The nature and evolution of humoral immune responses to influenza virus

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**General introduction**

**Influenza virus**

**Epidemiology**

Influenza is an infectious respiratory disease caused by influenza virus. Humans can be infected by influenza A and influenza B viruses; influenza C and influenza D virus also exist but they are not clinically relevant for the human population (1). Influenza A virus (IAV) can be subdivided in multiple subtypes and it is the only influenza virus type to cause occasional pandemics. Influenza B viruses form 2 lineages, the B/Victoria/2/87-like lineage and the B/Yamagata/16/88-like lineage, but are not further divided in subtypes (1–4).

Symptoms associated with influenza virus infection are characterized by a very sudden onset and include runny nose, cough, fever, sore throat, muscle pain and fatigue. In the most severe case, influenza virus infection leads to secondary bacterial infection of the lower respiratory tract and to severe pneumonia and it can as well lead to several non-respiratory complications (5). The WHO estimates that annual epidemics of influenza result in ~1 billion infections, 3 to 5 million cases of severe illness, and about 290 000 to 650 000 respiratory deaths (5). There are multiple risk groups for severe influenza disease. Very young children, who have not had multiple previous exposures to influenza viruses often experience more-severe symptoms and shed larger number of virus particles for longer periods of time. The elderly population is also at risk of more severe illness, probably because of immune-senescence or concurrent chronic illnesses. Finally, pregnant women, people with chronic pulmonary or cardiac conditions, diabetes mellitus or immunocompromising conditions are also considered risk groups for influenza (1,5–7).

**Virus structure**

All influenza viruses are enveloped negative-sense single-strand RNA viruses with a segmented genome (3). Influenza A and influenza B viruses have eight RNA segments which encode for RNA polymerase (PA, PB1, PB2), haemagglutinin (HA), neuraminidase (NA), nucleoprotein (NP), matrix protein (M1) and membrane protein (M2), the nonstructural protein (NS1), nuclear export protein (NEP), and finally PA-X and PB1-F2 (1–3). HA and NA are glycoproteins located on the outer part of the viral membrane and therefore they are the immunologically most relevant influenza virus proteins and the most antigenically variable. So far, 18 different subtypes of the HA protein (H1-H18) and 11 different subtypes of the NA protein (N1-N11) have been identified among IAV (2,4). The HA subtypes can be divided into two groups according to the phylogeny of the HA molecule. Within each group, the HA stalk is antigenically similar among different subtypes (see Fig 1) (8). HAs within the phylogenetic group 1 are H1, H2, H5, H6, H8, H9, H11, H12, H13, H16, H17 and H18 while HAs within the phylogenetic group 2 comprise H3, H4, H7, H10, H14, H15. The H1N1 and H3N2 IAV strains currently co-circulate in the human population (1,2,4,8,9) (Figure 1).

**Replication cycle**

The targets of influenza virus are epithelial cells of the respiratory tract in humans and other mammals and epithelial cells of the intestinal tract in birds (3,10,11).

In the first step of the replication cycle the HA on the surface of the virus binds to sialic acid present on glycoproteins or glycolipids on the cellular surface. Interestingly, HAs from human influenza viruses bind
preferentially to sialic acids linked by an α2,6 linkage to the rest of the oligosaccharide, whereas those from most of the avian influenza viruses bind preferentially to α2,3-linked sialic acids. The evolutionary reason for this phenomenon is likely to be that these bonds are the most abundant sialic linkages in the epithelial cells of the human upper respiratory tract and of the avian intestinal tract, respectively (1–3,12).

**Figure 1. IAV HA phylogenetic groups:** The left panel portraits the structure of the HA head and stalk from the influenza virus. HA is a trimeric glycoprotein located on the surface of influenza virus. Together with NA, the 2 represent the most immunogenic proteins of this pathogen. The right panel describes the two HA groups, according to which IAV strains are divided into two phylogenetic groups, **Group 1** and **Group 2**. The H1N1 and H3N2 IAV strains currently co-circulate in the human population (1,2,4,8,9). Image created with BioRender.com.

After binding, the virus is internalized in an endosome which undergoes acidification, thus triggering a conformational change in the viral HA that induces the fusion of the viral and endosomal membrane. At this point the genetic material of the virus is released in the cytoplasm and imported into the nucleus. Here, in the nucleus, the viral RNA undergoes replication. From the negative sense viral RNA, two types of positive strand RNA are transcribed: cRNA (together with proteins forming complementary ribonucleoprotein (cRNP) complex) which serves as template for synthesis of new vRNA and mRNA. At this point, positive-strand mRNAs are capped and polyadenylated and exported into the cytoplasm for translation into viral proteins. Moreover, NP and the three polymerase subunits are transported into the nucleus where they form, with the new vRNA, RNPs. These RNPs then leave the nucleus and, together with the viral proteins, are then transported to the plasma membrane where new virions get
assembled and leave the cell by budding. To be functional, newly synthesized HA needs to be cleaved into HA1 and HA2 polypeptides by cellular proteases present in respiratory and intestinal epithelial (1,3,12). The enzymatic activity of NA prevents binding of HA of new virions to sialic acid receptors on the membrane of the infected cells, thus facilitating viral spread (3,4,13).

**Viral genome mutation mechanisms**

Influenza virus has a negative sense single stranded **RNA genome**. A high mutation rate characterizes the influenza virus genome due to the lack of an efficient proof-reading mechanism of the viral RNA dependent RNA polymerase. Because of this particular feature, influenza virus continuously accumulates **point mutations** in its genome, a phenomenon that can lead to a gradual change of HA and NA and thus to immune escape. The phenomenon by which viruses, after accumulating certain mutations, acquire a selective advantage and increase in frequency in the world population is called “**antigenic drift**”. The advent of drifted influenza virus strains is responsible for the yearly recurrence of influenza virus epidemics. It is because of this phenomenon that influenza virus vaccines require frequent updates, to make sure the circulating virus and the vaccine are as antigenically similar as possible (1,2,9). In the last 10 years the vaccine strains for the 2 circulating IAV strains have been changed multiple times. In the last 10 years the H1N1 strain was changed 4 times while the H3N2 strain was changed 8 times. This seems to suggest that the rate of antigenic drift for H3N2 is slightly faster than that for H1N1(5).

Another major and unique feature of influenza virus is the fact that the influenza viral **genome** is **segmented**, which enables interchange of genomic RNA segments when two viruses of the same type but different strains simultaneously infect the same cell in a host. This enables a phenomenon called “**reassortment**” by which genomic segments are swapped between different virus strains. Reassortment, usually in animals, is one of two ways in which a major change of IAV can happen, associated with the advent of a very different HA and/or NA in a human pathogenic IAV strain. This major change is called “**antigenic shift**”. The second way in which antigenic shift may happen requires a virus, which has existed in animal species, to jump to the human population as it is, without modifications. In fact, IAV can circulate not only in humans but also in pigs, poultry, horses and wild migratory birds. Unpredictable global pandemic outbreaks can occur which involve influenza A virus strains of zoonotic origin (1,9).

Aquatic wild birds and poultry host influenza A viruses containing many of the HA (H1–H16) and NA (N1–N9) subtypes and are believed to be the **natural reservoirs** of influenza viruses (1,14). Influenza viruses from wild birds can infect through water or fomites marine mammals, poultry, pigs and domestic ducks (10,11,14). This happens through farming and live animal markets. Humans can be infected with poultry derived IAV like in the case of H5N1, H7N9 and H10N8. Humans can also be infected by pigs, which can act both as an intermediate host between birds and humans or as “mixing vessels” where reassortment happens. An example of swine derived IAV is the 2009 pandemic influenza A H1N1 virus (10,11,15). The IAV strains created via reassortment have the potential to cause pandemics because they are new and the human population is naïve to them (14–16).

Of the **four pandemics** of human influenza that have occurred in the last 150 years, the 1918 pandemic, which was caused by an influenza A H1N1 virus was the most severe and it is estimated to have caused more than 40 million deaths (17,18). Influenza A H2N2, H3N2 and H1N1 viruses caused the 1957, 1968 and 2009 pandemics, respectively. In 1977, influenza A H1N1 started to circulate again in humans without causing a pandemic, as the strain was similar to the H1N1 circulating before 1957. On the other hand, the 2009 pandemic influenza A H1N1 virus was antigenically very different to the previous
seasonal influenza A H1N1 viruses and replaced the previously circulating H1N1 in the human population.

**Figure 2. More than a century of IAV pandemics:** In the past 120 years, four pandemics of human influenza have occurred, with the 1918 pandemic causing more than 40 million deaths (17). Influenza A H2N2, H3N2 and H1N1 viruses caused the 1957, 1968 and 2009 pandemics, respectively. In 1977, influenza A H1N1 restarted circulation in humans without causing a pandemic (1). Image created with BioRender.com.

**Nomenclature**
Every influenza virus isolate is named according to nomenclature rules (5). First is the name of the type of influenza virus (A, B, C or D), followed by the host species from which the virus was isolated (if not specified, the isolate is considered human), the geographical location at which the virus was isolated, the isolate number and the year of isolation. An example of an influenza strain name would be -A/Puerto Rico/8/1934 (H1N1)-: this virus is an influenza A virus, isolated in Puerto Rico in 1934; the virus isolate has an HA from the HA antigenic subtype 1 and an NA from the NA antigenic subtype 1 (1,9,19).

**Immune responses to influenza virus**

**Innate response**
Immune responses are mainly divided in innate and adaptive responses. Innate immune cells mostly involve epithelial cells, Natural Killer (NK) cells, antigen presenting cells (APCs) like dendritic cells (DCs), neutrophils, monocytes/macrophages but also innate-like B cells. The main players of the adaptive responses are lymphocytes: T and B cells (20).

The innate immune response is the first line of defense against pathogens. Epithelial cells are the ones to be infected and to first meet the virus, they react by production of type I IFN and signal to the site of infection several immune cells.

During IAV infection, conserved viral components called pathogen associated molecular patterns (PAMPs) are recognized by host pathogen recognition receptors (PRRs), such as retinoic acid-inducible gene-I protein (RIG-I) and toll-like receptor (TLR) located inside or on epithelial cells. For IAV these
PAMPs are mostly represented by viral RNA which has some peculiar characteristics that are not shared by cellular RNAs. The PRRs that recognize viral single stranded RNA are TLR 7 and TLR8 (21).

It is known that both type I interferons (interferon-α (IFNα) and IFNβ) and type III interferons (IFNλ) can inhibit viral replication in epithelial cells (22). Pro-inflammatory cytokines released at the site of infection by epithelial cells in turn recruit NK cells and pro-inflammatory monocytes to the lungs, where they contribute to fight viral infection by killing infected cells. These host responses to infection are crucial for viral clearance, nevertheless an excess of these responses can lead to the so-called cytokine storm, which can in turn result in severe disease (1,23,24).

Signals derived from PRRs can induce the activation, maturation and migration of DCs to the lymph nodes. When they are in the lymph nodes, DCs can present antigens to T cells, thus initiating adaptive immune responses (25).

**Adaptive response: cell mediated**

Activation of CD4+ T cells initially relies on interaction between their T cell receptors (TCR) and peptide-MHC class-II complex on APCs. CD4 T cells can differentiate into various T helper (Th) subtypes (Th1, Th2, Th17, T follicular helper (Tfh)) and exert critical functions like the activation of CTLs and macrophages and the activation and differentiation of B cells into plasma and memory B cells. In particular T follicular helper cells (Tfh) stimulate B cells to produce antibodies [62]. Tfh contribute to the generation and maintenance of the germinal center reaction and induction of long-lived plasma cells and memory B cells (20,25,26).

Virus-specific CD8+ T cells are activated upon engagement of their TCR with peptide-MHC class-I complex on APCs. The main function of CD8+ cytotoxic T Lymphocytes (CTL) is to recognize and kill virus-infected cells and produce pro-inflammatory cytokines like IFN-γ. CD8 T cells, depending on the balance of cytokines in the lymph nodes, can differentiate into effector cytotoxic or memory CD8 T cells (20,25,26).

**Memory T cells** can be divided in different subsets; effector-memory (TEm), central memory (Tcm), and terminally differentiated T cells (TEMRA) which can be further divided depending on longevity and proliferation capacity. The different subsets present different characteristics: Tcm cells are able to persist in the periphery longer than other subsets like Tem (in mice) (27,28). On the other hand, in human studies, TEMRA cells have been observed to be the most predominant memory T cell population in tissues and peripheral blood (20,29–32). Importantly, TEM cells are crucial for sustaining the frequency of T resident memory (TRM) CD8 T cells in the lungs following influenza infection (20,25,32,33).

Because of the fact that T cells can target internal and therefore conserved viral proteins (NP,M1 etc..), T cell-mediated immunity is cross-reactive and can protect against infection with IAV of various subtypes (heterosubtypic immunity) and drift variants (25).

**Adaptive response: humoral**

When an influenza virus infection is initiated, naive B cells or pre-existing memory B cells are activated by recognizing the viral antigens and interacting with CD4+ T cells (6). A part of the activated B cells will differentiate into short-lived plasmablasts, which produce the first round of virus-specific antibodies. A second part of the **activated B cells** will migrate to the B cell follicles of secondary lymphoid tissue. Here, in the germinal centers Tfh cells provide B cells with important co-stimulatory signals and produce interleukin IL-21, thus stimulating the generation of high affinity-matured, long-lived plasma cells and
memory B cells (32,34,35). A small part of activated B cells will differentiate into long-lived plasma cells and migrate to the bone marrow where they will remain and produce antibodies (6,36,37). A second part of the activated B cells will become memory B cells which are long-lived, but cannot produce antibodies and will remain in the blood, spleen and lymph nodes for immune surveillance. When memory B cells re-encounter an antigen, they are rapidly re-activated and differentiate into plasmablasts (peaking ~7 days after infection) and more memory B cells (36,38,39). There is a significant difference between the influenza specific memory B cell compartment and the steady-state serum antibody repertoire. The memory B cell compartment contains a broader repertoire than the serum antibody compartment, allowing the B cells to recognize a wide variety of influenza epitopes. This allows for recognition of conserved epitopes on drifted or shifted strains and potentially, for cross-protection (6,37).

Influenza specific antibodies
Influenza specific antibodies mostly target external and variable viral proteins (1,2,4,40). HA and NA on the surface of the virus are the most accessible influenza virus proteins. They represent the main targets for antibodies induced by influenza virus infection and vaccination: HA reactive antibodies can interfere with entry of the virus into cells.

The head domain of HA is very variable and highly immunogenic (41–43). Antibodies directed against the HA head involve virus neutralization as a mechanism of action and are for the most part very strain specific. Only few neutralizing antibodies directed against the HA head can neutralize different strains and even different subtypes of influenza virus (37,42,44–46). Antibodies that are able to neutralize more strains of influenza virus are called broadly neutralizing antibodies (bNAbs). bNAbs directed against the HA head bind to conserved regions of this domain, for example the receptor binding domain pocket of HA which binds sialic acid, but they are rarely found (2,42,43).

In contrast, the stalk domain of HA is significantly more conserved than the head, but significantly less immunogenic (41–43). Antibodies binding to epitopes on the HA stalk domain are broadly cross-reactive, yet only a fraction of these antibodies is able to neutralize a wide variety of influenza strains (homosubtypic and heterosubtypic neutralization) (1,2,9). Unfortunately anti HA-stem antibodies are less commonly found compared to antibodies targeting the HA head, most likely because the stalk domain is immuno-subdominant and seasonal influenza viruses and vaccines do not always induce these broadly neutralizing antibodies (47). HA stalk-directed antibodies can also have a non-neutralizing mechanism of action and act via FcγR-dependent mechanisms: antibody-dependent cytotoxicity (ADCC) mediated by NK cells, complement-dependent cytotoxicity (CDC) as well as antibody-dependent cellular phagocytosis (ADCP).

NA reactive antibodies can prevent virus budding and egress from infected cells. These antibodies similarly inhibit viral release from the proteins that trap the virus via HA-sialic acid interactions on mucosal surfaces. Moreover, antibodies bound to NA at the surface of infected cells might aid in the clearance of the virus through FcγR-dependent mechanisms, just as HA stalk antibodies (1,2,4,40).

NA is known to be more conserved than HA but immune subdominant and outnumbered on the influenza virus particles by HA spikes 1:4 (13). Nevertheless, recent findings demonstrate that a quarter of the plasmablasts induced by natural influenza virus infection targets NA (40). In contrast, current influenza vaccines poorly display key NA epitopes and consequently rarely induce NA-reactive B cells (13,40). Moreover, it has been observed that NA antibodies are broadly cross-reactive and can bind multiple historic IAV strains within the same group or across the two IAV groups (13,40,48).
Finally, antibodies targeting internal and conserved viral proteins mostly act via FcγR-dependent mechanisms. The targets of these antibodies, proteins such as M2 are poorly expressed on virions but abundant on the surface of infected cells. These FcγR-dependent mechanisms are ADCC, ADP and ADCD (2,42,49–53). See chapter 2 for more information on influenza specific antibodies and their mechanisms of action.

**How to measure influenza-specific antibodies**

Several different assays are used to measure different types on influenza specific antibody titers. The serological assay employed to measure antibodies able to bind IAV proteins (binding antibodies) is an enzyme-linked immunosorbent assay (ELISA). Depending on the choice of coating of the experimental plates, antibodies to whole inactivated virus (WIV) or to single viral proteins (NA, HA, M2, NP) or specific protein domains (HA head, HA stalk) can be detected (2,4,54–56). This assay is very versatile and is able to capture cross-reactive antibodies that bind to conserved viral epitopes as well as strain specific antibodies targeting variable viral epitopes.

The two primary serological assays that measure neutralizing antibodies are the hemagglutination inhibition (HI) assay and the virus neutralization (VN) assay (2,56,57). 

HAI measures antibodies able to bind the viral HA and by doing so prevent agglutination of red blood cells (RBCs) induced by the binding of HA and sialic acids present on RBCs. Even though in literature HAI antibodies are sometimes referred to as a synonym of neutralizing antibodies, it is important to underline that only a fraction of antibodies measured via HAI are functionally neutralizing (56–58). VN assays on the other hand, measure antibodies able to prevent cellular infection in vitro by exerting neutralizing activity (4). Because of the fact that most of the antibodies measured via HI and VN target variable epitopes on the HA head domain and only rarely target conserved viral epitopes, these assays mostly measure strain specific antibodies.

Finally, NA-inhibiting antibodies, able to interfere with the enzymatic activity of the protein, can be quantified with the enzyme-linked lectin assay (ELLA). The antibodies measured via ELLA assay can be both strain specific and broadly reacting. See chapter 3 and 5 for more information regarding influenza serology assays.

**Influenza virus immune history**

Given that antibodies are the first line of defense against influenza virus, the magnitude and quality of the antibody repertoire in human subjects are very important determinants of disease outcome for influenza infections. Humans are exposed to several influenza viruses throughout their lifetime and most of the children by the age of 3 already encountered influenza virus at least once. Antibody responses to influenza virus are known to be long lasting. For example, antibodies raised to strains encountered in childhood can usually be detected in elderly individuals (1,53,58). On the other hand, vaccine elicited antibody responses are less broad and not as long-lived (6).

The severity of epidemic/pandemic influenza depends on multiple factors, including the virulence of the virus strain, the age of the subjects and the level of pre-existing immunity. It has been observed before that immune history influences the magnitude of the antibody response to newly encountered influenza virus strains (9,59). A clear understanding of how previous exposure to influenza virus affects antibody responses to vaccination and infection is of major importance, especially with regard to the development of a universal influenza vaccine able to protect the population against multiple influenza virus strains without the need for yearly update (1,2,9,59,60).
Figure 3. Serological assays for the detection of influenza virus specific antibodies: The serological assay employed to measure antibodies able to bind IAV external proteins is the ELISA assay (top left panel). HAI measures antibodies able to bind the viral HA and by doing so prevent agglutination of red blood cells (RBCs) induced by the binding of HA and sialic acids present on RBCs. Only a fraction of antibodies measured via HAI are functionally neutralizing. VN assays measure antibodies able to prevent cellular infection in vitro by exerting neutralizing activity. Finally, NA-inhibiting antibodies, able to interfere with the enzymatic activity of the protein, are quantified through ELLA. Image created with BioRender.com.

Original antigenic sin (OAS) and antigenic seniority (AS)
We know that immune memory acquired through past influenza exposure affects and shapes the response to subsequently encountered strains, but the way this happens remains partly obscure (61,62). Already in 1960 Thomas Francis and colleagues observed that the antibody response to influenza strains first encountered in childhood dominated the antibody response to influenza virus over time. Based on this observation, they phrased the “original antigenic sin” (OAS) theory (Figure 1). According to this theory, encounter with a new influenza virus strain predominantly leads to activation of memory B cells induced by previously encountered strains, rather than to activation of naive B cells. The response is therefore dominated by antibodies with high affinity to the old but possibly low affinity to the new strain (9,63–65).
Since 1960 this theory has been challenged multiple times and less outspoken terms for such a phenomenon were coined such as “antigenic seniority” (AS) or “immune imprinting” (9,58,66,67). The imprinting strain would then refer to the first antigenic virus variant encountered early in life which should have an impact on lifelong immunity in humans (56,58,68). The AS theory is significantly different from the OAS theory since it states that every new encounter with an influenza virus strain would induce a de novo immune response against the novel strain and a boost of antibodies against all past strains (6,37,56,68–70). Because of frequent boosting, the antibodies directed against the imprinting senior strain will be, according to AS, at the highest levels at any given moment, followed by antibodies to the next encountered virus and so on and so forth. The debate is open as to which theory (OAS or AS) is most representative of the reality of influenza virus immune memory. Independent of the outcome of this debate, it is in any way clear that influenza-specific pre-existing immunity can have both a positive and negative impact on the generation of a successful immune response upon encounter of a new influenza virus strain (Figure 1).

An example of a beneficial effect of influenza immune memory is a phenomenon called “protective HA imprinting”. This kind of imprinting implies that an individual’s first IAV infection would confer lifelong protection against severe disease from novel HA subtypes in the same phylogenetic group (9,69). Indeed, a recent publication showed that protective homologous HA imprinting provided 75% protection against severe infection and 80% protection against death for both zoonotic IAV H5N1 and H7N9 (69).

An example of a detrimental effect of influenza immune memory is a phenomenon called “antigen masking” or “negative antigenic interaction”. This phenomenon is most likely caused by pre-existing serum antibodies that mask virus antigens and hide them from B cell receptors, in this way impairing naïve B cell activation (60,71–74). It is noteworthy that vaccine effectiveness is strictly dependent on influenza pre-existing immunity. It has indeed been shown that revaccination with influenza virus vaccines in consecutive years can lead to reduced vaccine effectiveness in the second year (71–74).

Figure 4. Antigen masking: According to OAS theory, encounter with a new influenza virus strain predominantly leads to activation of memory B cells activated by previously encountered strains, rather than to activation of naïve B cells. This phenomenon would likely be caused by antigen masking: serum antibodies and memory B cells that mask virus antigens and hide them from B cell receptors, in this way impairing naïve B cell activation. (a) First encounter with IAV. (b) Subsequent encounter with homosubtypic IAV strain. (c) Subsequent encounter with heterosubtypic IAV strain. Image created with BioRender.com.
Scope of the thesis

The aim of this thesis was to study and better characterize IAV specific pre-existing immunity and its implications. Humoral immune responses are crucial for protection from IAV and for reduction of disease severity in infected hosts. For this reason, in chapter 2 we review current knowledge about the several different mechanisms of action of influenza-specific antibodies. We focus both on the neutralizing and the less known non-neutralizing responses. We describe multiple targets of IAV specific antibodies: the more immunogenic HA head and NA, but also the less immunogenic HA stem and internal viral proteins.

In chapter 3 we focus on two of the most commonly used methods to quantify influenza specific neutralizing antibodies, namely the hemagglutination inhibition (HAI) assay and the virus neutralization (VN) assay. The titers determined with these assays are currently used as a measure and an indication for vaccine effectiveness.

Both assays are characterized by high intra- and inter-laboratory variability. For the VN assay, variability is likely due to the use of different assay protocols which use different readouts. We therefore aimed at investigating which of the VN assay readout methods currently in use would be the most suitable choice to replace the more frequently employed HAI, given the numerous drawbacks of the latter. For this purpose, human serum samples were tested for the presence of influenza specific neutralizing antibodies against an H1N1 (49 sera) or H3N2 (50 sera) IAV, using four different infection readout methods for the VN assay (cytopathic effect, hemagglutination, ELISA, RT qPCR) and using the HAI assay. The results were compared by correlation analysis and by determining the level of agreement between assays and among VN readouts, before and after normalization to a standard serum.

This study could be useful to inform the scientific community, but also helped us to determine what assay to use in order to measure neutralizing antibodies in the next chapter.

In chapter 4, we investigate IAV immune memory and its evolution over-time in the human population and the impact that IAV pre-existing immunity may have on the encounter with a novel pandemic strain in the human hosts. We study pre-existing immunity against IAV by assessing the specific immune responses to 5 different historic IAV strains in 180 subjects from 3 different age cohorts: adolescents, adults and elderly (sampled between 2009 and 2017). The strains of choice cover the span of the last 100 years and they likely represent the strains that individuals possibly sequentially encountered during their life. We focus on measuring IAV binding (IgG), neutralizing, and NA-specific antibodies to gain a picture of the IAV specific antibody repertoire in humans and how it evolves over-time.

Finally, after gaining a more detailed idea of IAV specific immunity in humans, in chapter 5 we investigate in a mouse model the effect of sequential exposure to different influenza virus strains, either as live virus or as vaccine, on infection by a new virus strain. In particular, we focus on the mechanisms of cross-protection conferred by IAV pre-existing immunity, generated by sequential exposure to IAV. For this reason, in this study mice are sequentially infected or vaccinated with antigenically distinct virus strains belonging to group 1 and 2 IAV respectively, and immune responses possibly involved in cross-protection are investigated. IAV specific antibody responses and T cell immunity were evaluated to answer our research questions.
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