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Dott.ssa Erica Borgogni
Matricola: 033381

Tutor: Prof. Marco Vanoni

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Università degli Studi di Milano-Bicocca
Piazza dell'Ateneo Nuovo 1, 20126, Milano

Dipartimento di Biotecnologie e Bioscienze
Piazza della Scienza 2, 20126, Milano

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Abstract

Ogni anno nel mondo il virus dell'influenza A colpisce il 5-15% della popolazione, causando oltre mezzo milione di morti, in particolare nelle categorie più a rischio: bambini, anziani e soggetti immunocompromessi.
Il virus dell'influenza A infetta l'epitelio delle vie respiratorie superiori ed inferiori e generalmente la malattia si manifesta in modo brusco ed improvviso, con febbre alta, dolori osteomuscolari, congestione delle vie aeree e faringiti, che persistono per una settimana circa.
La polmonite batterica è la complicanza più frequente nei soggetti deboli, mentre più raramente sono riferite miocarditi, miositi ed encefaliti.
Il virus dell'influenza A appartiene alla famiglia degli Orthomyxoviridiae, è un virus a RNA non codificante, costituito da un rivestimento lipidico, *envelop*, in cui sono inserite le glicoproteine di membrana, l'emoagglutinina (HA) e la neuraminidasi (NA) fondamentali sia per il riconoscimento e l'ingresso del virus nella cellula bersaglio, sia per la propagazione della progenie virale. Sono anche le responsabili della risposta immunitaria dell'ospite. All'interno dell'envelop, il genoma virale è composto da otto segmenti genici che codificano per dieci-undici proteine. La classificazione del virus nei diversi sottotipi è possibile grazie all'emoagglutinina e alla neuraminidasi; ad oggi sono stati descritti sedici tipi di emoagglutinina e nove di neuraminidasi che combinandosi possono originare molti sottotipi diversi (H_nN_n).
Il virus dell'influenza A infetta diverse specie animali, anche se il principale reservoir di tutti i sottotipi sono le specie aviarie, in particolare quelle acquatiche, mentre l'uomo ed altri animali sono suscettibili solo ad alcuni sottotipi.
La vaccinazione rappresenta il metodo più efficace per prevenire l'influenza e le sue possibili gravi complicanze. Sia la vaccinazione che l'infezione inducono una risposta immunitaria in grado di garantire protezione a lungo termine, tuttavia la composizione del vaccino, a seguito delle raccomandazioni della World Health Organization (WHO), deve essere aggiornata ogni anno, poiché l'immunità conferita dall'infezione naturale o dalla vaccinazione, con un certo ceppo di virus influenzale, non conferisce necessariamente la protezione contro nuovi ceppi.
Questi frequenti cambiamenti sono dovuti a meccanismi in grado di generare una ricorrente variabilità del virus. Mutazioni minori, *antigenic drift,* si riscontrano continuamente; sono dovuti alla bassa fedeltà della RNA polimerasi virale e causano il continuo cambiamento dei ceppi circolanti. Maggiori modificazioni nel virus, dovute alla sostituzione di uno o più segmenti genici derivati da altri sottotipi virali, *antigenic shift*, si verificano più raramente e rappresentano la grande sfida per la salute pubblica, in quanto possono originare nuovi sottotipi, verso i quali la popolazione ha

protezione scarsa o nulla. Questo sarebbe in grado di causare un'epidemia a livello mondiale, una pandemia.
La più famosa e letale pandemia fu l'influenza spagnola (H1N1) che, tra il 1918 e il 1920, causò più di quaranta milioni di morti. Nel corso del XX secolo le successive due pandemie non furono così devastanti: l'Asiatica (H2N2) nel 1957 e l'influenza di Hong Kong nel 1968, anche se morirono milioni di persone, furono di minore gravità.
Queste pandemie sono state causate da un riassortimento tra virus aviari ed umani, poiché un virus aviario può infettare l'uomo causando anche gravi patologie, tuttavia può difficilmente trasmettersi efficientemente da uomo a uomo.
Recentemente ha destato una notevole preoccupazione la trasmissione all'uomo di ceppi aviari altamente patogenici (HPAI) di H5N1 in quanto potrebbero causare una nuova pandemia. A partire dal 1997 nel sud est asiatico, in occasione di epidemie di H5N1 nel pollame sono stati segnalati diversi casi di trasmissione diretta del virus all'uomo. Da allora il virus ha continuato a circolare, soprattutto in Asia, e ad oggi sono stati riportati più di quattrocento casi di infezione, con un tasso di mortalità superiore al 60%.
Una possibile nuova pandemia, causata da H5N1, potrebbe essere contrastata grazie all'impiego di un vaccino capace di indurre una risposta immunitaria forte e ad ampio spettro di specificità.
Gli studi clinici finora condotti hanno mostrato che due dosi di vaccino pre-pandemico, formulato con un potente adiuvante come MF59, sono in grado di indurre titoli anticorpali neutralizzanti, potenzialmente protettivi sia verso il ceppo contenuto nel vaccino, sia verso altri ceppi di H5N1. Questi studi hanno anche dimostrato che adiuvanti come i sali di alluminio hanno una limitata efficacia, se comparati con l'MF59: emulsione di olio in acqua, autorizzato in Europa per il vaccino anti-influenzale da oltre un decennio.
Nonostante, ad oggi, non siano stati ancora ben definiti i correlati di protezione per l'influenza aviaria, svariati studi nel campo della vaccinazione stagionale hanno chiaramente indicato che gli anticorpi correlano con la protezione ed in particolare un titolo anticorpale ≥ 40 nel test di inibizione dell'emoagglutininazione, HI, è considerato protettivo. Per quanto riguarda i ceppi di aviaria è stata dimostrata una buona correlazione tra titoli HI ≥ 40 e titoli di microneutralizzazione, MN ≥ 80. Inoltre l'analisi dei sieri, nei sopravvissuti all'infezione da influenza aviaria, mostra una buona correlazione di protezione con un titolo di MN ≥ 80.
Sulla base di queste considerazioni sarebbe necessaria una "pre-vaccinazione" con due dosi a tre settimane di distanza l'una dall'altra, durante il periodo pre-pandemico, seguita da una dose di richiamo da somministrare all'inizio della pandemia.
Attualmente è possibile stabilire l'efficacia della vaccinazione solo dopo la dose di richiamo, mentre non è possibile determinare se nella popolazione è stata sviluppata memoria immunologica prima della dose di richiamo.

Nel corso di un trial clinico pre-pandemico, abbiamo valutato sia la risposta anticorpale, che la risposta cellulo-mediata, al fine di identificare marcatori precoci di efficacia, che permettano di stabilire/valutare l'efficacia della vaccinazione dopo le prime due dosi.
Durante un trial clinico di fase II, individui adulti hanno ricevuto due dosi del vaccino a subunità A/Vietnam/1194/2004, seguite dopo sei mesi da una dose di richiamo; i soggetti hanno ricevuto o la formulazione senza l'adiuvante (non AdJ 15) o con l'adiuvante MF59 (MF59-H5N1).
Abbiamo osservato che una dose del vaccino con MF59 è sufficiente per espandere la popolazione dei linfociti $CD4^+$, che mostrano un fenotipo Th1, mentre una seconda dose è necessaria per espandere il pool delle cellule B della memoria H5N1 e per ottenere titoli anticorpali neutralizzanti.
Inoltre un aumento di tre volte delle cellule $CD4^+$ antigene specifiche, dopo la prima dose, predicono l'aumento dei titoli anticorpali MN $\geq$ 80 dopo la terza dose e la loro persistenza a sei mesi dall'ultima dose, con una precisione del 75% e dell'85% rispettivamente.
Sulla base di questo primo trial clinico, se questi dati saranno confermati su un maggiore numero di soggetti, suggeriamo che l'analisi della risposta $CD4^+$ antigene specifica può essere utilizzata per valutare l'efficacia della vaccinazione e potrebbe aiutare a saggiare diverse formulazioni vaccinali pre-pandemiche per stabilire quale di esse sia più efficace nell'indurre una potente risposta immunitaria.

Introduction

Classification

Influenza A viruses belong to the Orthomyxoviridae family and there are currently described three types of influenza virus, A, B and C wich are distinguished by antigenic differences in two of their internal proteins, nucleoproteins (NP) and matrix protein (M). The three types of viruses also differ in their pathogenicity and genome organization. Type A is found in a wide variety of warm-blooded animals (birds and mammals), whereas type B and C are predominantely human pathogens.
Influenza A viruses are subdivided further into subtypes based on their two surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA), and currently, 16 known types of HA and 9 known types of NA have been isolated from aquatic birds, the natural reservoir for all influenza viruses [1, 2].
Influenza A virus has also a standard nomenclature that includes virus type, species from which it was isolated, if not human, location at which it was isolated, isolate numbers, isolate year and HA and NA subtype: thus A/Panama/2007/1999 (H3N2) was isolate numbers 2007 of a human influenza A virus taken in the country of Panama in 1999 and it has a HA subtype 3 and a NA subtype 2 [3].
In humans since the beginning of the twentieth century only three HA (H1, H2, H3) and two NA (N1 and N2) subtypes have been associated with stable infection resulting in recurrent annual epidemics [4].

Reservoir

Influenza A viruses have been isolated from a limited number of different animal hosts including humans, birds, horses, whales, seals, mink and swine. Many host populations are only transiently infected or harbour only a small number of antigenic subtypes. There are various lines of evidence that the primary reservoir of influenza A viruses are the aquatic birds. The first indications that aquatic birds species could be the hosts of influenza came in the 1972 when antibodies to human influenza NA were identified in Australian pelagic birds [5].
The disease signs associated with influenza A virus infections in avian species vary considerably with the strain of virus and avian influenza can be classified on the basis of virulence. Highly pathogenic avian influenza (HPAI) viruses cause systemic lethal infection, killing birds as soon as 24 hours post infection, and usually within one week, whereas low pathogenic avian influenza (LPAI) virus rarely generate outbreaks of severe disease with a limited associated morbidity and mortality [2]. Without exception, all of the HPAI viruses belong to the H5 or H7 subtype and, for reasons that are

still unclear, there isn't an association between specific NA subtypes and high pathogenicity (HPAI viruses).
Many wild and domestic avian species are susceptible to influenza virus infection, although viruses that are highly pathogenic in one avian species might not be pathogenic in another [6]. The host factors that determines differences in susceptibility to avian influenza viruses in different avian species are unknown.
LPAI viruses replicate mainly in intestinal and respiratory organs, and are shed in the feces of infected birds. Therefore, transmission of viruses through the fecal-contaminated-water-oral route is an important mechanism of LPAI-virus dissemination among aquatic birds. High concentrations of HPAI viruses, which replicate systemically in poultry, are also shed in feces. However, these viruses are more readily transmitted among birds in densely populated flocks by the nasal and oral routes through contact with virus-contaminated materials. LPAI viruses cause localized infections in the respiratory and/or intestinal tract, resulting in mild or asymptomatic infection. In chickens infected with HPAI viruses, common symptoms include swelling of the microvascular endothelium, multifocal haemorrhages and thrombosis [6, 7]. HPAI viruses can replicate efficiently in vascular endothelial and perivascular parenchymatous cells, which aids viral dissemination and systemic infection.
All HA and NA influenza A subtypes have been identified in aquatic bird populations particularly in migrating water fowl, all 15 HA subtypes, except H13 can be isolated from wild ducks and most HA and NA subtypes also have been isolated from migratory shorebirds. It is likely that both ducks and shorebirds are key reservoirs of influenza viruses and that each may preferentially, although not exclusively, harbour distinct subtypes [8].
The separation of influenza reservoir depends not just on the host species but also on geographical location. The separation of different migratory routes of aquatic birds has resulted in the formation of geographically defined influenza gene pool of North American and Eurasian lineages [9]. Recent data show, however, that within North American avian populations circulate at least two sublineages, one of which is more similar to the Eurasian lineages than to the other North American Cluster [10, 11] suggested that this similarity could be the results of some degree of contact between birds of different flyways.
A plethora of other avian species also have been associated with influenza infection, although the importance of any one of these populations in the ecology and emergence of influenza is uncertain. Among domestic avian species, chickens and turkeys are the most frequently involved in outbreaks of HPAI-virus-related disease although often the appearance of influenza in these birds is associated in the contact with wild water fowl [12].
The extent of influenza in passerine birds, ratites and other domestic poultry and their contribution to the maintenance and emergence of influenza A

viruses is far from understood. Additional non-human reservoirs of influenza A viruses are found in mammalian species such as swine and horses. Only a limited numbers of viral subtypes have been repeatedly isolated from swine (H1N1, H3N2 and H1N2) and horses (H7N7 and H3N8), although other subtypes can be isolated periodically. Marine and semi aquatic mammals such as whales, seals and mink, also have yielded influenza viruses. The viruses of the marine mammals appear to be of avian origin and are probably the result of independent transmission from aquatic birds rather than circulating lineages within these hosts.

Structure and genomic organization

The virions can exhibit a variety of shape and sizes, ranging from fairly spherical particles of approximately 100 nm in diameter to elongated filamentous form of the virus [13, 14].
The virus particles consist of a host-derived lipid bylayer envelope in which the virus encoded glycoproteins HA and NA and M2 are embedded, an inner shell of matrix protein 1 and at the centre the nucleocapsids of the viral genome. Complexes containing the three viral polymerase proteins (PB1, PB2 and PA) are situated at the end of nucleocapsids. The virus particles formation occur at the surface membrane of infected cells where budding occurs from region of the membrane at which the viral glycoproteins have accumulated. The virus consist of eight unique segments of single stranded RNA, which are of negative polarity, each segment contains a coding region that encodes one or two proteins (Figure 2).

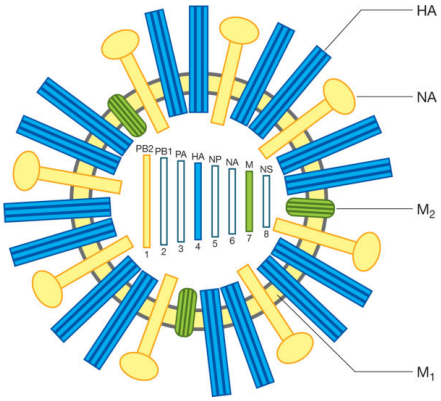

Figure 1. Schematic structure of the influenza A virus

The genome encodes at least ten recognized gene products: PB1, PB2 and PA polymerases, HA, NA, NP, M1, M2, NS1 and NS2 protein.

The three largest gene segments encode the subunits of the viral polymerase PB2, PB1 and PA, which are also named because their basic (PB2 and PB1) or acidic (PA) properties on isoelectric focusing gels. These are responsible for trascribing messenger RNAs (mRNAs), for synthesing positive sense antigenomic template RNAs (cRNAs) and for trascribing the cRNAs into gene segments (vRNAs) that are incorporated into progeny viruses.

Basic polymerase 2 (PB2, 2277 nucleotides, segment 1), it is a member of the protein complex providing viral RNA- dependent RNA plymerase activity. It is know to function during initiation of viral mRNA transcription as the protein which recognized and binds the 5' capI structures of the host cells mRNA for use as viral mRNA transcription primers. Endonucleolytic cleavege of these structures from host RNA is also at least in part a function of PB2. Newly synthetized PB2 proteins migrate to the nucleus of infected cells.

Basic polymerase 1 (PB1, 2271 nucleotides, segment 2), it function in the RNA complex as the protein responsible for elongation of the primed nascent viral mRNA and also as elongation protein for template RNA and vRNA synthesis. PB1 proteins localize in the nucleus of infected cells. PB1 gene also can encode a small pro-apoptotic mitochondrial protein that is translated in a different reading frame, PB1-F2)

Acidic protein (PA, 2148 nucleotides, segment 3), it also localizes in the infected cells nucleus and is a member of the RNA-dependent RNA polymerase complex along with PB1 and PB2, but its role in the viral RNA synthesis is unknown. PA might possess a transcriptase protease activity.

Two segments encode surface envelope glycoproteins that function as viral antigens:

Haemagglutinin (HA, 1698 nucleotides, segment 4), is an integral membrane protein and the major surface antigen of the influenza virus virion. It is responsible for binding of virions to host cell receptors and for fusion between the virion envelope and the host cell. It undergoes three kinds of posttranslational processing: proteolytic cleavage, glycosylation, and fatty acid acylation. Newly synthesized HA is cleaved to remove the amino-terminal hydrophobic sequence of 14 to 18 amino acids, which are the signal sequence for transport to the cell membrane. Carbohydrate side chains are added, whose number and position vary with the virus strain. Palmitic acid is added to cysteine residues near the HA carboxy terminus. The final processing step is cleavage of the HA into two subunits, HAl and HA2 uncleaved HA is called HA0), connected by disulfide linkages. This cleavage is accomplished by host-produced trypsinlike proteases and is required for infectivity because virus-cell fusion is mediated by the free amino terminus of HA2. The fully processed HA thus consists of HAl of (typically) about 324 amino acids plus variable carbohydrate, and HA2 of (typically) about 222 amino acids plus variable carbohydrate plus 3 palmitate residues. HA molecules form homotrimers during maturation. The

three-dimensional structure of a complete HA trimer has been determined and in essence, each HA molecule consists of a globular head on a stalk. The head is made up exclusively of HAl and contains the receptor-binding cavity as well as most of the antigenic sites of the molecule. The stalk consists of all of HA2 and part of HAl. The carboxy-terminal region of HA2 contains the hydrophobic transmembrane sequence and a terminal cytoplasmic anchor sequence where palmitate is attached. Although the amino acids making up the receptor-binding site, as well as cysteine and most proline residues, are highly conserved, the remainder of the HA molecule is highly mutable.

Neuraminidase (NA, 1407 nucleotides, segment 6), which is involved in budding of new virions from infected cells, it is also an integral membrane glycoprotein and a second major surface antigen of the virion. NA cleaves terminal sialic acid from glycoproteins or glycolipids. 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. NA is a tetramer, it is glycosylated and possesses an amino-terminal hydrophobic sequence which functions both as signal for transport to the cell membrane and as transmembrane domain; it is not cleaved away. The distribution of NA has not been conclusively resolved; immunogold-labeling experiments suggest that the NA tetramers are not evenly distributed over the virion envelope, as is HA, but aggregate into patches or caps. Like HA, NA is highly mutable.

The seventh segment encodes two proteins that share a short overlapping region: the matrix protein 1, M1, encodes the main component of the viral capsid, and M2, which is an integral membrane protein, functions as an ion channel.

Matrix protein 1 (M1, 756 nucleotides, segment 7) is the most abundant protein in the influenza virus virion. Matrix protein forms a shell surrounding the virion nucleocapsids, underneath the virion envelope. In the infected cell it is present in both cytoplasm and nucleus. It has no known enzymatic activities, although it has been speculated to play an important role in initiating progeny virus assembly.

Matrix protein 2 (M2, 291 nucleotides, segment 7) is a small transmembrane protein derived from an alternative splicing of M1 transcript. It is an integral membrane protein, whose membrane-spanning domain also serves as a signal for transport to the cell surface. It is present as a tetramer in large amounts on the infected cell surface, and a small amount is found in the virion. It is believed to act as a proton channel to control the pH of the Golgi during HA synthesis and to allow acidification of the interior of the virion during virus uncoating, that aids in virus disassembly during the initial stages of infection.

The eighth gene segment also encodes two proteins due to alternative splicing, these proteins were originally referred to as NS1 and NS2 because

they were thought to be nonstructural but NS2 has since been shown to be a component of virions.

Nonstructural protein 1 (NS1; 690 nucleotides, segment 8), has numerous functions, it is a regulator of both mRNA splicing and translation, and it also plays a critical role in the modulation of interferon responses to viral infection.

Nonstructural protein 2 (NS1; 363 nucleotides, segment 8) function to mediate the export of newly synthetized RNPs from the nucleus and as such, it is also referred to as nuclear export protein (NEP).

These proteins, particularly NS1, are abundant in the infected cell, NS1 primarily in the nucleus whereas 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.

Nucleoprotein (NP; 1,494 nucleotides, segment 5) is transported into the infected cell nucleus, where it binds to and encapsidates 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 the pattern of phosphorylation is host cell dependent and may be related to viral host range restriction.

Influenza virus life cycle

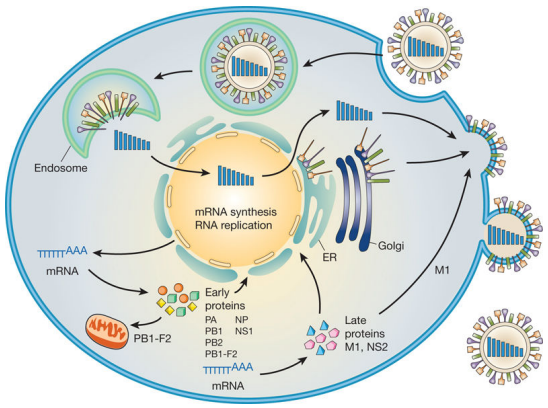

Figure 2. Schematic diagram of the influenza viral life cycle

Virus attachment

Influenza A viruses recognize N-acetylneuraminic (sialic) acid on the surface of host respiratory epithelial cells: sialic acid are nine carbon acidic monosaccharides commonly found at the termini of many glycoconjugates, thus they are ubiquitous on many cell type and in many animal species. The carbon 2 of the terminal sialic acid can bind to the carbon 3 or 6 of penultimate galactose residue of glycoproteins or glycolipids forming α-2,3- or α-2,6- linkages and resulting in unique steric configurations. The sialic acid moiety is recognized and bound by the HA on the surface of influenza virus which have a preferential specificity for either α-2,3- or α-2,6- linkages; the binding specificity of HA for glycoproteins and glycolipids on the host cells appears to be determinant of whether a particular subtype can infect humans therefore human viruses can only bind a receptors with an α-2,6- linkage, in contrast, avian influenza subtypes can only bind a receptor with an α-2,3- linkage.
In human tracheal epithelial cells have α-2,6- linkages predominate while α-2,3- linkage are more common in duck gut epithelium [15] whereas pig tracheal epithelium contain receptors with both α-2,3- and α-2,6- linkages [16] explaining why pigs are susceptible to both human and avian virus and this has led to the hypothesis of the pig as "mixing bowel": the permissiveness of the pig respiratory epithelium for both avian and human lineage influenza A strains is proposed to facilitate reassortment and the generation of new human pandemic strain with efficient human to human transmission.
Sialic acids with terminal α-2,3- are also present in human respiratory epithelium, thought in less abundance than those with α-2,6- linkages [17, 18]. Consequently, humans can be infected by avian influenza viruses albeit with overall less efficiency than by human strains [19-21]. The differential expression of sialic acids in the mammalian respiratory tracts may help to explain the low infectivity but high pathogenicity of some avian strains. In humans, α-2,3- linked sialylated proteins, while less abundant overall, are most prevalent in the lower respiratory tract (bronchioles and alveoli). The lungs are not as accessible to airborne virus particles as upper respiratory tract (nasopharynx, paranasal sinuses, trachea and bronchi) so avian virus infection is relatively rare in humans. However, when avian strains do infect the human lung, a severe and rapidly progressive pneumonia may result; in this clinical setting, fatality rates exceed 60% [22].

Virus Entry

Following attachment of the influenza virus HA protein to sialic acid, the virus is endocytosed, the acidity of the endosomal compartment is crucial to influenza virus uncoating in two ways. First, low pH triggers a conformational change in the HA, exposing a fusion peptide that mediates

the merging of the viral envelope with the endosomal membrane, thus opening a pore through which the viral RNPs are released into the host cell cytoplasm [23, 24]. Second, hydrogen ions from the endosome are pumped into the virus particle via the M2 ion channel. Internal acidification of the influenza virion via the M2 channel disrupts internal protein–protein interactions, allowing viral RNPs to be released from the viral matrix into the cellular cytoplasm [25].

Synthesis of viral RNA

Once liberated from the virion, RNPs are trafficked to the host cell nucleus by means of viral proteins' nuclear localization signals (NLSs), which direct cellular proteins to import the RNPs and other viral proteins into the host cell nucleus [26]. The nucleus is the location of all influenza virus RNA synthesis, both of the capped, polyadenylated messenger RNA(mRNA) that acts as the template for host-cell translation of viral proteins, and of the vRNA segments that form the genomes of progeny virus. The viral RNAdependent RNA polymerase, a component of the RNPs imported into the nucleus, uses the negative-sense vRNA as a template to synthesize two positive-sense RNA species: mRNA templates for viral protein synthesis, and complementary RNA (cRNA) intermediates from which the RNA polymerase subsequently transcribes more copies of negative-sense, genomic vRNA. Unlike host cell mRNA, which is polyadenylated by a specific poly(A) polymerase, the poly(A) tail of influenza virus mRNA is encoded in negative-sense vRNA as a stretch of five to seven uracil residues, which the viral polymerase transcribes into the positive sense as a string of adenosines that form the poly(A) tail [27-29]. Messenger RNA capping occurs in a similarly unique manner, in which the PB1 and PB2 proteins "steal" 5' capped primers from host pre-mRNA transcripts to initiate viral mRNA synthesis; this process is called "cap snatching" [30]. Once polyadenylated and capped mRNA of viral origin can be exported and translated like host mRNA. Nuclear export of vRNA segments, however, is mediated by the viral proteins M1 and NEP/NS2 [26]. M1 interacts with both vRNA and NP, and is thus thought to bring these two components together within the RNP complex; M1 also associates with the nuclear export protein NEP, which mediates the M1-RNP export via nucleoporins into the cytoplasm.

Synthesis of viral proteins

The envelope proteins HA, NA, and M2 are synthesized, from mRNA of viral origin, on membrane-bound ribosome into the endoplasmic reticulum, where they are folded and trafficked to the Golgi apparatus for post-translational modification. All three proteins have apical sorting signals that subsequently direct them to the cell membrane for virion assembly.

Although comparatively little is known about the translation and sorting of the non-envelope proteins, M1 is thought to play a role in bringing the RNP–NEP complex into contact with the envelope-bound HA, NA, and M2 proteins for packaging at the host cell membrane.

Packaging of RNA and assembly of virus

Influenza virus is not fully infectious unless its virions contain a full genome of eight segments. Previously, vRNA packaging was thought to be an entirely random process, in which vRNA segments are haphazardly incorporated into budding virus particles, and only those ending up with a complete genome become infectious; however, newer evidence suggests that packaging is a more selective process, in which discrete packaging signals on all vRNA segments insure that a full genome is incorporated into the majority of virus particles [31-33].

Virus budding and release

Influenza virus budding occurs at the cell membrane, probably initiated by an accumulation of M1 matrix protein at the cytoplasmic side of the lipid bilayer. When budding is complete, HA spikes continue to bind the virions to the sialic acid on the cell surface until virus particles are actively released by the sialidase activity of the NA protein. It possesses receptor-destroying activity, cleaving terminal sialic acid residues from cell-surface glycoproteins and gangliosides to release progeny virus from the host cell. In viruses with inactive or absent NA, or in the presence of neuraminidase inhibitors, virus particles clump at the cell surface and infectivity is consequently reduced. The NA also removes sialic acid residues from the virus envelope itself, which prevents viral particle aggregation to enhance infectivity [34, 35].
The NA is also thought to aid virus infectivity by breaking down the mucins in respiratory tract secretions and allowing the virus to penetrate through to the respiratory epithelium, and it may play a role in virus entry into respiratory epithelial cells [36]. Host antibodies to the NA, as well as neuraminidase inhibitors, prevent virus release from infected cells and thus inhibit viral replication.

Antigenic shift and drift

The genetic properties and ecological diversity of influenza A make it a classic example of a re-emerging virus; immunity induced by HA has been shown to increase host resistance to influenza and reduce the likelihood of infection and severity [37], however, such protection is not effective against newly emerging influenza viruses that contain antigenic variations, in particular in their surface glycoproteins, known as antigenic drift and shift [38]. Selection favors human influenza A strains with antigenic shift and

drift involving the HA and NA protein because these strains are able to evade neutralizing antibodies from priors infection or vaccination.
Antigenic drift refers to a minor change, such as amino acid substitution in HA and/or NA proteins, resulting in antigenic site change. Influenza A nucleic acid replication by the virus-encoded RNA-dependent RNA polymerase complex is relatively error-prone and these point mutation ~ $1/10^4$ bases per replication cycle in the RNA genome is the major source of genetic variation [39].
In contrast, antigenic shift is the formation of a new virus subtype with mixed HA and NA from different subtypes, The segmentation of the influenza A genome facilitates the reassortment among strains when two or more strains infect the same cells [40] and this lead to major genetic changes which are associated with pandemics because they originates the appearance of new influenza to which the population is immunologically naïve (Figure 3).

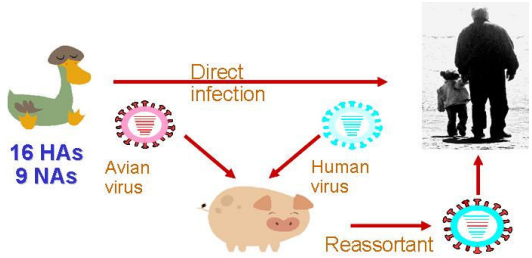

Figure 3. Mechanism of influenza A variation

In the 20th century, three novel influenza stains emerged to cause pandemics, in 1918, the Spanish Flu (H1N1), in 1957, the Asian Flu (H2N2) and in 1968, the Hong Kong Flu (H3N2) (Figure 4). These emerging viruses differed substantially in their overall mortality rates and apparent virulence for humans. Particularly the 1918 pandemic was the most severe with overall estimated numbers of death worldwide ranging from 50 to 100 million [41]. Although its clinical symptoms and pathological manifestations were largely confined to the respiratory tract [42] a large proportion of death occurred among healthy and immunocompetent adolescents and young adults [43].
Cloning of all of the protein-encoding mRNA sequences from the 1918 H1N1 virus has recently been completed using formalin-fixed pathology specimens and exhumed remains of an individual buried in Alaska [44-50]. An analysis of these sequences suggests that although they are not all identical to those isolated from contemporaneous influenza A viruses in birds [48], they nevertheless retain strong avian characteristics [49]. This suggests a relatively recent avian origin and only a relatively brief period of adaptation in humans or an intermediate mammalian host prior to the

pandemic. Based on in vitro studies, the HA from the 1918 H1N1 virus was capable of binding receptors having terminal 2–6 rather than 2–3 linkages of sialic acid [50].
The 1918 H1N1 virus and chimeric strains containing combinations of proteins from the 1918 and more a more recent H1N1 strain have also been produced. A recent study [51] with these viruses has revealed that the 1918 H1N1 strain has the unusual property of being able infect cell lines without the provision of exogenous proteases, such as trypsin, and to replicate to higher levels than more recent H1N1 isolates. Inoculation of the 1918 H1N1 strain into mice also resulted in higher viral load than in strains that lack either the 1918 HA and NA or the polymerase complex of proteins. Severe pulmonary pathology, lung infiltration by inflammatory cells and the hemorrhagic pneumonia, similar to what was observed in the lung of human victims of the 1918 pandemic [51]. Based on the results obtained with chimeric viruses, the HA, NA, and polymerase proteins are all critical for these pathogenic properties of the 1918 H1N1 strain.
In the last century other three less serius pandemics occurred: in the 1957 an H2N2 subtype, the Asian Influenza, replace H1N1, in the 1968 an H3N2, the Hong Kong influenza, replace H2N2 and in the 1977 H1N1 virus, the Russian Influenza, reappeared.
The 1957 virus consisted of HA (H2), NA (N2) and viral polymerase gene segment PB1 from an avian virus, with the other gene segments derived from a previously circulating human virus [52, 53]. The 1968 virus had an avian HA (H3) and PB1 in a background of human viral genes [52, 53] (Figure 4). Why both of these pandemics strains also carried the gene that encodes PB1 from an avian strain remain unclear but it could suggest that PB1 protein might play a role in viral transmissibility in humans.
The Russian influenza H1N1 strain was essential identical to those H1N1 that had circulated in humans in the 1950s [54]. This suggest that a previously frozen laboratory strain was reintroduced into general human population, most likely as the results of a viral challenge of military recruits in the Far East [55]. The magnitude of this outbreak was limited, as most the individual over 27 years of age had some immunological memory to the virus.

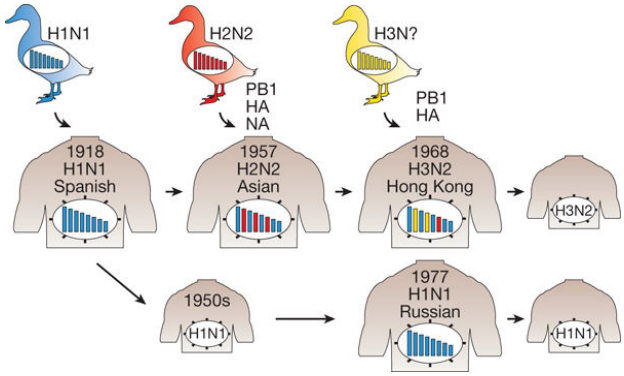

Figure 4. Past pandemic influenza viruses

Avian Influenza

Recently, purely avian influenza viruses, including H5N1, H9N2 and H7N7 subtypes, have been directly transmitted to humans, raising concern over the possibility of a new influenza pandemic among the world's immunologically naïve populations.

Historically, avian virus infection were largely limited to laboratory and occupational exposure [56-59] and most of these human infection resulted in conjunctivitis and, occasionally, mild respiratory symptoms. However in 2003 in the Netherlands, an outbreak of HPAI H7N7 virus resulted in 89 reported infections among veterinarians and individuals involved in poultry culling operations and few family members of cullers who had no exposure to infected poultry [60]. The majority of patients presented with conjunctivitis, a typical clinical sign of H7 human infection, although respiratory illness was seen in a few individuals, and in one, the disease progressed to a fatal pneumonia with acute respiratory distress syndrome [61]. In recent years, repeated outbreaks of both HPAI and LPAI have occurred in Europe and North America that have been associated with rare infections among humans exposed to infected poultry, primarily resulting in conjunctivitis or relatively mild respiratory illness [60, 62, 63]

The firs recorded direct transmission of avian influenza viruses to humans leadings to deaths occurred in 1997, when 18 people were infected during an outbreak of HPAI H5N1 in poultry markets in Hong Kong, six of whom died [64]. Since 1997, HPAI H5N1 viruses continued to circulate in Asia and reemerged in Hong Kong in 2003. Subsequently the HPAI H5N1 viruses have surfaced in poultry and wild birds across the Eastern hemisphere, also causing disease in several terrestrial carnivores [65-69] and humans [70]. In humans ~ 400 cases of HPAI H5N1 infection were detected to date with a fatal outcome in ~ 60% of infections (Figure 5).

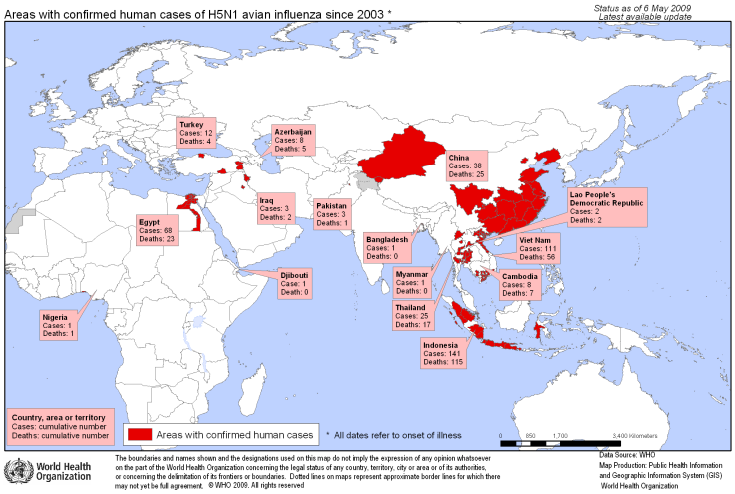

Figure 5. Areas reporting confirmed occurrence of H5N1 avian influenza in humans

The majority of human infections have been the result of exposure to H5N1 virus-infected poultry, but occasional family clusters have provided evidence for limited person-to-person transmission. The viruses isolated to date from humans are wholly avian in origin and lack the ability for sustained transmission in humans.

In general, patients infected with H5N1 viruses presented with fever, respiratory symptoms including cough and shortness of breath [61, 70, 71], gastrointestinal symptoms including diarrhea, vomiting and abdominal pain [72, 73], and in one pediatric case, neurological complications. Leukopenia and lymphopenia have been associated with a poor prognosis [73]. In severe cases, the clinical course of H5N1 influenza virus infection is characterized by rapidly, progressive bilateral pneumonia, requiring ventilatory support within days of onset [74-76]. At presentation, the pneumonia usually seem to be of primary viral origin without evidence of bacterial suprainfection. Complication include acute respiratory distress syndrome, renal dysfunction and moltiorgan failure. On the basis of reported cases, the mortality exceeds 60% with most patients dying of progressive respiratory failure. The average incubation period is 2–5 days, and for the fatal cases, death occurred approximately 9 days after symptom onset, a more rapid progression to fatal disease than seen in the first human cases in 1997. In contrast to the older age for fatal disease with seasonal influenza viruses, the highest case fatality rate among H5N1-infected patients is among individuals 10–19 years of age.

Whether and to what extent disseminated virus infection occurs and contributes to disease pathogenesis in people is uncertain. The presence of viral RNA or replicating virus in the blood of most patients, especially from those to died from infection [73, 77, 78] and the presence of replicating virus

in extrapulmonary tissues of some patients [79, 80] suggest the potential of H5N1 viruses to disseminate to other organs, similar to what occurs in animal. A few postmortem examinations suggest that actual replication in human non respiratory tissues, such as liver, lymph nodes, brain and placenta take place [81].

In addition to direct damage caused by viral replication, an intense inflammatory reaction in response to high amount of virus, possibly enhanced by H5N1 virus induced cytokine dysregulation [82-84] probably also plays an important part in disease pathogenesis (Figure 6).

Higher plasma levels of macrophage and neutrophil attractant chemokines and both proinflammatory and anti-inflammatory cytokines (interleukin-6, interleukin-10 and interferon-γ) have been observed in patients with influenza A H5N1, particularly in patient with fatal infection, than in patients with conventional influenza. Plasma levels of cytokines and chemokines correlate positively with pharyngeal viral loads suggesting that these responses are driven by high-level viral replications. In vitro experiments involving primary human macrophages and lung pneumocytes show differential up regulation of multiple cytokines by influenza A H5N1 virus as compared with human influenza viruses, indicating that viral hyperinduction probably contributes to hypercytokinemia.

The occurrence of mildly symptomatic and asymptomatic infections in individuals exposed to patients or poultry infected with H5N1 virus has been suggested by seroepidemiological studies after 1997 outbreak in Hong Kong [85, 86]. Serological studies during H5N1 outbreaks in Vietnam showed no evidence of infection in exposed health-care workers [87], whereas serosurveillance studies in a village affected with H5N1 in Cambodia showed no infection despite direct contact with poultry suspected of having H5N1 virus infection. These studies suggest inefficient transmission of H5N1 viruses to human beings, but additional studies in individuals exposed to infected birds are essential.

Characteristic laboratory results in patients infected with H5N1 influenza virus, especially those with severe virus, are an early onset of lymphopenia, with an inverted ratio of $CD4^+$ cells to $CD8^+$ cells. The depletion of lymphocytes might be secondary to virus induced apoptosis as suggested by in-vitro and murine experiments with H5N1viruses [88, 89].

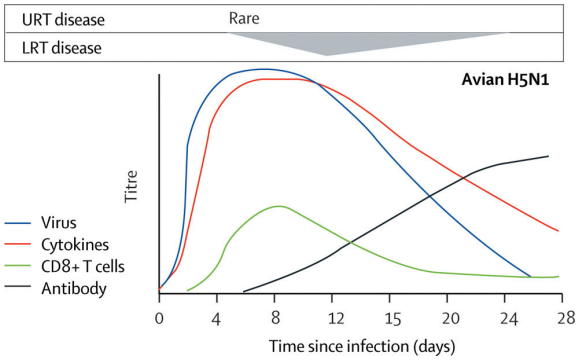

Figure 6. Course of human infection with highly pathogenic H5N1 ifluenza virus.
URT=upper respiratory tract. LRT=lower respiratory tract.
Presumed course of nasopharyngeal virus load (blue), cytokine response (red), antibody titer (black), and cytotoxic T-cell response (green), during infection with H5N1 influenza virus.
H5N1 infection predominantly causes pneumonia, which is usually complicated by acute respiratory distress syndrome. Infection is associated with prolonged shedding at high nasopharyngeal titres, and high concentrations of proinflammatory cytokines and chemokines. Neutralising antibody responses are detectable 14 or more days after the onset of symptoms. The cytotoxic CD8+ T-cell response peaks at 6-9 days but could potentially be extended because of prolonged exposure to viral antigens.

Pathogenicity

Multiple molecular determinants of pathogenicity of HPAI have been identified and these are important to better understand the disease caused by HPAI influenza virus in humans.

Haemagglutinin: HA is responsible for viral binding to host receptors, enabling entry into the host cells through endocytosis and subsequent membrane fusion.

HA binds to receptors containing glycans with terminal sialic acids, where their precise linkage determines species preference. A switch in receptor specificity from sialic acids connected to galactose in α2-3 linkages (avian) to α2-6 linkages (human) is the major obstacle for influenza A viruses to cross the species barrier and to adapt to a new host [90, 91]. On HA frameworks, as few as two amino acid mutations can switch human and avian receptor specificity.

Each monomer of HA is synthesized as a single polypeptide HA0 that is post translational cleaved by host proteases into two subunits HA1 and HA2 and this is essential for viral infectivity. LPAI viruses have a single Arg at the cleavage site of HA0 between HA1 and HA2. HAs are transported to the plasma membrane of epithelial cells of the avian respiratory and digestive tracts in an uncleaved form because cleavage is not mediated by proteases within the infected cells but is mediated by extracellular trypsin-like proteases that are in vivo still unknown. These proteases are secreted only by cells of respiratory and intestinal tract so these viruses, although fully infectious, replicate in limited cell types and are restricted in their ability to

spread in a host and cause anatomically localized infections with relatively mild symptoms.
HPAI viruses have a connecting peptide sequence of several basic amino acids which can cleaved intracellulary by ubiquitous subtilisin-like calcium-dependent proteases such as furin or PC5/PC6 [92]. These viruses can infect a broad range of host cells and in poultry cause an acute fatal systemic infection. Cleavage can be modulated by carbohydrate [93] and by amino acid structure of the cleavage loop [94], both of which can alter protease accessibility. Therefore HA cleavability is considered the main determinant of the tissue tropism of AI viruses [95] and differences in the tissue distribution of proteases and HA susceptibility to these enzymes can determine the outcome of virus infection.
Polimerase complex: the polymerase complex (PB2, PB1 and PA) is involved in replication and transcriptional activity, multiple amino acid substitution in the complex have been described, mainly in the PB2 that are important for adaptation of avian virus in human, enhance the polymerase activity and increase the virulence[96]. H5N1 viruses that are virulent in mice encode a lysine at position of 627 in PB2 whereas H5N1 viruses that are no virulent in mice, as well as other avian influenza A viruses strains, encode glutamic acid at this position [97]. However, this amino acid does not determine the cell tropism of the virus [98] but enhance viral growth in mice and probably in humans. This amino acid substitution was isolated from a patient whit a fatal infection of HPAI H7N7 in the Netherland in 2003 in contrast to viruses isolated from no fatal cases of conjunctivitis and mild infection. Likewise, many but not all of fatal HPAI H5N1 isolated in humans, five of eight isolates, show Lys627 in PB2 therefore there was no association with the presence of Lys at 627 and the clinical outcome. Interestingly three of four viruses without this substitution, but none with lysine at position 627, contained the substitution of aspartic acid to asparagine at position 701 that has been associated with adaptation of H7N7 viruses to mammalian cells [99] so this substitution may compensate for absence of lysine 627 in conferring enhanced viral polymerase activity and virulence in mammalian host.
These findings demonstrate that changes in only the polymerase genes of H5N1 are sufficient to dramatically alter its pathogenic potential, even changes in single segment PB2 and or PB1 attenuate the virus isolate's pathogenicity.
PB1-F2 is a protein encoded by an alternative reading frame in the PB1 gene; this protein target alveolar macrophages inducing apoptosis [100]. This results in down regulation of the host immune response and lead to secondary bacterial infections and virulence in mice [101]. All viruses that were highly pathogenic to mice had 66S in PB1-F2 whereas less virulent viruses had 66N, The N66S substitution resulted in more severe infection, higher virus titer and higher production of the cytokine IFN-γ and TNF-α in

the lung of infected mice [102]. Interestingly 66S was also present in the 1918 Influenza. Knock-out of PB1-F2 did not alter viral replication but enhanced clearance of the virus and reduced lethality in mice suggesting that PB1-F2 may play a role in viral pathogenesis [103].

NS1 An important aspect of viral pathogenesis is the ability of influenza virus to evade host immune response. Viral replication induce an innate immune response that is the first line of host defense and induces the production of IFNα/β which have an antiviral effect. The basis of the IFNα/β antagonistic properties of the NS1 relies on its ability to prevent IFN synthesis. NS1 prevent the activation of IFN transcription factor and also binds and inhibits the function of two cellular proteins that are required for the modification of 3' ends of cellular mRNA [104-106].

Nevertheless, the effects of NS1 protein on IFN response remain only partially understood and probably the viral infection antagonist activity shows an high degree of species specificity [107, 108].

In addition to blocking the IFN response, the NS1 protein of avian viruses binds cellular proteins and disrupts their function in regulatory pathways. The C-terminus of the NS1 protein contain a sequence motif, Glu-Ser-Glu-Val (ESEV), a PDZ ligand domain, capable of binding PDZ protein interaction domain of host protein. Many cellular proteins that contain this motif are involved in diverse cellular pathways including those that regulate protein traffic within the cells and those that maintain cell morphology and organization. In all the virulent H5N1 viruses isolated from humans and also in the 1918 virus there was a different PDZ binding sequence, Glu-Pro-Glu-Val (EPEV) that had stronger binding affinity to human protein PDZ domain whereas in low virulence influenza A viruses there is usually a different sequence without PDZ motif [109].

Pathology

Transmission of human influenza virus occurs by inhalation of infectious droplets or airborne droplet nuclei and, perhaps, by indirect contact followed by self-inoculation of the upper respiratory tract or conjunctival mucosa. The typical sign and symptoms of uncomplicated influenza are both local like nasal obstruction, cough, sore throat and systemic like headache, fever, chills, anorexia and myalgia. These sign and symptoms are due both to the damage at the site of virus replication and to the local and systemic release of cytokines and other inflammatory mediators [110, 111]. These symptoms persist for approximately one week. Pneumonia is a frequent manifestation of more severe infection. Rarely it can lead to myocarditis, encephalitis and other extrarespiratory tract disease. Viral infection is also associated with an increased incidence of subsequent otitis media [112] and influenza A pneumonia may be complicated by subsequent infection with bacterial pathogens such as *Staphilococcus aureus* [113]. Finally, influenza A infection is an important trigger of reactive airway disease in those with

preexisting asthma, and it may also promote allergic sensitization to envirolmental proteins [114, 115].
The most common complication of influenza is the extension of the viral infection distally to the lung, resulting in ***pneumonia***. In contrast to damage to the tracheo-bronchial epithelium in uncomplicated influenza, damage to the alveolar epithelium has severe consequences for the gas exchange function of the respiratory tract and it is due to the combination of the direct cytolytic effect of viral infection and the indirect effect of host response. Risk factor for development of viral pneumonia include lack of previous exposure to influenza virus with related surface glycoproteins, age grater than 65 years, pulmonary disease, cardiovascular disease and pregnancy. Viral pneumonia often occurs together with, or is followed by, bacterial pneumonia. Prior influenza virus infection may predispose the respiratory tract to bacterial infection by different mechanisms and vice versa, bacterial infection may enhance influenza virus infection [116].
Human influenza virus primarly infects and causes disease in the respiratory tract, however the infection is also associated with disease in other organs albeit to a lesser extent.
An important complication of influenza A virus infection is central nervous system dysfunction, that can take a number of forms, including influenza associated acute encephalopathy (IAAE), an uncommon neurological syndrome generally of children and adolescent and typically presents during the early phase of infection [117].
Myocarditis has been observed in association with fatal influenza in each of the three pandemics of the previous century and in the interpandemic periods but its pathogenesis is poorly understood. Myositis or myophaty is sporadically reported as a complication of influenza virus but again the pathogenesis is unclear.
Influenza pandemics cause higher morbidity and mortality rates than the seasonal epidemics during interpandemic period; this is mainly due the lack of specific immunity to the new virus, so that infection is more likely to result in complicated disease, in particular pneumonia. The characteristics of respiratory and extra respiratory complications do not differ in pandemic and interpandemic periods, but the proportion of infected people who develop complications is lower during interpandemic influenza.

The immune response to influenza A viruses

Influenza A viral infections elicit potent adaptive immunity and long term memory, the pathway by which protective immune responses to influenza develop is a multi-step process and it is based on the innate immune system.
Invading influenza A viruses are detected in the host environment by “pattern recognition receptors” (PRR) [118]. In the past, the molecular target was considered to be double-stranded RNA recognized by TLR3, a PRR [119, 120]. A role for TLR3 was questioned, however, given that the

concentration of ds RNA is unlikely to be sufficient to signal TLR3 [121] and it is now considered that influenza A virus infection does not generates ds RNA at all [122]. Instead, the influenza A virus polymerase generates ssRNA with an uncapped, 5' phosphate that serves as molecular signature identified by the immune system. The cytoplasmic RNA-helicase, RIG-1 but not MDA-5 is responsible for influenza A virus recognition, which occurs independently of viral replication [122-125]. In addition to RIG-1, TLR7 is implicated in influenza A virus detection, expressed in the endosomal compartments of plasmacytoid dendritic cells and B-cells, TLR7 detects influenza A virus ssRNA [125, 126]. The participation of multiple PRR in the surveillance of influenza A viruses may reflect cell type-specific roles. Once the influenza A virus is recognized, PRR initiate multiple signaling cascades that facilitate both innate and adaptive immunity to enable viral eradication.

Innate immunity

Innate immunity directed against influenza A virus provides an immediate and rapid response to the pathogen. The infiltrate of innate immune cells is comprised mainly of natural killer (NK) cells, neutrophils and macrophages. The NK cells represents the major innate response element and are detected in the infected lungs as early as 48 hours following influenza A infection [127, 128]. Protection is thought to be mediated by both cytokine production, interferon (IFN) γ and tumor necrosis factor (TNF) α and direct cytotoxicity of virus infected cells.

Together with NK cells, neutrophils also contribute to influenza A virus clearance trough the secretion of an array of pro-inflammatory molecules that serve to limit viral replication [129-131]. Finally, alveolar macrophages (AM) are also present in the innate pulmonary infiltrate, although initially they form anly a small contribution, but are recruited in large numbers later by the T cells response. AM represent the major phagocytic cell type resident in the lung [132], acting to scavenge influenza A virus driven antigen [133], they also secrete pro-inflammatory cytokine, including TNF-α, interleukin (IL) 1β, IL-6 and IFN-α/β [134, 135] together with the chemokines macrophage inflammatory protein (MIP) 1a, monocyte chemotactic protein (MCP)-1, RANTES and IFN-inducible protein (IP-10) [136-138]. The AM can also modulate adaptive T cell immunity to influenza A viruses [139]. Therefore, multiple immune cell types provide immediate innate defense against the influenza A viruses. The pulmonary infiltrate releases a torrent of innate immune molecules that are considered to limit influenza A virus infection. A long list of cytokines and chemokines are potentially involves. A major player is type I IFN, representing the most potent cytokine attack against the virus [140]. So potent is the IFN response that influenza A viruses encode a protein NS2 to disable this pathway. Nasal and pulmonary IFN rise rapidly following influenza infection and act to

directly limit viral replication and induce further cytokines and or chemokines secretions that enhances recruitment and activation of multiple immune cell types, it is important to enhance macrophage function, promote antigen presentation by antigen presenting cells and to modulate adaptive immunity. Plasmacytoid DCs are the major producers of type I IFN in response to many viruses, including influenza A viruses [141-144].
Other cytokines implicated in Influenza A virus immunity include TNF- α, IL-6, IL-1, IL-18 and IL-12 whereas chemokines with defined roles in influenza A virus immunity inclube MIP-1α and CCR5. Finally, while cytokines and chemokines are important in the immune control of influenza virus infection, their contribution can be detrimental as they elicit potentially fatal cytokine shock [145]. Therefore, particularly early on, potent inflammatory anti-viral activitie may be dangerous than protective, to the deleterious impact upon lung pathology.
Other important factors are also collectins, a collagen-like lectins, that are constitutively present in the fluids that line the respiratory tract. Collectin family members, the surfactant proteins A (SP-A) and SP-D together with mannan binding lectin (MBL) contribute to influenza virus clearance via a number of mechanism [146].
They inhibit hemagglutination and viral infectivity, in addition, MBL enhance the complement mediates lysis of infected cells, SP-A and SP-D promote the binding and the uptake of influenza virus by neutrophils and SP-A promotes the opsonization and phagocytosis of influenza A virus by the AM population. The sensitivity of different influenza A viral strains to collectin-mediated defense correlates with the degree of glycosilation of the HA glycoproteins.
Defensin are cationic peptides produced by both leukocytes and epithelial cells, they can exert direct microbial activity or promote immunity by chemotactic agents.

Dendritic Cells

DCs enable pathogen-derived antigens to be presented in a context that facilitate successful T cell immunity. Specialized in antigen presentation, the DCs facilitate the acquisition of antigen, processing and presentation of antigenic peptides in the context of host major histocompatibility complex (MHC) molecules and the provision of costimulatory signals. Immunity to influenza A virus infection requires DC for both primary and secondary T cell responses.

$CD8^+$ T cells

Effector $CD8^+$ T cells, cytolytic T lymphocytes (CTL) are important in the normal clearance of influenza viruses [147].

$CD8^+$ T cells are primed, activated and expand in the lung draining lymph nodes during the first week or so after primary infection [148]. Activated $CD8^+$ T cells then traffic to the respiratory airways and the infected lung to mediate viral clearance. At the site of infection, $CD8^+$ T cells target virus infected cells that express peptide derived from influenza virus protein associated with MHC class I. Once their target antigen is recognized, $CD8^+$ T cells exert multiple effectors function. Cytokine such as IFN-γ, TNF-α and IL-2 are secreted by influenza virus specific $CD8^+$ T cells [149]. In addition $CD8^+$ T cells mediate direct cytolysis of influenza A virus infected target cells by the exocytosis of cytolytic granules that contain perforin and granzymes [150, 151] and /or through the expression of FasL [152-154].
Following influenza virus clearance, virus specific $CD8^+$ T cells decrease in number, a plateau is reached approximately two months following infection.
It has been demonstrated that $CD8^+$ T cells contribute to protective immunity and are largely directed to epitopes within conserved viral protein, like nucleoprotein and matrix protein) and that are shared by the various subtypes of influenza A, including H5N1 [155-157]. In the presence of serum antibodies of proper specificity, induced by vaccination or previous infection, CTL immunity may be redundant; however, under circumstances in which pre-existing humoral immunity immunity in the population is not or poorly protective against the circulating outbreak strain, as in the case with antigenic drift variants or the introduction of a pandemic influenza virus subtype, the induction of cross-protective CTL may be relevant [158].

$CD4^+$ T helper cells

Virus specific $CD4^+$ T cells are important participants in influenza immunity. $CD4^+$ T helper cells do not have an antiviral effector function themselves but play a crucial role in the control and regulation of the humoral and cell mediated immune response. A vigorous, heterogeneous $CD4^+$ T cell response is elicited following influenza virus infection. Again, the process of clonal expansion and differentiation is initiated in the lung draining lymph nodes, with the peak response in the respiratory airways occurring 6-7 days following infection [159]. T helper cells recognized peptides that result from protein degradation in the endosomes and are complexed with MHC class II molecules. Several subsets of T helper cells are distinguished based on the cytokines they produce. T helper type 1 cells (Th1) preferentially produce IFN-γ and IL-2 whereas Th2 preferentially produce IL-4 and IL-5. These cytokine responses contribute to the regulation of both antibody and cytotoxic T lymphocyte response to vaccination. While both Th1 and Th2 provide T cell help for antibody production an increased Th1 relative to Th2 response to vaccination is needed for optimal stimulation of CTL responses [160].

The absence of $CD4^+$ T cells during primary influenza virus infections leads to a significant reduction in the size and magnitude of the secondary response and impaired viral clearance [161, 162].
Until recently, the spectrum of influenza virus $CD4^+$ T cells epitopes was much less well characterized than the panel known for the $CD8^+$ subset.

Humoral Immunity

Humoral immunity provides host defense through B lymphocyte secretion of antibody. Influenza virus elicits a diverse spectrum of antiviral antibody response. Natural antibodies present the first line of antibody mediated defense. These are low-affinity antibodies that restrict early virus dissemination and promote the recruitment of viral antigens to the secondary lymphoid organs [163, 164]. Natural antibodies reduce the overall load of influenza virus and, as such, are required for optimal specific IgG antibody response. Although natural antibodies are involved in the primary response to influenza virus, they are not required for optimal protection from secondary challenge [165]. While natural antibodies clearly display anti-viral properties, effective virus clearance requires the induction of neutralizing antibodies. Such neutralizing antibodies can be rapidly induced and possess high affinity (or avidity) for viral antigen.
$CD4^+$ T helper cells contribute to humoral immunity by promoting B cells differentiation into immunoglobulin class-switched, antibody secretion cells. In most studies, the production of anti influenza virus antibody is $CD4^+$ T cells dependent [166-169]. Classically, $CD4^+$ T cells help involves the recognition of viral antigen and the delivery of an activation signal to the B cell via TNFR family member, CD40. Of interest, $CD4^+$ T cells can help B lymphocytes by non cognate interactions that do not require specific influenza A virus antigen recognition.
Protective antibodies target antigenic structure exposed on pathogen surface. It is generally accepted that antibodies directed against the influenza virus HA is the major correlate of protection. The HA is the viral receptor-binding protein and antibodies that are directed to the epitopes located in or in close proximity to the receptor binding site, can prevent the binding of the virus to its receptors. This way the virus is neutralized efficiently and the induction of sufficientely high serum titers of virus strain specific antibodies by vaccination or infection will protect subjects from subsequent infection. The protective effect of HA-specific antibodies has been demonstrated both in experimentally infected animals and humans [170].The protective effect of HA-specific antibodies is used as a correlate of vaccine efficacy and vaccines are registered every year when they fulfill the minimal EMEA/FDA requirements according to the serological outcome of vaccination and potency of the vaccine (>15 μg HA per vaccine strain). Typically, the serological outcome of vaccination is measured with the hemagglutinin inhibition (HI) assay and an HI titers ≥ 40 is considered a protective titers.

For optimal vaccine efficacy, it is essential that there is a close antigenic mach between the vaccine strains and the epidemics strains. Since the HA of seasonal influenza virus strains exhibit extensive antigenic drift, these virus escape from recognition by virus specific antibodies and therefore the vaccine strain need to be updated almost annually [171].
Obviusly for pandemic influenza, vaccine strains must be selected that afford the broadest possible antibodies responses. For example, currently H5N1 strains continue to circulate from antigenically distinct clades and ideally, a vaccine would protect against all of these antigenic variants. It is virtually impossible to predict which of these variants may cause a pandemic outbreak.
To increase the immunogenicity of inactivated influenza vaccines adjuvants have become available. The use of these adjuvants not only increases the antibody response to the vaccine strain, it also results in a more broadly reactive antibody response [172-174]. Another important advantage of the use of these adjuvants is that less antigen is required for the induction of protective antibody responses, which is especially relevant with regard to the envisaged shortage of vaccine doses during a next influenza pandemics.

Treatment and prevention

There are two main methods of influenza prophylaxis: the use of antiviral drugs and vaccines. Several drugs are licensed in USA for both influenza prophylaxis and treatment: the M2 ion channel inhibitors (amantadine and rimantadine) and NA inhibitors (zanamivir and oseltamivir). These antiviral drugs provide a useful adjunct to influenza vaccine but vaccinations remains the keystone of prophylaxis and it is the primary methods for preventing influenza and its severe complication. Vaccination is associated with a reduction in influenza related respiratory illness and physician visits at all ages, in hospitalizations and deaths among high-risk persons, otitis media among children, and work absenteeism among adults.
The three current types of vaccines are inactivated vaccine, attenuated vaccine and vector based vaccines. In recent years development of cell-culture based vaccines is an attractive approach to the production of inactivated vaccine production.
Since 1940s inactivated vaccine are produced by propagation of the influenza virus in embryonated hen's egg. The allantoic fluid is harvested, and the virus is concentrated and highly purified, then inactivated with formaldehyde or β-propiolactone. Separation of the HA and NA by means of detergent such as Tween 80 or Triton N101 produced split virus or subunit vaccine. Influenza vaccine may contain trace amounts of residual egg proteins and thus should not be administered to persons who have anaphylactic hypersensitivity to eggs. The vaccine is available in whole, split (chemically disrupted) and subunit (purified surface glycoproteins) formulations, which are administered intramuscularly or subcutaneously.

The vaccine potency is measured in terms of micrograms of HA per vaccine dose. General experience suggested that split and subunit vaccine are less pyrogenic and immunogenic than whole-virus vaccine [175] nevertheless whole influenza vaccine is associated with more frequent side reactions. In children younger than age 9, split or subunit preparation are preferred to reduce reactogenicity, and two half doses are recommended given at least one month apart.

The degree of protection after vaccination is dependent on antigenic match between the vaccine strains and those circulating in the community, the age of vaccine recipient and their previous history of influenza. Inactivated vaccines are 60-100% effective in the prevention of morbidity and mortality but they may have reduced effect in young (immune naïve) and elderly (decreased immune function) and in years of poor antigenic match.

Moreover the serological response to inactivated vaccine depends on the previous experience of the recipient to infection by viruses of the same subtype of influenza virus as that present in the vaccine. For example whereas a single subcutaneous injection of H1N1 vaccine gave as good response than two doses prior to 1957, the advent of the new pandemic H2N2 virus produced a different effect. Thus Holland et al. [176] demonstrated that two doses at an interval of 2 or more weeks produced a better response to one dose and in this regard the vaccine induced immune response was much inferior to that noted before the change in virus subtype.

Vaccination with inactivated vaccine results in both local and systemic responses and within 2 weeks of vaccination, 90% of vaccines have protective antibody titers.

In the event of the unexpected appearance of a pandemic strain, vaccination is the best approach for limiting the impact of infection at a public health level. Safe and immunogenic inactivated H5N1 vaccines have been developed but the changing antigenicity of circulating strains of H5N1 viruses calls for the development of vaccines able to elicit cross-clade immunogenicity. For sub-virion vaccines without adjuvants, naïve individual, who have not received a priming dose, require two doses of an adjuvanted vaccine to elicit high, potentially protective antibody titers. Alum adjuvants have not consistely improved the responses to H5 vaccines whereas oil in water adjuvants, such as MF59, are highly effective, allow antigen sparing and induce cross-reactive antibody responses.

The antibody level required for protection against human influenza H5N1 illness is unclear and data are extrapolated from the experience with interpandemic influenza (HI titers ≥ 40). Interestingly it has been shown that even if the persitance of protective antibody titers is limited, boosting with a single dose of a vaccine made with either the homologous strain [174] or with viral antigen from another clade are effective in people who had received two priming doses several years before.

Despite the fact that the tenet in influenza immunology has been that neutralizing antibodies are the only component of immunity that contribute to protection several studies in animal suggest that cell-mediated immunity might be important in protection from influenza infection, including lethal strain such as H5N1 and H7N7 subtypes [177-179], and preventive vaccines that generate cell-mediated protection deserve consideration [180].
Although no studies have been done so far to specifically document the role of cellular immunity in protection against H5N1 infection in humans beings, studies in animal support the notion that generation of robust and sustained memory T-cell responses that can respond early during H5N1 infection could be key to success of vaccine deigned to stimulate heterosubtipic protection [181].

Objectives

In 1997, the increase in outbreaks of highly pathogenic avian influenza (HPAI) in poultry and the occasional transmission of these viruses to humans has caused great concern for the emergence of a new influenza A virus pandemics. Protection from avian H5N1 influenza virus could be achieved by vaccines capable of eliciting sustained and broadly cross-reactive immune responses.

All clinical studies so far have shown the need for two doses of adjuvanted pre-pandemic flu vaccines to achieve potentially protective neutralizing antibody titers against avian H5N1Vietnam. In addition all clinical studies have shown in influenza a limited efficacy of alum compared to oil in water emulsions, such as MF59, an adjuvant with excellent safety record licensed from more than a decade for seasonal flu vaccines in Europe.

Usually to evaluate vaccine efficacy and its capacity to induce protection only a few serological criteria are considered: specific antibody titer that are present after a month following vaccination and their persistence in the course of time.

Nevertheless vaccine protection cannot be ascribable only to the present of antibody but it is very important to remember and to consider other component of immune system like T lymphocytes CD4 and CD8 and B memory lymphocytes.

The knowledge of these complex mechanisms those are essential to develop a right immune response is a fundamental principle during formulation and optimization of a vaccines candidate.

During a phase II study was assessed the efficacy of two different vaccine formulation with or without adjuvant and with different antigen concentration in order to identify condition to induce a better immune response. We evaluate both antibody and cellular-mediate response.

Typically, the serologically outcome of vaccination is measured with hemagglutination inhibition (HI), single radial hemolisis (SRH) and virus microneutralization (MN) assays the latter providing more information on the functionality of the antibodies.

Several research have shown the fundamental importance of T cells in response in infection and vaccination by either mediating direct protection or by providing “help” to other arms of the immune system.

Among the different assays that can be used to measure T-cell responses (ICS by FACS, Elispot, Elisa and Proliferation) we choose ICS by FACS since it allows the simultaneous analysis of multiple parameters like frequency and functionality of antigen specific T cell.

The analysis of human memory B-cells repertoire is fundamental during vaccination: in response to antigenic stimulation, specific B cells undergo clonal expansion, class switch and somatic hypermutation leading to the

selection of antibodies with increased affinity. Some of these cells differentiate to plasma cells that secrete antibodies whereas other become memory B cells that persist for the lifetime.
Specific memory B cells can be identified by flow cytometry using fluorescent antigen, by Elispot or by LDA. We choose the latter method since it allow bothe the enumeration of antige-specific memory B cella and the analysis of the fine specificity, cross reactivity and neutralizing capacity of the antibodies produced.
Thus the goal of this study was to establish reproducible assays to evaluate the magnitude and functional capacity of B cells and T cells responding to H5N1 influenza vaccine and to examine the adjuvant effect of MF59 compared to no adjuvant on cell mediated immune responses.

Materials and Methods

Study protocol

This was a phase-II, randomized, controlled, observer-blind, single-center study, carried out in Italy from 2006 to 2008. The study protocol (Registered at Clinical Trials.gov as: NCT 00382187) was in accordance with the Helsinki Declaration and Good Clinical Practice principles, and approved by the local Ethics Committee. The vaccine was a monovalent H5N1 subunit from the A/Vietnam/1194/04 influenza virus obtained by reverse genetics (NIBRG-14) and grown in hens' eggs. Forty healthy adults (63% male 38% females, mean age 34.8 years, range 24-50) were enrolled and randomized in a 1:1:1 ratio to 3 groups. All groups were similar in respect to age, sex and ethnicity. All enrolled subjects gave their written informed consent and none of them had serious health problems, history of allergies to vaccines' components, nor was under immune-suppressive therapy. Vaccines were administered at day 1, 22 and 202 in the deltoid muscle in a volume of 0.5 mL using coded pre-filled syringes. One group received 15 µg of plain H5N1 (Non-Adj-15; N = 13); another group received 7.5 µg of H5N1 adjuvanted with MF59 (MF59-7.5; N = 14; Aflunov™); the third group received 15 µg of H5N1 adjuvanted with MF59 (MF59-15; N = 13). As in previous studies [174, 182-185] MF59 was mixed with the antigen at a 1:1 (v:v).
Immunogenicity assays were carried out on coded specimens collected at baseline, 3 weeks after the 1st and the 2nd immunization (days 1, 22 and 43 n = 13, 14, 13 from the Non-Ad-15, the MF59-7.5 and the MF59 group, respectively); 3 and 6 months after the 2nd immunization (day 130 n = 13, 14, 12 and day 202 n = 12, 13, 12); 3 weeks and 6 months following the booster dose (day 223 n = 11, 13, 12 and day 382 n = 11, 12, 10).

Peripheral blood mononuclear cells preparation

PBMC were isolated by Ficoll gradient (Amersham Pharmacia, Uppsala, Sweden) centrifugation of heparinized blood within 6 h after bleeding and either used immediately or frozen at –150°C. PBMC were thawed in PBS containing 20µg/ml DNase (SIGMA-Aldrich), washed and diluted in complete medium (RPMI with 100 units/ml penicillin, 100 µg/ml of streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, and 0.1 mM non-essential amino acids; Invitrogen). Average viability of thawed PBMC samples was >90%.

Analysis of antigen-specific T cell response.

The antigen-specific T cell response was assessed by stimulating PBMC with H5N1 or the following pools of peptides (18-mers overlapping by 10): I) pool of 70 peptides spanning the whole H5 A/Vietnam/1194/2004 protein; II) pool of 50 peptides spanning the regions of H5 conserved among the strains A/Vietnam/1194/2004, A/Indonesia/5/2005 and A/Duck/Singapore/97; III)
pool of 18 peptides spanning the regions of H5 unique to A/Vietnam/1194/04; IV) a pool of 18 peptides spanning the regions of H5 unique to A/Indonesia/5/2005; V) pool of 29 peptides spanning the regions of H5 unique to A/Duck/Singapore/97. All peptides were synthesized at the Institute of Biochemistry (University of Lausanne, Switzerland) and purified to 90% by HPLC.
PBMC were stimulated with the different peptide pools (final concentration of each individual peptide: 2.5 μg/ml) or H5N1 (final concentration: 1 μg/ml) in the presence of 1 μg/ml of anti-CD28 and anti-CD49d mAbs (Becton Dickinson). Brefeldin A was used at 5 μg/ml (Sigma-Aldrich). PBMC cultures in medium alone, or in the presence of 1 μg/ml of an anti-CD3 mAb (Becton Dickinson) were included in each assay as negative and positive control. PBMC were stained with the LIVE/DEAD aqua viability marker (Invitrogen), fixed and permeabilized with the cytofix/cytoperm kit (BD Biosciences) and incubated with Pacific blue-anti-hCD3, PerCP-Cy 5.5-anti-hCD4, FITC-anti-hIFN-γ, APC-anti-hIL-2 and PE-anti-h-IL-13. For each samples $1x10^6$ events were acquired using a LSRII instrument (BD Bioscience) and analyzed with FlowJo software (TreeStar, San Carlos, CA). Response to medium was subtracted from responses in stimulated samples for each of the response patterns.

Enumeration of H5N1-specific memory B cells (MBC)

Frequencies of MBC were determined by the ELISA-coupled limiting dilution assay. PBMC were plated in 0.2 ml of RPMI with 5% FBS in serial 2-fold dilutions, 6 replicates per dilution, starting from $8x10^5$ PBMC/well, in 96-well U-bottom plates containing 2.5 μg/ml of a phosphorothioate CpG-ODN (tcg tcg ttt tgt cgt ttt gtc gtt; Primm Milano, Italy), 1:10,000 fixed *Staphylococcus aureus* Cowan Antigen (SAC, Calbiochem), 1:100,000 pokeweed mitogen extract (Sigma), and 1,000 U/ml rhIL-2 (Proleukin, Novartis). Parallel control cultures of PBMC were run in medium alone. On day 10 individual supernatants where collected and kept at –20°C until tested in ELISA for their content in H5N1-specific and total IgG. ELISA assays were run on Maxisorp plates (Nunc) pre-coated with either H5N1 (A/Vietnam/1194/04, 5 μg/ml in PBS pH 7.5) or a polyclonal sheep IgG fraction against-whole human IgG (Sigma, 2 μg/ml in PBS pH7.5), and

developed with an alkaline-phosphatase-conjugated anti-human γ-chain polyclonal sheep antiserum (1:4000 in PBS 0.05% Tween 3% BSA), followed by incubation with the substrate p-nitrophenylphosphate (Sigma). Wells displaying at 405 nm an OD ≥ 0.4 (total-IgG) or an OD ≥ 0.45 (H5N1-IgG) (5-fold higher than the blank OD) were considered positive. The PBMC dilution containing one antibody-secreting cell precursor was derived by applying the Reed and Muench algorithm [186] to the distribution of antibody positive and negative wells among replicates. Frequencies of H5N1-IgG secreting cell precursors (H5N1-IgG MBC) were expressed as % of the total IgG MBC precursors measured. Numbers of subjects analyzed were: 13, 14, 13 at day 1; 11, 14, 13 at day 22; 11, 11, 12 at day 43; 8, 8, 9 at day 202; 11, 12, 12 at day 223 and 11, 11, 10 at day 382 for the Non-Adj-15, the MF59-H5N1 7.5 and the MF59-H5N1 15 group, respectively.

Titration of neutralizing antibodies

Pre and post vaccination sera were heated at 56°C for 30 min. Titers of H5N1-specific antibodies were determined by microneutralization assay applying an ELISA endpoint reading (reference 6 in the article), and using the homologous /Vietnam/1194/2004 NIBRG-14 recombinant vaccine strain. Sera were tested in duplicate, in serial two-fold dilutions. Titers are expressed as reciprocal value of the highest dilution giving ≥ 50% neutralization of virus growth. Control sera from humans and sheep immunized with the vaccine A/Vietnam/1194/2004 strain were included in each assay. A titer of 10 was assigned to sera that gave a negative result at the first (1:20) dilution.

Descriptive Statistics

Descriptive statistics were calculated by vaccine group using the Statistical Analysis System (SAS) software version 9.1 (SAS Institute, Cary, NC). MN antibody titers were Log_{10} transformed. For the three vaccine groups, GMTs, GMRs and their 95% CIs were computed by exponentiation (base 10) the least squares means and 95% CIs of the Log_{10} titers. These were obtained from a one-way ANOVA with a factor for vaccine group.
Comparisons within each vaccination group between values at day_0 and at day_x were done by the Wilcoxon test for dependent variables (WinSTAT™ for Excel 2002). Comparisons between groups were done by one-factor ANOVA applying the Least Significant Difference *post hoc* test for multiple comparisons (WinSTAT™ for Excel 2002). Differences in the contribution of each cytokine subsets to the total population H5-specific and H5N1-specific $CD4^+$ T cells were analyzed by the Kruskal-Wallis H-test, followed by the Wilcoxon test for pair-wise comparisons (WinSTAT™ for Excel 2002).

Results

Induction of broadly reactive H5-CD4$^+$ T cells

Forty healthy adults were randomly assigned to 3 groups and immunized with two doses of either 15 µg of H5N1 (Non-Adj-15) or MF59-adjuvanted H5N1 at 7.5µg or 15µg of antigen (MF59- H5N1 7.5 and 15). To directly assess priming to H5 we analyzed the T cell response after in vitro stimulation with a library of peptides spanning the whole H5 A/Vietnam/1194/2004 protein (H5-CD4$^+$ T). In parallel we analyzed the T cell response to H5N1, the antigen preparation present in the vaccine (H5N1-CD4$^+$ T). CD4$^+$ T lymphocytes were analyzed by polychromatic flow cytometry to simultaneously measure the frequency of CD3$^+$ CD4$^+$ T lymphocytes and the synthesis of three cytokines (IL-2, IFN-γ, IL-13). This approach allows the enumeration of antigen-specific T cells and a detailed analysis of their functionality [187].

We first enumerated total antigen-specific CD4$^+$ T cells by summing the frequency of CD4$^+$ T cells producing all non-overlapping permutations of the cytokines tested. Low frequencies of H5-CD4$^+$ T cells were detected in the pre-immune samples (mean 164 in 10^6 total CD4$^+$ T cells; Figure 1A).

In subjects that received the plain vaccine (Non-Adj-15) the frequency of H5-CD4$^+$ T lymphocytes increased only1.4-fold after the 1st and 2nd dose; did not increase further after booster vaccination and contracted to values indistinguishable from baseline 6 months following booster immunization (day 382) (Figure 1A). Remarkably, a single dose of either of the MF59-adjuvanted formulations (day 22) induced more than a 3-fold increase in the frequency of total H5-CD4$^+$ T lymphocytes; total H5-CD4$^+$ T cells increased only modestly after the 2nd dose (day 43) but remained 2-fold above baseline 3 and 5 months later (day 130 and 202). The booster immunization with MF59-H5N1 expanded the total H5-CD4$^+$ T cells to values 4-and 8-fold above baseline and 2-fold above the frequency observed after the first 2 doses (day 223; Figure 1A). Of note, 6 months after booster immunization with MF59-H5N1, total frequencies of H5-CD4$^+$ T cells remained above baseline (day 382; Figure 1A).

We then analyzed the frequency of CD4$^+$ T cells upon in vitro stimulation of PBMC with H5N1 (H5N1-CD4$^+$ T). Few H5N1-CD4$^+$ T cells were detected in the pre-immune samples (mean 219 in 10^6 total CD4). In volunteers that received the plain H5N1 vaccine the frequency of H5N1-CD4$^+$ T cells increased 1.5-fold after the first 2 doses, reached values 3-fold above baseline after booster immunization (day 223) but contracted to baseline frequencies 6 months afterwards (day 382; Figure 2).

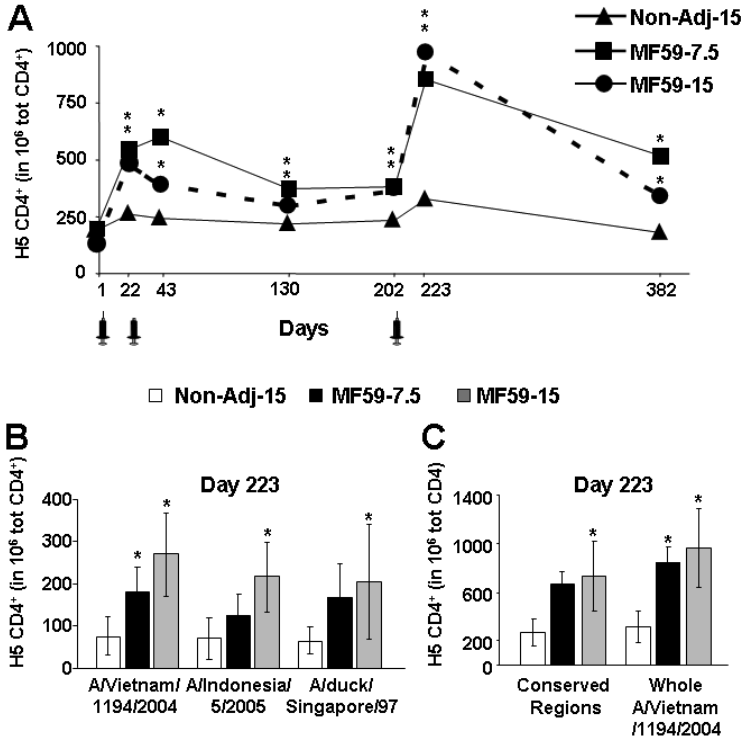

Figure 1. One dose of MF59-H5N1 induces the expansion of H5-specific $CD4^+$ T cells, reacting to antigenically-distinct H5 proteins.
(**A**) Mean frequency of $cytokine^+$ $CD4^+$ T lymphocytes following *in vitro* stimulation of PBMC with a library of peptides spanning the whole H5 A/Vietnam/1194/2004. (* = significantly, $p< 0.05$, different from baseline; Wilcoxon test for dependent variables). **(B-C)** Mean frequency (with 95%CI) of $cytokine^+$ $CD4^+$ T lymphocytes after *in vitro* stimulation of PBMC with pool of peptides spanning: **(B)** the regions non-conserved or **(C)** conserved between the indicated H5 strains, or the whole H5 A/Vietnam/1194/2004. (* = significant, $p< 0.05$, differences compared to the Non-Adj-15 group; one-factor ANOVA with LSD *post hoc*). PBMC were taken at the indicated time point from subjects vaccinated with: non-adjuvanted H5N1 at 15 μg/dose (**triangles or white bars**); MF59-adjuvanted H5N1 at 7.5μg/dose (**squares or black bars**); MF59-adjuvanted H5N1 at 15 μg/dose (**circles or gray bars**).

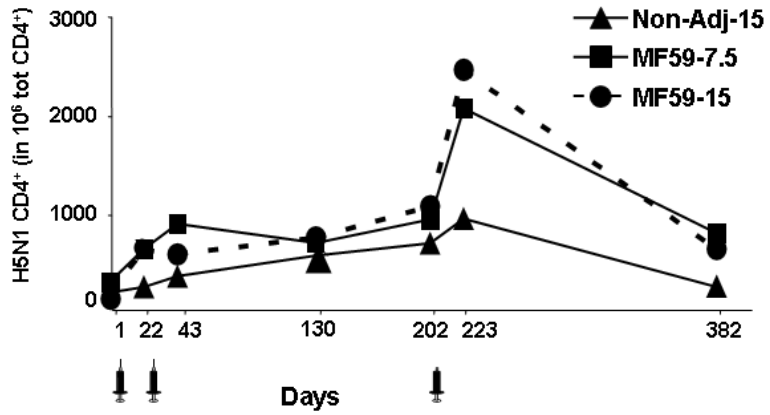

Figure 2. One dose of MF59-H5N1 induces a stable pool of H5N1-specific $CD4^+$ T cells.
Mean frequency of cytokine$^+$ $CD4^+$ T lymphocytes following *in vitro* stimulation of PBMC with H5N1, the antigen preparation of the vaccine (H5N1-$CD4^+$ T). At all time point frequency H5N1-$CD4^+$ T cells in the groups of subjects vaccinated with MF59-adjuvanted H5N1 at 7.5μg/dose (**squares**), or MF59-adjuvanted H5N1 at 15μg/dose (**circles**), but not in subject vaccinated with the non-adjuvanted H5N1 at 15μg/dose (**triangles),** were significantly higher than at baseline ($p< 0.05$; Wilcoxon test for dependent variables).

By contrast, in subjects vaccinated with either adjuvanted formulation, total H5N1-$CD4^+$ T cells increased 2 to 3-fold after the 1st dose, modestly after the 2nd dose, greatly expanded in response to booster immunization (8-fold and 15-fold in the MF59-H5N1 at 7.5 and 15, respectively) and remained 3-fold above baseline 6 months afterward (Figure 2).
We then tested if total $CD4^+$ T cells, induced by vaccination with clade 1 H5N1 A/Vietnam/1194/2004, reacted with H5 of different clades (http://www.who.int/csr/disease/avian_influenza/guidelines/nomenclature/en/index.html).
We compared the T cell response to in vitro stimulation with pools of peptides spanning either conserved or non-conserved regions of H5 A/Vietnam/1194/2004 (clade 1), A/Indonesia/5/05 (clade 2.1) and A/duck/Singapore/97 (clade 0-like). Vaccination with MF59-H5N1, but not with the plain vaccine, expanded H5-$CD4^+$ T cells that responded with similar potency to peptide pools spanning the non-conserved regions of drifted H5 (Figure 1B). In addition, total $CD4^+$ T cells strongly responded to stimulation with peptides spanning the regions of H5 conserved between the 3 strains tested (Figure 1C).
In conclusion, the plain H5N1 vaccine expands H5- or H5N1-specific $CD4^+$ T cells only modestly. A single immunization with MF59-H5N1 is sufficient

to prime high frequencies of antigen-specific $CD4^+$ T cells that markedly expand in response to booster immunization and recognize H5 of different clades.

H5-$CD4^+$ T cells display an effector/memory Th1 phenotype

We then analyzed the relative proportion of Th1 (IFN-γ) and Th2 (IL-13^+) T cells within H5- and H5N1-$CD4^+$ T lymphocytes [188, 189]. The frequency of IL-13^+ cells within H5 or H5N1-$CD4^+$ T lymphocytes was extremely low and slightly decreased following vaccination, suggesting that neither the plain nor the MF59-adjuvanted formulation induced a Th2 response (Figure 3 A,B).

We then analyzed at the single cell level the production of IL-2 and IFN-γ. After immunization with either the plain or the MF59-adjuvanted vaccines, the $CD4^+$ T cell response was dominated by lymphocytes producing IL-2 but not IFN-γ (IL-2^+ IFN-γ^-), which constituted up to 70% of the total H5- and H5N1-$CD4^+$ T cells (Figure 3 A,B). Double positive $CD4^+$ (IL-2^+ IFN-γ^+) T cells were less represented in all vaccinees (average 20% of the H5- and H5N1-$CD4^+$ T cells after the first two doses). After booster immunization, H5-$CD4^+$ (IL-2^+ IFN-γ^+) T cells increased only modestly, whereas double positive H5N1-$CD4^+$ (IL-2^+ IFN-γ^+) T cells increased to 40% of the responding lymphocytes. Single positive H5- or H5N1-$CD4^+$ (IL-2^- IFN-γ^+) T cells never contributed more than 5-10% to the total $CD4^+$ response throughout the study (Figure 3 A,B).

In conclusion, the $CD4^+$ T cell response to H5 peptides and H5N1 protein is dominated by lymphocytes synthesizing IL-2 but not IFN-γ, a $CD4^+$ T cell sub-population with memory potential but limited effector functionality [187, 190]. Mature Th1 effector/memory lymphocytes, synthesizing both IL-2 and IFN-γ account for 20% of the responding T cells and increase to 40% of H5N1-$CD4^+$ T cells after booster immunization. $CD4^+$ T cells producing IFN-γ but not IL-2, a more terminally differentiated population with limited memory potential, are less represented (5-10%) [187, 190]. A comparative analysis of the proportion of each $CD4^+$ T cell subset to the H5- or H5N1-specific $CD4^+$ T cell response showed no significant variation in response to the plain or MF59-adjuvanted vaccines (p-value>0.1).

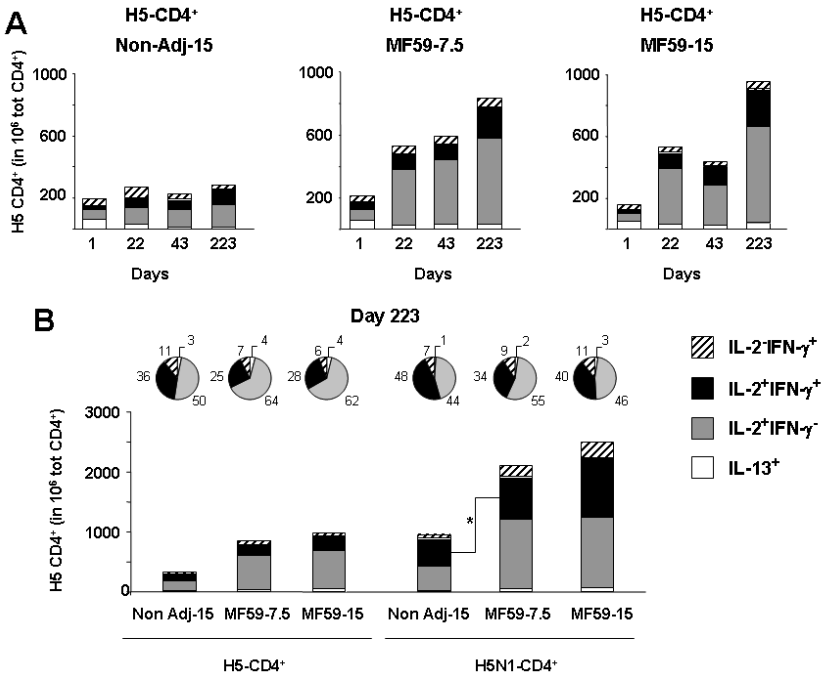

Figure 3. Functional characterization of $CD4^+$ T cells induced by vaccination with H5N1 A/Vietnam/1194/2004.
Frequency of IL-13^+ (white bars), $IL2^+$IFN-γ (gray bars) $IL2^+$IFN-γ^+ (black bars), or $IL2^-$IFN-γ^+ (striped bars) $CD4^+$ T lymphocytes after a short *in vitro* stimulation of PBMC with (**A-B)** a library of peptides spanning H5 A/Vietnam/1194/2004 (H5-$CD4^+$ T) or (**B**) the H5N1 subunit, the antigen preparation of the vaccine (H5N1-$CD4^+$ T) (**B**) The pie charts represent the relative contribution (%) of each $CD4^+$ T subset to the total response to H5 and H5N1. No significant variation in the proportion of IL-13^+ / IL-2^+ IFN-γ^- / IL-2^+ IFN-γ^+ / IL-2^- IFN-γ^+ was associated to the plain or the MF59-adjuvanted vaccine formulations (p-value > 0.1. Kruskal-Wallis H-test, followed by theWilcoxon test for pair-wise comparisons) An asterisk marks the only significant variation observed (Wilcoxon test p = 0.049).

Expansion of H5N1 memory B cells

Before vaccination, the mean frequency of H5N1-IgG memory B cells (MBC) was ≤ 1% of total IgG-MBC in all groups (Figure 4). In subjects vaccinated with plain H5N1, only minor changes in the frequency of H5N1-IgG MBC were detected throughout the study (Figure 4).
In contrast, a significant expansion of H5N1-IgG MBC was observed after 2 doses of the MF59-H5N1 vaccines (mean values at day 43 of 5.2% and 3.1% in the MF59-H5N1 at 7.5 and 15 μg, respectively; Figure 4). In both MF59-adjuvanted groups H5N1-IgG MBC greatly expanded upon booster

immunization (mean value at day 223 of 11% in both MF59-H5N1 groups). Six months later (day 382) about 60% of subjects in both MF59-H5N1 groups maintained frequency of H5N1-IgG MBC 4-fold above baseline (mean values at day 382 of 11% and 9.5% in MF59-H5N1 7.5 and 15 μg, respectively; Figure 4).
In conclusion 2 doses of MF59-H5N1 vaccine, at either 7.5 or 15 μg, prime a large and stable pool of H5N1-MBC that further expands upon boosting and persists for at least 6 months.

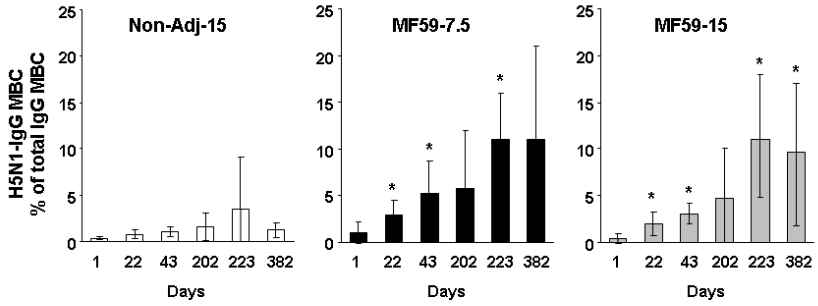

Figure 4. Two doses of MF59-H5N1 are required to expand a large and stable pool of H5N1-IgG memory B cells.
Mean frequency (with 95%CI) of circulating H5N1-IgG memory B cells (MBC) as % of total circulating IgG MBC; (* = significant, $p < 0.01$, different from baseline; Wilcoxon test for dependent variables).

Neutralizing antibody responses

Before vaccination, most subjects had MN titers below the limit of detection. As observed in previous studies [182, 184], a single dose (day 22) did not induce an increase in MN titers, irrespective of the formulation tested (Figure 5A).
In subjects vaccinated with the plain formulation MN titers rose only slightly above baseline after the 2nd dose and upon booster immunization, but contracted to baseline values 6 months afterward (Figure 5A; geometric mean ratio above baseline (GMR) were 1.6 at day 43; 2.1 at day 223 and 0.9 at day 382)).
Conversely, after 2 doses (day 43) of either adjuvanted vaccines, MN titers rose significantly above baseline (GMR=8.1 and 5.8 in the MF59-7.5 and MF59-15 groups respectively) and 64% and 38% of subjects had MN titers ≥ 80 (Figure 5B).
Following booster immunization (day 223), in either MF59-adjuvanted groups MN titers sharply increased above baseline (GMR=17 and 34 in the

MF59-7.5 and MF59-15 groups, respectively) and 92% and 83% of subjects had MN titers ≥ 80 (Figure 5B). Six months after boost (day 382), in both MF59-H5N1 groups MN titers remained > 5 fold above baseline and 55% of vaccinees maintained MN titers ≥ 80 (GMR=5.6 and 7 in the MF59-7.5 and MF59-15 groups, respectively; Figure 5).
In conclusion 2 doses of MF59-adjuvanted-H5N1 vaccine are required to induce high, boostable and sustained MN antibody responses.

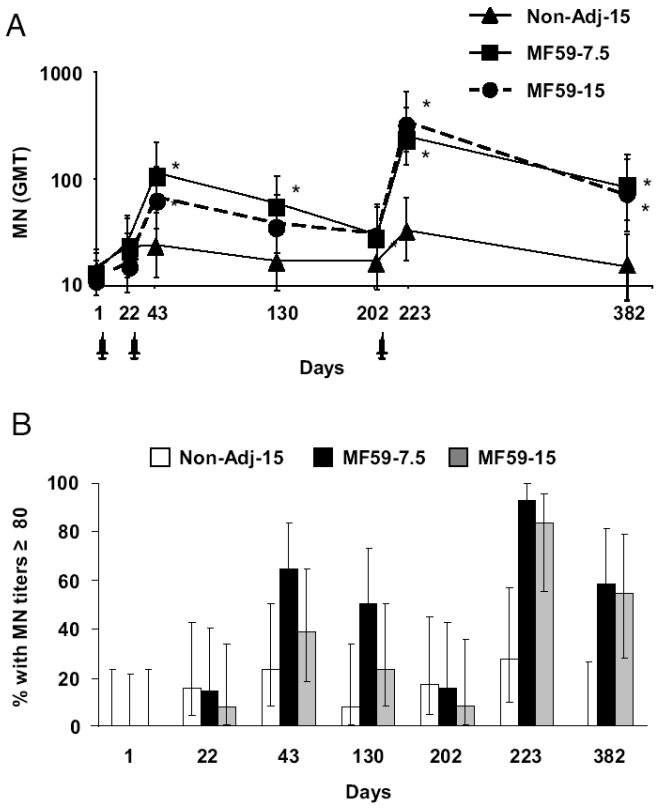

Figure 5. Two doses of MF59-H5N1 are required to induce sustained neutralizing antibodies.
(A) Geometric mean titers (GMT) with 95% CI of circulating antibodies neutralizing the homologous A/Vietnam/1194/2004 NIBRG-14 recombinant virus in subjects vaccinated with Non-Adj-15 (triangles), MF59-7.5 (squares), MF59-15 (circles). (* = significant, $p < 0.01$, different from baseline; Wilcoxon test for dependent variables). **(B)** Percent of subjects displaying MN antibodies titers above the potentially protective threshold of 1:80 in the Non-Adj-15 (white columns), MF59-7.5 (black columns), MF59-15 (gray columns).

Early $CD4^+$ T cells expansion predicts long term MN response

Looking for an early predictor of long-term antibody responses we studied the earliest meaningful time-points for H5-$CD4^+$ T cells, MN titers and H5N1-IgG MBC frequencies.

Figure 6A shows the relationship between the fold increase of total $cytokine^+$ H5-$CD4^+$ T cells, measured at day 22, and MN titers measured at day 223. A rank-correlation analysis of the data indicated a significant correlation between frequency of total H5-$CD4^+$ T cells and MN titers (Spearman $\rho=0.60$, p-value $< 10^{-4}$). Furthermore, a ≥ 3 fold increase in H5-$CD4^+$ T cells always associated with high MN titers. More specifically, a ≥ 3 fold expansion of H5-$CD4^+$ T cells at day 22 significantly associated (Fisher test, association p-value $< 10^{-3}$) with an MN titer ≥ 80, the proposed threshold of protective antibodies [191], with a predictive accuracy and specificity of 75% and 100%, respectively (Table 1). A similar correlation was found at day 382 (Figure 6B, Spearman $\rho=0.54$, p-value $< 10^{-3}$), with association p-value $= 10^{-4}$ and both predictive accuracy and specificity of 85%.

The choice of a 3-fold increase of H5-$CD4^+$ T cells as a predictor for MN $\geq$ 80 at days 223 and 382 was supported by the association analysis shown in Figure 7, where the association p-values are shown for all the range of cut-offs of H5-$CD4^+$ T cells at day 22. Only for a fold-increase ≥ 3 of H5-$CD4^+$ T cells we observed a highly significant association (Fisher p-value $< 10^{-3}$) with MN titer ≥ 80 at both day 223 and 382.

A similar analysis showed that MN titers ≥ 40 at day 43 were associated with MN titers ≥ 80 at day 223 (Fisher p-value $< 10^{-3}$), and predicted MN response at day 223 with an accuracy of 78% (Figure 8 and Table 2). However, no MN titer ad day 43 associated or predicted an MN titers ≥ 80 at day 382 (Fisher p-value $> 10^{-2}$).

Finally, frequency of H5N1-IgG MBC at day 43 weakly associated with MN titers ≥ 80 at day 223 and 382 (Fisher p-values $= 9 \cdot 10^{-3}$, $2 \cdot 10^{-3}$ respectively), with a predictive accuracy of 68% at day 223 and 78% at day 382 (Figure 9 and Table 3).

A comparison of the various predictors tested (Table 4) shows that total H5-$CD4^+$ T cells 3 weeks after the 1^{st} dose are the earliest and most accurate predictor of a protective neutralizing antibody response and its persistence.

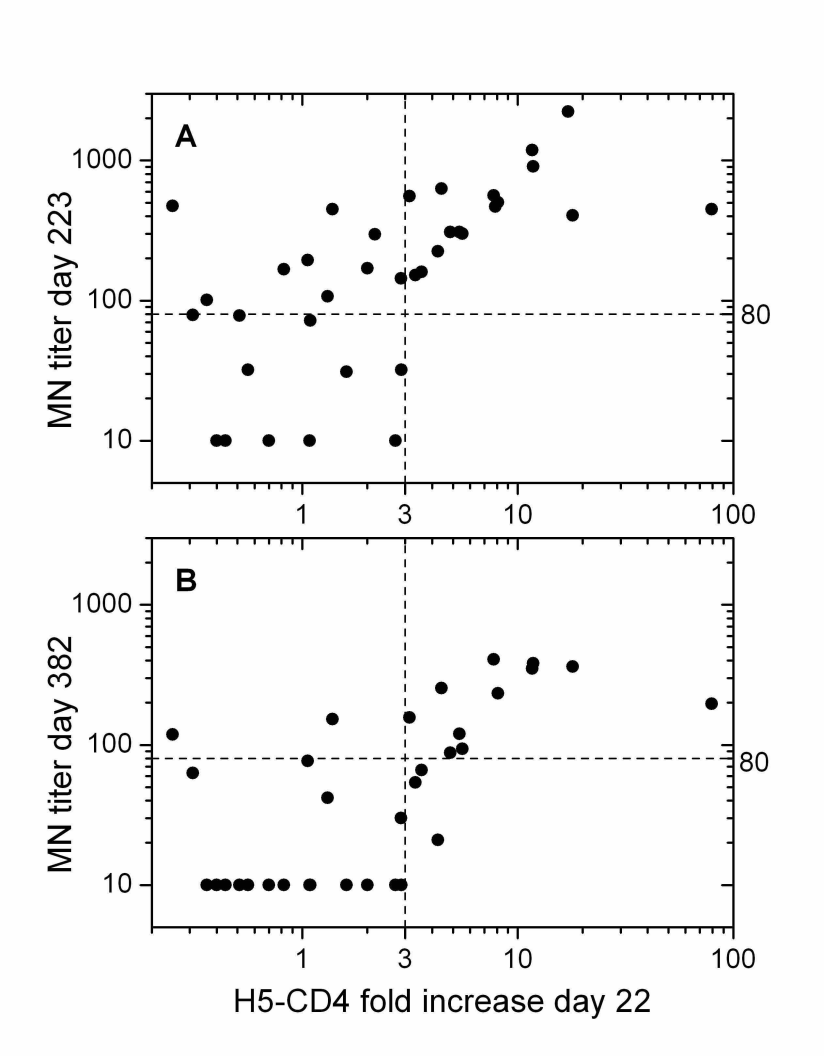

Figure 6. Association between expansion of H5-CD4$^+$ T cells after the 1st dose and MN titers at later time points.
For each subject, the MN titer at day 223 (A) or 382 (B) is plotted versus the H5-CD4$^+$ T cells fold increase at day 22 over pre-immune. Horizontal dashed lines indicate the value of MN titer = 80, the proposed threshold of protective antibodies. Vertical dashed lines indicate the value of H5-CD4$^+$ T cells 3-fold increase.

		MN titer day 223		
		≥ 80	< 80	
CD4 fold rise (day 22 / baseline)	≥ 3	16	0	PPV 100%
	< 3	9	11	NPV 55%
p-value = 0.0005		Sens. 64%	Spec. 100%	**Accuracy 75%**

		MN titer day 382		
		≥ 80	< 80	
CD4 fold rise (day 22 / baseline)	≥ 3	11	3	PPV 79%
	< 3	2	17	NPV 90%
p-value = 0.0001		Sens. 85%	Spec. 85%	**Accuracy 85%**

Table 1: Expansion of H5-CD4$^+$ T cells ≥ 3 fold at day 22 predicts MN titer ≥ 80 at days 223 and 382.
In each 2x2 contingency table Sensitivity (Sens.), Specificity (Spec.), Positive Predictive Value (PPV), Negative Predictive Value (NPV), Accuracy and two-tailed Fisher exact association test p-value are shown (see Methods).

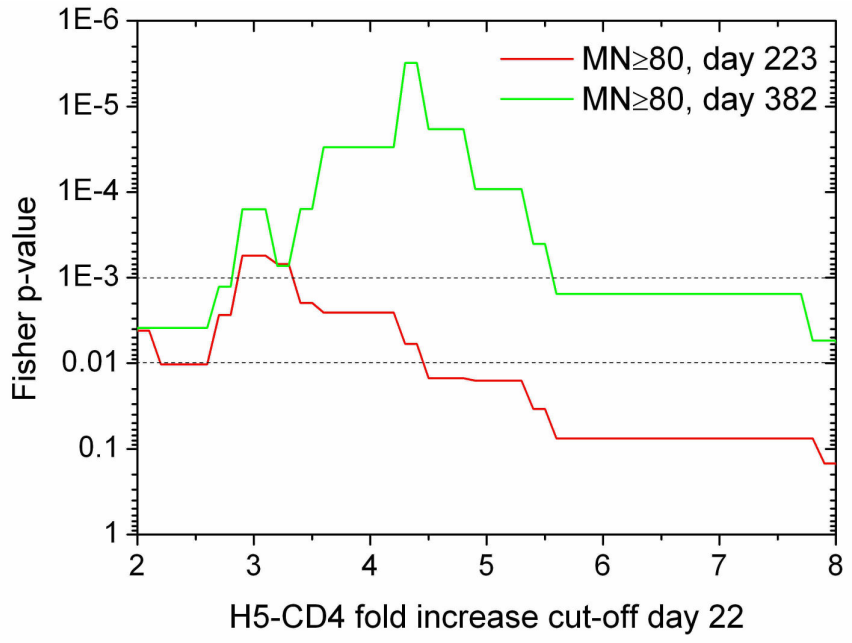

Figure 7. Association between expansion of H5-CD4$^+$ T cells after the 1st dose and MN titers ≥ 80 at later time points.
Shown are the Fisher's exact test two-tailed p-values for the association between the whole range of fold increases over pre-immune in H5-CD4$^+$ T cells at day 22 (≥ cut-off values depicted on the *x*-axis) and MN titers ≥ 80 at day 223 (red line) and 382 (green line). The association was considered significant for $p < 0.01$ and highly significant for $p < 0.001$ (dotted lines). A ≥ 3-fold increase of H5-CD4$^+$ T cells after the 1st dose significantly associates with MN titers ≥ 80 at all time points investigated

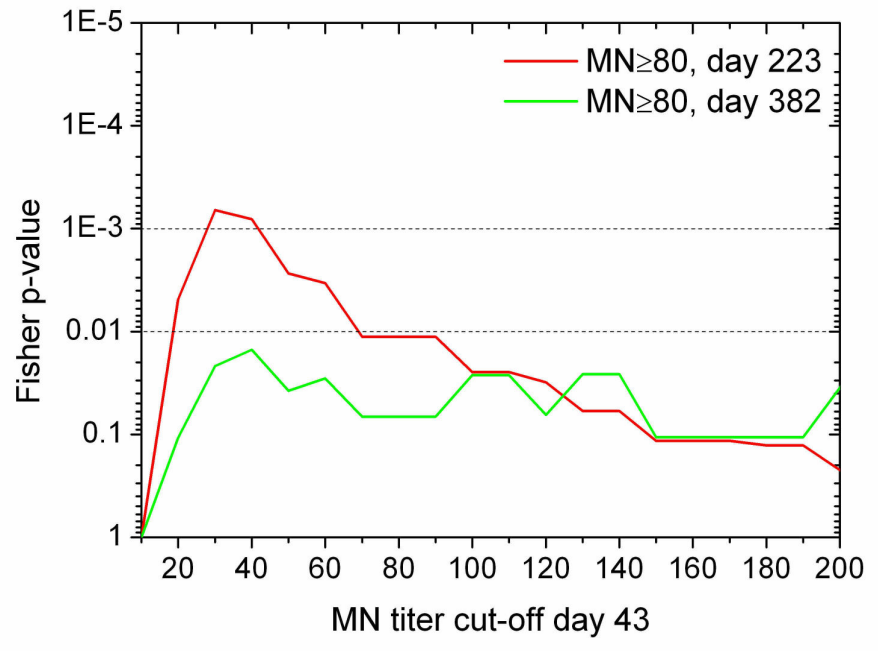

Figure 8. Association between MN antibody titers at day 43 and MN titers ≥ 80 at later time points.
Shown are the Fisher's exact test two-tailed p-values for the association between MN antibody titers at day 43 (≥ cut-off values depicted on the *x*-axis) and MN titers ≥ 80 at day 223 (red line) and 382 (green line). The association was considered significant for $p < 0.01$ and highly significant for $p < 0.001$ (dotted lines). MN titers ≥ 40 at day 43 are significantly associated with MN titers ≥ 80 at day 223, but no cut-off for MN titers at day 43 is associated with MN titers ≥ 80 at day 382.

		MN day 223		
		≥ 80	< 80	
MN day 43	≥ 40	20	4	PPV 83%
	< 40	5	11	NPV 69%
p-value = 0.0007		Sens. 80%	Spec. 73%	**Accuracy 78%**

		MN day 382		
		≥ 80	< 80	
MN day 43	≥ 40	11	13	PPV 46%
	< 40	2	14	NPV 88%
p-value = 0.04		Sens. 85%	Spec. 52%	**Accuracy 63%**

Table 2: MN titers ≥ 40 at day 43 predict MN titer ≥ 80 at day 223 but not at day 382.
In each 2x2 contingency table Sensitivity (Sens.), Specificity (Spec.), Positive Predictive Value (PPV), Negative Predictive Value (NPV), Accuracy and two-tailed Fisher exact association test p-value are shown (see Methods).

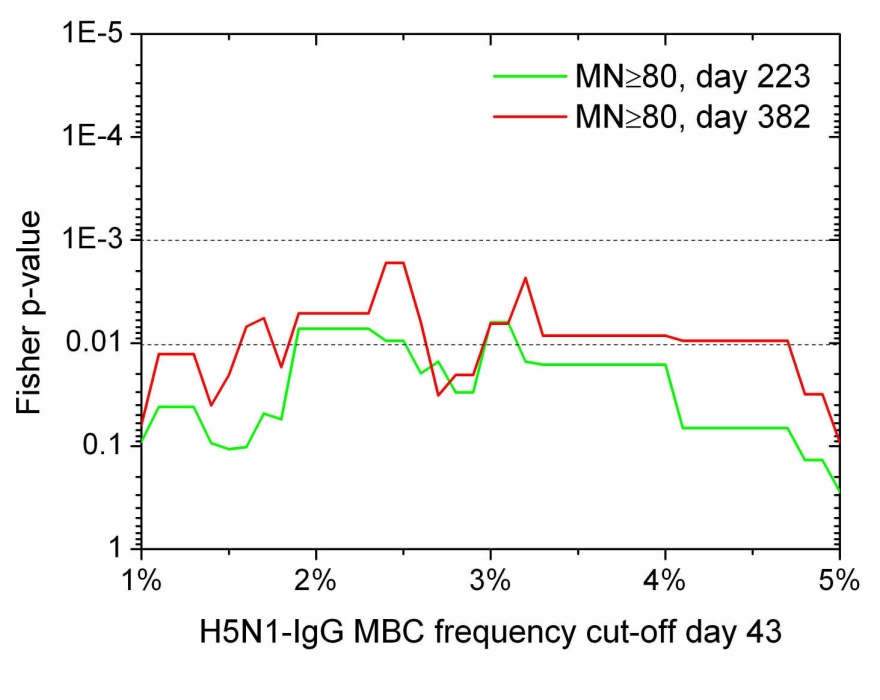

Figure 9. Association between Frequency of H5N1-IgG MBC at day 43 and MN titers ≥ 80 at later time points.
Shown are the Fisher's exact test two-tailed p-values for the association between frequency of H5N1-IgG MBC at day 43 (depicted on the *x*-axis) and MN titers at day 223 (red line) and 382 (green line).

		MN day 223		
		≥ 80	< 80	
MBC % day 43	≥ 2.5	14	2	PPV 88%
	< 2.5	11	13	NPV 54%
p-value = 0.009		Sens. 56%	Spec. 87%	**Accuracy 68%**

		MN day 382		
		≥ 80	< 80	
MBC % day 43	≥ 2.5	10	6	PPV 62%
	< 2.5	3	21	NPV 88%
p-value = 0.002		Sens. 77%	Spec. 78%	**Accuracy 78%**

Table 3: At day 43 frequency of H5N1-IgG MBC ≥ 2.5% predict MN titer ≥ 80 at day 223 and 382.
In each 2x2 contingency table Sensitivity (Sens.), Specificity (Spec.), Positive Predictive Value (PPV), Negative Predictive Value (NPV), Accuracy and two-tailed Fisher exact association test p-value are shown (see Methods).

Predictors:		**Endpoint: MN ≥ 80**	
		day 223	**day 382**
$CD4^+$ T cells	day 22	0.006	0.001
	day 43	0.96	0.04
MBC	day 22	1	1
	day 43	0.11	0.02
MN titer	day 22	1	1
	day 43	0.008	0.48

Table 4: Significance of associations between predictors and efficacy endpoints.
Shown is the Bonferroni-corrected Fisher exact test p-value for the association between different predictors ("$CD4^+$ T cells" = ≥ 3-fold expansion of H5-$CD4^+$ T cells over baseline, "MBC" = frequency of H5N1-IgG MBC ≥ 2.5%, "MN titer" = MN titer ≥ 40) and the efficacy endpoint of MN titer ≥ 80 at days 223 or 382.

Discussion

The spread of avian influenza in wild birds and poultry, the identification of cases of direct transmission to humans, and its very high fatality rate have created the specter of a new pandemic. The development of a vaccine capable of eliciting a broad memory immune response, rapidly boostable at the start of the pandemic, is considered the best strategy to provide a first line of defense to the population largely naïve to avian influenza. Limitations in developing such a vaccine come from the need to induce a broad memory response in a naïve population together with the lack of an early immune-marker predictive of vaccination take rate measured as development of immunological memory.

Unlike seasonal influenza, for which vaccine efficacy in humans has been tested both in protection and challenge studies, for avian influenza there is limited knowledge of the immunological correlates of protection. Comparison of HI and MN assays for seasonal and avian influenza strains showed a good correlation between HI titers of 1:40, considered protective in seasonal influenza, and MN titers of 1:80. Such studies together with results from the analysis of sera from patients who recovered from avian influenza infection support the use of an MN titer of ≥ 80 as an efficacy endpoint for avian influenza vaccines [191].

To better characterize the immune-response to avian flu vaccines and to search for early markers predictive of induction of immune-memory, we analyzed the kinetics, magnitude, and quality of the antibody and cell-mediated responses to an avian flu vaccine, plain or adjuvanted with MF59, in a three doses prime-boost phase II clinical trial. We found that only the MF59-H5N1 vaccine, containing 7.5 or 15 μg of H5N1 antigen, expands H5N1-$CD4^+$ T cells with a Th1 effector/memory phenotype (IL-2^+ IFN-γ^-/ IL-2^+ IFN-γ^+), generates a large pool of H5N1-IgG MBC, and high and sustained titers of neutralizing antibodies. Moreover, in subjects immunized with MF59-H5N1 both cell-mediated and antibody responses are strongly boosted by a 3rd dose given 5 months after priming indicative of the induction of immunological memory. Of note the frequency of H5N1-IgG MBC measured after three doses of MF59-adjuvanted vaccines are comparable to those observed after seasonal influenza vaccination [192, 193].

We found that the $CD4^+$ T cell response is measurable after a single immunization with either MF59-adjuvanted formulations whereas 2 doses are required to induce a measurable increase in MN antibodies and memory B cells.

A $\geq$ 3-fold increase in the frequency of total cytokine$^+$ H5-$CD4^+$ T cells after the 1st dose (day 22) predicts the rise of MN titers ≥ 80 after booster

vaccination and their maintenance 6 months later with 75% and 85% accuracy, respectively.
The other parameters studied also showed some correlation but it was never as good as the one observed with total cytokine$^+$ H5-CD4$^+$ T cells. For instance, after the second dose, at day 43, MN titers ≥ 40 were predictive of MN titers ≥ 80 at 3 weeks after boost, but not of their maintenance 6 months later. Similarly, at day 43, frequency of memory B cells ≥ 2.5% predicted the rise and persistence of MN titers ≥ 80 after booster immunization, despite less accurately than H5-CD4$^+$ T cells at day 22.
We therefore conclude that a ≥ 3-fold increase in the frequency of total H5-CD4$^+$ T cells after the 1st dose was the earliest and most accurate predictor of the rise of MN antibodies to potentially protective titers after booster vaccination and of their maintenance 6 months later.
Given that CD4$^+$ T cells are not directly responsible for antibody production and that their expansion is the earliest measurable immunological parameter predictive of the neutralizing antibody response, we suggest that, if confirmed on a larger subject database, CD4$^+$ T cell responses after the 1st immunization could be used as an early measure of pre-pandemic vaccination take-rate and become a valuable tool for comparing the effectiveness of different vaccine formulations.
The predictive association of CD4$^+$ T cell responses and antibody responses is not surprising since CD4$^+$ help is required for the optimal activation and early clonal expansion of B cells, for the initiation and maintenance of germinal centre reaction and, ultimately, for the generation of long-lived plasma and memory B cells [194-198]. Indeed the groups that had a significant increase in the total CD4$^+$ T cell response after the 1st dose had significant increase in memory B cells after the 2nd and the 3rd dose (Figure 4). In addition to their helper function, several preclinical studies suggest that influenza-specific CD4$^+$ T cells can accelerate recovery via a direct effector function [199-201].
We also assessed the cytokine profile of antigen specific CD4$^+$ T cells elicited by vaccination. To this aim we analyzed at a single cell level synthesis of IL-2, IFN-γ and IL-13 as prototypic of Th1 (IL-2 and IFN-γ) and Th2 (IL-2 and IL-13) polarized responses [188, 189]. The ability of antigen specific CD4$^+$ T cells to produce IL-2 was also used as a predictor of the ability of CD4$^+$ T cells to survive in vivo and proliferate upon challenge [187, 190].
We choose IL-13 over IL-4 since it has been reported that all CD4$^+$ T cells that produce IL-4 also produce IL-13 and that production of IL-13 is more sustained over time [202]. In addition the effects of IL-4 and IL-13 on human B cells are largely similar since IL-13 does enhance production of IgM, IgG and IgA [203, 204] . Such analysis showed that the frequency of H5-specific CD4$^+$ T cells synthesizing IL-13 is extremely low and decreases

upon vaccination suggesting that neither the plain nor the MF59-adjuvanted formulation induce a Th2 response.
This analysis also showed that the antigen specific $CD4^+$ T cell response is dominated by lymphocytes synthesizing IL-2 but not IFN-γ a $CD4^+$ T cell sub-population with memory potential but limited effector functionality [187, 190] followed by mature Th1 effector / memory lymphocytes (synthesizing both IL-2 and IFN-γ $CD4^+$ T cells producing IFN-γ but not IL-2, a more terminally differentiated population with limited memory potential, were less represented in all groups [187, 190].
In conclusion the CD4 response we detected after vaccination is dominated by IL-2^+ IFN-γ^- T cells with only 20-40% of lymphocytes synthesizing both cytokines, indicating that limiting the analysis to IFN-γ does not allow a full measure of the elicited T cell response and that $CD4^+$ T cells elicited by pre-pandemic vaccination retain memory potential. It is also of interest that the difference in the $CD4^+$ T cell response between subjects vaccinated with the plain versus the MF59-adjuvanted vaccines was limited to the frequency of the responding $CD4^+$ T cells with no significant impact on their cytokine profile, suggesting that formulation with MF59 affects the magnitude but not the quality of the elicited T cell response. These findings in humans are in contrast with studies in BALB/c mice where MF59 has been reported to induce a Th2 response [185], thus emphasizing the need of testing several mouse strains to characterize adjuvant activity.
Finally the finding that $CD4^+$ T cells primed by vaccination with clade 1 H5N1 (A/Vietnam/1194/2004) react with H5 proteins of different clades (clade 0-like and clade 2) supports the use of MF59-adjuvanted pre-pandemic vaccines to induce broadly reactive $CD4^+$ T cells that could exert their helper and effector function toward antigenically distinct H5 strains.
Although the implementation of pre-pandemic vaccination will require complex cost-benefit analyses, the data presented collectively support the use of MF59-adjuvanted vaccines to prime the population against a variety of avian influenza viruses.

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