assays combined within a single microplate well. A green reporter laser (532nm) in the array reader simultaneously excites a fluorescent reporter tag (PE) bound to the detection antibody in the assay. The amount of green fluorescence is proportional to the amount of analyte captured in the immunoassay. Extrapolating to a standard curve allows quantification of each analyte in the sample.
We have determined cytokines concentration as quadruplicate of each sample following the manufacture's indications.

3.13 RNA labeling, microarray hybridization, data acquisition and analysis

RNA was extracted from the virus particles using RNeasy Mini Kit (Qiagen). All the labeling reactions and hydridizations were performed following the Two-Color Microarray-Based Gene Expression Analysis protocol (Agilent Technologies). Images were acquired using the ScanArray Express microarray scanner (Perkin Elmer).
Microarray images were first analyzed using the Feature Extraction 9.5 software (Agilent Technologies), and the data were then transferred to the BASE 1.2 database/analysis software (Saal *et al.*, 2002). For each spot, local background was subtracted and spot intensities were normalized by the mean fluorescence intensity for each channel. Spots with a signal-to-noise ratio ≤ 3 in both channel were filtered. The average intensity ratio of each spot from experimental replicates was estimated by geometric mean and the accuracy and statistical significance of the observed ratios were determining using the Student's t-test spots with less than two values in the same time point were considered "not found" and we assigned a Log_2 ratio of zero. Only genes having t-test p-values lower than 0.05 and average intensity ratios greater than 3 (Log_2 ratio ≥ |1.5|) in at least one time point were selected. Hierarchical clusterin was performed with TMEV 3.1 software (Saeed *et al.*, 2003) on the Log_2 ratio

transformed dataset applying the Euclidean distance matrix and the average linkage clustering method. Some genes appear more than once in clusters because they are represented by multiple unrelated probes in the Agilent 44k whole human genome array. Functional analysis was performed using Gene Ontology (GO) and Ingenuity Pathway Analysis (IPA).

4. RESULTS

4.1 REVERSE GENETICS

The study of viruses and their interactions with host cells and organisms has benefited greatly from the ability to engineer specific mutations into viral genomes, a technique known as Reverse Genetics (RG). Positive-strand RNA virus genome manipulations were the first to be performed, partly because the viral genome is also mRNA sense. In this case, the simple transfection into susceptible cells of plasmids, or of RNA transcribed from plasmids, containing positive-strand RNA virus genomes, results in the recovery of infectious virus.

On the contrary, the genomes of the negative-strand RNA viruses have been less amenable to artificial manipulation for several reasons; first, precise 5' and 3' ends are required for replication and packaging of the genomic RNA; second, the viral RNA polymerase is essential for transcribing both mRNA and complementary, positive-sense antigenome template RNA; third, both genomic and antigenomic RNAs exist as viral ribonucleoprotein (RNP) complexes.

As previously described, the Influenza A is a segmented, single strand, negative sense RNA virus. Therefore, introduction of the genomic RNAs into cells could not result in the formation of infectious virus. In fact, to generate viral proteins and particles, the negative-sense viral RNAs (vRNAs) of influenza A viruses (IAV) need first to be transcribed into positive sense mRNA molecule, a function that can only be performed by the viral polymerase complex. (Fig. 5).

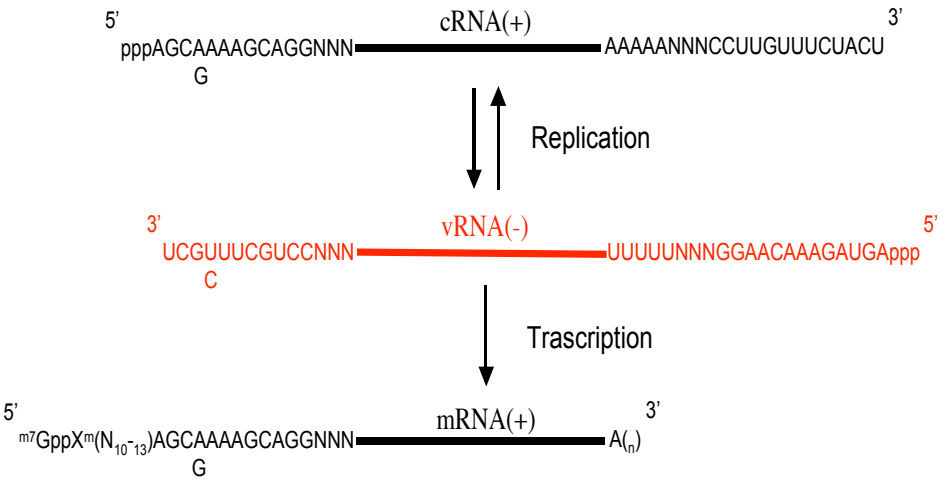


Figure 5 Cycle of viral RNA. When vRNAs molecules encapsidated with the four viral polymerase complex proteins (RNPs: PB1, PB2, PA and NP) penetrate the cell nucleus, the associated proteins begin to transcribe vRNAs into mRNAs (who gives rise to the viral proteins) and cRNAs positive sense used as a template to generate more negative sense vRNA and continue the cycle (adapted from Palese and Shaw, 2006).

On these bases, the first RG system made for the Influenza A virus, put right by Palese and colleagues, presumed the transfection of eukaryotic cells with RNP complex generated by *in vitro* vRNA synthesis in the presence of purified polymerase and NP proteins (Luyties *et al.*, 1989; Enami *et al.*, 1990). Afterwards Neumann *et al.*, developed a method based on transfection of cells with plasmids that contains cloned influenza virus cDNAs flanked by RNA polymerase I promoter and terminator (that allowed the reconstitution of RNP complexes). Both these approaches needed a subsequent infection with a helper virus which provided the remaining viral proteins and RNA segments, resulting in the generation of infectious viruses (Neumann *et al.*, 1994; Flick *et al.*, 1996; Zhou *et al.*, 1998) (Fig. 6).

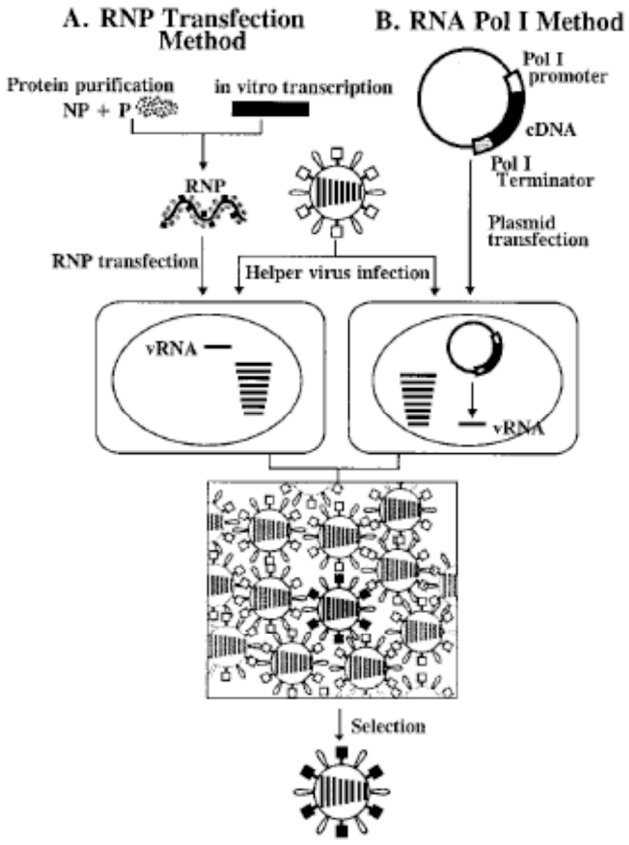


Figure 6 Schematic diagram of established reverse genetics systems. In the method *A*, cells are transfected with RNPs assembled with *in vitro* synthesized vRNA and purified NP and polymerase proteins; then infection with helper virus follows. In the method *B*, cells are transfected with a plasmid carrying an RNA polymerase I promoter, a cDNA encoding the vRNA to be rescued, and the RNA polymerase I terminator. Intracellular transcription by RNA polymerase I yields synthetic vRNA, which is packaged into progeny virus particles upon infection with helper virus. With both methods, transfectant viruses (i.e., those containing RNA derived from cloned cDNA) need to be selected from the helper virus population (adapted from Neumann *et al.*, 1999).

The disadvantage of these previous systems was related to the need for a helper virus, which must be selected against in order to isolate the rescued virus.

Approximately a decade after the birth of reverse genetics system, Fodor *et al.* and Neumann *et al.*, reported the generation of influenza viruses entirely from cloned cDNAs. Fodor's team used a mixture of eight plasmids in which individual cDNAs were inserted between a human polymerase I promoter (polI) sequence and a genomic ribozyme sequence of hepatitis δ virus (to ensure the precise 5' and 3' end of the vRNA) and four plasmids expressing PB1, PB2, PA, and NP under the control of the adenovirus type 2 major late promoter (Fodor *et al.*, 1999).

Finally Hoffmann and colleagues developed a system in which only eight plasmids were required. In this system a plasmid named pHW2000, was engineered with a human RNA polymerase I promoter and a mouse RNA polymerase I terminator, respectively at 5' and 3' ends. Externally to these elements, a cytomegalovirus (CMV) polymerase II promoter and the polyadenylatation site of the gene encoding bovine growth hormone (BGH) were inserted at the 5' and 3' ends. The cDNAs segments derived from vRNA sequences were cloned between promoters and terminators, in the negative orientation relatively to the polI promoter (Hoffmann *et al*., 2000) (Fig. 7). In this way, while the polI promoter gives rise to the viral RNA negative sense (-vRNAs) that contains the non coding regions (NCR) at 5' and 3', the CMV promoter drives the transcription of 5' cap and 3' poly-A tailed mRNA, then translated into viral proteins. As results of tranfection into eukaryotic cells, they got the formation of infectious virus derived entirely from only eight plasmids (Fig. 7).

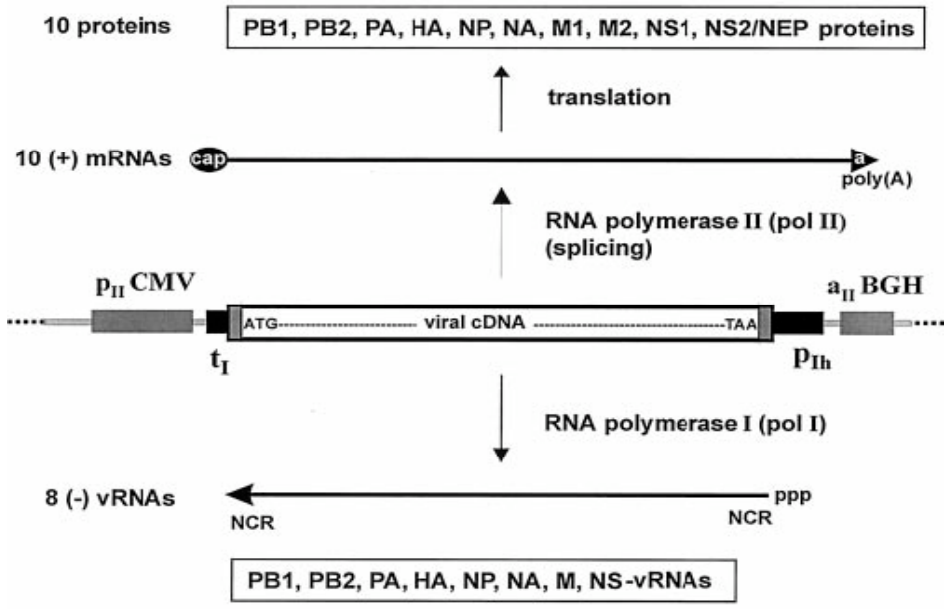


Figure 7 The polI–polII transcription system. The cDNA corresponding to each of the eight influenza proteins was inserted between the pol I promoter (p_{Ih}), a mouse polI terminator (t_I), a polII promoter from CMV and a polyadenylation signal (a_{II} BGH) (adapted from Hoffmann *et al*., 1999).

As RG allows the generation of infectious viral particles starting from cloned cDNA, it is a powerful technique to explore the biological effects of mutations that can be easily introduced

into the cloned viral sequences.

The aim of my thesis was to explore the use of the reverse genetics technology for the generation and production of new Influenza vaccines.
In particular, we wanted:

- to set up a reverse genetics system that could work in MDCK cells, the canine cell line of choice for vaccine production;
- to identify influenza virus strains with optimal growth characteristics in MDCK cells which could then be used as backbone for seed virus production using RG;
- to create a library of HAs and NAs molecules from both seasonal and potentially pandemic strains by cloning these molecules into the RG rescue plasmids;
- to test the compatibility of these HAs and NAs molecules for the generation of functional viral particles;
- to test the effects of single aminoacid sostitutions in the HA molecule on virus-host interactions.

4.1.1 Establishment of the Reverse Genetics System

To set up the RG system, we first developed a rescue plasmid, named pKS10. As the Hoffmann's pHW2000 plasmid, our engineered vector contains the human polI and CMV polII promoters, a mouse terI terminator and a polyadenylation site. A double BsmBI restriction site was introduced between the pol I promoter and the terminator sequence, to allow proper cloning of the viral segments (Fig. 8).

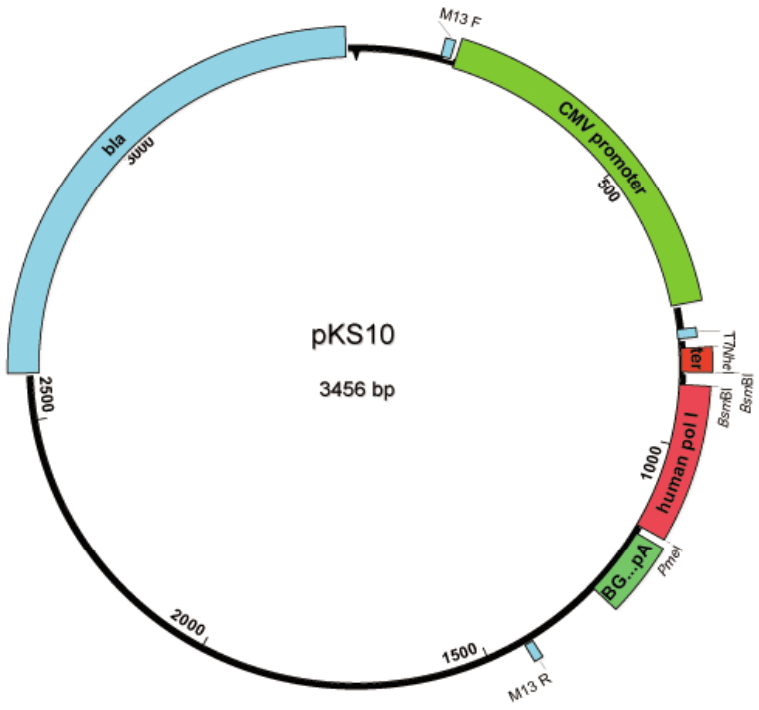


Figura 8 A representation of our rescue vector.

As starting point for the establishment of our RG system, we chose two influenza virus strains:

- A/WSN/33 (H1N1), a high yield donor strain, widely used in animal studies;
- A/PR/8/34 (H1N1), a strain well adapted to growth in embryonated chicken eggs and currently used as the master strain for the production of inactivated vaccines.

After RNA extraction from viral particles, we obtained the cDNAs by RT-PCR (details in Materials and Methods). The primer chosen for the in vitro reverse transcription, FLU 07, has the following sequence: **AGCAAAAGCAGG**. It represents an "universal primer" for all eight flu segments, as its sequence is complementary to the very 3'end of the (-)ve sense vRNAs, a sequence present in all eight influenza A segments. Then, using a specific set of two primers for each influenza segment, we amplified from cDNA the eight viral segments. As the primers were designed to have BsmBI sites at the 5' ends, we subsequently digested the amplified DNA with BsmBI and ligated them into pKS10. The BsmBI restriction enzyme was chosen because it cuts outside its recognition site, in an asymmetric fashion on the two DNA strands, thus allowing a precise insertion of the viral fragments downstream of the polI promoter,

without the addition of any exogenous nucleotide that would hamper their recognition by the viral polymerase (Fig. 9).

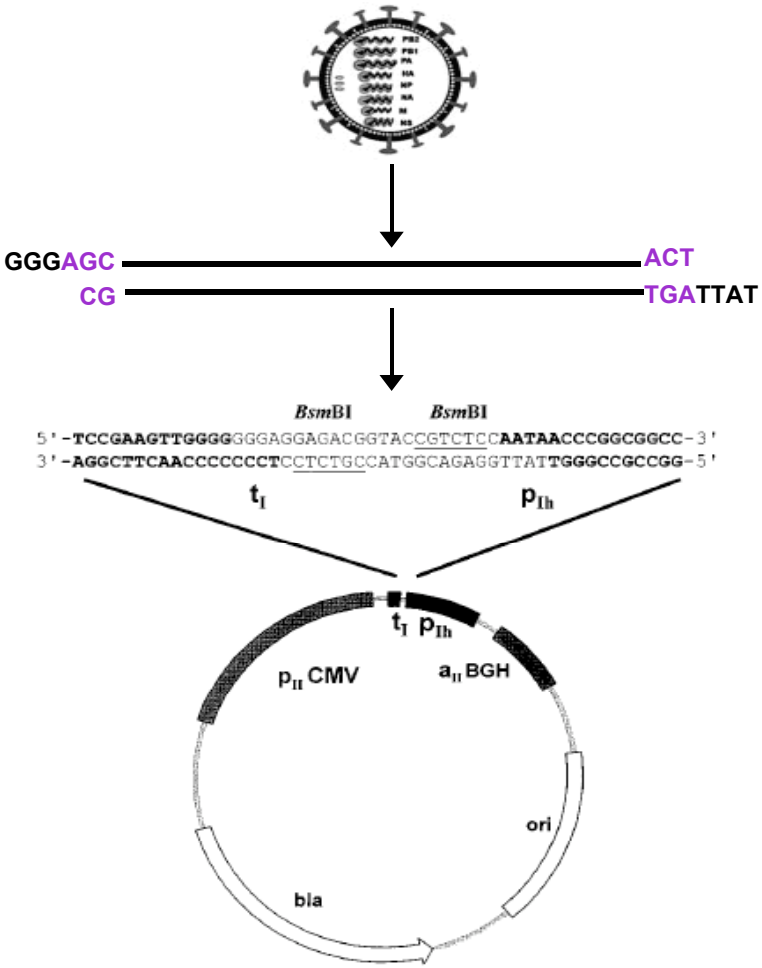


Figura 9 Schematic representation of our cloning strategy (adapted from Hoffmann *et al.*, 1999).

The colonies were first screened by restriction digestions and then sequenced to exclude to presence of unwanted mutations generated by the PCR reaction.

In this way, we cloned the eight viral segments PB1, PB2, PA, NP, NS, M, NA and HA from A/PR/8/34 (H1N1) and A/WSN/33 (H1N1). In addition, we cloned the fragments encoding HA and NA from the following series of influenza strains, some of which have been used in recent years for vaccine preparation:

- A/New Caledonia/20/1999 (H1N1)
- A/Panama/2007/1999 (H3N2)

- A/New York/193/2003 (H3N2)
- A/VietNam/1194/2004 (H5N1).

4.1.2 Generation of reassortant viruses

Once cloned all the eight Flu proteins, we used these plasmids to transfect a co-culture of 293T and MDCK cells. The presence of the human embryonic kidney 293T cells is related to the species-specificity of the polI promoter (Heix and Grummt, 1995), that requires the use of cells line derived from humans, as 293T, or monkeys, as the African green monkey kidney cells Vero. However, 293T are not currently approved for human vaccine production and Vero cells have low productivity for many influenza strains. On the contrary, the canine cell line MDCKs support the efficient growth of influenza viruses and is currently used to produce seasonal and pandemic influenza vaccines (Fig. 10).

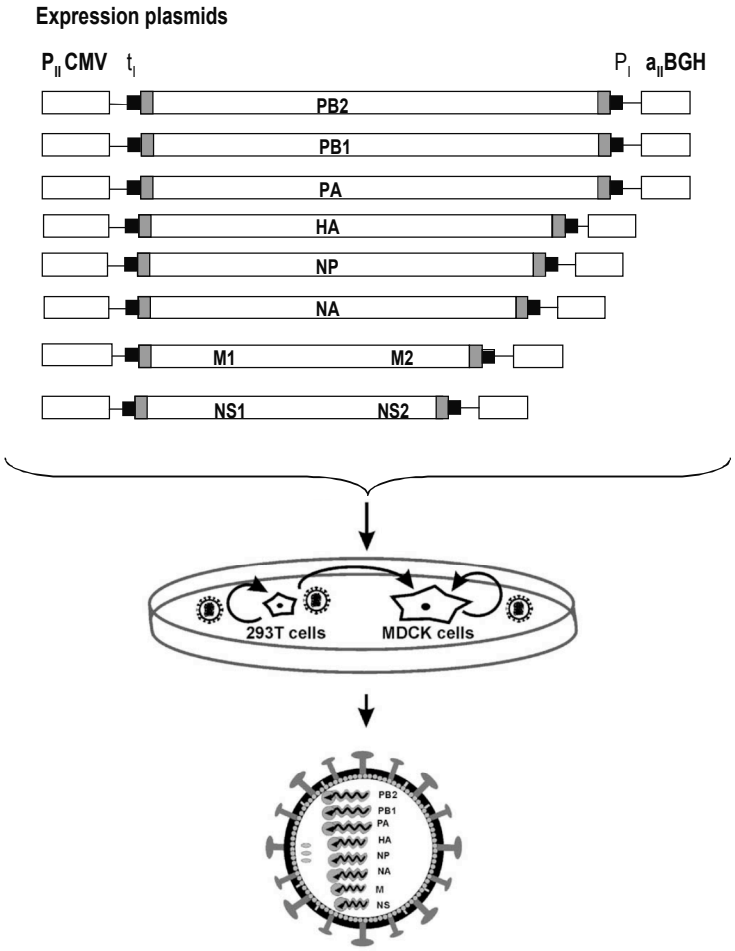


Figure 10 Rescue of RG virus. A co-culture of 293T and MDCK cells is transiently transfected by permeabilization with lipofectamine, enabling the eight plasmids carrying the viral cDNAs to penetrate the cells. Viruses rescued in the 293T cells after transfection are fully infectious and can then penetrate MDCK cells and expand (adapted from Hoffmann *et al*., 2002).

The first virus that we rescued by transfection was the wild type A/WSN/33 (H1N1). The choice of this strain as reference strain in our experiments, was dictated by its characteristics of high productivity. After transfection, supernatants from the colture were collected at different time-points, and titration assays were performed to measure the infectivity of the virus (Fig. 11) (for details, see Materials and Methods).

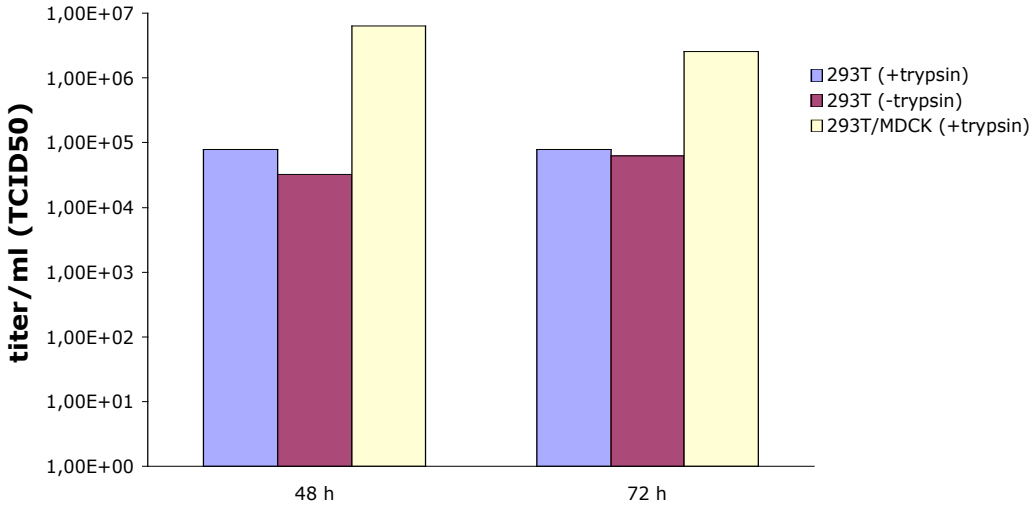


Figure 11 Eight plasmids containing the eight different viral segments were used to transfect 293T cells in the presence or absence of trypsin. The same plasmids were also used to transfect a co-culture of 293T/MDCK cells. The virus was harvested at 48 and 72 hours, and the virus titer determined as $TCID_{50}$ (tissue culture infectious dose 50, see Materials and Methods) on MDCK cells.

To define the ideal conditions for the rescue, we performed the first transfections in different conditions. The same amounts of plasmids were used to transfect either 293T alone or a co-culture of 293T and MDCK, in the presence or absence of trypsin. The addition of trypsin to cultures in vitro is known to increase virus yields, as trypsin hydrolizes the HA molecule into two subunits, thus allowing virus fusion with host cell membranes. As expected, the titers were higher in co-cultured 293T/MDCK cells, in the presence of trypsin.

The subsequent step was the passaging of the rescued virus in MDCK cells to test the virus ability to propagate in culture (Fig. 12).

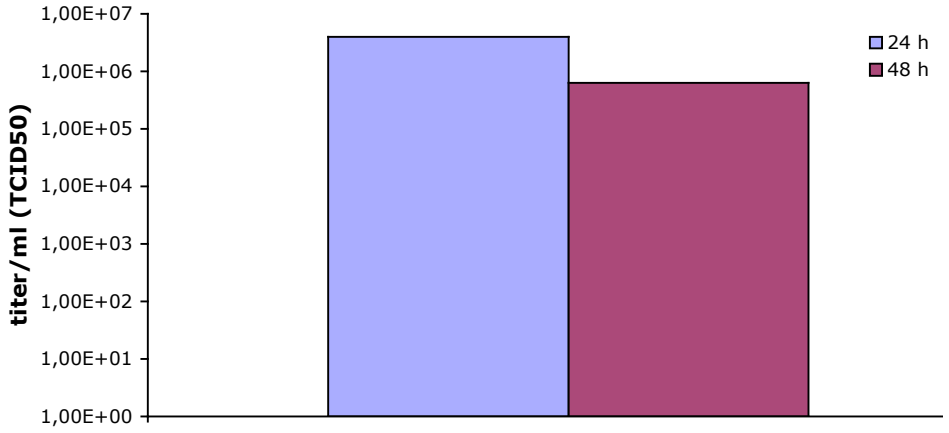


Figure 12 Supernatants from rescue experiments were used to infect naïve MDCK cells; at 24 and 48 hours post infection, supernatants were collected and titrated on MDCKs.

These results shown in Figure 12 demonstrated that an infectious, fully functional A/WSN/33 virus could be rescued in culture from transfected plasmids, and then serially passaged in MDCK cells to reach significant titers.

Once set the system for the wild type A/WSN/33, we try to generate reassortant viruses, in which each single viral segment from the A/WSN/33 strain was substituted by the corresponding segment from a different but closely related strain, the A/PR/8/34**.** The rescue of the A/WSN/33 wild type was done in parallel as control.

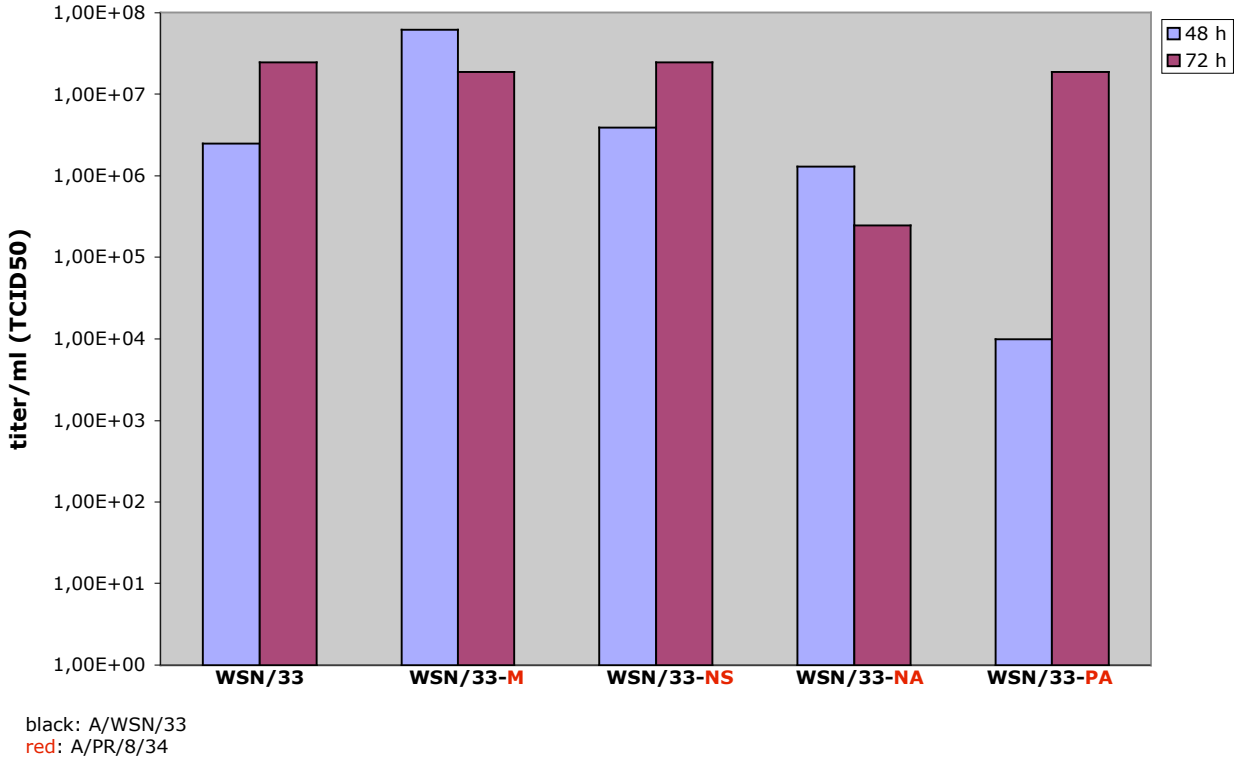


Figure 13 Rescue of reassortant viruses. In this case A/WSN/33 wild type is used as reference strain to compare reassorted viruses. Each reassortant was generated by replacement of the plasmids that contain the M, NS, NA or PA segments from A/WSN/33 with the corresponding segments from A/PR/8/. The A/WSN/33 wild type is used as reference strain. Viral titers were measured after 48 and 72 hours.

The data shown in Figure 13 clearly demonstrate that reassortant viruses carrying viral segments from different strains can be generated and propagated by RG. In the case of M and NS, the swapping of viral segment between two close strains did not have any impact on the virus titers, while in the case of PA and NA, the substitution caused a 1-2 log decrease and/or a delay in virus yields.

In these first experiments, we have used two related strains of viruses, both belonging to the H1N1 type. The strain A/New York/193/2003 is apart of the H3N2 type, evolutionary very distant from the H1N1 viruses. We cloned the HA molecule from this strain and attempted to rescue a reassortant virus with this HA segment and the other 7 segments derived from the A/WSN/33 virus. As shown in Figure 14, this reassortant virus could still be rescued, but its

ability to expand in culture was greatly hampered, as its titers were considerably lower than those of the reference A/WSN/33 wt, and never exceeded 10^3/ml (Fig. 14).

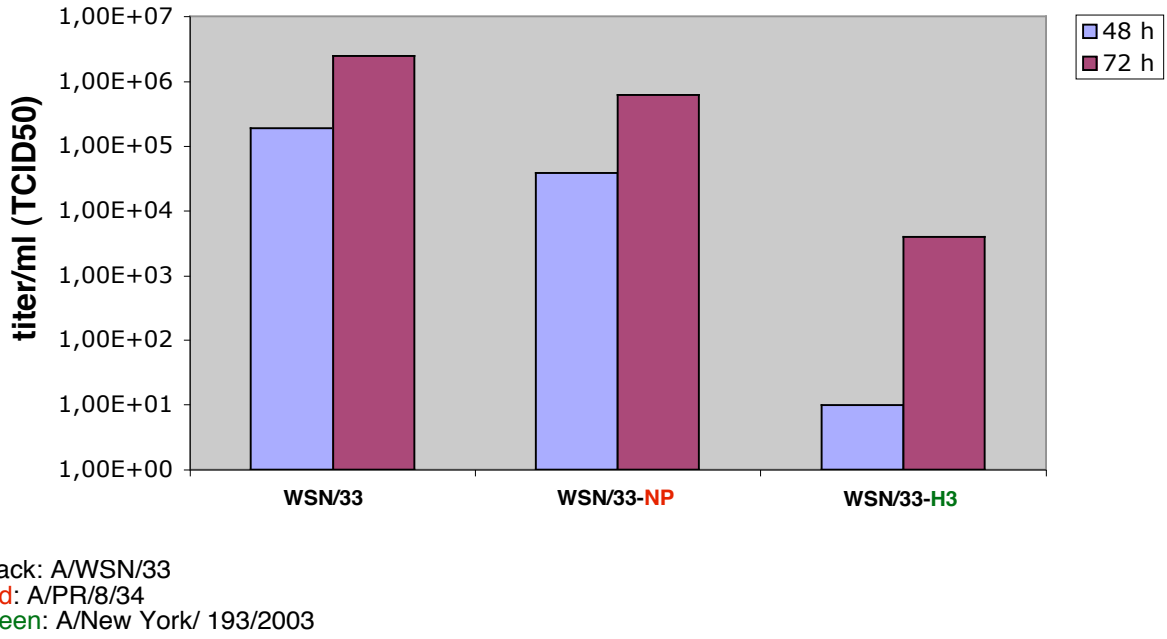


Figure 14 Reassortant viruses were generated by substitution of an internal protein as the nucleoprotein with the corresponding protein from A/PR/8/34 or by substitution of the native HA with HA from A/New York/193/2003.

Similar results were obtained when the A/WSN/33 HA was substituted with the HA molecule of a different H3N2 strain, the A/Panama/2007/1999 (Fig. 15), indicating that the rescued virus although infectious, is not fully functional. It is possible that the H3 and the N1 molecules are not compatible when expressed on the same virions, and indeed virus carrying this combination of molecules have been rarely isolated.

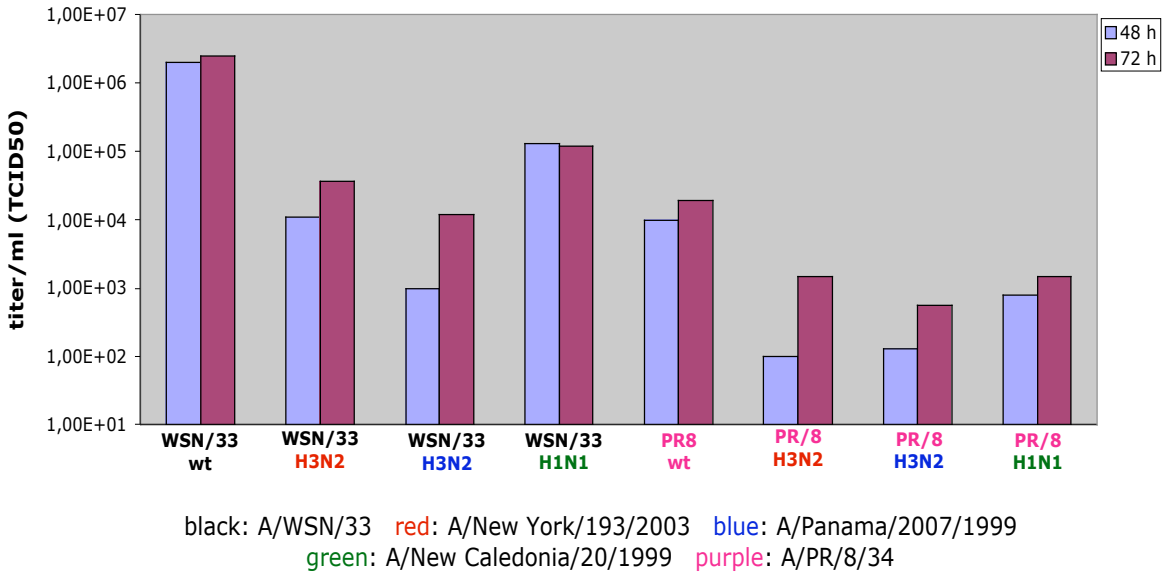


Figure 15 Rescue of reassortant viruses. A/WSN/33 and A/PR8/8/34 have been used as reference

In conclusion, we have developed an eight plasmid-based Reverse Genetics system that allows the rescue of viruses in human cells. This system can be used to create reassortant viruses, whose fitness and ability to expand in culture can be determined as virus yield.

4.1.3 RG vector for FLU production in MDCK cells

The Reverse Genetics system requires the use of a polymerase I promoter to generate precise vRNA molecules, as any additional nucleotide could not be tolerated by the viral polymerase. Since the pol I promoters are species-specifics and the canine MDCK cells are one of the few cell lines that have been licensed for influenza virus production, we tried to develop an RG system that could work in canine cells. Although the polI promoter sequences are not well conserved between different species, by comparing sequences upstream of rDNA, we have been able to identify in the canine genome a region that correlate with the human polI promoter that we have been used in the experiments described so far (Fig. 16).

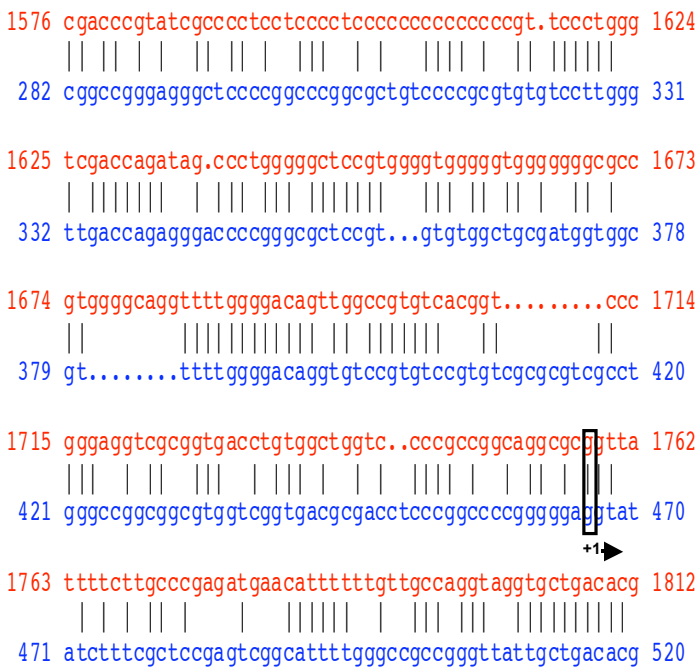


Figure 16. Comparison between a human, in blue, and a canine, in red, polI promoter sequences. The +1 indicates the hypothetical starting point for transcription.

Once identified this 230bp hypothetical minimal promoter, we tested its functionality. For this purpose, we cloned the GFP (green fluorescence protein) gene flanked by 5' and 3' non-coding regions (NCR) from Influenza A virus in the negative orientation under the control of the identified promoter. As positive control, we also cloned the GFP in the positive orientation under the control of a strong CMV promoter. We then used these construct to transfect MDCK cells, together with plasmid that can drive the expression of PB1, PB2, PA, the three components of Influenza virus polymerase, and of NP. We reasoned that, if our canine pol I promoter was active in MDCK cells, it should give rise to antisense transcripts of the GFP gene. These antisense molecules contain viral NCR at the 5' and 3' ends and therefore can be used as templates by the viral polymerase to generate positive sense transcrips that, in turn, can be translate into protein (Table 1; Fig. 17).

	A	B	C
PKS10/PB1	+	+	-
PKS10/PB2	+	+	-
PKS10/PA	+	+	-
PKS10/NP	+	+	-
Canine polI_GFP	-	+	-
pMAX_GFP	-	-	+

Table 1 The different combinations of plasmid used to transfect the MDCK cells. A, B and C refer to the imagines below.

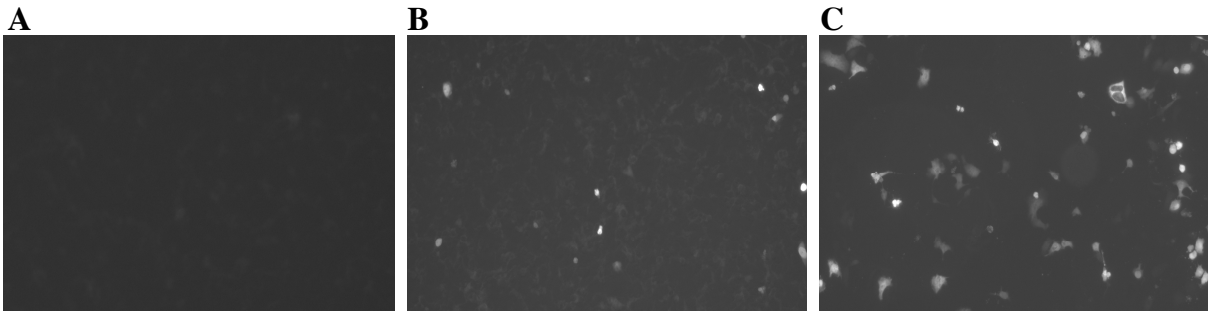


Figure 17 MDCK cells are transfected with the combination shown in table 1 and analyzed for GFP expression by florescence microscope at 48 hours post transfection.

A high percentage of cells transfected with the plasmid pMAX_GFP (C, positive control) were highly positive for GFP expression, indicating a good efficiency of transfection.

As expected, no positive cells were detected after transfection with the pKS10 plasmid driving the expression of PB1, PB2, PA and NP from WSN/33 under the CMV promoter (A, negative control). However, when these four plasmids were cotransfected with the plasmid carrying the GFP gene in the negative orientation under the polI promoter, we could detect fluorescent

cells (B). This result indicates that the identified sequences are recognized by the canine polI polymerase and can drive the transcription of downstream sequences.
After this evidence, we created a new rescue vector, by substituting the human polI promoter sequences with the canine polI promoter in our pKS10, and named this new plasmid pKS11 (Fig. 4 in Materials and Methods).

4.1.4 Development of a Reverse Genetics System for canine MDCK cells

Our original aim was to develop a Reverse Genetics system for MDCK cells that could be used for the generation of seed viruses for vaccine production.
For this purpose, we needed to identify Influenza virus strains with optimal growth characteristics in MDCK that could be used as donors of the 6 internal genes (PB1, PB2, PA, NP, M, NS) for the generation of highly efficient seed virus backbones. These 6 internal segments could then be combined with the HA and NA molecules from the new seasonal strains that emerge any year, as indicated by the WHO, to generate the desired seed virus.
In collaboration with Novartis Marburg we identified two strains from human clinical isolates:

- A/New Caledonia/20/1999 (H1N1; indicated as #105)
- A/Wisconsin/67/2005 (H3N2; indicated as #003)

that grow at high titers, over multiple passages, in MDCK cells.
We have previously demonstrated that reassortants between evolutionary distant strains can be hampered in their ability to propagate in culture. For this reason, we decided to develop two backbone systems, based on strains belonging to either the H1N1 or the H3N2 class, that are currently the influenza classes most widespread in the human population.
We cloned and sequenced all the viral segments from these two strains into the pKS11 plasmid , using the primers described in Materials and Methods.
An assay similar to the GFP assay (Table 2; Fig. 18) described before was then performed to test the functionality of the viral polymerase and of nucleoprotein.
In this experiment, we substituted one by one the three components of the viral polymerase and NP from WSN/33, that have been shown to work in RG, with the corresponding PB1, PB2, PA an NP fragments cloned from strain #003 or #105. The appearance of green fluorescence in the cells indicates the functionality of the cloned fragment.

	A	B	C	D	E	F	G	H
Canine polI_GFP_ter	+	-	+	+	+	+	+	+
pEGFP	-	+	-	-	-	-	-	-
pKS10/PB1 (WSN)	-	-	+	-	+	+	+	+
pKS10/PB2 (WSN)	-	-	+	+	+	+	+	+
pKS10/PA (WSN)	-	-	+	+	-	+	+	+
pKS10/NP (WSN)	-	-	+	+	+	-	-	-
pKS11/PB1 (#003)	-	-	-	+	-	-	-	-
pKS11/PA (#003)	-	-	-	-	+	-	-	-
pKS11/NP (#003)	-	-	-	-	-	+	-	-
pKS11/PA (#105)	-	-	-	-	-	-	+	-
pKS11/NP (#105)	-	-	-	-	-	-	-	+

Table 2 Different combinations of plasmids were used in to transfect MDCK cells. GFP expression was analyzed 48 hours post transfection. The letters refer to the picture below.

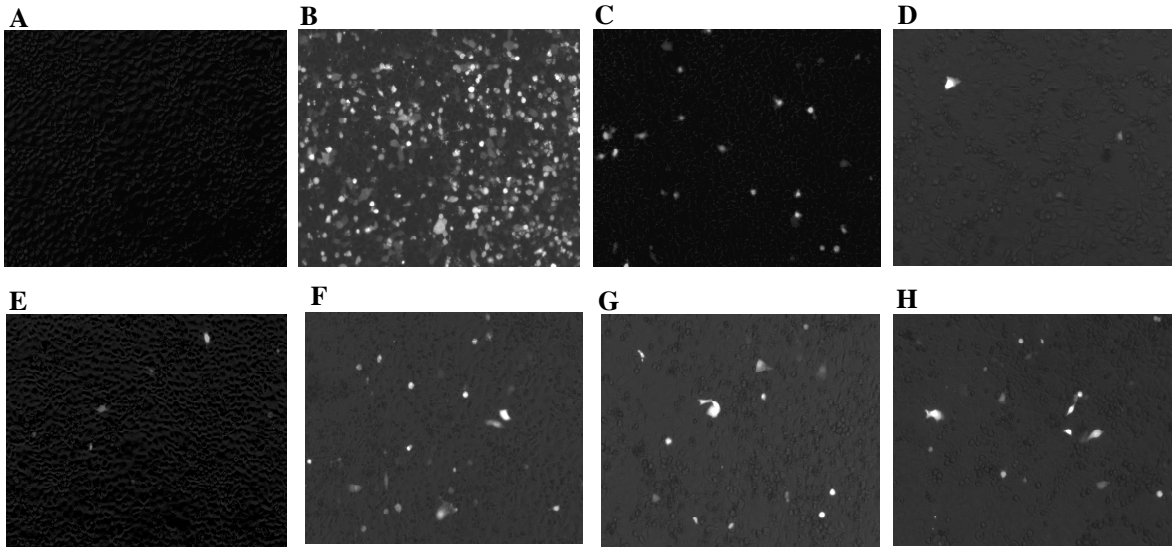


Figure 18 MDCK cells were transfected with the combination of plasmids shown in the table 2 and analyzed for GFP expression by florescence microscope at 48 hours post infection.

These cloned fragments were then used to transfect directly MDCK cells, allowing the rescue of infectious particle (Fig. 19)

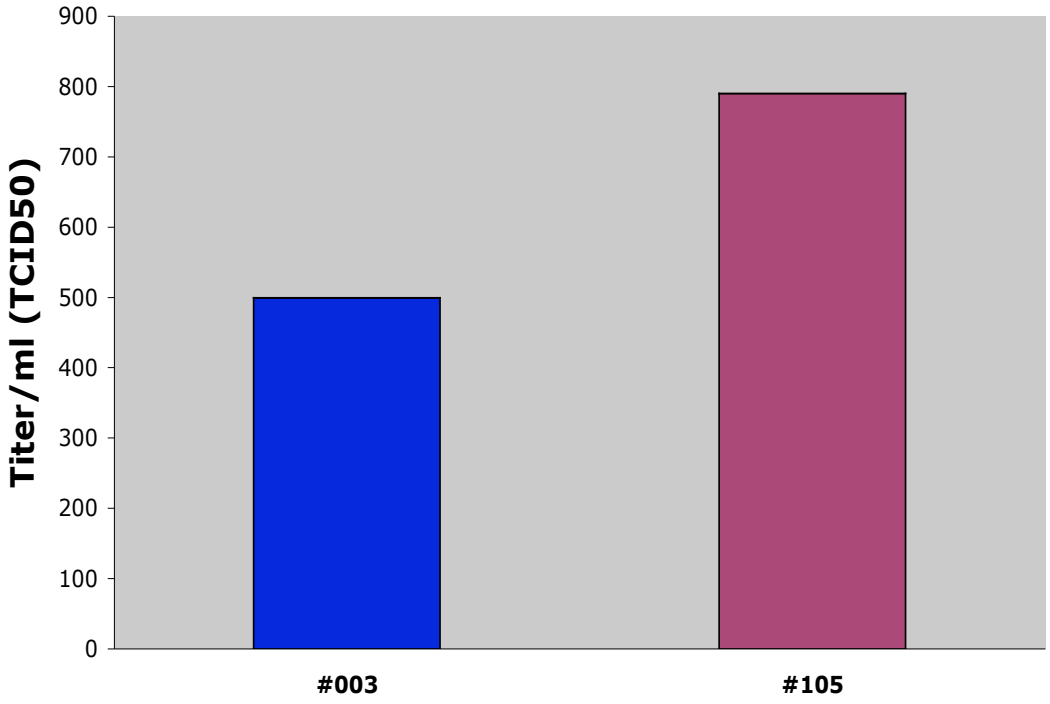


Figure 19 Rescue of reassortant viruses. In this we compare the titer between A/New Caledonia/20/1999(#105) and A/Wisconsin/67/2005 (#003) at 72 hours.

In conclusion, we have developed a Reverse Genetics System that would allow the quick generation of seed virus for vaccine production.

4.2 FLUINNATE

Novartis Vaccines & Diagnostics was part of an European network, FLUINNATE, whose general aim is to identify and characterize the essential viral and host factors that determine the outcome of infection, with regards to both viral replication fitness and host adaptation process and defense mechanisms. FLUINNATE deals with human, avian and swine influenza virus strains, some of which have been generated by Reverse Genetics (RG) entirely from plasmids. Single and multi-segment reassortant viruses have been produced and characterized with respect to growth kinetics, interferon response and disease potential. These projects will provide new information important for better understanding emerging influenza viruses and for generating efficient control measures against these devastating pathogens.

Limited available data suggest that receptor specificity and sialidase activity of influenza viruses can affect innate immune responses to the virus and thus determine its pathogenicity.
It is well known that Influenza viruses attach to target cells via interactions of the viral hemagglutinin protein with sialyloligosaccharide moieties of cellular glycoconjugates. As sialic acids are ubiquitously expressed on the surface of most avian and mammalian cells, in addition to infect susceptible cells, influenza viruses can bind to a variety of other cell types, including cell of the immune system, leading to significant biological responses. As part of the FLUINNATE project, my aim was to test if and how receptor specificity and sialidase activity can affect immune responses to the virus and thus determine its virulence and pathogenicity.
To address these questions, in collaboration with Mikhail Matrosovich at the University Of Marburg, we used RG to generate two viruses, R1 and R2, that share the six genes of the internal viral proteins of the laboratory virus strain A/WSN/33 (H1N1) and harbor the HA and NA genes from the human pandemic virus A/Hong Kong/1/68 (H3N2) (HK/68). These two viruses differ only for two aminoacids in the HA receptor-binding site: R1 contains the original HA molecule of the pandemic virus A/Hong Kong/1/68, while R2 contains mutations at HA codons 226 (a Serine-S- in human- and a Glycine-G- in avian-like viruses) and 228 (a Leucine- L-in human- and a Glutammine-Q- in avian-like viruses) that reverted the HA aminoacidic sequence (226L/228S) to the consensus sequence of the avian virus HA (226Q/228G). These mutations were chosen because of the essential role of amino acids in

position 226 and 228 for the HA-mediated receptor specificity and because it has been described that the human pandemic virus HK/68 emerged from an avian precursor (Matrosovich *et al.*, 2000) (Fig. 20).

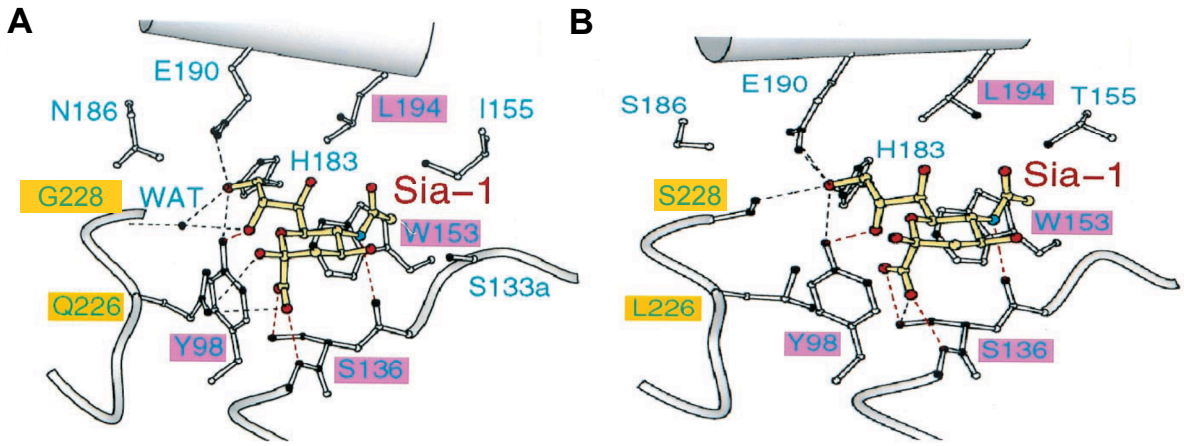


Figure 20 Conserved and variable interactions of avian-specific (A; A/Duck/Singapore/3/97 (H5N1)) and human-specific (B; A/Hong Kong/1/68 (H3N2)) influenza virus subtype HAs with sialic acid. Red highlighted areas, conserved residues; yellow highlighted areas, mutated residues; yellow backbone, sialic acid (Sia-1); red, oxygen; blue, nitrogen; filled HA atoms contact Sia-1; dashed lines, hydrogen bonds; red dashes, conserved hydrogen bonds (adapted from Ha *et al.*, 2001).

It is now well established that the presence of specific aminoacids in specific position of the hemaglutinin molecule determines both the host range and the cellular tropism of different influenza virus strains, as avian influenza viruses bind preferentially to cell-surface receptors containing terminal sialyl–galactosyl residues linked by 2–3-linkage [Neu5Ac(α2–3)Gal], whereas human viruses bind to receptors which contain terminal 2–6–linked sialyl–galactosyl moieties [Neu5Ac(α2–6)Gal] (Paulson, 1985; Nobusawa *et al.*, 1991; Connor *et al.*, 1994; Matrosovich *et al.*, 1997).

With the experiments that follows we explored whether small variations in the HA receptor-specificity could also influence the onset and the breath of the innate immune response to infection.

4.2.1 Natural Killer cells recognize and lyse P815 infected cells

HA molecules expressed on the surface of influenza virus-infected cells are involved in the functional recognition of infected cells by Natural Killer (NK) cells. In fact the natural cytotoxicity receptors (NCRs) NKp44 and NKp46, two glycosylated proteins expressed on the surface of NK cells, carry sialic acid residues that can bind HA molecule on the surface of infected cells, mediating their lysis by NK cells.

As we wanted to investigate how different HA/NA specificities can modulate innate immune, we first tested whether small alterations in HA specificity could modify the ability of NK cells to recognize and lyse infected cells. For this purpose, we performed cytotoxicity assay using as effectors highly purified human NK cells, and as target the P815 cell line, a mouse lymphoblast-like mastocytoma cell line.

To perform these experiments we first rescued the R1 and R2 viruses described above by reverse genetics and then expanded and titrated these viruses as described in Materials and Methods.

Purified NK cells were obtained as described in Materials and Methods. Briefly, PBMCs (Peripheral Blood Mononuclear Cell) were separated from peripheral blood by Ficoll-Paque density gradient centrifugation and then incubated with RPMI8866, a human B cell line usually used as a feeder cell line for the in vitro expansion of NK cells. After 10 days, NK cells were purified by depletion of the magnetically labeled $CD3^+$ cells (after depletion cells were >95% $CD56^+$, $CD3^-$, $CD19^-$, $CD14^-$).

Target cells P815 were infected with the R1 or R2 viruses (m.o.i. of 0.5), or mock infected. After an overnight incubation to allow expression of viral proteins, both infected and non infected cells were labeled with CFDA SE (Carboxyfluorescein diacetate, succinimidyl ester), a compound that enters into the cells by diffusion and is then cleaved by intracellular esterase enzymes to form an amine-reactive product. This product gives a detectable fluorescence that can not any more diffuse through the plasma membrane, as it covalently binds to intracellular lysine residues and other amine sources. We then incubated the NK cells (effector) and the P815 cells (target) at different effector/target ratios for 4 hours, with the number of target cells kept costant in every condition. The amount of lysis was determined as disappearance of fluorescent cells using the following equation: % of lysis = (the number of CFDA SE^+ cells

acquired in the control without NK cells) – (the number of CFDA SE$^+$cells in the sample)/(the sample of CFDA SE$^+$ cells in the control) x 100 (McGinnes *et al.*, 1986) (Fig. 21).

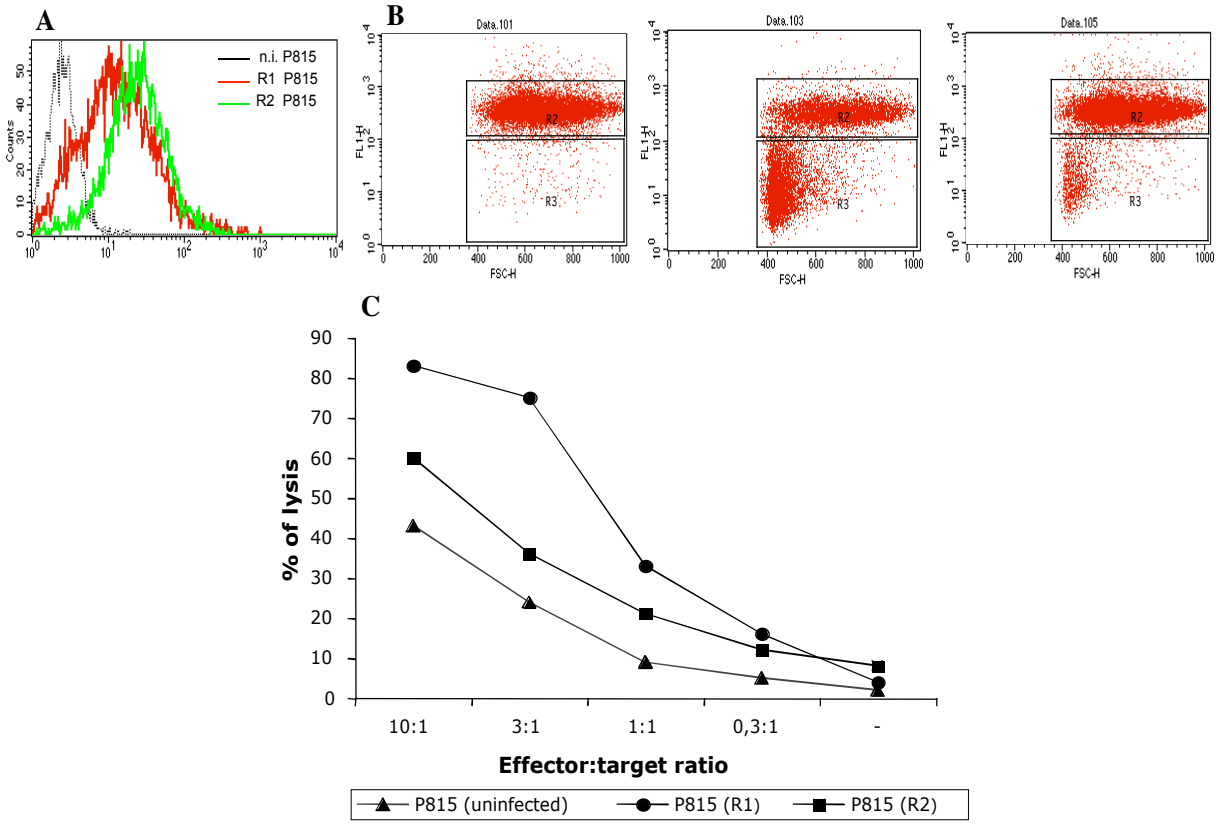


Figure 21. NK cell specific killing is modulated by different HA specificities. The experiment was performed plating NK cells (effectors) with a fixed number of fluorescent P815 cells (target) infected with R1 or R2 virus or not infected, at different ratio in order to evaluate the capacity of the effector cells to recognize and lyse target infected cells. (A) P815 infection by R1 and R2 was determined 18 hours post infection by intracellular staining with an anti-NP (viral nucleoprotein) antibody. (B) Dot plots of three representative P815 samples, in the presence or absence of effector cells. (C) The % of lysis was determined as described in the text.

As shown in Figure 21, NK cells can lyse non-infected P815 with an efficiency that can reach 43%, at high E:T ratio. However, infected P815 were lysed at higher efficiency compared to non-infected ones, for almost any E:T ratio considered. Moreover, R1 infected cells appeared to be significantly more prone to lysis than R2 infected cells.

The dot plots shown in Figure 21 B display in gate R2 live, fluorescent P815, and in gate R3 the dead fluorescent cells. Proceeding from left to right, it should be noted how, in the control without effector cells, almost all fluorescent P815 are in the R2 gate, with very few events in R3. The presence of effector cells at 10:1 (middle) or at 1:1 (right) cause a decrease in the number of cells in R2 and the appearance of cells in R3. These dot plot data were used to draw graphs similar to the one shown in Figure 21 C. These results, repeated in several experiments, indicate that the receptor binding properties of HA molecules cells can affect the ability of NK cell to recognize and lyse infected cells.

To demonstrate that the observed increase in killing recognition is mediated by interaction between the hemagglutinin on infected cells and sialic acid on NK cell receptors, we repeated the experiment described above pre incubating the NK cells with a mixture of neuroaminidase from *Vibrio Choleare* and *Arhtrobacter ureafaciens*. These enzymes are able to "shave off" the sialic acids from the surface of cells, thus reducing the cell capability to interact with HA molecules.

To determine the reduction in sialic acid levels before and after neuraminidase treatment, NK cells were incubated with the lectins DIG-MAA agglutinin (digoxigenin-*Maackia amurensis*) and DIG-SNA agglutinin (digoxigenin- *Sambucus nigra*) that bind 2–3- and 2–6- linked sialic acids respectively. These lectins were digoxigenin-labeled, allowing the identification of the positive cells by FACS analysis by mean of an anti-digoxigenin-FITC antibody (Figure 22 A)

As evident from the histogram, the neuraminidase treatment significantly reduced but yet did not abolish the amount of bound lectins. We tried different conditions of neuraminidase treatment, but were enable to eliminate completely the sialic acid from the cellular surface without damaging the cells irremediably.

After the neuraminidase treatment, NK cells were extensively washed and then incubated with fluorescent P815 cells, R1 or R2 infected or mock infected, at different E:T ratio.

Again, the specific lysis was determined according to the formula: % of lysis = (the number of CFDA SE^+ cells acquired in the control without NK cells) – (the number of CFDA SE^+cells in the sample)/(the sample of CFDA SE^+ cells in the control) x 100 and reported into graphs.

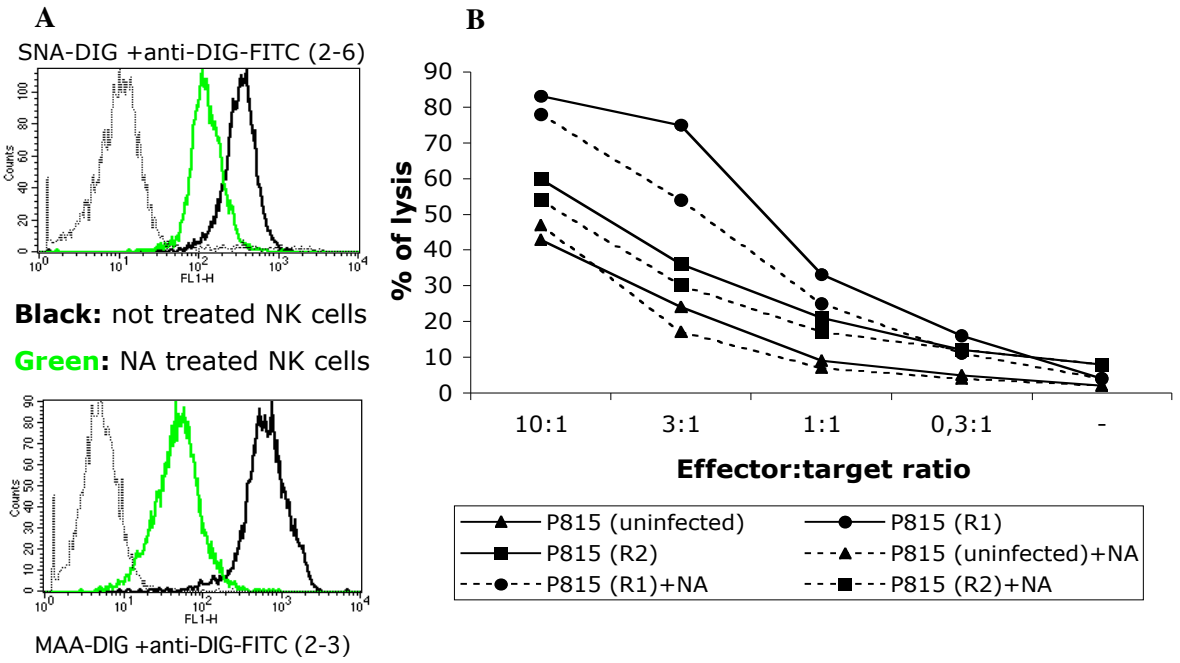


Figure 22 Lysis of infected cells by NK cells is mediated by HA-sialic acid interaction. (A) The level of sialic acid on NK cell surface, before and after neuraminidase treatment, was determined by staining with DIG-MAA or DIG-SNA followed by a FITC-conjugated anti-DIG Ab, to identify α2,3Gal or α2,6Gal moieties respectively. (B) Flourescent P815 cells infected or non infected, were incubated with NK cells for 4 hours. The % of specific lysis was determined as described and reported into the graph.

Again, P815 infected cells were killed more efficiently that not infected ones and, among the infected targets, R1 infected were lysed better than R2 infected P815 (Fig. 22 B).
However, when effector cells were treated with neuraminidase before setting up the cytotoxicity assay, these difference became less evident or disappeared, indicating that the increased killing of infected cells is mediated by binding of sialic acid moieties on the surface of NK cells with hemaglutinin molecules on the surface of target cells (Fig. 22 B).

4.2.2 Cytokines production by influenza virus infected HTBE cells

As described above, the avian influenza viruses bind preferentially to cell-surface receptors containing terminal sialyl–galactosyl residues linked by 2–3-linkage [Neu5Ac(α2–3)Gal], whereas human viruses bind to receptors which contain terminal α2–6–linked sialyl–galactosyl moieties. In agreement with this finding is the fact that human tracheal

epithelial cells contain mostly SA α2,6 Gal, while the duck gut epithelium, where avian viruses preferentially replicate, possesses mostly SA α2,3Gal sugar moieties. It is a general opinion, although not formally proved, that an alteration of the receptor specificity can be sufficient for the emergence of human pandemic viruses from avian progenitors, as alteration in HA specificity could cause changes in virus tropism.

With our work, we tried to demonstrate that receptor specificity modification could contribute to the emergence of pandemc viruses also by influencing the ability of the host cells to mount early anti-viral responses.

To address this question, we used primary, differentiated cultures of human tracheo-bronchial epithelial cells (HTBE). These cultures resemble human airway epithelium, both morphologically and functionally, as they are pseudo-stratified and polarized, and contain different types of cells including ciliated, secretory, and basal cells (Matrosovich *et al*., 2004). HTBE cultures therefore represent an invaluable substrate to study the biology of viral infections in primary target cells. Preserving the physiology of the human respiratory epithelium, HTBE cultures allow studies on local innate immune responses at various levels, including secretion of mucus, interferons and cytokines. Influenza virus-infected airway epithelial cells express both pro- and anti-inflammatory mediators and antioxidants. At the same time, they can undergo apoptosis, resulting in airway epithelial injury, thus recapitulating the full course of infection.

The hemagglutinin of the pandemic virus A/Hong Kong/1/68 (H3N2) (HK) differs from its putative avian precursor by 7 amino acid substitutions. By reverse genetics, Matrosovich's group has generated the complete recombinant virus rHK and its HA variants with amino acid reversions back to the ancestral avian sequence (Table 3). Among these variants, the double mutant rHK-R2 and the seven aa mutant (rHK-7aa) have a typical avian-virus-like receptor-binding specificity due to substitutions L226Q and S228G, while the wt rHK and the five aa mutant rHK-5aa possess human-virus-like receptor specificity (Table 3).

Virus	Amino acid substitutions in the HA	Receptor specificity
rHK-R1	Wt (human pandemic virus)	Human (2-6)
rHK-5aa	I62R, N81D, K92N, S193N, G144A	Human (2-6)
rHK-R2	**L226Q, S228G**	Avian (2-3)
rHK-7aa	I62R, N81D, K92N, S193N, G144A, L226Q, S228G	Avian (2-3)

Table 3 Summarizing table of viruses obtained by Reverse Genetics. Here we have generate the wt recombinant virus A/Hong Kong/1/68 (rHK) and its HA variants with amino acid reversions back to the ancestral avian sequence. Among these variants, the double mutant rHK-R2 and the seven mutant (rHK-7aa) had a typical avian-virus-like receptor-binding specificity due to substitutions L226Q and S228G.

Our collaborators have already demonstrated that HA mutations in position 226/228 influence virus tropism. In fact, when HTBE cultures are infected at the air liquid interface (ALI) with rHK-R1 or rHK-R2 recombinant viruses, rHK-R1 infected mainly non ciliated cells, while its mutant rHK-R2 preferred ciliated ones (Fig. 24).

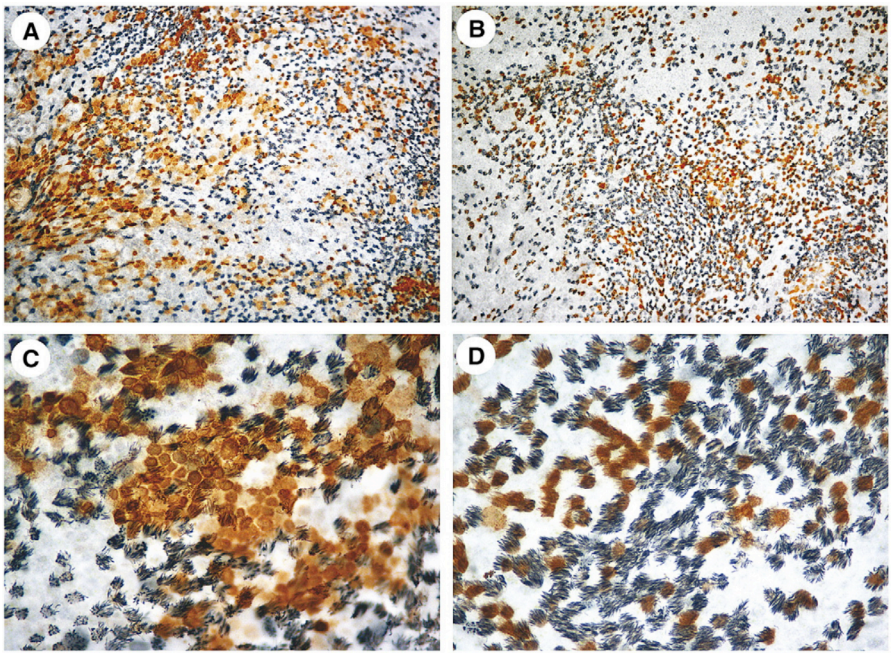


Figure 24 Human-like viruses infect preferentially non ciliated cells, while avian-like viruses show a preferential tropism for ciliated cells. Patterns of infection by recombinat rHK-R1 (A, C) and rHK-R2 (B, D). HTBE were infected at multiplicity 0.04, fixed 24h post infection, and double immunostained for virus (brown) and cilia of ciliated cells (gray). Objective 10x (A, B) and 40x (C, D) (adapted from Matrosovich *et al.*, 2007).

To test the hypothesis that virus interactions with sialic acid receptors may play a role in innate antiviral immunity, we infected HTBE cultures with the viruses described above and collected samples from both the apical and the basolateral side of the cultures, at different time-points post infection. Virus titers in the apical supernatants were determined using an infectivity assay in MDCK cells (Fig. 25), and concentrations of about 50 pro- and anti-inflammatory mediators and chemokines were measured using multiplex bead assay.

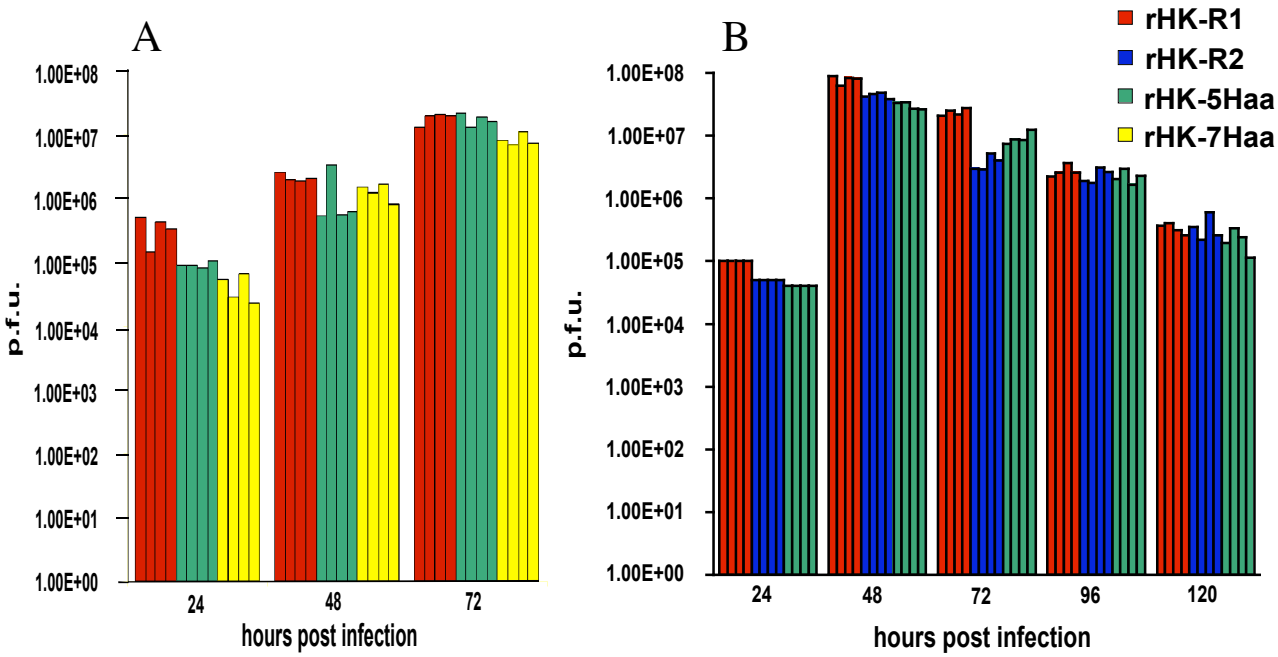
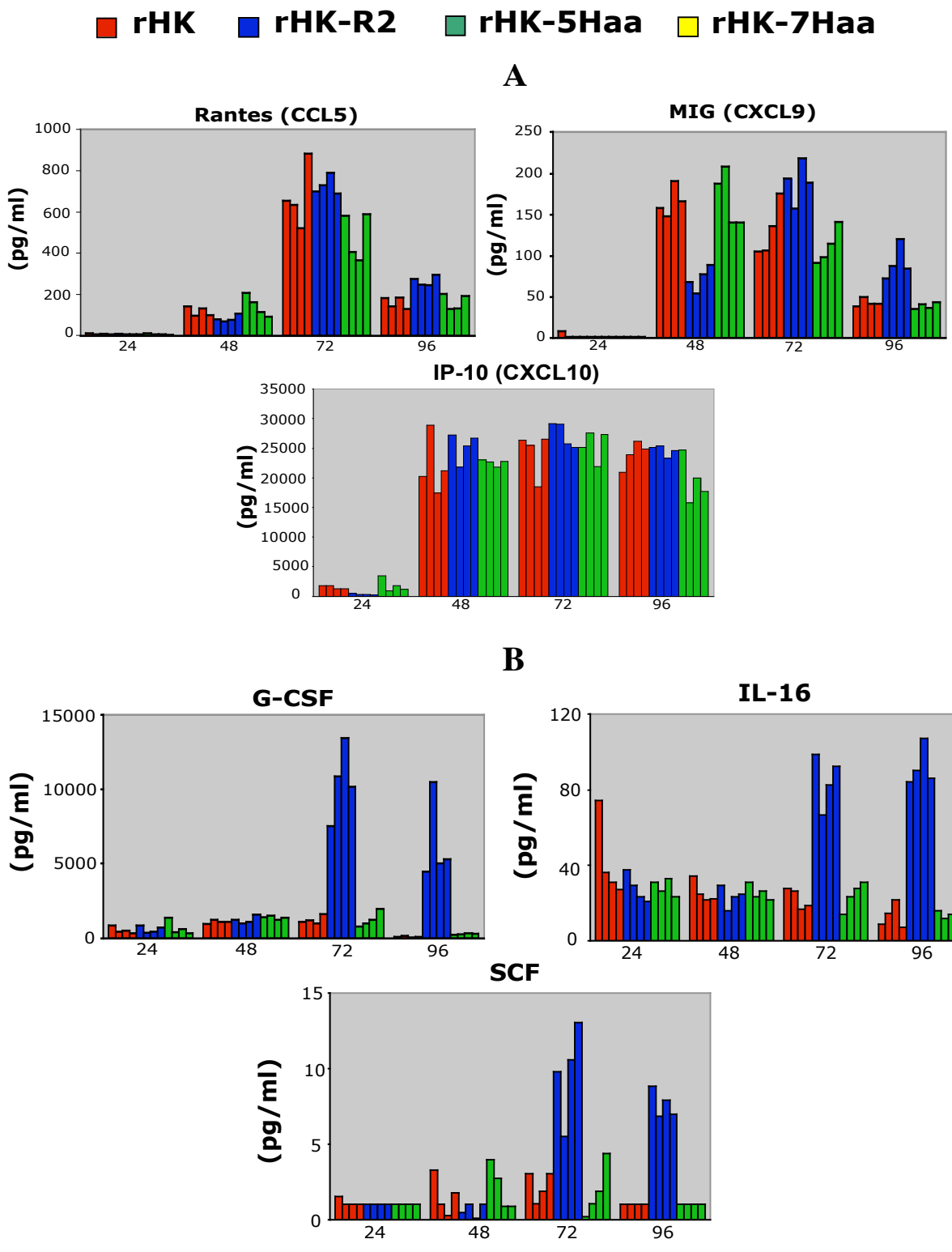


Figure 25 HAE cultures were infected apically with rHK and its HA mutants with 200 (A) and 1000 p.f.u (B) . After 24, 48, 72, 96, 120 hours, supernatants were collected from apical (shown) and basolateral sides. Virus titers were determined in MDCK cells using an infective assay.

The concentrations of most cytokines progressively increased at the apical side of the cultures in the course of the infection. Many cytokines, including T-cell-attracting chemokines such as IP-10 and RANTES, were induced to similar levels by different viruses (Fig. 26). However, some mediators were induced significantly stronger by the avian-like viruses rHK-R2 and rHK-7aa as compared to rHK and rHK-5aa. In particular, avian-like viruses stimulated a higher release of potent chemo-attractants of innate immune cells, such as G-CSF and IL-8, shedded adhesion molecules (CD25, VCAM-1, ICAM-1), and pro-apoptotic factors (TRAIL) (Fig. 26).


rHK
rHK-R2
rHK-5Haa
rHK-7Haa
A
Rantes (CCL5)
(pg/ml)
1000
800
600
400
200
0
24
48
72
96
MIG (CXCL9)
(pg/ml)
250
200
150
100
50
0
24
48
72
96
IP-10 (CXCL10)
(pg/ml)
35000
30000
25000
20000
15000
10000
5000
0
24
48
72
96
B
G-CSF
(pg/ml)
15000
10000
5000
0
24
48
72
96
IL-16
(pg/ml)
120
80
40
0
24
48
72
96
SCF
(pg/ml)
15
10
5
0
24
48
72
96

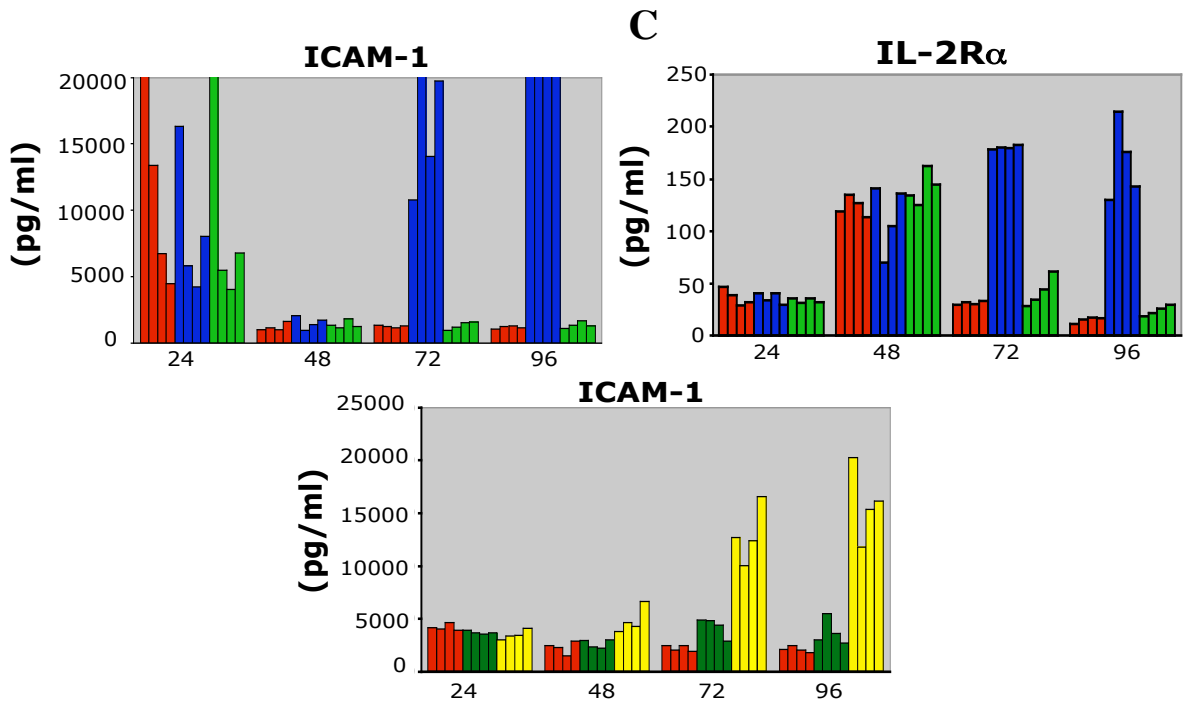


Figure 26 Cytokine expression in the course of infection. (A) T cell chemo-attractants: IP10, Rantes, CXCL9; (B) Chemo-attractants for innate immune cells: G-CSF, IL-16, SCF; (C) Shedded adhesion and surface molecules: ICAM-1, IL-2Rα (CD25); (D) Inflammation: IL-6, IL-1α The value on the x line is expressed in hours.

Remarkably, the patterns of secreted cytokines differed between the apical and basolateral sides of the cultures. Whereas avian-like viruses typically induced similar or higher levels of cytokines at the apical side than did rHK and rHK-5aa, the human-like viruses were stronger inducers of basolaterally secreted mediators (Fig. 27; Table 4).

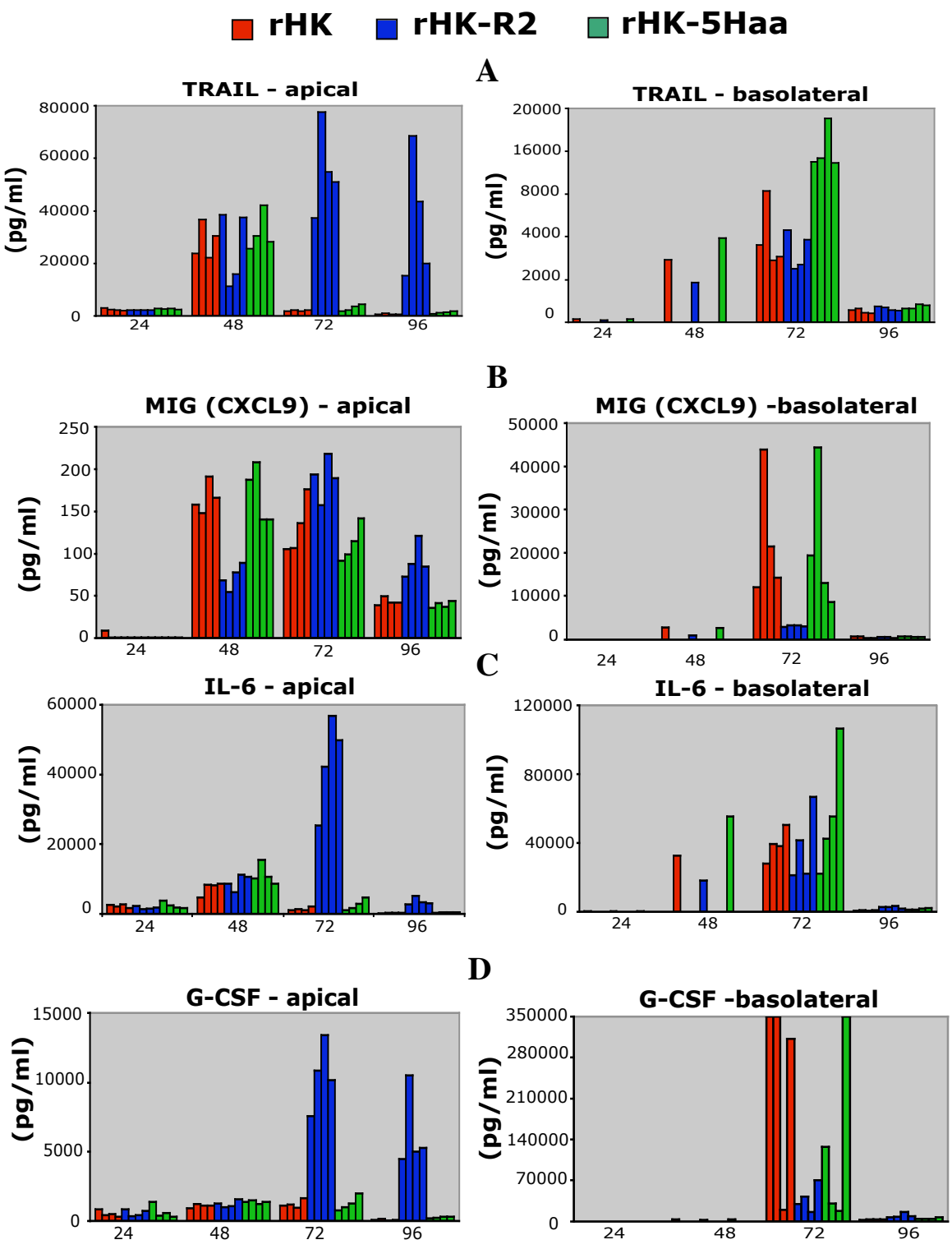


Figure 27 Expression of proapoptotic (A), T cell attracting (B), inflammation (C) and chemo attractants for immune cells (D) cytokines, in the apical and basolateral supernatants. The value on x line is expressed in hours.

CYTOKINE	APICAL	BASOLATERAL
MIF IL-18 MIG IL-12 p40	rHK = rHK-R2	rHK > rHK-R2
IL-9 MIP-1β IP-10 LIF MIP-1α IFN-2α CCL27 (CTACK) MCP-1 (CCL2) M-CSF IL-12 p70 TNF-α SCF IL-3 IL-16 Eotaxin Basic FGF GRO α	rHK = rHK-R2	rHK = rHK-R2
SDFα1 (CXCL12) IL-2 rα IFN-γ VEGF HGF TRAIL IL-10 IL-8	rHK < rHK-R2	rHK = rHK-R2
G-CSF ICAM-1	rHK < rHK-R2	rHK > rHK-R2
VCAM-1 IL-6	rHK < rHK-R2	rHK < rHK-R2
IL-1α IL-1β IL-1rα RANTES	rHK > rHK-R2	rHK>rHK-R2
IL-17	rHK > rHK-R2	rHK<rHK-R2

Table 4 Comparison of the levels of cytokines secreted by HTBE cells infected by human- and avian-like viruses tested on samples collected from the apical and basolateral sides.

Overall, avian-like viruses induced more cytokines related to inflammation, apoptosis and recruitment of innate immune cells, on the apical side.

Basolaterally, the opposite was found, that is to say avian-like viruses induced less cytokines.

In conclusion, these data provide a first experimental evidence that receptor specificity of influenza viruses can significantly affect patterns of innate immune responses in human airway epithelium. Further studies are required to determine the physiological meanings of our observations and the effects of the observed differences in determining the host range and the pathogenicity of different influenza viruses in humans.

4.2.3 Microarray analysis

To clarify epithelial cell responses to viruses with different receptor specificities, we performed a comprehensive analysis of gene expression in airway epithelial cells upon IV infection, using the Agilent gene chip technology.

HTBE cultures were infected from the apical side with the wt rHK or the rHK-7aa mutant. We used either live or β-propiolacton-inactivated viruses, the latter ones to test the effects of viral surface protein binding to target cells and eliminate any contribution from cellular mechanisms that could detect replicating viral nucleic acids.

Eight hours post-infection, RNA from triplicate cultures was extracted and labeled for microarray analysis. The resulting gene expression data were normalized directly to that of mock-infected cultures. Sequential Student's t-tests (infection versus mock) were then performed to identify genes significantly differentially expressed ($\geq$1.5 Log_2 change; $p \leq 0.05$) in at least one group. These results were then analyzed by Ingenuity Pathways Analysis (IPA). IPA is a database and analysis-system used for a better understanding of:

- gene expression and protein-protein interactions within the context of metabolic or signaling pathways;
- how proteins operate and form pathways;
- analyze data sets from the perspective of gene families.

Our analysis strategy, which identified genes both up- and down-regulated during rHK and rHK-7aa infection, resulted in a list of 700 genes with significantly changed expression. Hierarchical clustering analysis (by gene) revealed several groups of coordinately expressed genes in different prominent functional clusters, including a cellular stress and immune response gene network cluster and a cluster related to IFN signaling (Fig. 28: red=up-regulated, green=down-regulated).

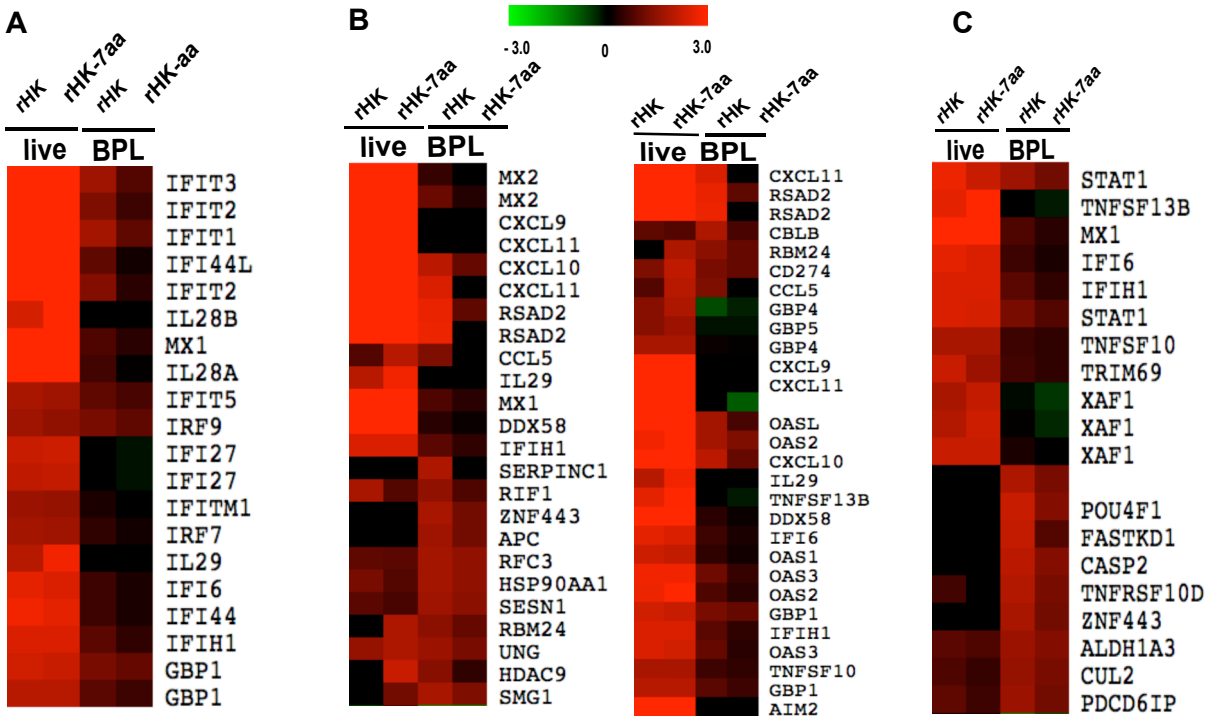


Figure 28 One-way hierarchical clustering of genes selected by IPA (Ingenuity Pathway Analysis) from three innate immunity-related gene clusters (red=upregulated, green =downregulated) Each sample was analysed versus mock infected-cells (merge of triplicates; $\log_2$ ratio>1,5; p value<0,005). (A) IFN-responsive genes; (B) stress and immune response genes; (C) cell death-associated genes.

IRGs are a large family of IFN-signaling and IFN-stimulated immune mediators with pleiotropic downstream functions in innate antiviral responses and host defense. Figure 28 shows that many IRGs, such as IFI44, MX1, MX2, OAS1, OAS2, were significantly up-regulated in infected relative to mock-infected HTBE.

CXCL10 (IP-10), CXCL9 and CXCL11 gene expression was also significantly up-regulated in infected cultures. CXCL10 is a potent chemo-attractant for activated Th1 lymphocytes and natural killer cells and is thought to play a role in the temporal development of innate and adaptive immunity in concert with type I and II IFNs.

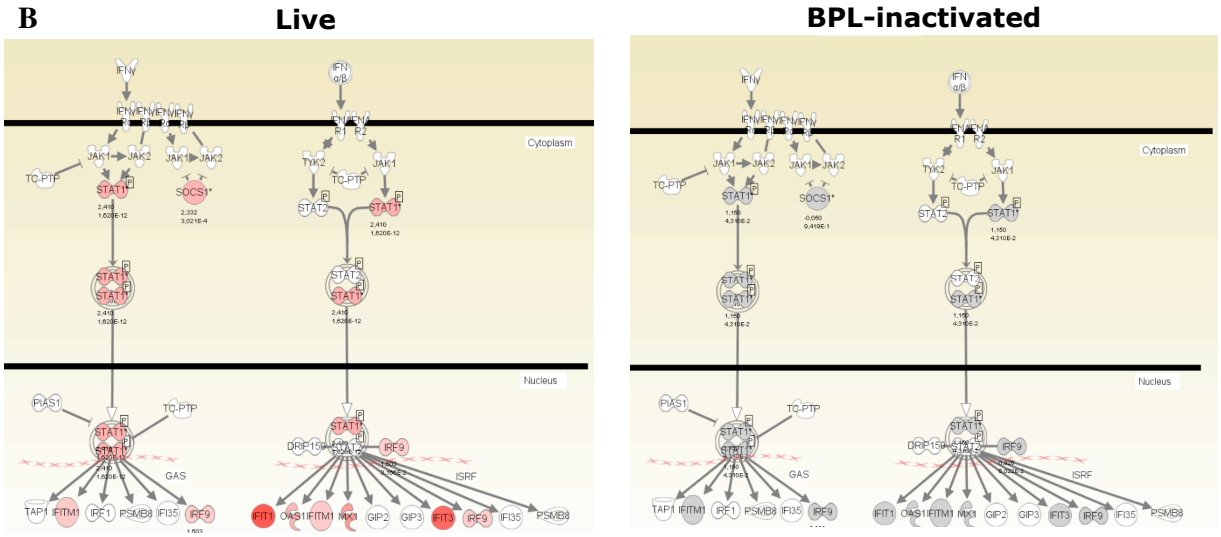


Figure 29 Activation of the IFN-pathway upon infection with live or BPL inactivated virus. Gene Ontology modeling of the IFN-pathway the microarray analysis of gene expression data from HTBE infected with live or BPL-inactivated rHK virus (red, upregulated; white, genes present in the pathway but not in our experimental samples; gray, genes present in our samples but not significantly up- or down-regulated).

These clusters of genes were then analyzed with Gene Ontology (GO). The three organizing principles of GO are cellular component, biological process and molecular function. A gene product might be associated with or located in one or more cellular components; it is active in one or more biological processes, during which it performs one or more molecular functions. Therefore, GO classifies a gene product according to its behavior in a given cellular context. Figure 29 shows an example of such analyses, for the cluster of genes associated to type I IFN-signaling. As expected, these genes were generally highly up-regulated in cultures infected with live viruses, while treatment with BPL-inactivated viruses led to less pronounced induction.

As there were no obvious differences between rHK and rHK-7aa viruses, we performed meta-analyses of our results with Gene Ontology (GO) (Fig. 30). The three organizing principles of GO are cellular component, biological process and molecular function. A gene product might be associated with or located in one or more cellular components; it is active in one or more biological processes, during which it performs one or more molecular functions. Therefore, GO classifies a gene product according to its behavior in a given cellular context.

These analyses, summarized in Figure 30, revealed an overall stronger response to live avian-virus-like rHK-7aa compared to live human-virus-like rHK. However, this stronger response could not be easily pinned down to a particular gene or group of genes. For this reason, we are currently performing more detailed analyses to better characterize and validate the pathways differentially modulated by viruses with different receptor specificities.

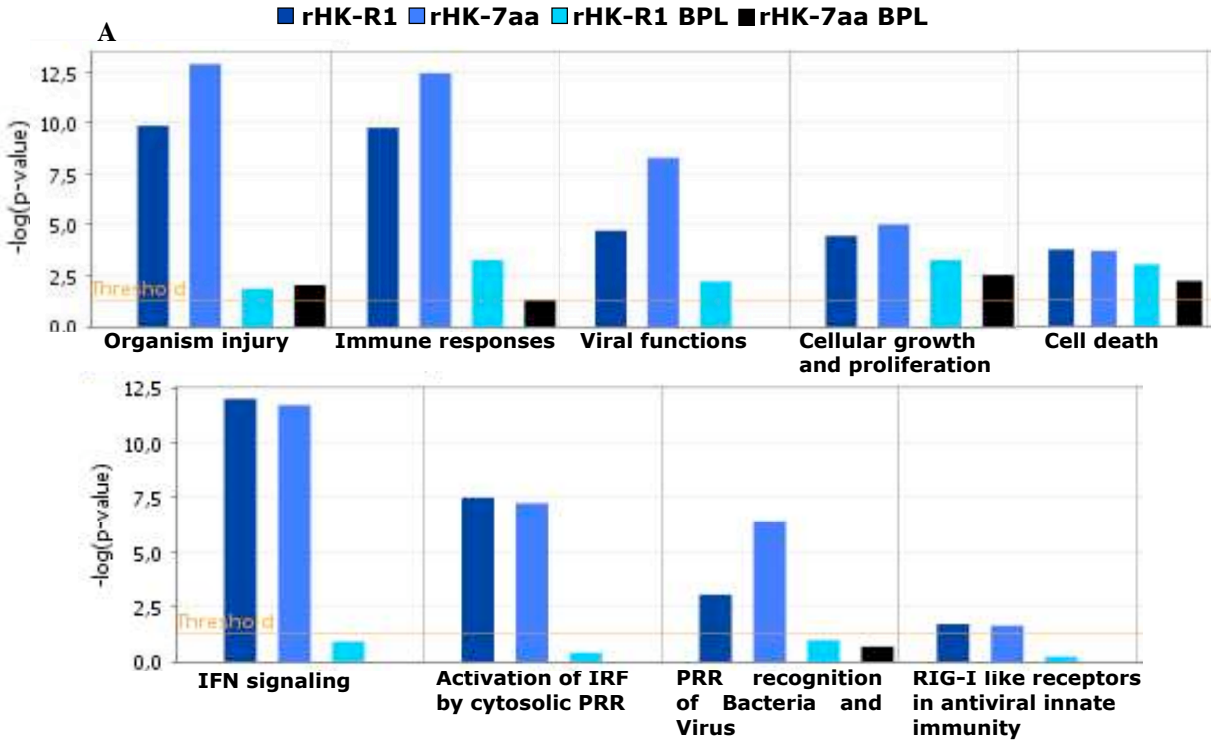


Figure 30 Schematic representation of the microarray data by the GO pathway modeling.

5. DISCUSSION

Influenza virus vaccines still remain the major control means against influenza disease.

The antigenic drift that involves HA of circulating influenza viruses require periodic replacement of the vaccine strains during inter pandemic periods. For this reason, every year the World Health Organization (WHO) publishes semiannual recommendations for the strains to be included for the Northern and Southern Hemispheres and in the United States Food and Drug Administration (FDA) determines in February which vaccine strains should be included in the following winter's vaccine in order to allow sufficient time for manufacture. Unfortunately, these recommendations are not always optimal.

The influenza vaccines currently licensed consist of embryonated egg-grown and formaldehyde-inactivated preparations, which, after purification, are chemically disrupted with a nonionic detergent. The introduction of the zonal ultracentrifuge, a technology developed by Gerin and Anderson during the Sixties, has given an improvement to vaccines permitting to reduce the eggs contamination and pyrogenicity deriving from whole virus. The content in HA for one dose of vaccine consists in 45 μg (15 μg from each HA of the three antigenic component), that is the amount of purified virus obtained from the allantoic fluid of one infected embryonated egg. This means that one dose corresponds to one embryonated egg.

The second class of vaccines approved by FDA consists of cold-adapted attenuated vaccines. By this approach developed by the Maassab's team (Maassab *et al.*, 1990) and adapted by Murphy and colleagues (Murphy and Coelingh, 2002), the influenza virus was passaged several times at 25°C in tissue culture (chicken kidney cells) and in embryonated eggs, resulting in the development of a temperature-sensitive, highly attenuated master strain. These cold-adapted influenza virus vaccines are easily administered by nasal spray inducing local mucosal neutralizing immunity and cell-mediated responses.

Current influenza vaccines, as mentioned, are based on technologies developed in the 1960s. They consist of partially purified preparations of influenza viruses grown in embryonated eggs and are constituted by 3 components: an H1N1 (hemagglutinin [HA] subtype 1; neuraminidase [NA] subtype 1), an H3N2 influenza A virus, and an influenza B virus. For example, the 2009–2010 vaccine formulation recommended for northern hemisphere is made up of the

A/Brisbane/59/2007 (H1N1), A/Brisbane/10/2007 (H3N2), and B/Brisbane/60/2008 viruses.
The breakthrough of reverse genetics techniques now allows the rescue of infectious influenza virus from plasmid DNAs transfected into tissue culture cells.
This technology permits the construction of high-yield 6:2 seed viruses by mixing the 6 plasmid DNAs from a good-growing laboratory strain with the HA and NA DNAs obtained by cloning relevant genes from currently circulating viruses. In this way, the appropriate seed viruses could be generated and distributed to the manufactures within 1-2 weeks, a much shorter period compared to the time required for performing the lengthy and laborious classical reassortant techniques. Moreover, the RG approach allows the generation of viruses directly in MDCK cells, a cell line approved from WHO for influenza vaccine production, removing egg passages (and subsequent adaptation) necessary for the actual vaccines.
In addition to accelerate the timeframe to obtain seed viruses for annual production, a RG approach would have several other advantages over the present manufacturing procedure. First, it would standardize the seed viruses to be used. Second, it would be safer, as the DNA cloning process would eliminate any adventitious agent present in the original viral isolate. Finally, in the case of the highly pathogenic H5 strains, viruses with that HA (containing a multibasic HA1/HA2 cleavage site) kill embryonated eggs, making it difficult to use eggs as growth substrate.

The aim of my project was to establish a RG system for the generation of new seed viruses for Influenza vaccine production.
So far, two strains have been widely used in RG studies: the A/WSN/33 (H1N1), a strain with high productivity and widely used in animal studies, and the A/PR/8/34 (H1N1) that is used for production of commercial Influenza inactivated vaccines because adapted to growth in eggs. First of all, according to the knowledge of abundance of virus yield given by the A/WSN/33, we cloned its genome in our home-developed RG vector (pKS10), an engineered plasmid constructed on the bases of the published pHW2000 plasmid developed by Hoffmann (Hoffmann *et al.*, 2000), the father of modern Reverse Genetics. We cloned the eight viral segments, each one coding for one of the eight proteins from WSN into pKS10 and used the eight plasmids to transfect a co-culture of 293T/MDCK cells, in the presence of trypsin. The use of this enzyme that hydrolyses HA helps the virus to entry host cells membrane, while the

use of 293T, that is a human cells line, it is necessary because of species specificity of the polymerase I promoter (Heix and Grummt, 1995). In this way the virus is rescued in 293T and expands in MDCK cells. As expected, higher titers were obtained rescuing the virus in co-cultured cells plus trypsin, rather then 293Ts alone (regardless of addition of trypsin), while no difference was observed extending the time of incubation at 72 hours relatively to 48 hours. Subsequently we used supernatant of rescued virus to test its ability to propagate in MDCK cells, thus reaching even higher yields.

Once set up the system and tested its functionality for A/WSN/33, we generate reassortant viruses by substitution of each viral fragments of WSN with the corresponding ones derivated from the A/PR/8/34 strain, a different but closely related H1N1 strain. Comparing the titers of the reassortants we showed that while the WSN harboring M and/or NS from PR8 have not significant difference to the reference strain, in the case of NA or PA substitutions there was a decrease of virus production, in the range of 1-2 log, for NA and PA respectively. Nevertheless, these data demonstrated that reassortant recombinant viruses can be rescued.

We then used WSN or PR/8 as backbones for generation of reassortant viruses carrying HA and/or NA from evolutionary different strains, such as the A/New York/193/2003 or the A/Panama/ 2007/1999, both belonging to the H3N2 type. In these cases the viruses shown no good propensity to be rescued, confirming their incapacity to give rise to high titers also after expansion in MDCK. So, even though these reassortant viruses could be rescued, their ability to expand in culture was greatly hampered, indicating that the rescued virus although infectious, is not fully functional. These results could indicate that certain combinations of HA and NA molecules are not compatible when expressed on the same virions, and indeed virus carrying combination such as H3N1 have not been isolated, although the H3N2 and H1N1 subtypes are presently co-circulating.

Once established that RG can be used for the generation of reassortant viruses in human cell lines, we try to develop a rescue system for MDCK cells that, as previously mentioned, are a canine cell line approved by WHO for the production of influenza virus vaccines.

The reverse genetic technique is based on the use of plasmids that carry polymerase I promoters to generate viral RNA molecules. Unfortunately the polymerase I promoter are not well conserved between different species, so to apply the system to canine cells, we had to use a canine polymerase I promoter. By genomic sequence comparison, we identified a

hypothetical minimal sequence in the dog genome that could correspond to a polymerase I promoter and tested its functionality in MDCK cells. Once defined the functionality of the promoter, we engineered a new rescue vector (pKS11) by substituting the human with the canine promoter.

According to the policy of WHO with regard to use of MDCK for growing seasonal seed virus and in collaboration with Novartis Vaccines in Marburg, we identified two Influenza strains from human clinical isolates that grow to high titers in MDCK cells, also after multiple passages.

The choice of developing two seed viruses starting from two strains, the A/New Caledonia/20/1999 (H1N1; #105) and the A/Wisconsin/67/2005 (H3N2; #003), was done on the basis of the results described above. In fact, we have seen that when we combine the backbone (the six internal genes) from a H1N1 virus type with HA and NA molecules derived from a H3N2 type, the resulting reassortant virus was somehow hampered in its ability to propagate in culture. As the strains actually circulating in the human population belong to the H1N1 and H3N2 sub-types, we decided to generate two different seed viruses.

In conclusion, we have developed a canine polI-driven RG system that work in MDCK cells and it is based on the internal genes of two strains that have been described as good-growing strains in MDCK cells. This system could represent an alternative approach to generate cell culture-adapted virus strains for future influenza vaccines.

Influenza viruses attach to target cells via multivalent interaction of the viral hemagglutinin with sialyloligosaccharide of cellular glycoproteins or glycolipids.

Human, avian and swine influenza viruses have different receptor-binding specificity that correlates with biological properties such as host range and tissue tropism. These distinct receptor-binding phenotypes are maintained by many selective pressures including availability of receptors on target cells and neutralization by competitive inhibitors (e.g. mucins) and contribute to the creation of barriers for interspecies transmission, limiting the emergence of new influenza outbreaks and pandemics.

Sialylglycoconjugates are ubiquitously expressed on the surface of most avian and mammalian cells. Therefore, in addition to mediate infection in susceptible cells they are responsible for

binding of viral particles to a variety of cells types, including cells of the immune system. Polyclonal activation of B-lymphocytes (Butchko *et al.*, 1978; Anders *et al.*, 1984), deactivation of neutrophils (Abramson, *et al.*, 1986; Cassidy, *et al.*, 1989) and stimulation of inflammatory responses are some of the biological responses that have been described in response to the binding of viral particles to the surface of immune cells. However, specific correlations between the receptor-binding characteristics of viruses and their ability to influence host immune response have not yet been fully investigated.

The aim of my project was to test if and how receptor specificity can affect immune responses to influenza virus and thus determine its virulence and pathogenicity. To address this question, we have used the reverse genetics technology to generate a panel of viruses that share the six internal genes and the NA gene and differ only by some aminoacids in the hemagglutinin receptor-binding site.

Natural killer cells, as well as cytotoxic T lymphocytes, are major components of the cellular mechanisms that lead to the destruction of foreign, tumor or infected cells. NK cell activation is regulated by the balance of signals received through activating and inhibitory surface receptors. Inhibitory receptors bind major histocompatibility complex (MHC) class I proteins, allowing NK recognition of target cells with decreased MHC class I expression. NK cells also express lysis triggering receptors specific for non-MHC ligands, including CD16, NKG2D and the natural cytotoxicity receptors NKp46, NKp44, and NKp30.

The influenza virus HA protein is known to be among the ligands recognized by both NKp46 and NKp44, and this interaction may contribute to NK activation by influenza virus-infected cells (Arnon *et al.*, 2001). The binding of influenza virus HA to NKp46 was reported to be mediated mainly via sialic acids (SA) carried by NKp46. Moreover, alterations in HA glycosylation associate with alterations of the HA-SA binding properties and with variation in natural cell lysis of infected cells (Owen *et al.*, 2007). All these data suggest that HA proteins that bind with different affinities to SA may have a differential ability to trigger NK cell activation. In line with this hypothesis, we found that cells infected with viruses bearing avian-like (R2) HA molecules activated a lower level of NK cell lysis than did cells infected with their human-like counterpart (R1). This differential killing was mediated by interaction between hemagglutinin molecules on infected cells and sialic acid molecules expressed on the

NK cell surface, as pre-treatment of NK cells with neuraminidases reduced the overall lysis of target cells, abolishing any significant difference in the efficiency of killing of cells infected with the different viruses.

Evidence from both murine and human studies indicate that NK cells are rapidly activated following influenza virus infection and make an important contribution to control of early virus replication as well as promoting the induction of the adaptive immune response. Therefore, it is conceivable that viruses bearing HA proteins that have a reduced affinity of binding to activating NK receptors may have in vivo replicative advantage and cause more severe pathology.

Using a reverse genetic approach, we have been able to study differences in NK activation in response to different viruses, based solely on changes in the virus surface genes for HA. It is plausible that influenza viruses also utilize mechanisms involving other viral genes to evade NK cell activation, and these might vary between viral strains. For example, recent studies have shown that the NKG2D ligand MICB is up-regulated in influenza virus-infected macrophages and that this can mediate NK cell activation (Sirén *et al.*, 2004). It is also possible that some influenza virus isolates up-regulate MICA/MICB and activate NK cells via NKG2D, whereas other influenza viruses suppress the up-regulation of stress-inducible molecules such as MIC, thus differentially influencing the NK cell ability to lyse infected cells.

Primary lung and airway epithelial cell cultures have been used to investigate cellular responses to infection with many respiratory viruses, including influenza A virus, respiratory syncytial virus (RSV), human coronaviruses, human parainfluenza virus type 3, and adenovirus.

Unlike conventional transformed cell lines, fully differentiated primary airway cultures mirror the in vivo tissue in their organization into multilayered, polarized cultures consisting of different cell types, including secretory (Clara), goblet (mucous), ciliated, and basal cells.

An important aspect of influenza virus infection of airway epithelial cells is the interaction between the virus hemagglutinin protein and the corresponding receptor on the host cell. Although the precise nature of the viral receptor is incompletely defined, influenza viruses target glycosylated oligosaccharides that terminate in sialic acid residues. These residues are

bound to glycans through α 2,3, or α 2,6, linkage by sialyltransferases that are expressed in a cell- and species-specific manner. Influenza viruses primarily target airway epithelial cells via α 2,3- or α 2,6- type receptors, but the distribution of these receptors in many species is uncertain and may be a significant factor influencing infection.

The preference of receptor binding can be altered by changes in specific HA amino acids that can influence host specificity and tropism. Indeed, mutations of avian influenza virus HA genes have been shown to alter binding preference from the α2,3- to the α2,6-linked SA and are thought to have contributed to the 1918 Spanish, 1957 Asian, and 1968 Hong Kong pandemics.

Though many cell lines are capable of supporting influenza virus infection, continually passaged cells are transformed and therefore have different genetic programs regulating important features such as localization of receptors and innate host responses.

For all these reasons, respiratory epithelial cells cultured under physiologic conditions that allow for full differentiation into the cell types representative of the native airway may provide not only a better infection model but also a reasonable surrogate of the in vivo situation to study epithelial innate immune responses to virus infection.

Successful infection of the respiratory tract by viruses is the result of a balance between pathogen virulence, host receptor expression, innate and acquired immune responses. Cytokines produced during influenza A virus infection can mediate pathology and disease severity, as it has been demonstrated for infections with avian H5N1 viruses, where enhanced cytokine production has been associated to the increased mortality observed in patients.

In our study, we have used differentiated, primary human tracheo-bronchial epithelial cultures to investigate epithelial cytokine responses to influenza A virus infection focusing mainly on the contribution of HA binding properties in regulating innate immune responses.

By infecting HTBE cultures with different viruses and analyzing samples from the apical and basolateral sides of the cultures at different time-points post infection, we showed that a wide array of cytokine are produced in response to infection. Many cytokines, including T-cell-attracting chemokines such as IP-10 and RANTES, were induced to similar levels by different viruses, while others were induced significantly stronger by the avian-like viruses as compared to their human-like counterpart. In particular, chemo-attractants of innate immune cells, such as G-CSF and IL-8, shedded adhesion molecules (CD25, VCAM-1, ICAM-1), and pro-

apoptotic factors (TRAIL) were strongly induced in response to infection with avian-like viruses. Remarkably, most of the cytokines analyzed were released in a directional, polarized manner during HTBE infection.

Polarized secretion of cytokines during virus infection has been reported for similar primary differentiated airway systems, and this again highlights an advantage of primary airway systems over cell lines as the latest often lack the unique properties of polarization and cell type heterogeneity that primary differentiated cultures can provide. In addition, polarized cytokine secretion in primary cultures provides a working model to study how immune cells might be directionally recruited to the site of infection in vivo.

In conclusion, our data indicate that the first innate immune responses to influenza virus infection in airway epithelium can be significantly determined by the binding properties of the virus hemagglutinin molecules.

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7. PUBLICATIONS

Pirada Suphaphiphat, Bjoern Keiner, Heidi Trusheim, Stefania Crotta, Annunziata Barbara Tuccino, Pu Zhang, Philip R. Dormitzer, Peter W. Mason, and Michael Franti. "Human RNA Polymerase I-Driven Reverse Genetics for Influenza A Virus in Canine Cells". Accepted *Journal of Virology* (published online 13 January 2010).

Pirada Suphaphiphat, Michael Franti, Armin Hekele, Anders Lilja, Terika Spencer, Ethan Settembre, Gene Palmer, Stefania Crotta, Annunziata Barbara Tuccino, Bjoern Keiner, Heidi Trusheim, Kara Balabanis, Melissa Sackal, Mithra Rothfeder, Christian Mandl, Philip R. Dormitzer, and Peter W. Mason. "Variation in the growth of 2009 H1N1 pandemic influenza viruses determined by a quasispecies in the HA gene". Submitted to *Journal of Virology* .