Exploring the Field of Respiratory Virus Preventions

How to Deal with Future Pandemics

A Doctoral Thesis by

Elias Tjärnhage

Institute of Clinical Medicine

Faculty of Medicine

University of Oslo

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I will conclude these acknowledgements by circling back to my lack of knowledge when entering this PhD work. I am so grateful to my supervisor, Dr. Gunnveig Grødeland, who believed in me and gave me a chance despite me having no real experience with neither tissue cultures, working with mice, nor immunology as a field. I hope you agree that it turned out ok.

Thank you for all your guidance over these years!

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Abbreviations

Ab	Antibody
ACE2	Angiotensin converting enzyme 2
ADCC	Antibody dependent cellular cytotoxicity
ADCP	Antibody dependent cellular phagocytosis
Ag	Antigen
AID	Activation induced deaminase
APC	Antigen presenting cell
ASC	Antibody secreting cell
BCR	B cell receptor
BCL6	B cell lymphoma 6
BLIMP-1	B lymphocyte-induced maturation protein-1
BM	Bone marrow
CA07	A/California/07/2009 (H1N1)
CD	Cluster of differentiation
CDR	Complementarity determining region
CLR	C-lectin type receptor
COV	Coronavirus
CSR	Class-switch recombination
DAMP	Damage associated molecular pattern
DC	Dendritic cell
ELISA	Enzyme-linked immunosorbent assay
ELISpot	Enzyme-linked immunosorbent spot assay
ER	Endoplasmatic reticulum
Fc	Fragment crystallizable
FcR	Fc receptor
FDC	Follicular dendritic cell
Fv	Fragment variable
GC	Germinal center
HA	Hemagglutinin
HC	Heavy chain
HPAI	Highly pathogenic avian influenza
HTS	High throughput sequencing
IAV	Influenza A virus
IBV	Influenza B virus
ICOS	inducible costimulatory
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
iNKT	Invariate natural killer T cells
IRF4	Interferon regulatory factor 4
ITAM	Immunoreceptor activating tyrosine motif
ITIM	Immunoreceptor inhibitory tyrosine motif
LAIV	Live attenuated influenza virus
LC	Light chain
LD	Lethal dose
LLPC	Long-lived plasma cells
LN	Lymph node
LTK	lymphocyte tyrosine kinase
mAb	Monoclonal antibody
MBC	Memory B cell
MDCK	Madin-Darby canine kidney
MERS	Middle east respiratory syndrome
MHC	Major histocompatibility complex
MNA	Microneutralization assay
mRNA	Messenger RNA
MZ	Marginal zone
MΦ	Macrophage
NA	Neuraminidase
NHEJ	non-homologous end joining
NIP	4-hydroxy-3-iodo-5-nitrophenylacetyl
NK	Natural killer
NLR	NOD-like receptor
NGS	Next generation sequencing
NP	Nucleoprotein
PAMP	Pathogen associated molecular pattern
PB	Plasma blast
PC	Plasma cell
PR8	A/Puerto Rico/8/1934 (H1N1)
φNA	Psuedovirus neutralization assay
RBD	Receptor binding domain
RBS	Receptor binding site
RLR	RIG-like receptor
SA	Sialic acid
SARS	Severe acute respiratory virus
scFv	Single chain Fragment variable
SCS	Subcapsular sinus
SHM	Somatic hypermutation
SLO	secondary lymphoid organs
TCR	T cell receptor
TFH	Follicular helper T cell
TH	Helper T cell
TLR	Toll-like receptor
TMPRSS2	Transmembrane protease serine 2
TNF	Tumor necrosis factor
WHO	World Health Organization

List of papers included in this thesis

Paper I

Elias Tjärnhage, Diamond Brown, Bjarne Bogen, Tor Kristian Andersen, Gunnveig Grødeland.

Trimeric, APC-Targeted Subunit Vaccines Protect Mice against Seasonal and Pandemic Influenza

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Paper II

Elias Tjärnhage, Thea Kristin Våtsveen, Melinda Raki, David Nemazee, Hesso Farhan, Ludvig Munthe, Gunnveig Grødeland.

Repurposing ALK Inhibitors as Broad-Spectrum Antivirals Targeting Lymphocyte Tyrosine Kinase (LTK)

Submitted

Paper III

Elias Tjärnhage, Khang Le Quy, Taissa de Matos Kassahara, Victor Greiff, Gunnveig Grødeland.

Influenza H1N1 Strains Differ with Respect to their Potential for Dominating Immune Formation during Secondary Viral Exposures

Manuscript

Sammanfattning på svenska

Pandemier orsakade av luftburna virus utgör ett allvarligt hot mot mänskligheten. Vi såg det tydligt under den nyliga SARS-CoV-2 pandemin som utbredde sig över världen 2020. Många miljoner människor miste livet trots stora ansträngningar att hantera pandemin och begränsa effekterna. Nu är situationen mer hanterbar även om det fortfarande sker en smittspridning. Det är dock fortfarande mycket vi inte vet om hur vi bäst ska hantera en eventuellt kommande pandemi. En ökad förståelse för hur virus, vaccin och vårt immunförsvar samspelar kommer vara viktigt för vår framtida förmåga att vara bättre förberedda och ha ett bättre skydd. En viktig del i en hantering av virussjukdomar är behovet av bättre profylaktiska (t.ex. vacciner) och terapeutiska (t.ex. antiviraler) behandlingar.

De senaste 100 åren har vaccin mer eller mindre bestått av försvagade eller inaktiverade versioner av sjukdomsframkallande virus. Det har i många fall gett väl fungerande vaccin, trots att man inte haft en detaljerad förståelse över vilken typ av vaccinrespons man har velat åstadkomma. Den senaste SARS-CoV-2 pandemin visade hur viktigt det är att kunna designa och anpassa vacciner baserade på särskilda antigener som kan leda till skyddande responser.

Vaccinutveckling är fortfarande i huvudsak baserad på långa perioder av tester och utvärderingar. Detta gör oss sårbara för nya virus under perioderna där vaccinerna utvecklas och framställs. Derför har utvecklingen av antivirala läkemedel gett en ny och viktig behandlingsmetod för allvarliga virussjukdomar där vacciner inte ger ett fullgott skydd eller inte är tillgängliga. Tyvärr är majoriteten av dagens antivirala läkemedel effektiva endast mot en typ av virus och det gör dem sårbara för mutationer i viruset som leder till att läkemedlen förlorar sin effekt. Detta gör att dessa läkemedel kan vara av begränsad nytta i framtida pandemier med okända virus. Det behövs därför en ny klass av antivirala läkemedel som är aktiva mot fler typer av virus.

Vidare, inom utvecklingen av vaccin och antiviraler, saknas det också mer grundläg-

gande kunskap om hur vårt immunförsvar reagerar på virus som kan mutera snabbt. Till exempel vet vi inte detaljerna om hur det bildas nya immunresponser eller om det främst sker en återkallelse av tidigare minnesresponser när vi utsätts för återkommande varianter av samma virus. Vi vet inte heller hur detta i sin tur påverkar utfallet av vaccineresponsen. Denna typ av kunskap är mycket användbar för en mer rationell utformning av framtida vacciner, då det kan användas för att fokusera immunresponsen dit den är mest användbar. Det är också viktigt för att kunna förutse en mer grundläggande immunförsvarrespons mot nya versioner av virus vi blivit exponerad för tidigare.

Denna avhandling kan beskrivas med två övergripande teman:

1. Utveckling av förbättrade behandlingar mot virus, som influensa och coronavirus. Vi utvecklade och testade ett APC-målstyrt hemagglutinin subenhets vaccin som bibehåller den naturliga trimer strukturen som man ser på influensavirusets yta. Vi testade också en panel av läkemedel för dess förmåga att förhindra replikation av både influensa- och koronavirus. Detta var en såkallad ”drug repurposing” strategi där läkemedel som tidligare var gjorda för att förhindra utsöndring av protein i cancerceller blev testade för deras förmåga att förhindra utsöndring av virusproteiner i infekterade celler. Detta skulle leda till en indirekt behandling av virusinfektioner utan att virusspecifika mekanismer behövs.

2. En mer grundläggande undersökning om hur en individs tidigare exponeringar för influensavirus påverkar senare responser mot andra influensavirus. Detta var fokuserat på B- cellers roll i germinalcenter, med extra fokus på inverkan av specificiteten hos B-cellreceptorsekvenser, där vi såg hur olika influensavirusstammar har väldigt varierande förmåga att prägla immunförsvaret och dess förmåga att svara på och förhinda nya infektioner med andra varianter av influensavirus.

Summary in English

Pandemic outbreaks of respiratory viruses potentially pose a great danger for society, as was recently exemplified by the global outbreak of SARS-CoV-2 in early 2020. Our ability to respond is reliant on our understanding of how viruses, vaccines and the immune system interact. Even though we have been able to endure every pandemic so far, sometimes at high cost to human lives, there are many knowledge gaps that should be filled to improve future responses.

One aspect that should be improved is available treatments, both prophylactic and therapeutic. For about a century, vaccines have typically contained weakened or inactivated versions of the pathogen, without specifically designing vaccines to raise the most desirable type of immune responses. The recent SARS-CoV-2 pandemic demonstrated the importance of vaccines based on selected antigens, but vaccine development is still mostly empirical in nature.

Antiviral therapeutic drugs are a more recent invention and are crucial for treating severe disease when vaccines are not enough or simply not available. However, currently available antivirals are mostly specific for a particular virus and susceptible to emerging escape mutations, which means that they may be of limited use in the emergence of a novel virus with pandemic potential. Therefore, new classes of broadly acting antivirals targeting conserved pathways of pathogen replication should be developed.

More fundamentally, there are knowledge gaps on key interactions between variable viruses and the immune system. As an example, the extent to which de novo responses versus recall responses are initiated against sequential exposures to variants of a virus, and how this affects vaccine efficacy, is not fully understood. Such knowledge will be useful for future rational vaccine design, both to overcome restrictive primary imprinting and to generate relevant immune responses. In the face of a potential new pandemic, such knowledge will also be important for meaningful forecasts on baseline protection against a new viral variant.

The content of this thesis can be split into two parts:

1. Development of improved treatments against variable viruses such as influenza and SARS-CoV-2. Here, we developed and evaluated an APC-targeted hemagglutinin subunit vaccine that maintains the naïve trimeric structure seen on the viral surface and could therefore maintain all five main epitopes found on hemagglutinin. In addition, we also evaluated a panel of drugs for their ability to inhibit two families of viruses, influenza viruses and coronaviruses. This was a drug repurposing strategy where the drugs were host-directed and prevent protein secretion, with the aim being to prevent secretion of functional virus from infected cells.

2. Studying immune imprinting of highly variable influenza viruses, and how this can affect subsequent immune responses to an infection. Here, the focus was on B cells during germinal center responses, and the impact of specific B cell receptor sequences, where we found distinct differences between influenza strains ability to imprint the immune system and dominate future responses to varying influenza strains.

Introduction

1. Respiratory viruses responsible for pandemics

Infectious diseases pose a continuous threat to human health, and viral respiratory infections are responsible for a large proportion of the disease burden from infectious diseases(1). Two families of respiratory viruses have been responsible for global pandemics in the last 100 years. Namely, influenza and coronaviruses. Influenza have historically posed a greater threat, but this hegemony was broken with the recent SARS-COV-2 pandemic.

1.1 Influenza virus

Influenza is a respiratory disease caused by infection with the influenza virus. It is unknown when the first infection occurred(2,3), but the start of our modern experience with influenza was the 1918 pandemic termed "Spanish Flu"(4). Interestingly, the influenza virus was not isolated until 1933(5).

The influenza virus is a negative-sense single-stranded encapsulated RNA virus that is part of the orthomyxoviridae family, and there are four genera of influenza virus: influenza A, B, C and D. Influenza A (IAV) and B (IBV) are currently circulating in humans. While IBV is mostly restricted to human infections (with some exceptions where IBV have been isolated from animals(6)), IAV has its primary reservoir in birds. Besides avian viruses, IAV circulates in many mammals, including humans. IAV and IBV also differ in their ability to reassort their genes (see below), and this in combination with the zoonosis of new viruses means that IAV constitutes the greater pandemic threat.

Influenza genomic structure

The genome of IAV and IBV are divided into eight separate strands of RNA. Most strands contain one gene each, while the smallest RNA strand has multiple genes(7). The three largest genes encode the proteins basic polymerase 2 (PB2), basic polymer-

ase 1 (PB1) and acidic polymerase (PA), respectively. Together they form the virus' RNA-dependent RNA polymerase complex. The following genes encode different structural proteins. Hemagglutinin (HA) is the major surface protein located in the viral membrane and is required for viral entry into new host cells(8). Neuraminidase (NA) is the other main surface protein, and is important for release of newly formed virus particles(9). Nucleoprotein (NP) is associated with the RNA genome. The remaining genome encodes matrix protein (M1), membrane protein (M2) and other non-structural proteins (NS1). M1 helps form the membrane matrix and M2 is an ion-channel. The fractured nature of the influenza genome allows for reassortment of the genes in cells infected by more than one virus, and where RNA strands can be combined in new ways(10). This creates the possibility that new subtype combinations could be formed and is called antigenic shift. In addition to antigenic shift, there is also antigenic drift. Antigenic drift is a process where mutations accumulate during the replication to slowly change the sequence of the viral proteins, ultimately escaping previously established immune responses. NA and HA are particularly prone to such antigenic drift(11).

Influenza classification and nomenclature

The two major surface proteins: HA and NA form the basis for the classification of IAVs. There are 18 known HA subtypes (H1-18) and 11 known NA subtypes (N1-11) that combined make up the HxNx subtypes. However, the similarity between HA subtypes have led to a clustering of IAV subtypes into two overarching groups, either group 1 (H1, H2, H5, H6, H8, H9, H11, H12, H13, H16, H17 and H18) or group 2 (H3, H4, H7, H10, H14 and H15). The strain nomenclature for IAV is then A/[Place of isolation]/[Isolate number]/[Year of isolation](Subtype). For example: A/Puerto Rico/8/1934(H1N1).

Influenza B viruses are not classified based on subtypes like IAVs but rather into two antigenically different lineages, either B/Victoria/2/1987-like or B/Yamagata/16/1988-like. The limited host spectrum of IBV explains this reduced diversity as compared to IAV, and also reduces the risk of antigenic shift into previously unknown combinations that could constitute potential pandemic threats(12).

Influenza origin and natural reservoirs

Influenza viruses are thought to have originated in wild birds, and birds are still the main reservoir of IAV, with HA 1-16 all being found in birds(13). The origin of HA 17 and 18 are more uncertain as they are found in bats and differ more from the other subtypes(14). Thus far, only H1, 2 and 3 have circulated in humans, with H1N1 and H3N2 currently co-circulating(15). Influenza is thought to have spread from wild birds to domesticated birds such as ducks and poultry, and then to pigs before jumping to humans(16). The ability of pigs to get infected with influenza viruses of both avian and human origin is important, since the close proximity between pigs and humans on farms increases the risk of zoonosis(17). In addition, pigs can be a reassortment host for the generation of new viruses, as was seen in the 2009 pandemic. The currently circulating H1N1 strains originate from the 2009 pandemic, the so-called "swine flu", where a reassortment of IAV genes occurred in swine, likely somewhere in central Mexico(18–20). Besides seasonal flu, the threat of highly pathogenic avian influenza (HPAI) such as H5Nx and H7Nx is of utmost concern for future human outbreaks. So far, no human-human spread has been noted from these HPAI, but there have recently been transmission of H5N1 in Spanish mink(21). Further, H5N1 has been detected in cats and dogs(22–24). Of concern, the cases of human infection from domesticated birds have shown much higher mortality rates than current human seasonal influenza strains(25,26), but it is not clear if this will be the same should one of these viruses acquire the ability of human to human transmission.

Influenza viral proteins and life-cycle

The two surface proteins HA and NA are the two proteins involved in cellular entry and release during the viral replication cycle. Their importance and exposed nature mean that they are therefore important antigens in immune responses against influenza. HA is a homotrimeric glycoprotein with an extracellular domain of around 60 kDa per monomer, a transmembrane region, and a short intracellular domain(27). The extracel-

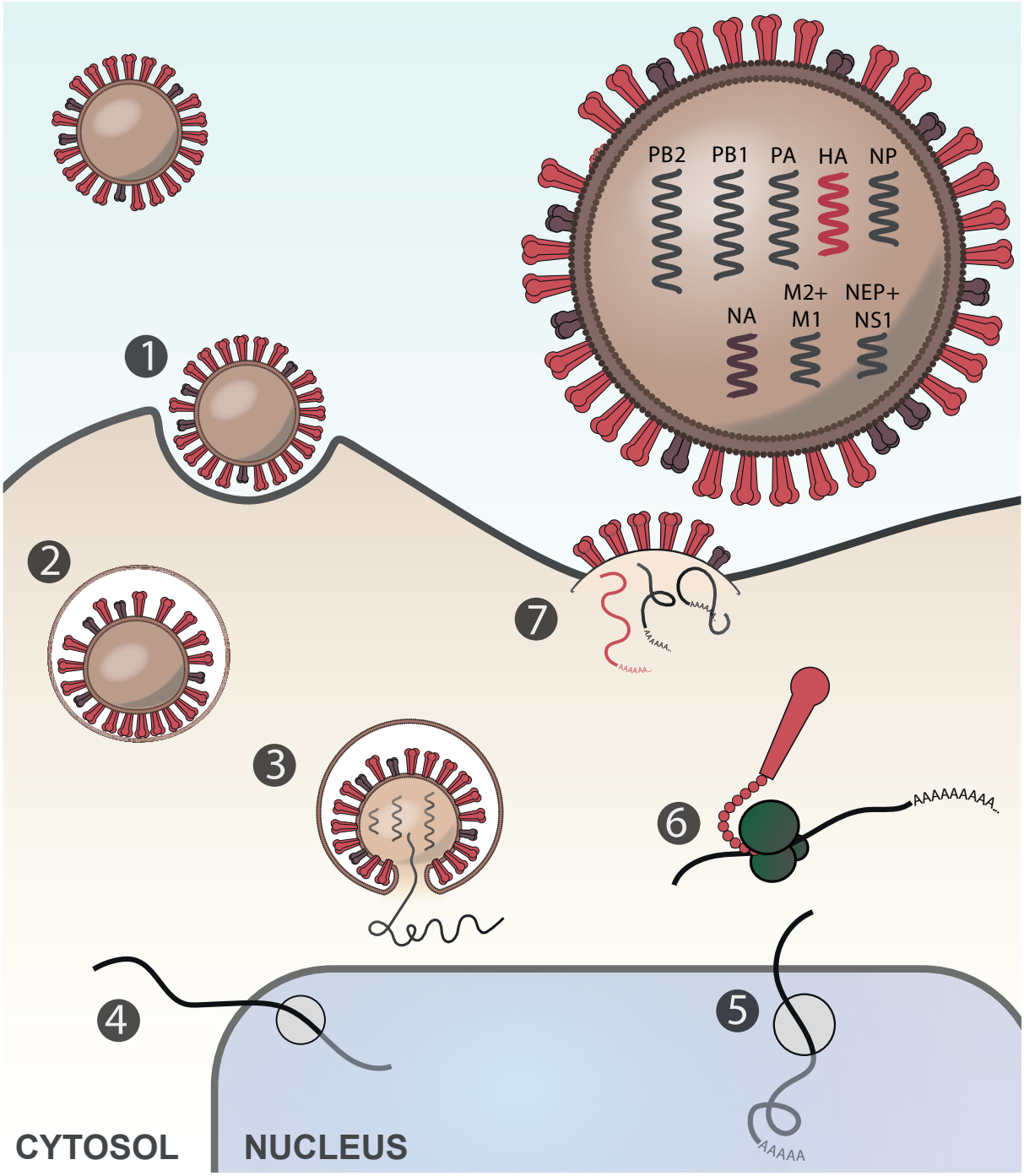

Figure 1: Influenza viral life cycle

Influenza A virus is a membrane enveloped RNA virus with a segmented genome. The genome consists of eight RNA segments that together encode all viral components. During the infection of a cell, the HA attaches to sialic acid on the surface (❶). The virus is then endocytosed (❷) and due to the acidification of the vesicle trigger a conformational change in HA leading to fusion with the vesicle membrane (❸). The released viral RNA segments are then transported to the host nucleus where they are replicated (❹) and transcribed into mRNA and transported to the ER (❺). In the ER, the viral mRNA is then translated to and any post-translational processing such as the cleavage of HA into two polypeptides takes place (❻). Lastly, the viral proteins and genome is packed into new virions that bud off the host cell. Here, NA deglycosylates sialic acid to facilitate the release of newly produced viruses (❼).

lular HA domain is composed of a membrane proximal stem region and a distal head region. The stem region is more conserved between influenza strains (e.g. within the overarching groups 1 and 2 described above), whereas the head that contain the sialic acid binding domain, also known as the receptor binding site (RBS), is more prone to mutations and antigenic drift(28). In the post-processing of synthesized HA, a proteolytic cleavage of the extracellular domain splits the HA into two polypeptide chains that remain associated. The HA1 domain then contains the head region and the HA2 domain then contain the stem and membrane region(29,30).

Neuraminidase is a homotetrameric deglycosylation enzyme with a monomeric molecular weight of around 60 kDa(9). Each monomer of the NA has an enzymatic site, but tetramerization is important for active catalytic functions as recombinant monomers display very low activity(31). The substrate for NA is sialic acid on the cell surface. Cleavage of SA is important in the release of newly formed viral particles that would otherwise remain bound to the cell via HA. NA cleavage of SA have also been shown to play a role during the infection stage of the replication cycle(32,33).

The life-cycle of influenza viruses begins with the infection of a suitable host cell, normally epithelial cells in the upper respiratory tract(34). This is initiated by the binding of HA to sialic acid on the surface of the host cell. The binding preferences of human and avian HA differ slightly, as human-circulating HA preferentially bind sialic acid with an α2,6 linkage, whereas avian HA prefers an α2,3 linkage(35,36). After binding of virus to the membrane and internalization by endosomes, the acidification of the endosomes triggers a pH-dependent conformational change in the HA that induces fusion of the viral membrane with the host membrane, resulting in viral release into the host cell cytoplasm. After trafficking of viral ribonucleoprotein complexes into the host nucleus, the negative-sense RNA is transcribed into a positive-sense RNA, that after polyadenylation and capping, is ready for translation into new viral proteins. After translation and proper post-processing of all virus proteins, the components are assembled, and newly formed viruses are budded off from the host membrane. In order to efficiently release

the virus from the cell, NA trims surface sialic acid to prevent attachment of newly formed influenza virus to the originating cell surface, thereby facilitating the spread of newly formed virus to other cells (Figure 1).

Immune responses against influenza virus

The exposed nature of HA and NA makes them targets for antibody responses. HA is considered more immunogenic than NA, although this is mostly occurring in vaccines rather than natural infection(37). The head region of HA is in turn also more immunogenic than the stem region(38). Antibody responses towards the head of HA make up the majority of neutralizing antibodies, mainly due to their ability to block the binding between HA and sialic acid by binding to the RBS(39–41). Antibodies towards the more conserved stem region are more rare, but can be neutralizing by preventing the pH-induced conformational change required for membrane fusion(42,43). When elicited, they can be capable of neutralizing a wide range of strains and subtypes, so are therefore an example of broadly neutralizing antibodies (bnAbs)(44,45). However, bnAbs are not restricted to stem epitopes, there are conserved head epitopes capable of neutralizing influenza infections(46). Similarly, NA-binding antibodies can also neutralize influenza virus by preventing NA-function(47).

In addition to neutralizing antibodies, non-neutralizing antibodies are also important for clearing influenza viruses and virus-infected cells. These mechanisms are more detailed described in section 2.2.

Vaccine designs against influenza viruses

Because of the importance of the HA protein in the replication of influenza virus, vaccines against influenza have traditionally had the goal of inducing neutralizing anti-HA IgG titers(48). More recently, the relevance of inducing T cell responses, especially towards more conserved intracellular proteins such as the nucleoprotein, have also been

raised(49,50). This would allow for a broad baseline protection that would resist seasonal antigenic drift as well as more drastic antigenic shift.

When this work started in 2019 our focus was on influenza. This changed with the emergence of SARS-CoV-2 and its world-wide spread in 2020, so the research shifted to include SARS-CoV-2 as well.

1.2 Coronavirus

Coronaviruses were first isolated from humans in the 1960's(51). The name coronavirus stem from early electron micrographs where the virus particles had a distinct "halo" around them, like the corona around the sun.

Coronaviruses can be divided into four genuses, alpha, beta, gamma and delta, that all are a part of the orthocoronavirinae subfamily. Some coronaviruses circulate in humans as part of "common cold", namely alpha (e.g., 229E) and beta (e.g., OC43) coronaviruses. In addition to common cold viruses, there have been three outbreaks of beta coronaviruses that either could have become or became pandemic. The first was the Severe Acute Respiratory Syndrome (SARS) corona virus (SARS-CoV) that was first identified in the Guangdong Province in China in late 2002(52–54). The virus then mostly spread to surrounding Asian countries with a handful of cases in other countries around the world. The mortality rate varied from country to country but the total mortality rate was 10% during the first nine months(55). The virus was not so easily transmitted between humans, so viral spread could be fairly easily contained(56,57). In 2012, we saw the first cases of Middle East Respiratory Syndrome coronavirus (MERS-CoV)(58–60), outbreaks that have since repeatedly occurred in the Middle East with mortality rates around 36%. 84% of the laboratory confirmed cases since 2012 have occurred in Saudi Arabia, with a slightly higher related mortality rate of 39%(61). Lastly, we have the recent pandemic caused by SARS-CoV-2(62), marking not only the largest outbreak of any coronavirus, but also the largest pandemic of any virus since the influenza pandemic of 1918.

Corona virus genomic structure

Like influenza virus, coronaviruses are single-strand RNA membrane encapsulated viruses. However, unlike influenza, the coronavirus genome is a single positive-sense RNA molecule that encodes all viral protein components over different reading frames(63). The largest reading frames, ORF1a+b, encodes 16 non-structural proteins involved in functions such as viral RNA replication, protease activity to cleave transcribed polypeptides into distinct proteins, anchoring of components to membranes, and acting as modulators of host cell pathways(64). The second largest reading frame encodes the protruding membrane protein spike involved in cellular entry. The remaining reading frames encode proteins such as envelope (E) and membrane (M) proteins that are important for the structure of the virus, and nucleocapsid (N) – an RNA binding protein that packs the viral RNA genome, along with some smaller structural and accessory proteins.

The fact that the coronavirus genome is encoded on only one gene segment removes the possibility of reassortment between different coronaviruses. This means that, unlike influenza viruses, coronaviruses cannot undergo antigenic shift to produce new reassortant viruses. The implication is that the virus is a bit more predictable as compared to influenza, since it has to acquire new functions by accumulating the necessary mutations over time. However, gradual evolution may still surprise us due to a large natural coronaviral reservoir in bats and other species.

Coronavirus Spike protein and clinical developments

The spike protein has been the target for early vaccine development against coronaviruses(65–68), including SARS-CoV-2(69,70). The spike protein is similar in function to HA on influenza in that it binds to the target receptor (angiotensin converting enzyme 2, ACE2) on the surface of the host cell, and following a conformational change leads to cellular entry and fusion of the viral and host membrane(71). The tip of the spike houses the receptor binding domain (RBD), and that has been a major focus for vaccine

development as antibodies that block the binding between RBD and ACE2 can prevent host infection. However, the RBD is also a site where many escape mutations have taken place to circumvent antibody binding. This is clearly exemplified by the number of mutations in the RBD, with the alpha, beta, gamma and delta having between 1-3 mutations and the first omicron variant having 15 mutations(72).

The conformational change in Spike required for cellular entry is mediated by two sequential proteolytic cleavages. The first is mediated by host furin proteases(73) and separates the spike into S1 and S2 subunits, and the second is mediated by membrane-anchored proteases, such as Transmembrane protease, serine 2 (TMPRSS2), and triggers fusion of virus and the host membrane(74). This has led to development of TMPRSS2 inhibitors for SARS-CoV-2 therapeutics(75). However, the most successful therapeutics against SARS-CoV-2 have been nucleoside analogues acting as RNA dependent RNA polymerase inhibitors such as Remdesivir(76) (see section 3.2).

2. The immune system

What happens when a virus tries to infect a new host? It will first have to reach a site where there are suitable host cells to infect, and evade the protective barriers placed by the host to prevent this. These barriers are both physical, cellular and molecular, and can be collectively described as the immune system.

2.1 An introduction to the immune system

The immune system is a collective name for all cells and tissues involved in preventing harmful pathogens from taking hold and causing damage to us. It starts with physical barriers like the skin and mucous membranes, which in addition to being a site for residing immune cells prevents infection by a lower than physiological pH and a general hostile environment for pathogens(77). Mucous membranes are located at sites where foreign material can enter, such as airways and lungs, in addition to gastrointestinal and reproductive tracts. Although skin and mucosa are important parts of the immune system, the main workhorses are the immune cells capable of clearing both pathogens and pathogen-infected cells. Immune cells are often divided into two compartments, the innate and the adaptive immune system. The innate immune system is very rapid to respond and recognize general features of pathogens with predefined receptors. The adaptive is slower to respond initially, but recognize specific antigens with clonally unique receptors. Immunological memory is also maintained by the adaptive system.

The differences between the innate and the adaptive immune system allow for a cooperative response. The adaptive system with the B cells and T cells is more specific and better at clearing infections, but this specificity takes time to develop. The ability of the innate immunity to respond quickly is therefore important during early phases of an infection. Here, the innate immune system can either clear the pathogen itself or limit the initial spread while signaling for activation of the adaptive immune system. Recognition of general pathogen features is sufficient to start the innate response, and release signaling molecules to activate surrounding immune cells. When the initial infection

has been detected and somewhat hampered by the innate immunity, antigen presentation by innate immune cells to the adaptive immune cells then provide the necessary keys for activation of an adaptive immune response.

Dendritic cells (DCs) are innate immune cells that continuously sample their surroundings for foreign material, which they present on their surface for recognition by adaptive immune cells. Natural killer (NK) cells are specialized in destroying infected cells, while macrophages (MΦ) are specialized in phagocytosis of foreign material such as virus particles(78). Both of these functions can be enhanced by effector functions from adaptive immune responses.

After an infection has been cleared, a unique ability of the adaptive immune cells is to maintain immunological memory against encountered pathogens. Upon re-exposure to the pathogen, formed memory cells can respond quickly and neutralize the infection in cooperation with the innate immunity, providing the core concept of "immunity" (Figure 2).

My PhD work has mainly focused on the adaptive immune system, and particularly on B cells. I will therefore go into more details of the adaptive immune system. That being said, the innate immune responses are key to shaping initial adaptive immune responses, and I will therefore first provide a general introduction to the innate immune system, while also highlighting the important links between adaptive and innate immunity.

2.2 The innate immune system and its role in adaptive immune responses

Innate cell receptors

The essence of innate immunity is the ability to react quickly and dispatch pathogens. Key to this functionality is a set of germline-encoded receptors expressed on innate immune cells. These can be classified into different pattern-recognition receptors (PRRs), that recognize pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs). They recognize generic foreign material such as

microbial lipoproteins or microbial and viral DNA/RNA, or other markers associated with cell-damage as a result of pathogen infection (Reviewed in (79)). There are several classes of PRRs: Toll-like receptors (TLRs), NOD-like receptors (NLRs), RIG-like receptors (RLRs) and C-type lectin receptors (CLRs), where TLRs are heavily involved in viral infections (reviewed in (80,81)). Activation of TLRs can result in secretion of pro-inflammatory signals, such as interferons (IFNs), communicating the infection to the nearby cells. In turn, such signals activate surrounding immune cells, both innate and adaptive. One important result of TLR signaling is the upregulation of major histocompatibility complexes (MHC) – a class of molecules fundamental for tying the whole immune system together.

MHC, a link between innate and adaptive immunity

MHC molecules are crucial for the overall function of the immune system due to their ability to present antigens to T cells, which require MHC displayed antigens for their activation. MHC molecules come in two main classes, MHC class I (MHCI) and class II (MHCII), with slightly different structures and function. MHCI is expressed on all nucleated cells, and continuously sample fragments of the proteome produced by the cell to provide a "fingerprint" of that cell. MHCII is, on the other hand, only expressed on professional antigen presenting cells (APCs) and some epithelial cells that sample surrounding extracellular proteins. Fragments of these proteins are then subsequently displayed on MCHII.

MHCI is composed of two chains, one membrane bound polymorphic α-chain and a non-polymorphic β2-microglobulin chain (B2M) that together form the peptide-binding interface(82). During the regular protein synthesis of the cell some proteins are being degraded into peptides and transported to the ER where the empty MHCI complex is located. The transported peptide is then loaded onto the MHCI molecule, and the whole complex is transported to the cell surface (Reviewed in (83)). This provides a continuous display of proteins being produced in the cell. In the case of an infection, peptides derived from invading intracellular pathogens will similarly be loaded on MHCI and

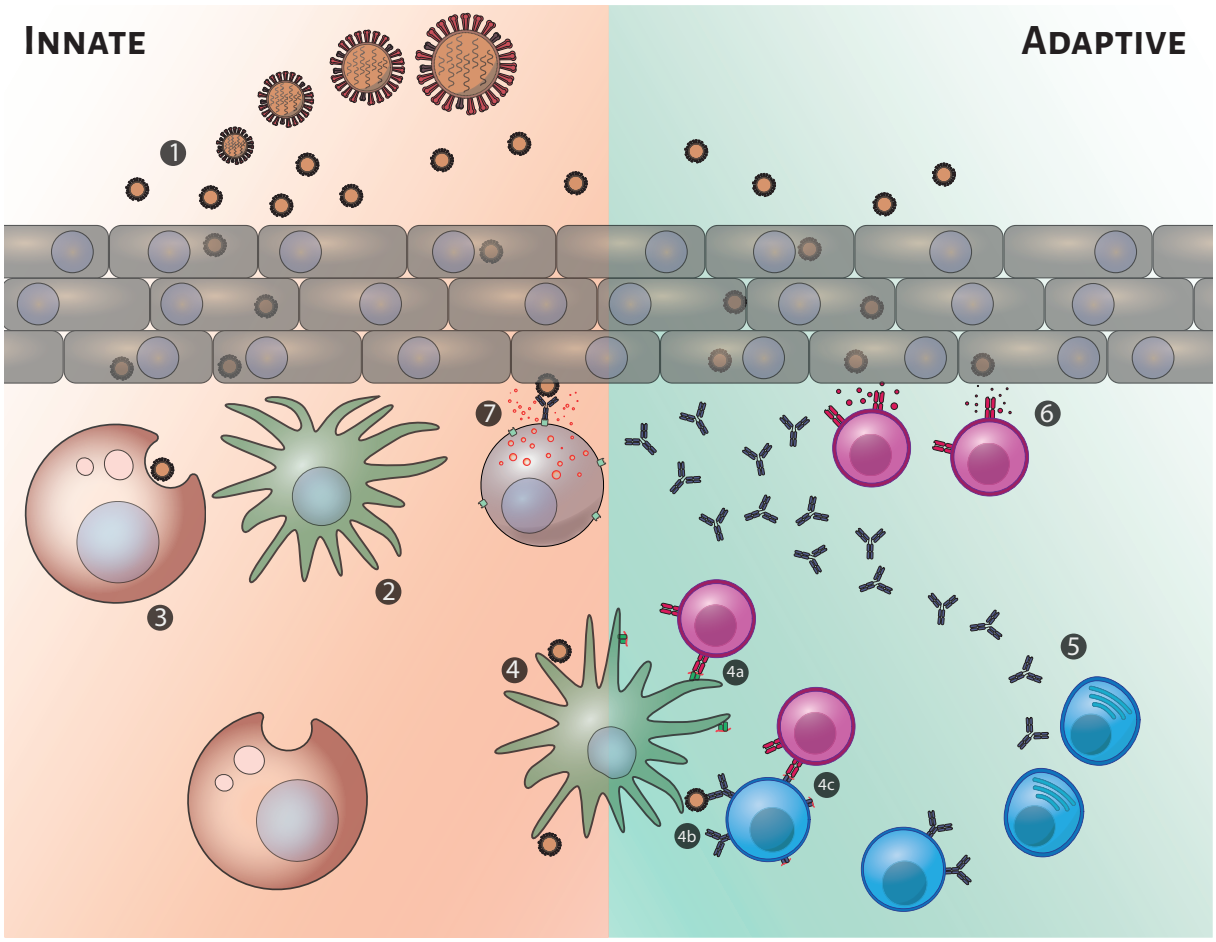

Figure 2: Innate & adaptive immune system overview

There are a number of events that takes place combat an infection. After an infection (❶), surveying cells and professional APCs such as DCs (❷) and MΦs (❸) detect and capture pathogens and pathogen material. Professional APCs then migrate to secondary lymphoid organs and present the antigens to T and B cells (❹). Here, peptide antigen fragments are portrayed on MHC molecules and presented to T cells (❹a), while whole antigens displayed on the surfaces are presented to B cell (❹b). B cells that recognize the antigen can internalize it and display peptide fragments on MHCII for CD4 T cell help (❹c). B cells that receive T cell help can undergo SHM and transition into LLPCs secreting antibodies such as IgG (❺). In addition to the B cells and CD4 T cells, CD8 T cells can detect virus-infected cells based on MHCI display and release granules that promote apoptosis of the infected cells and limit the spread (❻). Finally, the secreted IgG molecules can either bind free virus particles and mark them for clearance or bind infected cells expressing the viral proteins on the surface, marking them as infected for NKs cells to target and destroy (❼).

exported to the cell surface. These peptide-MHCI (pMHCI) complex can be recognized by other immune cells as a sign of infection, ultimately resulting in the destruction of the infected cells.

MHCII consist of two membrane bound polypeptides, an α-chain and a β-chain, together forming a heterodimer. MHCII is expressed on professional APCs like DCs, MΦs

and B cells in addition to epithelial cells. They acquire surrounding proteins through endocytosis where the proteins are degraded to smaller peptide fragments in lysosome. These peptides are then transported to antigen-processing compartments together with unbound MHCII molecules. Here, the sampled peptides will replace the structurally important invariant chain from the peptide binding cleft on MHCII(84). After peptide loading, the peptide-MHCII (pMHCII) complexes are translocated back to the surface for presentation.

Above is described what are considered the classical presentation pathways. In addition to these, there are cross-presentation pathways where extracellular-derived peptides are trafficked into the ER instead of the antigen-processing compartments. Here, they can be loaded onto MHCI instead of MHCII(85,86). Certain subsets of DCs have been shown to be more involved in antigen cross-presentation than others, one being CD8 and XCR1 expressing DCs (Reviewed in (87–89)).

This central role of MHC molecules in orchestrating both innate and adaptive responses via different types of T cells (see section 2.3) accentuates how antigen presentation is fundamental for immune responses. However, antigen peptide presentation by MHC is not the only type of antigen presentation by the innate immune system, whole protein antigens can also be presented on the surfaces of certain APCs like DCs, and this is important in the mounting of a B cell response.

B cell antigen presentation and Ab effector functions

Unlike T cells, B cells do not require the antigen to be processed and displayed in order to recognize it and become activated (see section 2.3). However, surface-bound antigens have been shown to be more effective in activating B cells, and are important for persistent germinal center reactions(90,91). Two cell types capable of presenting antigens to B cells are DCs and MΦs, and in particular follicular dendritic cells (FDCs) and subcapsular sinus (SCS) macrophages(92,93). The exact mechanisms are not always known, but a number of receptors are known to play a role, including complement

receptors CR1/CR2 (CD21/CD35) and Fc-gamma receptor IIB (FcγRIIB) (see below), which are capable of displaying immune complexes (i.e. antibody bound antigens) on the cell surface(92,94).

Antibodies (Ab, or Immunoglobulin, Ig) produced by the B cells might be seen as the hallmark of the adaptive immune response and are undoubtedly an important part in dealing with infections. The structure of antibodies will be described in more detail below.

In some cases, antibodies can neutralize infections by simply binding and blocking proteins, such as preventing cellular entry of a virus(95–98). However, an important part of their effects comes from recruiting different effector cells and molecules based on their Fc region. These effector functions stem from the innate immune system, where MΦs bind Ig coated particles and phagocytose them, while NK cells can be activated by Igs bound to antigens and release their cytotoxic payloads. Again, this relationship highlights how the interaction between the adaptive and the innate immune system is crucial.

Immunoglobulin isotypes and structures

In mammals such as humans and mice, there are four isotypes of IgG, two isotypes of IgA, and one isotype each of IgE, IgD and IgM, each with their unique Fc regions (Figure 3&4). The different Fc regions have different affinities for a range of Fc-receptors (FcRs) expressed on immune cells. For example, there are four IgG-specific FcRs (FcγRs) in mice (FcγRI, FcγRIIB, FcγRIII and FcγRIV) and six in humans (FcγRI, FcγRIIA, FcγRIIB, FcγRIIC, FcγRIIIA and FcγRIIIB)(99). These receptors can have either activating or inhibiting effects depending on their cytoplasmic domain which may associate with or contain an immunoreceptor tyrosine-based activation motif (ITAM), or an immunoreceptor tyrosine-based inhibitory motif (ITIM)(reviewed in(99–102)). The only inhibitory FcγR in both mouse and human is FcγRIIB, and it can be expressed on B cells, DCs and monocytes. While FcRIIB binding can result in inhibitory signaling

within the cell, FcRIIB can also be used to present IgG-bound antigen to surrounding cells(103–105). Remaining FcγRs all result in some type of activation signaling within the cells, but the consequence of such signaling varies for different cell types and environments.

Antibody effector functions

Antibody-dependent cellular toxicity (ADCC) is an important process initiated by an antibody binding to infected cells and the resulting attraction of cytotoxic cells. FcγRI, FcγRIIA and FcγRIIIA can all mediate ADCC, and where FcγRIIIA on NK cells is classically considered the main receptor involved(106,107). Human IgG1 and IgG3 have high affinity for FcγRI and FcγRIIIA, and are therefore the IgG subclasses traditionally associated with ADCC(108)(Figure 5). Another important effector function is antibody dependent cellular phagocytosis (ADCP, also described as antibody-dependent opsonization). Here, antibody-coated particles (for example viruses) can be engulfed and phagocytosed by innate cells such as macrophages and neutrophils(109,110). FcγRIIA/B is important in mediating ADCP and is highly expressed on monocytes and neutrophils. hIgG3 is particularly potent at mediating ADCP(111,112) (Figure 5)

Lastly, antibodies can also activate the complement system. The complement system is a large and complex network of interacting components that I will not describe in detail, but rather refer to comprehensive overviews(113–115). However, the important link between antibody effector functions and complement activation include the classical activation pathway, and this is mostly initiated by IgG1 and IgM. They can activate the C1 complex components and the subsequent pathway ensues(116).

In sum, antibodies are potent mediators of protection due to their wide range of effector functions. They can block infection, trigger the destruction of infected cells and mediate the clearing of foreign material. This central role in clearing infections is a large reason for why humans have measured antibody levels as a benchmark for protection against infectious diseases since their discovery.

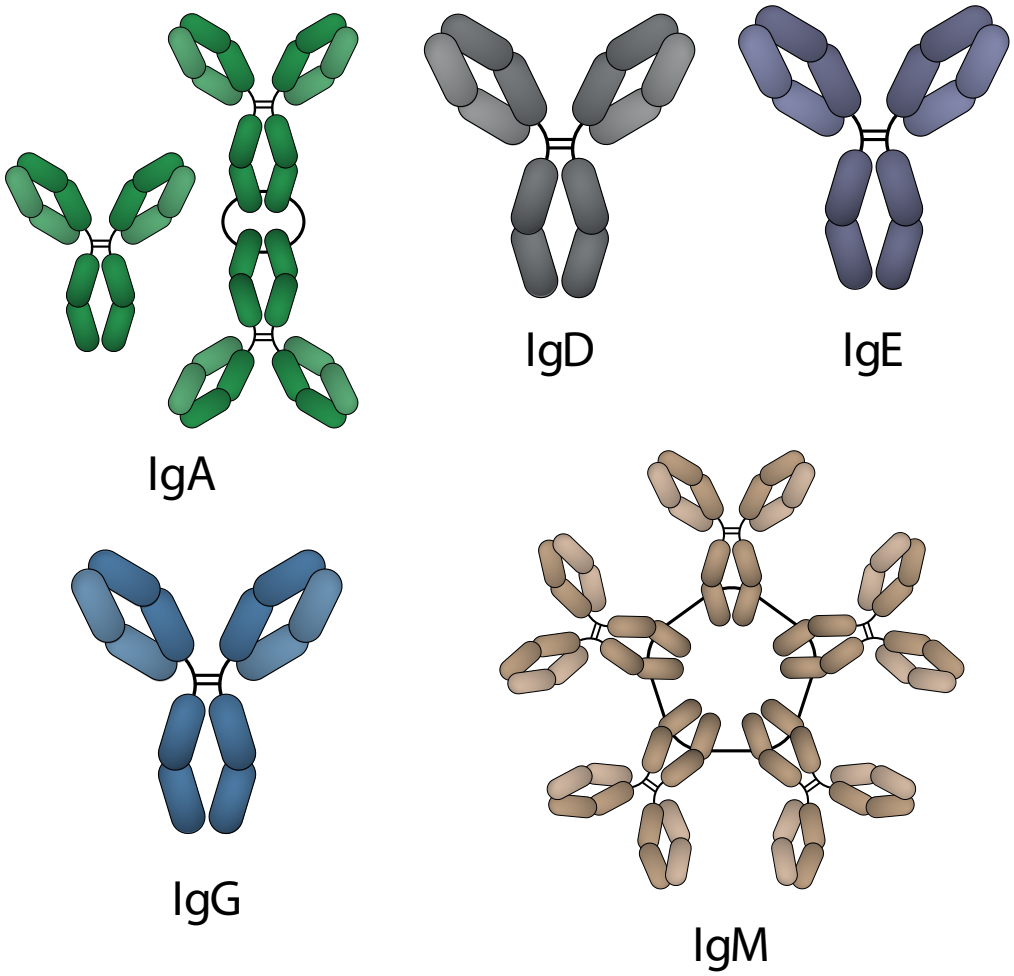

Figure 3: Immunoglobulin isotypes and structures

There are five classes of immunoglobulins in humans and mice. The five subtypes are: IgA, IgD, IgE, IgG and IgM. Different subclasses have different roles, IgD and IgM are expressed on naïve B cell and are often involved in early and extrafollicular responses, unlike IgA, IgE and IgG that requires class-switching. IgA have to forms and are traditionally associated with mucosal immune responses. IgE is often involved in parasite and helminth infections as well as allergic reactions. Human and murine IgG have four isotypes and is the first immunoglobulin isolated and is associated with a system immune response. IgG is therefore often found in serum but can be present in other sites.

2.3 The cells of the adaptive immune system

The characterizing feature of the adaptive immune system is the ability to accurately recognize a specific pathogen, and form long lasting memory responses against them. The specificity arises from clonally unique B cells receptors (BCR) and T cell receptors (TCR) that are formed first by a combinatorial assembly of genes to form highly diverse receptor binding sites, and secondly in a maturation process involving random muta-

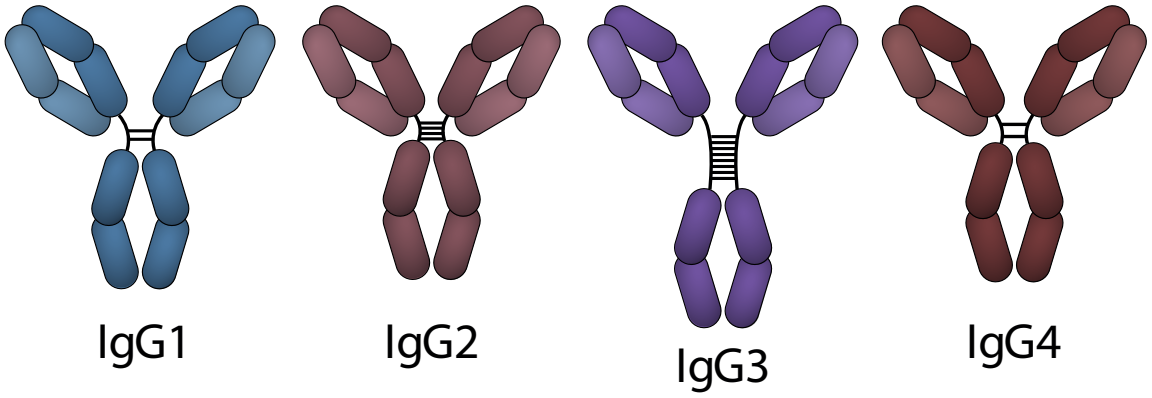

Figure 4: Immunoglobulin G isotypes and their structure

Humans have four isotypes of IgG: IgG1, IgG2, IgG3 and IgG4. They differ in their C domain structures which affects their affinities for different Fcγ-receptors and thereby their effector functions. Mice also have four isotypes, but they are named IgG1, two IgG2a/b/c depending on the strain, and IgG3, also with differing affinities for receptors.

tions of the antigen binding site. The affinity between B cells and the antigen can then be further improved during the primary response in germinal centers (GC). During a secondary response, specific memory B and T cells can rapidly become re-activated and differentiate into effector cells. B cells can also undergo the same affinity maturation process a second time, increasing the affinity for the antigen even further.

2.3.1 B cells

Protective immunity, such as that of successful vaccinations, are often measured by the induction of high antibody titers in the serum and the ability of these antibodies to neutralize the pathogen. The basis for these effector responses is B cell maturation, which is not one of the main topics for my thesis. I will therefore spend the following sections describing important steps and processes crucial for understanding adaptive immune responses that should be considered when developing new vaccines.

B cell development and BCR structure

The name “B cell” originates from the avian organ “bursa of Fabricus” where the cells were first identified, and that has no equivalent mammalian organ(117). In mammals,

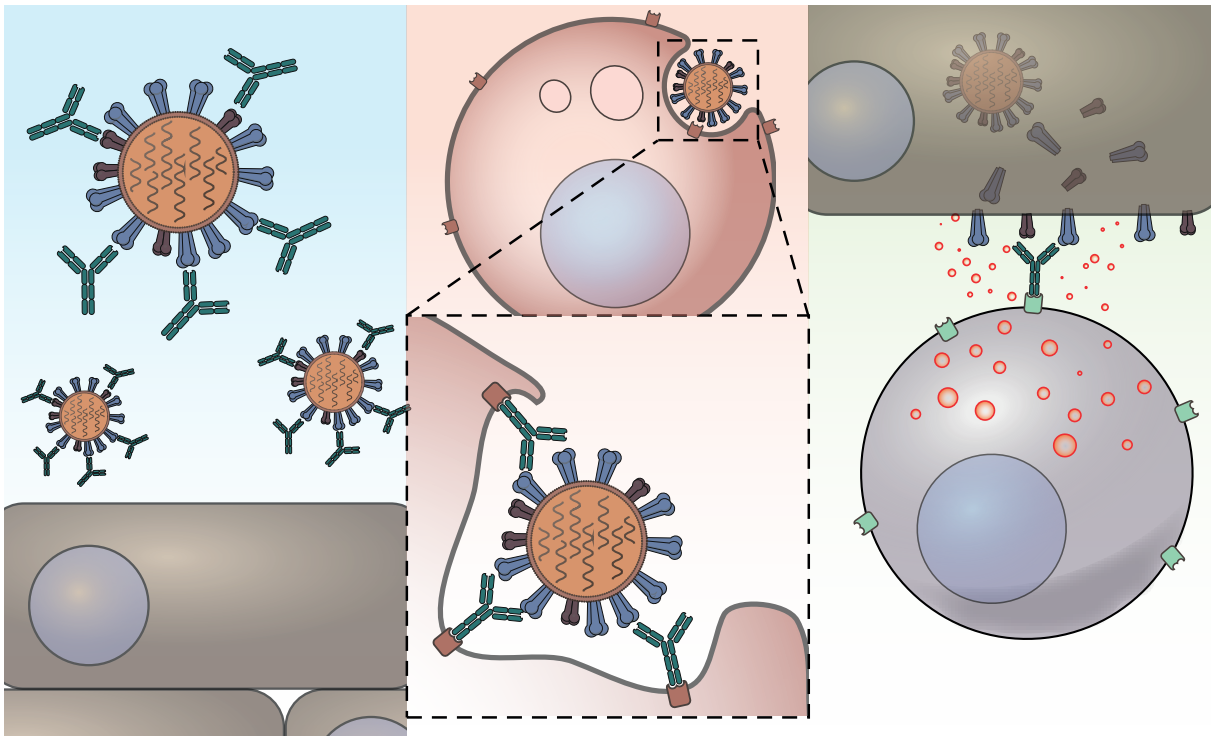

Figure 5: Antibody effector functions

Antibodies can help neutralize viruses in multiple ways. First, they can block proteins on the virus surface and thereby prevent their ability to infect the host cells. Second, the coating of virus particles can trigger the encapsulation and phagocytosis by macrophages and other cell types specialized in antibody-dependent phagocytosis (ADCP) by FcγR activation. Lastly, they can recognize viral proteins expressed on the surface of infected cells. This can trigger antibody-dependent cellular cytotoxicity (ADCC) by cell such as NK cells, once again by FcγR activation. Not illustrated here is also their role in complement activation via the classical pathway.

B cells originate in the bone marrow from hematopoietic stem cells, together with T cells. B and T cell development is a large field of study on its own, and not something I have spent time on during this thesis. I will therefore summarize key steps and then refer to the following reviews for an more comprehensive overview: (118–120). During the initial maturation in the bone marrow, the B cell develop the B cell receptor (BCR) in a stepwise fashion for each chain in a process called V(D)J recombination. After the completion of V(D)J recombination and maturation in the bone marrow, the B cells express a combination of IgD and IgM BCRs and migrate out in circulation. These mature but naïve B cells then circulate throughout the body ready for an encounter with their cognate antigen and subsequent activation.

V(D)J recombination

The BCR shares the same structure as soluble immunoglobulins with two heavy chains (HC) and two light chains (LC) forming the antigen recognition sites at the interface of their variable (V) domains. The only difference is the membrane-proximal constant (C) region in the BCR that anchors it to the B cell and these differences rise from alternative splicing. The maturation of the BCR begins with the HC. Here, one diversity (D) gene segment is selected and joined with one joining (J) gene segment, in a process that involves double-stranded DNA breaks triggered by the recombination activating gene protein complex (RAG)(121,122). RAG recognizes conserved sequences motifs named recombination signal sequences adjacent to the V, D, and J segments(123). This double stranded break is then repaired by a non-homologous end joining (NHEJ) (or alternative end joining) process (reviewed in (124)). The process is then repeated for the joining of the V-gene segment and the DJ-segment in the HC and then for the V-segment and J-segment in the LC. Importantly, this process of NHEJ introduces random base-pairs at the sites of repair. These can either lead to deletions, substitutions or introductions of new amino acids in the antigen-binding sites, contributing to the enormous diversity of the antigen-binding capabilities of BCRs.

As mentioned, a mature but naïve B cell starts with a combination of IgD and IgM expression. However, upon activation they can undergo a transition to either IgG, IgA or IgE, and the cell will start to produce the soluble form of the immunoglobulin instead. This process of transitioning to IgG, IgA or IgE is called class-switch recombination (CSR), and occurs prior to the GC reaction (see below).

B cell activation

Even though B cells in theory can bind antigens in any format and in circulation, it is mainly antigens displayed on the surface of presenting cells in secondary lymphoid organs (SLO) that lead to activation(90,91) (Reviewed in (125–127)). This activation of B cells is enhanced by the clustering of the antigens in an immunological synapse

structure between the B cell and the antigen presenting cell. Here, clustering of antigen leads to increased signaling through B cell co-receptors whose ligands are expressed on the surface of the APC(128–130). After BCR activation, the BCR-Ag complex is internalized by clathrin-coated vesicles and transported to antigen processing and MHCII loading vesicles(131). Here, as the name implies, peptides from the bound antigen is processed and loaded on MHCII for display to CD4 T cells. In this instance they are follicular helper T (TFH) cells, see section 2.3.2 s, also present in the SLOs. Display of antigen-derived peptides to cognate CD4 T cells is important in the complete activation of B cells, where stimulatory signals by the CD4 T cell leads to further activation and entry into GC structures.

Secondary lymphoid organ and germinal center organization

As antigen activation of the B cells is mainly taking place in the SLOs, antigen transport here from the sites of infection is important. The antigen can either drain there on its own if the antigen is small enough(132), or it can be captured and presented by one of the surveilling APCs such as DCs or SCS MΦs, which then migrate to the draining lymph node from the site of infection with the antigen(133). While this takes place in all SLOs, the following description will focus on the architecture of the draining lymph nodes.

The entry point of antigens to the lymph nodes is the subcapsular sinus (SCS), which lines the lymph nodes and where afferent lymph vessels drain. Inside the SCS lies the medulla, surrounding the T cell zone, which in turn harbors B cell follicles(134). The initial activation of B cells usually takes place at this interface where DCs or SCS MΦs present antigen. After antigen recognition, the B cell presents the processed and MHCII bound antigen to the T cells at the T cell-B cell border between the T cell zone and the B cell follicles. After it receives the T cell help, it can migrate into the B cell follicle and enter the germinal center substructure. The GC contain two zones, a light zone (LZ) and a dark zone (DZ). The LZ harbors follicular dendritic cells (FDCs), which continuously present antigen to GC B cells, as well as TFH cells that provide T cell stimulation, and

the dark zone contain proliferating B cells. (Figure 6). The germinal center reaction will be discussed in more detail below.

For a more comprehensive overview of lymph node and other SLO architecture, I refer to the following reviews: (127,135–138).

Germinal center reaction, somatic hypermutation and affinity maturation

After the initial activation of naïve B cells and subsequent entry into the GC, the B cells start to proliferate and undergo a process called somatic hypermutation (SHM) (139,140). SHM is a process where the enzyme Activation-Induced cytidine Deaminase (AID) removes amine groups on cytidines in the antigen recognition region of the BCR, resulting in an uracil mismatch with the corresponding guanosine(141). This triggers the DNA repair mechanisms which can incorporate new base pairs or mutate the region, resulting in new clones (Reviewed in (142,143)). These new clones then migrate to the light zone for testing of the new BCR against the antigen displayed on FDCs, and subsequent internalization and MHCII presentation to TFH cells after successful antigen recognition. After receiving TFH help, the B cells return to the dark zone to undergo a new round of proliferation, SHM and then testing in the light zone (Figure 7). This repeated process is called affinity maturation due to the overall increase in affinity that is observed between the B cells and the antigen as the GC reaction progresses(144–146). This increase in affinity is a result of a selection of higher affinity clones over clones with lower affinity and can proceed for several months(147), but many also decline after around 14 days(148). The explanation for this selection of high affinity B cell clones has been that there is a competition for binding to the antigens on the FDCs, where the high affinity BCRs outcompete the low affinity BCRs(149,150). However, some experimental results correlate better with an alternative idea to explain affinity selection, and where TFH help is limiting and only the B cells with the highest expression of cognate peptide-MHCII survives(151–155). More recently, this has been challenged further as studies have shown that it is not only B cells with the highest affinity BCRs that survive(156,157). Lower affinity MBCs can emerge from the GC, and prolonged GCs

result in BCRs with varying affinities. The field of GC dynamics and clonal selection is still developing, so for a more comprehensive overview I recommend the following reviews(146,158).

Before the B cells enter the GC and undergo the affinity maturation process, a secondary process where the activated GC B cells transition into other subclasses than IgM and IgD occurs(159). This process is called class-switch recombination (CSR), and is similarly to SHM initiated by AID(141), but this time upstream of the Cμ and Cδ, and upstream of the new C-gene segment. This facilitates the irreversible removal of the C-segments in between(124). in GCs, class-switching is dependent on a combination of BCR signaling, and stimulation of cytokines such as CD40 (provided by the TFH cells). This can be seen as CD40 knockout mice are unable to mount IgG, IgA and IgE responses against thymus dependent antigens(160) and addition of antigen increases CSR rate over LPS-only stimulated B cells in vitro(161).

Germinal center cell fate and exit

During the SHM cycle, GC B cells can instead of reentering the DZ exit the GC as either MBCs or antibody secreting plasma blasts (PB) and ultimately long-lived plasma cells (LLPC). The differentiation of B cells into LLPCs and MBCs is important as the infected host will need enough ASCs to supply antibodies to combat the infection, but also enough MBCs for protection against future infections. The affinity between BCR and antigen has been identified as important for this selection, and where high affinity B cells preferentially transition into LLPCs(162–164). However, more recent studies have explored this more mechanistically and challenged this dogma. Today we believe that deciding factors also include antigen availability(165) and GC timeline, with memory B cells being generated earlier in the GC reaction than PCs(166,167). There is also evidence that subclass development and differentiation fate are linked(168). As an example, IgE BCR expression appear to promote PC differentiation over MBC(169,170).

Genetic control over B cell fate is largely done by the transcription factor B lympho-

cyte-induced maturation protein-1 (BLIMP-1, Prdm1), which represses B-cell lymphoma 6 (BCL6) – a transcription factor for GC entry – and interferon regulatory factor 4 (IRF4), which lead to PC formation(171–175). LLPCs then migrate to the bone marrow where they reside in special niches and provide a supply of Abs(176) crucial for clearing the agents triggering the immune response(177). During this transition into LLPCs, the convention has been that the PCs downregulate the expression of membrane bound surface immunoglobulins in favor for alternatively spliced secreted immunoglobulins. However, this has been shown to not be true in all instances. For example, IgA expressing PCs in the gut retain surface IgA(178,179), and both IgE and IgM expressing PCs respond to antigen stimulation(180,181).

Memory B cells and secondary infections

Memory B cell formation in GCs could be considered one of the most important steps in the immune response, as this defines the continued protection against a similar infection. Unlike most LLPCs, MBCs can circulate in the body and await a new encounter with the same (or similar) antigen leading to reactivation of the MBCs. However, MBCs also migrate to the bone marrow, although their exact role there is not fully known(182). Our current understanding of MBCs role in the immune system is that they can be rapidly reactivated upon re-exposure to the same antigen. However, their role in secondary infections is less straight-forward than initially thought. The question of whether MBCs are capable of re-entering GCs or not is covered in the following series of reviews: (183–185). In brief, a major role of MBCs is to rapidly differentiate into early PBs upon reactivation without entering GC reactions. This would generate PBs with higher affinities than PBs from naïve B cells as they have been "pre-selected" during the first exposure. One determining factor for rapid PB differentiation over GC entry appears to be the affinity between the BCR and the antigen(186), but there have also been indications that expression of markers CD73, CD80 and CD273 (=PD-L2), or MBCs class-switching to IgG increases the likelihood of PB differentiation(187–189). Correspondingly, IgM-positive MBC may preferentially seed secondary GCs(188).

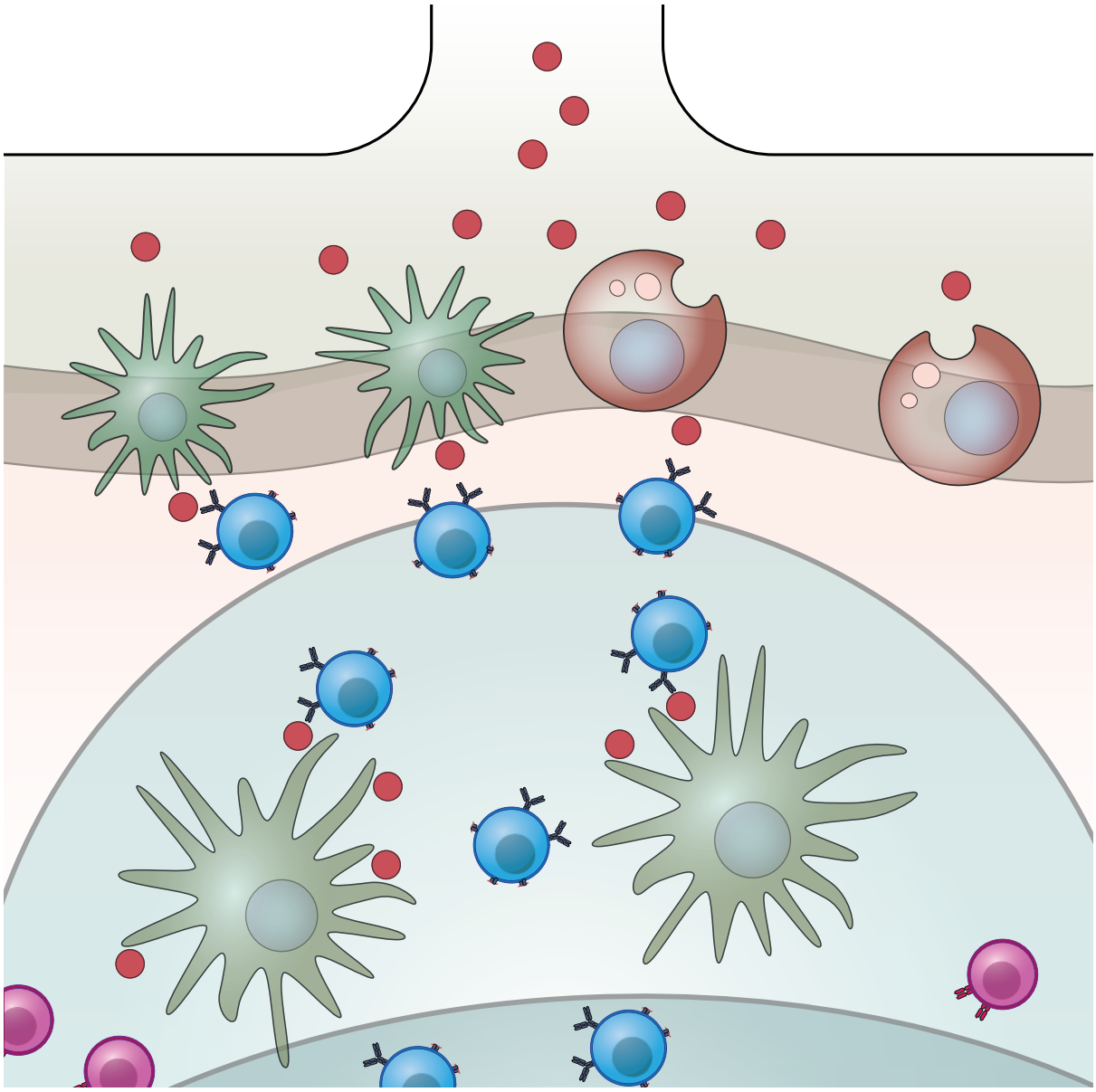

Figure 6: Antigen transport to lymph nodes and germinal centers

Antigen can either drain to the lymph nodes on own or be transported by APCs such as DCs and SCS MΦs through afferent lymph vessels. Here, they are transported to the B cells via the surrounding SCS and medulla. B cells then enter the B cell follicles with the antigen where it can be transferred to FDCs. After being activated and receiving T cell help, the B cells can enter the germinal center and undergo SHM, affinity maturation and CSR.

GC-independent B cell activation

While the above description has focused on GC reactions in lymph nodes and its role in B cell activation, B cells can also become activated and differentiated to ASCs without receiving T cell help. Such extrafollicular responses are often seen with multivalent BCR cross-linking by highly repetitive antigens, such as surface antigens on virus

particles and bacterial flagella, or requires co-stimulation by TLR signaling(190–192). This process is GC-independent, so does not lead to SHM and affinity maturation. The short-lived PBs that are formed may, however, be important for the early response and protection(193).

Preexisting immunity and original antigenic sin

In some instances, B cell responses may appear to be focused on pre-established specificities. This was observed by Dr. Thomas Francis Jr. during his early work with influenza virus and influenza vaccines. He noticed that patients had high serum titers against previously circulating influenza strains, and he saw how serum antibodies towards the first exposure of influenza seemed continuously maintained and boosted to higher levels than subsequent exposures(194,195). Dr. Francis Jr. summarized this in a concept called "Original Antigenic Sin" (OAS). This sparked a discussion of how immunological (B cell) memory functions, and how this can affect vaccine responses against antigenically drifted viruses. Dr. Francis wrote that the presence of these antibodies does not appear to diminish the capability to generate an immune response after a new vaccination with a seasonal influenza vaccine, but it can have an effect on how well someone deals with an infection after that(196). The theory behind this have been that B cells from the initial exposure are continuously being reactivated by the antigenically drifted viruses, and the reactivity towards that initial strain is therefore maintained at a high level(197,198). That this is something that occurs is well documented(199,200), but the exact mechanism behind it has not been well described.

Immune Imprinting

Another alternative theory that have emerged is termed "immune imprinting" or "antigenic seniority"(201,202). Where OAS is stricter in its description of how the very first infection is defining all subsequent responses, immune imprinting is more generous and describes how the whole immune history plays a role in imprinting subsequent immune responses. Here, the early infections are important but not defining. It has been shown

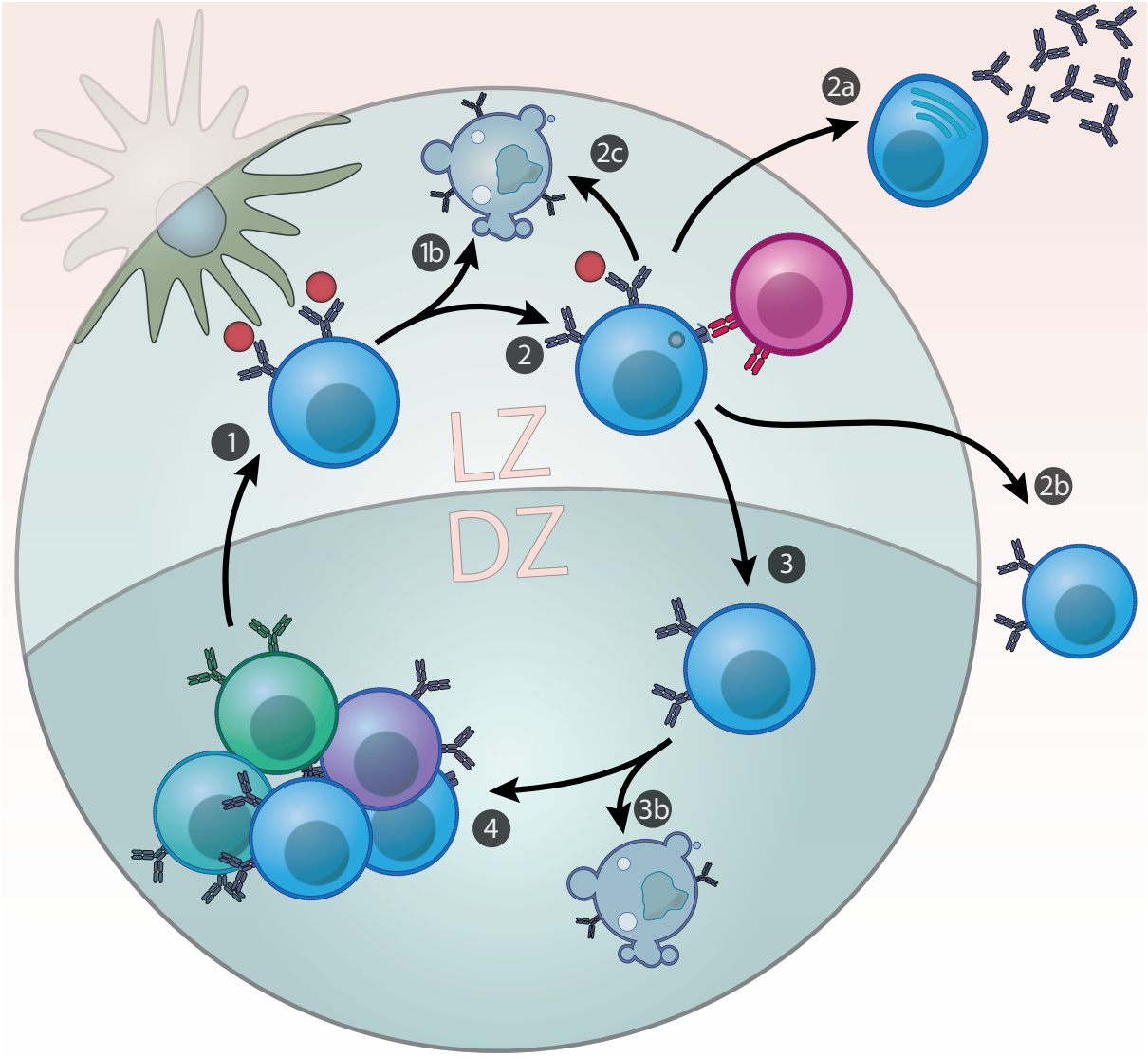

Figure 7: Germinal center reaction overview

The germinal center reaction facilitates increased affinity for the antigen by repeated cycles of proliferation and testing against the antigen. Starting with the entry into the LZ, B cells test their BCR against antigens displayed on FDCs (❶). There are then two fates, if the B cell fails to recognize the antigen it undergoes apoptosis from a lack of survival signals (❶b), or it can internalize the antigen after successful recognition. Internalization leads to MHCII display for TFH cells (❷) where lack of pro-survival signal again leads to apoptosis (❷b). After successful TFH help, the B cells can either exit the GC as antibody secreting plasma cells (❷b) or circulating memory B cells awaiting a new exposure (❷b). Alternatively, the B cells can receive signals promoting entry to the DZ and undergo SHM (❸). Here, if the SHM results in a non-functioning BCR gene, they once again undergo apoptosis (❸b). However, productive SHM lead to further proliferation before they enter the LZ again and repeat the cycle (❹).

that vaccinations and live virus infections have different effects on immune imprinting, where viral infection lead to long-term imprinting while vaccinations do not(203,204).

OAS and immune imprinting are discussed more in depth in conjunction with our findings in the General Discussion below.

Studying B cells

Our ability to study B cells have increased steadily with the continuous advancements of biotechnology. Hybridoma technologies allowed for studying the clonality of B cells(205). Methods such as flow cytometry allowed for more detailed phenotypic analysis than previous immune cytochemistry and histochemistry. Sequencing technologies advanced from sanger sequencing to high-throughput sequencing (HTS, also known as next generation sequencing, NGS) which allowed for sequencing of wide repertories of cells(206). The first studies utilizing HTS of immune cell repertoires were published in 2009 and showed the potential HTS unlocks(207–210). However, these initial techniques could not provide heavy-light chain pair information that is crucial for understanding B cell (and T cell) specificities. The development of single cell RNA sequencing technologies such as droplet emulsion RT-PCR allowed for paired chain information in 2013(211). Since then, single cell RNA sequencing has been used for studying the everything from B cell responses from influenza infections(212) to autoreactive B cells in autoimmune diseases(213) to tumor infiltrating B cells in breast cancer(214).

2.3.2 T cells

T cells originate from the same hematopoietic precursor as B cells, but they leave the bone marrow and migrate to the thymus where they complete their maturation, hence the "T" in T cells is from "Thymus/thymocyte". The defining feature of T cells is their T cell receptor (TCR), a dimeric membrane bound glycoprotein consisting of one α-chain and one β-chain. The TCR is similar in structure to the BCR, where the α-chain is analogous to the light chain of BCRs and the β-chain is analogous to the heavy chain of the BCR in that they contain a variable (V) domain, a (D)J-segment and a constant (C) domain. Similar to BCR, the large diversity in different specificities is generated by

V(D)J recombination(215). However, while the BCR recognize a wide range of antigens, the TCRs only recognize peptides bound to MHC molecules. The class of MHC they recognize define the T cell, where CD8 expressing T cells recognize MHCI bound peptides and CD4 expressing T cells recognize MHCII bound peptides. In CD8 T cells, the CD8 co-receptor is clustered with the TCR on the cell surface and binds the side of the MHC class I. This co-receptor binding stabilizes the TCR-MHC complex and is required for proper cell signaling by aiding in recruitment of signaling kinases(216). The corresponding co-receptor for MHC class II is CD4.

T cells are important for clearing infections in multiple ways, and are therefore important for vaccine evaluation and design. For this thesis, their role in orchestrating immune responses will be more relevant, but their ability to limit infection as effector cells is also relevant to some extent. The T cells' ability to recognize peptide from processed antigens allow them to recognize internal proteins otherwise unavailable to the immune system. In viruses, these intracellular regions are often more conserved between strains due to less evolutionary pressure to mutate. T cells are therefore uniquely qualified for providing a broad response towards multiple strains that otherwise might escape B cell immunity(217,218). The recent SARS-CoV-2 pandemic demonstrated this nicely, with the T-cell responses raised by the mRNA vaccines mediating protection against severe disease even after substantial antigenic drift(219) (see section 3.1).

Below, I will describe the conventional αβ-T cells in the context of immune formation.

CD8 T cells

Cytotoxic T-cells express CD8, and can limit pathogen replication by instructing infected host cells to self-destruct. This is important for protection against intracellular pathogens such as viruses. If a cell is infected by a virus, and begin producing viral proteins, peptides from these viral proteins will be presented on MHCI, as described in section 2.2. Since the presentation of these peptides will be a sign of infection, these foreign peptides can be recognized by CD8 T cells and trigger TCR-signaling. As a

result of this signaling cascade, effector T cells can release pro-apoptotic granules containing perforins and granzymes to induce apoptosis of the infected cell, as well as direct cell-to-cell contact and Fas-mediated cell death. As an additional function, CD8 T cell activation results in release of cytokines such as TNFα and IFNγ to signal the infection to surrounding cells(220,221).

CD4 T cells

CD4 T cells are (mostly(222)) not cytotoxic, but rather recognizes MHCII-presented peptides on APCs (see section 2.2). Upon activation, they can secrete stimulatory cytokines to surrounding immune cells, both innate and adaptive, and are therefore referred to as "helper" T cells (Th cells)(223,224) This "helper" role makes CD4 T cells central in orchestrating essentially the entire immune response. This conducting of the immune response is to a large extent done by different secretory cytokine profiles that define different CD4 T cell subsets. The first two subsets identified were Th1 and Th2 cells, characterized by the expression of IFNγ and IL-4, respectively(225,226). Since then, there have been more subsets identified and the borders between subsets blurred(227), but Th1, Th2 and regulatory T cells (Treg) are still commonly used to classify T cell responses after an infection or vaccination. Here, Th1 responses are traditionally associated with a cellular immune response meant to target intracellular pathogens, whereas Th2 responses traditionally are associated with humoral responses meant to target extracellular pathogens. Treg cells are important for both limiting autoreactive immune responses and to return the system to homeostasis(228).

As described above, Th cell stimulation is crucial for B cells entering the GC reaction. This stimulation is provided by a different subset of Th cells, T follicular helper cells. TFH cells are characterized by expression of BCL6 and CXCR5(229). During the GC reaction in the LZ, B cells that have internalized the antigen and are displaying peptides on their MHCII molecules are subjected to receive pro-survival signals from the TFH cells such as CD40L and ICOSL, promoting B cell proliferation(230). Failure to receive

this pro-survival signaling results in apoptosis of non-productive GC B cells. T cell help has been suggested as one of the major limiting factors for GC progression(231).

3. Vaccines, vaccination and other treatments

3.1 Influenza vaccines and their conception

After the initial isolation of influenza virus in 1933 by W. Smith, C. H. Andrews and P. P. Laidlaw(5), a series of advancements were made in the propagation and isolation of the virus in the late 1930's and early 1940's(232). This ended up with the first influenza vaccine being developed on behalf of the US military during WWII, by an effort led by Dr Thomas Francis Jr.(233). Francis is therefore often seen as the creator of the standardized influenza vaccine together with Dr Jonas E. Salk, who was working as a medical intern with Francis before he then joined him in the influenza vaccine efforts(234). This vaccine was an equal mix of formaldehyde-inactivated influenza A and B viruses concentrated from chicken egg allantoic fluid(233).

Since the licensing of Francis's influenza vaccine to the general population in 1945, the seasonal influenza vaccine has remained mostly the same. The inactivating agent has changed from formaldehyde to beta propiolactone, and the virus composition has been updated to include four virus strains: two IAV strains, one H1N1 and one H3N2, and two IBV strains, one Yamagata lineage and one Victoria lineage(235). The trivalent inactivated influenza vaccines (TIIVs) based on egg-production that have dominated the market for the past decades are now being replaced with similar quadrivalent inactivated influenza vaccines (QIIVs), but alternatives have also emerged. In 2003, FluMist was approved by the FDA for clinical use(236). This is a live attenuated influenza vaccine (LAIV) given as a nasal spray, containing influenza virus that has been adapted to grow at cold temperatures(237). Ten years later, in 2013, the first recombinant protein vaccine against influenza was approved, Flublok(238). This marked a breach with the more traditional influenza vaccines based on virus growth in either eggs or cell-cultures, as Flublok only contained HA proteins that were recombinantly produced and purified from cell cultures. However, due to different immunogenicity of protein subunit vac-

cines and QIIVs/LAIVs, protein subunit vaccines often require adjuvants for increased immunogenicity. Flublok therefore contain squalene as an adjuvant(239).

mRNA/DNA vaccines

Another type of vaccines that has recently received approval for use in humans are vaccines based on genetic material like DNA and mRNA. Unlike previous vaccines, mRNA/DNA vaccines contain genetic material encoding selected antigen(s). Upon vaccination, transfected host cells will transcribe and/or translate vaccine DNA/mRNA into protein antigens that are secreted for recognition by the immune system (Reviewed in (240–242)). This technology was brought into the spotlight as the first two vaccines approved against SARS-CoV-2 were mRNA vaccines(69,70). Later, the pandemic also produced the first approved DNA vaccine for human use(243).

The delivery of genetic vaccines requires a bit more thought than conventional vaccines. For DNA vaccines in humans, plasmid DNA is often injected by jet injectors that force the DNA into the cells of the skin by pressurized air(244). Another way to deliver genetic vaccines is leveraging technologies for transfecting cells in a laboratory setting: by either microencapsulation in lipid nanoparticles that merge with the cell membrane(69,70), or encapsulation in viral vectors that insert genetic material into host cells(245).

There are a number of pros and cons with genetic vaccines as compared to the conventional virus-based vaccines. One disadvantage is a more complex cold-chain storage, with mRNA often requiring ultralow storage temperatures of lower than -20°C compared to the fridge temperatures (2-8°C) required for conventional vaccines(246). However, DNA vaccines are in general more stable than mRNA vaccines, often being stable at 2-8°C, thereby mitigating the cold-chain issue(247,248). Genetic vaccines share most of the same advantages of recombinant protein subunit vaccines, such as the ability to specifically select relevant antigens, and the possibility of engineering the antigen structure. This can, for example, increase antigen stability(249–252), or fuse

antigen to extra domains steering the vaccine to antigen presenting cells for increased immunogenicity(253–256).

APC-targeted DNA vaccines against influenza

Previously, DNA vaccines targeting antigen to selected receptors on APC have been developed here at the University of Oslo. This began with vaccines aiming to raise responses against tumor idiotypes(255), and have since been developed also for infectious diseases such as influenza(256). Key to the vaccine structure is a targeting unit that selectively will steer produced vaccine proteins to APC for increased antibody and T-cell responses(256–258). Importantly, depending on the particular receptor chosen as a target for vaccine steering, responses can be polarized to different types(259,260).

Further, the vaccine structure promotes dimerization of two vaccine monomers to allow for bivalent antigen display, which has been shown to be important for efficient immune formation(261).

Correlates of protection and desired immune responses

Historically, successful vaccine responses have been based on measuring serum antibody titers. The gold-standard correlate of protection against influenza have been a hemagglutination inhibition (HAI) titer of 40 or more(262). This historical focus on serum immunoglobulins, and by extension B cells responses, is still seen in the vaccine authorization process, where neutralizing antibody responses are required and T cell responses are not mentioned(263). Fortunately, this view of desired vaccine responses is gradually changing. Neutralizing antibodies are important, but the protective potential afforded by non-neutralizing antibodies and T cell responses should not be overlooked, particularly for protection against variable viruses(264–266).

3.2 Therapeutic treatments

Antiviral drugs

One major disadvantage with vaccines against emerging pandemic viruses is the fact that they require time to develop, and the development is dependent on knowledge of the particular virus emerging. This causes an intrinsic delay in the time from discovery to a vaccine being available. This delay is a vulnerability in our current ability to combat new pandemics. While the advent of the SARS-CoV-2 pandemic saw the fastest vaccine development time ever, the development time still took around 12 months before emergency use approvals were issued. During this year, close to two million lives were lost(267). This gap from virus emergence and to vaccine deployment could be filled with therapeutic treatments such as anti-viral drugs.

Mechanisms of current antiviral drugs

In most cases, development of new antiviral drugs has as a prerequisite that the virus is from a particular family, or shares familiarity with viruses of known replication mechanisms.

There are multiple steps in the viral replication cycle that could be interrupted by antiviral drugs. This includes cellular entry, translation/transcription of viral genetic material, virion assembly and release. For example, neuraminidase inhibitors (NAIs) such as oseltamivir prevent proper influenza virion release(268), while CCR5 antagonists and reverse transcriptase inhibitors in anti-retroviral therapies (ARTs) against human immunodeficiency virus (HIV) prevent infection and insertion of genetic material, respectively(269,270). Prior to the emergence of SARS-CoV-2, we had experience from both SARS-CoV-1 and MERS-CoV, and viral RNA-dependent RNA polymerase inhibitors (Such as Remdesivir = GS-5734) had previously been shown to be effective against these earlier coronaviruses in vitro(76,271). They were therefore a promising early candidate against SARS-CoV-2(272). Despite this early promise, Remdesivir was

later found to barely have an effect in preventing disease progression and no effect if the patient was already ventilated(273).

There is also an additional risk with antiviral drugs, mutations in the target viral enzyme or receptor can render antiviral therapies obsolete. For example, NAI resistant escape mutants of influenza have been isolated from patients since the early 2000's(274,275). This has revealed the need of other types of antiviral therapies.

Broad spectrum antiviral drugs

Broad-spectrum family-independent viral inhibitors would allow for rapid treatment after a newly emergent pandemic. Many current broadly active viral inhibitors are nucleoside analogues that inhibit viral polymerases. They have been developed against multiple classes of viruses, including (HIV), herpes simplex virus (HSV), hepatitis B virus (HBV) and influenza virus, in addition to the aforementioned Remdesivir(76) (reviewed in (276)).

Another approach is targeting host cell mechanisms required for viral replication (reviewed in (277)). Here, the rationale is that by targeting common host cell pathways you do not need to know the exact mechanisms of the viral replication beforehand, in addition to circumventing the possibility for the virus to mutate the binding site of the anti-viral compound. As an example, picolinic acid was recently shown to be effective against an array of enveloped viruses by interfering with membrane fusion(278).

Lymphocyte tyrosine kinase as antiviral target

In the second paper in this thesis, we explored host-targeted antiviral drugs that would work against multiple virus families. We considered lymphocyte tyrosine kinase (LTK) as interesting due to recent discoveries of its role in protein transport and association with Golgi and ER(279,280). Interestingly, LTK is required for coat protein complex II (COPII)-dependent trafficking from the ER to the Golgi. Both influenza and coronaviruses appear to be reliant of transport through ER exit sites (ERES)(281,282), sug-

gesting that interfering with ER function upstream could prevent proper viral transport. LTK is a receptor tyrosine kinase (RTK) with familiarity with other oncogenic RTKs like anaplastic lymphoma kinase (ALK)(280). Therefore, inhibitors of LTK are already available. Ceritinib(283), crizotinib(284), entrectinib(285), ensartinib(286), brigatinib(287), lorlatinib(288), and alectinib(289) all display some inhibitory activity against LTK. Some of these inhibitors showed early promises, warranting progression into animal models where they also had an effect.

To sum up, broadly acting antivirals are crucial for improving the pandemic response. Host-directed antivirals are one encouraging avenue to achieve this.

Aims of the study

With over 750 million cases of COVID-19 and close to 7 million deaths as a result of it (as of August 2023(260)), this emphasizes how global pandemics are one of the greatest threats to human health. Our current tools for overcoming a pandemic are based on limiting the spread by social measures and developing vaccines as rapidly as possible. However, we can better prepare for future pandemics by developing new and improved vaccines and therapeutics. Improved therapeutics include effective and broadly acting drugs that can be used against a wide range of viruses immediately after an outbreak. A next generation of vaccines could be designed to induce specific responses, thereby minimizing non-productive responses, and improving overall effectiveness. However, in order to design these improved vaccines, more knowledge of how the immune system respond to certain antigens is needed. This includes repeated exposures of similar antigens such as those of seasonal epidemics of influenza and common colds.

The aims of the thesis presented here are thus:

Specific Aims

- Design and evaluate an APC-targeted influenza vaccine with native trimeric HA structure.
- Evaluate the necessities of including trimer-stabilizing domains in vaccines.
- Exploring the possibility of repurposing available cancer therapeutics as broad-acting host-directed antivirals in vitro and in vivo.
- Study the effect and immune imprinting of multiple influenza infections on the B cell repertoire in wild-type mice.
- Investigate the role of specific BCR sequences obtained from secondary GC reactions in differently imprinted mice and how they interact with HA as antigen.

Summary of Research

I: Trimeric, APC-Targeted Subunit Vaccines Protect Mice against Seasonal and Pandemic Influenza

Elias Tjärnhage, Diamond Brown, Bjarne Bogen, Tor Kristian Andersen, and Gunnveig Grødeland.

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In this paper, we present a new APC-targeted influenza vaccine that utilizes trimeric HA as antigen. We wanted to explore whether the trimerization of HA, mimicking the natural HA structure on the virus surface, would improve the vaccine response. Known HA antibody epitopes are located both within the HA monomers and at their interphases, so the presence of a trimerized antigen should facilitate formation of more diverse antibody responses as compared to HA monomers. Thus, we linked selected APC-targeting domains to HA, with or without a trimerization domain. Interestingly, it seemed that HA in this format trimerized equally well both with the trimerization domain and in its absence. Following DNA vaccination of mice, we observed that improved immune responses and protection against disease was dependent on steering of the trimerized antigen to APC.

After validating the trimerized DNA vaccine format for homologous protection against influenza A/PR/8/1934 (H1N1), we mixed HAs from H1, H5 and H9 influenza viruses into the same vaccine bolus. The trivalent mixture gave good responses against all subtypes included in the mix, and also protected against a lethal challenge with RG14 [reassortant PR8 with HA from A/Viet Nam/04/2005 (H5N1)]. This showed that the vaccine format is suitable as both a seasonal vaccine, where known circulating strains can be included in a multivalent mixture, and as a pandemic vaccine, where protection against HPAIs is accomplished.

Lastly, we also converted the vaccine into a protein vaccine. Protein vaccines can be

advantageous over DNA vaccines in some instances. For one, they have historically been easier to get approval for, especially in the western market. The composition of different antigens can also be easier to control in protein vaccines, as different antigen translation rates can skew the ratio in mixed DNA vaccines. This is important for seasonal influenza vaccines with more than one subtype included.

We then wanted to benchmark our protein vaccine with the only approved recombinant influenza protein vaccine, Flublok. Here, our protein vaccine was either comparable or surpassed Flublok on measures such as elevated serum IgG, equal cellular responses and complete protection against a viral challenge. This reinforces the notion that APC-targeting improves the vaccine efficacy over HA alone.

II: Repurposing Lymphocyte Tyrosine Kinase Inhibitors for Therapeutic Treatment of Influenza and SARS-CoV-2 in Mice

Elias Tjärnhage, Thea Kristin Våtsveen, Valgerdur Björnsdottir, David Nemazee, ..., Ludvig Munthe, and Gunnveig Grødeland.

Submitted

In this paper we explored drug repurposing of ALK inhibitors for use as broadly acting antivirals against influenza and SARS-CoV-2. Multiple clinical ALK inhibitors have demonstrated ability to inhibit the related kinase LTK, involved in ER and Golgi trafficking. Therefore, we wanted to test whether they could interfere with viral protein trafficking in infected cells and act as host-directed antivirals. Based on published gene expression data, we could see how LTK was expressed to higher degrees than ALK in both lung and gastrointestinal tissue, both common sites for viral infection. This supported the idea of repurposing ALK inhibitors as LTK inhibitors against viruses further.

Selected LTK inhibitors were first evaluated in vitro against SARS-CoV-2 and different seasonal and potentially pandemic influenza strains. Here, the majority of LTK inhibitors had an effect against all viruses tested, but three candidates stood out: ceritinib, crizotinib and entrectinib. This led us to move forward with these for in vivo protection studies in mice. Here, the inhibitors were tested against challenges with influenza H1N1 (PR8 and CA07) and SARS-CoV-2. Despite observations of incomplete protection, which means that drugs need further development, ceritinib and crizotinib stood out from the rest in regards to protection against SARS-CoV-2 and H1N1 influenza viruses, respectively.

These results are a promising start for the development of universal acting antivirals targeting the host protein transport pathways. Both the dose of drug and delivery method needs to be further optimized. Currently these were given orally, but delivery to the

upper airways might be more relevant. Delivery to the airways might also lower the dose required to achieve the effect.

Universal antiviral therapies would have been invaluable during the outbreak of SARS-CoV-2 where they could have been used to reduce the mortality while the vaccines were being made. Having access to broad antivirals should therefore be highly prioritized as a strategy in preparation for the next potential pandemic emergence.

III: Influenza H1N1 Strains Differ with Respect to their Potential for Dominating Immune Formation during Secondary Viral Exposures

Elias Tjärnhage, Khang Le Quy, Taissa de Matos Kasahara, Victor Greiff, and Gunnveig Grødeland.

Manuscript

In this paper, we investigated the mechanisms shaping antibody responses following repeated exposures to variable viruses. More specifically, we evaluated how the initial exposure can affect antibodies and B-cell responses following a secondary influenza exposure. In brief, mice were primed with a sublethal dose of influenza H1N1, either PR8 or CA07, and then given a secondary homologous or heterologous infection 6 weeks later. An evaluation of the induced serum antibodies demonstrated that priming with CA07 would more potently dominate secondary antibody formation as compared to priming with PR8. Further, we observed an increase of PD-L2posCD80pos HA-specific memory B-cells and a corresponding decrease in the PD-L2negCD80neg memory B-cells following a first exposure to CA07 as compared to PR8. This suggested a difference in the viruses' ability to induce memory B-cells that bear a particular potential for differentiation into antibody secreting cells or re-enter GC.

Lymph nodes were harvested two weeks after a secondary influenza infection, HA specific B cells were isolated, and single-cell RNA sequencing of their BCRs were performed. Here, we saw a broad repertoire of HA binding CDR sequences, in line with the multiple of B-cell epitopes available on HA. Based on the number of mutations and degree of clonal expansion, we then selected candidate sequences for expression as the corresponding antibody proteins. Importantly, the observed that in vitro binding profiles corresponded to the sequence annotations. Further, all the selected PR8 specific sequences could neutralize PR8 virus in vitro, albeit at different concentrations, and all but one of the CA07 specific sequences could neutralize the CA07 virus. The selected

cross-reactive sequence could not neutralize either, reinforcing the notion that it recognized a conserved, non-neutralizing epitope.

Method and experimental discussions

Vaccine design

Antigens begun being coupled to targeting moieties such as Abs in the 1980's and this targeting towards immune cells and APCs led to increased immunogenicity(246,283). During the 1990's, the targeting moieties were expanded from Ab-based domains to chemokines as well(247).

Previously, APC-targeted DNA vaccines have been demonstrated to increase efficacy in mice(248), ferrets, pigs, and rhesus macaques(284,285). These APC-targeted vaccines contained a dimerization unit that promoted bivalent display of antigens and targeting units, as such forming an X-shaped protein(249). The implication of this X-shape is two monomeric antigens being presented in the vaccine. However, HA is displayed on influenza virions as a trimeric protein, and there are known B-cell epitopes located at the interphase between monomers in this structure(286,287). Thus, we here designed novel APC-targeted vaccines aiming for trivalent HA display (paper I). First, we made constructs where antigen was linked directly to an APC-specific targeting moiety via a short linker sequence. To some of the vaccines we also added a small trimerization domain(242). Interestingly, we did not see an improvement in vaccine protein secretion with the trimerization domain present, but rather the opposite, significantly more vaccine protein was produced when the trimerization domain was omitted. In vivo immune responses from the two vaccines formants were for the most part identical. There were no significant differences in either serum IgG titers or cellular responses. This also reiterated by equal protection against viral challenges (paper I).

DNA and protein vaccination of mice

The trimeric HA vaccines in paper I were mostly tested as DNA vaccines. The reason was that DNA allows for easy construction and up-scaling, enabling rapid evaluation

in pre-clinical mouse models. However, simple injection of DNA delivery does not necessarily lead to uptake and production of the corresponding proteins. For the DNA vaccines to be efficiently processed, we electroporated the injection site to facilitate DNA uptake by very briefly opening up pores in the cells surrounding the electrodes, allowing the DNA to enter the cells(288). Other approaches that could have been used include injecting the DNA with a jet of pressurized air(237), or encapsulation in lipid nanoparticles(289).

In addition to DNA, we also converted the vaccines to protein-based vaccines for benchmarking vs established vaccines. This was done to further test the whether the increase efficacy of APC-targeting translates into clinical relevance. The comparison included Flublok, another recombinant protein based influenza vaccine(231), and Pandemrix, an inactivated virus vaccine designed for pandemic use(290). This benchmarking highlighted the need for adjuvants with recombinant protein vaccines, something not required with DNA vaccines. In addition to improving the immune response, the inclusion of adjuvants can have other benefits by modulating the immune response in specific directions further(291). However, adjuvants are often the cause of side effects such as pain and fever many experience during vaccinations(292), so omitting them could lead to better vaccination experiences.

Viral challenges

The best way to test the efficacy of a vaccine or antiviral therapeutic in mice is through live viral challenges. This was therefore performed in most experiments regarding vaccine development and therapeutic testing in papers I and II. In addition, viral challenges were utilized in all immune imprinting studies in paper III, to mimic the natural way of repeated influenza infections.

All infections were performed by anesthetizing the mice and then pipetting virus suspension intra nasally, making sure the suspension entered the lungs. Here, the viral dose can be controlled to modify the outcome of the challenge. For vaccine and therapeutic

testing, a lethal dose of virus is desired to test the efficacy of the treatments under severe conditions. A dose of 5×LD50 was most often used for this, as this results in the death of control mice if left unmonitored, while effective treatments should protect the mice (Paper I and II). In contrast, the infections in paper III were not meant to be lethal so a much lower dose of ~0.5×LD50 was used instead. This results in some weight-loss but should not lead to a severe disease and death.

During the challenges, all mice were monitored and weighed daily. If a mouse lost more than 20% of their initial bodyweight, it is assumed that they would not be able to survive and are therefore euthanized to prevent further suffering. This is important as infection challenges are among the most ethically questionable experiments we perform, and it is important to try to minimize the necessary suffering. With this it is also important to stick to the three Rs of animal studies: Replace, Reduce, and Refine. Replacing is difficult to achieve for infection studies so more emphasis is placed on reducing the numbers to a minimum and refining the assays to get the most out of each experiment.

Measuring immune responses in mice

Measuring immune responses in mice have been essential for the present work (papers I-III). There is only so much in vitro experiments can tell you about immunology because the immune system is so vast and immune responses so complex. Animal models are therefore often required in to embrace this complexity. However, the use of animal models can expose other potential biases, such as differences in immune systems between species. This needs to be taken into account when comparing experimental results from mice with human responses in the literature. Examples include differences in immunoglobulin chain gene locus and usage (~95% λ-LC in mice vs ~33% λ-LC in humans) or differences in lymphocyte and leukocyte concentrations in blood (~75-90% Lymphocytes in mice vs ~30-50% lymphocytes in humans)(293). Despite this, animal models are still the best way to study many facets of the immune system.

Antibody responses in mouse sera

Immune responses in the serum are among the most readily available to study. Blood can be sampled from the mice at regular intervals and the most serum components can efficiently be analyzed by enzyme-linked immunosorbent assays (ELISAs) (or similar methods). We mostly looked at serum immunoglobulins and did this by adsorbing the antigen in microtiter wells and then detecting the bound immunoglobulins. This allows for analysis of different immunoglobulin isotypes by using specific secondary antibodies, and you can thus gain a representation of the B cell responses taking place in the mouse. However, this is only a representation and can give indications, i.e., degrees of IgG vs IgA class-switching occurring in the B cell compartment. For more accurate analyses of cellular events, the cells themselves need to be directly studied (see below). Due to their usefulness, ELISA measurements of serum IgG reactivity against the relevant antigens have been included in all immune response studies.

In addition to the quantitative evaluations of antigen specific IgG in mouse sera, we also measured antibody quality with respect to their capacity for neutralization. This was done by microneutralization (MNA) of live virus. Many perform neutralization studies using psuedovirus assays (φNA), but we wanted to make sure the neutralization would be representative for a natural infection and therefore used the native viruses. Either method involve incubating the serum with the virus before adding it to a suitable cell line and measuring viral infection of the cells. The amount of virus components in the cells, or lack thereof caused by antibodies in the serum preventing viral infection, can then be read out. While MNA utilize the genuine virus of interest, φNA often uses replication deficient viruses (often lentiviruses) with a reporter gene (e.g., GFP or luciferase) and components from the virus of interest that facilitate cellular entry added (such as HA from influenza or spike from coronaviruses). For the MNA, the read-out used was detection of viral nuclear proteins such as nucleoprotein for influenza or nucleocapsid for SARS-CoV-2. φNA assays therefore only provide information of whether the serum can prevent cellular entry by blocking the HA for example whereas MNA

titers include all components of the virus. However, none of these provide information about other effector functions capable of clearing the virus such as ADCC or ADCP.

B cell responses in secondary lymphoid organs

B cells are one half of the adaptive immune response and their capacity to maintain a memory for rapid activation during new infections is paramount to the immune system. Studying B cells is important for pinning down their function in different types of responses. Measuring cellular B cell responses is very often done by flow cytometry, and this have been central to the studies regarding pre-existing immunity and B cell responses (Paper III).

Plasma cells and plasma blasts are very important as they are the "end goal" of B cells and produce most of the soluble antibodies. LLPCs and PBs have distinct surface marker expressions (e.g., CD138) that can be used to separate them from other B cell lineages However, it is more difficult to stain antigen reactive LLPCs since IgG expressing PCs start to downregulate their BCRs(294,295). In order to stain for antigen reactivity, you need to stain intracellularly which require fixation and permeabilization of the cells. If you are only interested in the binding profile of the plasma cells this does not pose a problem. However, if you want to sequence the BCRs, for recombinant antibody production or other reasons, fixating the cells limits the ability to sequence the RNA in the cells as most sequencing technologies currently requires non-fixed cells. Because we have had the sequence of the antigen specific BCRs as the goal, we focused on MBC and GC B cells in lymph nodes instead of PB and LLPCs.

As Memory B cells are supposed to be reactivated during a new infection, they need to be available, and many MBCs are therefore continuously circulating in the blood stream. Circulating MBCs are difficult to isolate in large numbers from mice due to the small volume of blood, we therefore harvested spleens as a proxy due to the perfusion of blood through the spleen. However, the frequency of MBCs in the spleen is still low, with the vast majority being naïve B cells. This forces a screening of large amount of

splenocytes to gather enough memory B cells. We therefore started with an enrichment step by magnetic isolation, prior to flow cytometry to facilitates this.

In addition to the low numbers of accessible MBCs, there is also unclear consensus regarding their classification in mice. In humans, MBCs are classified as CD27 positive, but CD27 is not an MBC marker in mice. Similarly, activated B cell start expressing CD38 in humans whereas both naïve and MBC express CD38 in mice(296,297). This makes distinguishing between naïve B cells and MBCs in mice difficult. One strategy is to distinguish between non-class-switched B cells (i.e., expressing IgD and IgM BCRs) as naïve, and class-switched (i.e., IgG, IgA or IgE expressing) as MBCs. However, IgM expressing MBCs exist and are quite common in both humans and mice(183,298). CD73, CD80 and CD273 are sometimes used when studying MBCs in mice, but they have been shown to describe different phenotypes of MBCs(191,299). After going back and forth, we decided to use CD38 positive and IgD negative as a definition of MBCs in our studies (paper III), not excluding either IgM negative or positive cells.

In contrast, germinal center B cells are easier to isolate as they are confined to draining lymph nodes. In addition, GC B cells are fairly prevalent and can be clearly defined by surface markers such as CD38low, FASHigh, and GL7High(300). However, it is important to select the correct draining lymph nodes. For respiratory infections, we isolated B cells from the mediastinal lymph nodes in the lung cavity (paper III) while we isolated B cells from inguinal and/or mesenchymal/iliac/lumbar(?) lymph nodes after intradermal or intramuscular vaccination. In cases where the antigen draining is less clear, the specific draining can be validated in advance by either simply testing for antigen reactivity in different lymph nodes, or by administering a dye that can be traced(301).

The ultimate role of B cells is dictated by their antigen specificity, a high number of CD27pos MBCs (in humans) will not protect an infected person unless they also bind the correct antigen. For isolation of antigen specific B cells, a good antigen probe is required. In our testing, the combination of well-expressed and monobiotinylated HA used in conjunction with the high valency Klickmers® significantly improved the num-

ber of isolated cells over streptavidin-based HA probes. This was crucial for achieving a sufficient number of cells for the single cell sequencing of antigen specific B cells.

T cell responses in mouse spleen

On the other half of the adaptive immune response, we have T cells. They are important for clearing infected cells and intracellular pathogens like viruses and for coordinating the immune response with different cytokine profiles. While the BCR binds its cognate antigen, the TCR is restricted to binding peptides in the context of a relevant MHC molecule. In order stain for TCR reactivity directly, you need to know the correct combination of peptide and MHC molecule for that TCR. This requires knowledge regarding the peptide epitope(s) beforehand unless peptide pools with all their respective MHC molecules are produced. This increases both the complexity as well as the cost of the assay significantly.

T cell response are therefore often measured by detecting different cytokines or combinations of cytokines associated with different T cell roles as an indirect measure of TCR activation. Measuring cytokine secretion is often done in two main ways, through ELISpot or flow cytometry. ELISpot is very sensitive and a robust method and is often used to describe T cell responses and was used the most for the studies in this thesis (paper I). However, ELISpot is often limited to a single (or sometimes two) analyte(s) per well unlike flow cytometry where you can stain for multiple cytokines and activation markers simultaneously. We therefore stimulated the T cells by adding antigen in the form of peptides, either single peptides or pools, or whole proteins. However, the antigens need to be processed by APCs before presentation to the T cells. When we measured T cell responses from murine spleens, adding splenocytes is sufficient as the splenocytes contain a mixture of T cells and APC (such as B cells and DCs). Because these methods do not measure direct antigen reactivity, they always require unstimulated cells as a baseline. In addition, irrelevant stimulation controls are also necessary to

correct for the levels of general activation or other cytokine secreting cell types (such as IFN-γ secreting NK cells in an IFN-γ ELISpot).

Antiviral selection, testing and delivery

Testing of the repurposed ALK/LTK inhibitors in paper II was done both in vitro and in vivo. In vitro testing is similar to a viral micro neutralization assay with the drugs added instead of the serum (see above). Here, drugs were incubated together with virus and susceptible cells and viral infection was measured by detecting viral nuclear proteins in the cells. It is important that the cell lines used express the relevant receptors needed for the infection by the virus while also expressing LTK in this case. Madin Darby Canine Kidney (MDCK) cells are commonly used for influenza studies due to their ability to propagate the virus, something not all cell lines are capable of. Similarly, Vero E6 cells are commonly used for SARS-CoV-2 studies as they express ACE2 and can be infected(302,303). However, since none of these are human or mouse, the transferability of the results to pre-clinical models could be limited here, where the homology of LTK is important. However, if the inhibitors have an effect here where they might be suboptimal, the effect could be larger in cells expressing higher levels of the correct LTK such as many lung tissue cell types.

Things are less straight-forward for in vivo protection. High amounts of drugs are needed to achieve the desired dose in animals, on the scale of 0.1-1mg drug per day per animal. In contrast, when Zykadia (the brand name for ceritinib) is given in humans the dose is between 150-300 mg per day. This problem is exacerbated by the solubility of the drugs in aqueous solutions differing widely between the drugs. This sets a limit on how concentrated the drugs can be in such small volumes. Oral gavage is among the methods which can deliver the largest volumes while also being the delivery routes of the drugs in humans. The drugs were therefore given orally here. However, we also tried intranasal delivery of the drugs to get them to the site of infection. Here, a 10-fold higher dose with a tenth of the volume was used. However, this was not feasible due to

the high viscosity of the drug solution leading to complications. This would also pose a challenge due to a majority of the drugs being insoluble at the required concentrations.

Bioinformatics

For the pre-existing immunity project (paper III), we wanted to look at a combination of antigen reactivity and their corresponding BCR sequence while also gathering the role of the B cell in the lymph node. To achieve this, we needed to perform bioinformatic analysis of single cell RNAseq data acquired from GC B cells in lymph nodes 14 days after a secondary exposure to influenza virus. For this, 10X Genomics single cell sequencing platform was used. 10X Genomics provide some tools for analysis of the data, but it has several limitations if you want more advanced analysis than basic clustering based on gene expressions. Another limitation we had to work around was the mouse reference genome, as 10X Genomics use a mouse reference genome from C57BL/6 mice whereas we used BALB/c mice in our studies. This meant that mapping the sequencing reads had to be done using a custom BALB/c reference genome. This was not an issue for the general gene expression RNA data since they could be mapped well enough to get the correct gene, but the more sequence specific BCR sequencing needs a correct reference for determination of mutations and so on. This was generated based on the OGRDB(304). The BCR alignment was performed in MiXCR(305), while most of the rest of the single cell data was analyzed in R, using packages from Seurat(306) and Immcantation(307).

General Discussion

Current needs in the fight against pandemic viruses

Like all previous global pandemics in the last century, the next pandemic will likely be caused by a highly variable respiratory viruses such as influenza- or coronaviruses. Due to their high mutation rate and often large animal reservoirs, the risk of a transmission event to humans increases(308). This has some implications for how we should plan future prophylactic vaccine strategies. Current vaccines against influenza and SARS-CoV-2 contain the major surface antigens HA and Spike, respectively, and aim for the induction of neutralizing antibodies(97,309). While the neutralizing antibodies are effective only against a very narrow range of viral variants, they also constitute the only type of immunity that can prevent infection altogether. As such, the same strategy may be of relevance also against the next emergence with a pandemic potential, but this implies that it will take time after the virus emerges and before a vaccine is available for the population.

A broadly effective antiviral drug could be key for remedying disease during the gap period before protective immunity can be raised by vaccination in the population. Antivirals can alleviate the most severe cases of disease, and also be used preventively in high-risk situations(310). For these antivirals to be “universal” they need to be able to prevent viral replication without interacting directly with specific viral components, but rather by utilizing universal host pathways used by most if not all viruses, and in particular these potentially pandemic variable viruses(271).

Vaccines are the best way to end a pandemic, in that they can raise long-lasting immunity in the population(311,312). However, influenza and SARS-CoV-2 continuously undergo mutations, which means that current vaccine strategies will only be effective for a limited time. The current option is to repeatedly update vaccines to account for the antigenic drift, but it is not fully understood how the repeated exposure to similar viral

variants will influence formation of new responses. A major aim for the present work was to provide more insight into this. Further, one could seek development of new vaccines able to award broad protection against a wide range of viral variants(313–315). Such an endeavor starts with considering which parts of the virus are more accessible for the immune system, and whether these are rapidly changing or more conserved. The viral surface antigens contain immunodominant sites that typically are located in, or in the vicinity of, the binding site initiating viral entry into host cells. However, these sites are also typically prone to mutations(316,317). In paper I, we developed a vaccine aiming for presentation of an increased number of available B-cell epitopes, and as such potentially also increasing the protective breadth.

Antiviral drugs against influenza and SARS-CoV-2

The purpose of antiviral therapeutics is to disrupt and prevent the ability of the virus to replicate in the host. Unlike a vaccine response, antivirals can in theory inhibit any of the viral mechanisms such as cellular entry/fusion(262), viral transcription(318) or viral release(261). Most of the currently approved therapeutics have been designed for targeting of a selected viral enzyme. For influenza, the currently available therapeutics can either block neuraminidase [Oseltamivir(319), Peramivir(320), Zanamivir(321)] or viral polymerase [Baloxavir marboxil(322,323)] activity. In addition, there used to be antivirals aiming for blocking of the M2 ion channel [Amantadine and rimantadine(324,325)], but these are no longer in use due to the widespread resistance caused by mutations in in the M2 gene(326). These are all influenza specific, targeting regions that may mutate to cause resistance. In fact, Oseltamivir is already under threat from an increase in resistant strains bearing three point-mutations in NA(268). It is therefore important to have an array of broad-spectrum antivirals available.

Both classes of antiviral therapeutics approved against SARS-CoV-2, protease inhibitiors [Nirmatrelvir/ritonavir(327)] and polymerase inhibitors [Remdisivir(264,265,328) and Molnupiravir(329)], are broad spectrum to some degree, in that they were devel-

oped against other viruses first, and later repurposed for SARS-CoV-2. Polymerase inhibitors and nucleoside analogues are a common approach for developing broadly acting antivirals(269), because many RNA viruses first require transcription to coding mRNA for viral protein translation. They therefore share some RNA polymerase activity, despite not necessarily being similar enzymes in structure. The nucleoside analogues work by introducing lethal mutations in the replicating viruses(269), but this also raises concerns regarding safety as they can potentially cause mutations in the host cells as well(330). These types of broad-spectrum antivirals also do not address the issue of resistance mutations. In contrast, fears were raised among the public that it would lead to more variants(331).

Host-targeted antivirals

Host-targeted antivirals represent a potential future alternative to broad-spectrum antivirals targeting the viral replication machinery(332,333). This is a promising idea that has recently received more attention. Gassen et al. showed how autophagy modulation can inhibit SARS-CoV-2 growth in vitro(334). Wagoner et al. found that combining the established SARS-CoV-2 antivirals with host protease TMPRSS2 inhibitors resulted in a more potent drug regime(335). Reed et al. demonstrated that a drug compound against HIV could work by interacting with host proteins involved in viral assembly(336). These host-targeted antivirals address the issue with escape mutations, but not necessarily the pan-virus activity. Some of the pathways are used in more than one virus family, for example, TMPRSS2 is also involved in HA cleavage needed for influenza pathogenicity(337), and could therefore potentially be effective against influenza as well.

Our strategy for achieving broad spectrum antiviral inhibition was to target host mechanisms in the protein transport from the ER(272), with the hypothesis that infected cells with high levels of viral protein production would be affected to a larger extent than non-infected cells. The accumulation of viral proteins in these cells would then lead to cellular stress and apoptosis, thus preventing further spread of the virus. By

starting with repurposed drugs with the desired mechanism and already approved for clinical use, you could expedite the drug discovery process. This strategy is different from previously published strategies that often start with genome screening(338) or protein-protein interaction network analysis(333,339) for viral-host interactions, often with a single virus as starting point.

Challenges with vaccine design

Current vaccine research and design have progressed far from the first influenza vaccines from the 1940's. The use of only one antigen in subunit vaccines allow for an immune response tailored towards a selected part of the virus. However, most seasonal influenza vaccines are still inactivated virus vaccines. Although, commercially approved recombinant protein vaccines against influenza are subunit with HA as the only antigen, supplemented with an adjuvant exists(232). In paper I, we saw that the inclusion of an APC-targeting domain onto recombinant protein subunit vaccines increased levels of circulating antibodies long-term, as compared to the commercial vaccines. This was also achieved with a lower vaccine dose than the commercial vaccine. One explanation for this would be an increased "local concentration" around the APCs facilitated by the targeting compared to non-targeted vaccines that rely on natural draining.

While most influenza vaccines given are inactivated influenza vaccines, it is important to have a variety of different vaccine formats available. mRNA and DNA vaccines allow for shorter development times(340) and slightly different responses, often skewed towards IgG2a over IgG1, and with improved cellular responses(341,342), compared to protein vaccines. In addition, mixing vaccine formats might be beneficial for overall protection(343), with a combination of mRNA and Adenoviral vectored vaccines leading to increased neutralization and T cell reactivity(344). It can therefore be advantageous for vaccine platforms, such as the one in in paper I, being usable in both DNA and protein vaccines without any substantial modifications, as this can facilitate the vaccine production of both versions.

The goal of most vaccines is to induce neutralizing antibodies, but neutralization requires the correct BCR sequence to bind the neutralizing epitopes on the antigen. It has been shown that certain combinations of V(D)J genes are favored against certain epitopes, and this affects the neutralizing response(345,346). It is therefore important that the vaccine antigen is recognized by the germline sequence for it to enter the GC and mature and attain these affinities. However, this can be challenging as the germline repertoire is very broad, and the affinity for the antigen can be low(347). Therefore, vaccines strategies designed to generate specific antibody responses need to take this into account. One example of a strategy designed to induce a specific response against a conserved broadly neutralizing epitopes is germline targeted vaccines(348–352). Other strategies use antigen combinations that mainly include the target epitopes, like the HA stem(353–355).

However, the examples of subunit vaccines above still only use a single antigen. Being able to compose vaccines with specific responses towards multiple components of the virus is a potential next development step in vaccine design. Here, a T cell based response, using APC-targeting, adjuvants, different vaccine format or other means, can be directed to intracellular and conserved regions while antibody responses are induced against exposed epitopes.

Epitope availability and vaccine structure

B cells have the theoretical ability to bind everywhere on the antigen surface. Despite this there are regions that are more immunodominant than others, and some epitopes are more favorable for protection than others(286,356).

There are different approaches for this dilemma, some vaccines aim to include a large number of epitopes(357), while others focus on specific, broadly neutralizing epitopes. The germline targeting vaccine design mentioned above is one example of how responses against a specific epitope can represent the goal of a vaccine. In germline targeting strategy, antigens have been designed specifically for binding of the germline

BCR responsible for the desired high affinity antibody response. Subsequent antigens can then be designed to further steer the response in the desired direction by specifically interacting with the newly affinity matured BCR(349). However, this focusing of antibody responses towards a single epitope increases the vulnerability to escape mutations, even though the single epitope originally was selected for its high degree of conservation. In addition, issues could also arise from epitope masking by the circulating Ab's, preventing a new GC response after boosting(358,359). Lastly, even though there are shared clonotypes between individuals(360), populations are not homogenous and differences can impact the vaccine response(361). It has been shown that ethnicity can affect both humoral and cellular responses from vaccinations(362), and two groups can have significant different responses against one antigen, but not the other(363)

As mentioned above, the choice of antigen itself is of course important for epitope availability, but there are more factors affecting epitope responses. One is how the structure of the antigen is presented in the vaccine. Viral surface proteins are often multimeric in nature, but this is sometimes overlooked in vaccine design and development. For example, HA and Spike are trimeric, while NA is tetrameric. It has been shown that trimer-stabilized HA is more immunogenic than HA without such stabilization(243,364), and this could be due to the availability of interface epitopes present only in the trimeric HA(365). In contrast, monomeric HA have been proposed to expose epitopes that are normally hidden but become exposed during "conformational breathing" during cellular entry(365,366). Vaccine antigen structure should therefore be considered and explored during development of new vaccines.

When we incorporated the trimer-stabilized HA into an APC-targeted vaccine format, we did not observe any substantial increase in vaccine effectiveness compared to vaccines without a trimerization domain (Paper I). A bit surprisingly, we observed trimeric vaccine structures even in the absence of the trimer stabilizing domain. This indicates that interphase between monomers in the stem and head domain of HA is enough for it to spontaneously form trimers without a transmembrane domain or external trimeriza-

tion domain present. However, there is still a monomeric fraction in these APC-targeted HA constructs, which suggests that the trimers are not completely stable. it is possible that the stability of the trimeric vaccine construct has less impact in a DNA vaccine format where there is a potential for a short time from vaccine secretion in electroporated cells to antigen uptake by immune cells.

Similarly, an additional trimerization domain did not seem necessary in the Spike mRNA vaccines against SARS-CoV-2 while they did include some stabilizing mutations(71,367). This highlights that one strategy might not be strictly superior to the other when it comes to epitope availability, but rather that it is important to understand different vaccine formats and their dynamics in the vaccinated individual.

Going forward, vaccine design should consider epitope presentation for the different vaccine strategies, and make sure that relevant responses are achieved in as a diverse population. This could be achieved by including whole antigens in a native conformation (such as trimeric HA/Spike) or by other means such as artificially selecting certain epitopes in peptide vaccines(368) or in sub-domain vaccines, such as HA-stem vaccines(353).

The research in this field have so far focused mainly on B cell epitopes, since it has been the epitope-binding antibodies that have been the focus. However, recent findings have shown how the interaction between T cell epitopes and B cell epitopes are important for B cells selection(369). In addition, since TFH is crucial for GC-dependent responses, effective T cell epitopes should not be overlooked. We do not presently know the best way to design vaccines that supply the best epitopes for the B cells while also activating the correct TFH cells. What we do know, is that B cells recognizing a particular epitope must be able to present the correct peptide antigen to the TFH cells and receive proper stimulation in the GC LZ(223). This creates another dimension of epitope specificities that needs to be considered when designing vaccines.

Preexisting immunity and original antigenic sin

The whole discussion of which epitopes are targeted by vaccines is influenced by who the person receiving the vaccine is. One point is the genetic background that is highly diverse both within HLA molecules and germline receptor genes, and as predisposes the population for great heterogeneity. On top of this, comes the influence from previous antigenic exposures. It is one thing to generate a specific type of response in an immunologically naïve recipient (that often is an inbred mouse in a controlled research setting) vs someone who has been serially exposed to a wide array of pathogens. For RNA-viruses in particular, an individual will likely have been repeatedly exposed to different viral variants with different degrees of similarity to the vaccine. Even though Original Antigenic Sin or immune imprinting is often not detrimental to boosting through new vaccinations, the question is at present whether we can "re-engineer" the imprinting of previous infections by specific vaccine designs.

The "holy grail" of vaccine development have for a long time been the elicitation of broadly neutralizing antibodies that are capable of neutralizing all variants of a virus(370). For influenza, this is often manifested as neutralizing responses towards the stem of HA due to its conserved nature. There are numerous ongoing efforts aiming for the creation of broadly reactive responses against influenza stem epitopes by vaccination by stem-only antigens, and these can mount broadly neutralizing responses in animal models(353,354,371). These vaccines aim to overcome the higher immunogenicity of the HA head that can otherwise dominate the response and mask the broadly reactive stem component. However, it is not known whether these can overcome previous imprinting against the variable head domain. It is also not known whether a new exposure to the virus, where the head is present again will overwrite any more recently developed vaccine induced responses against the stem.

The effect of previous exposures to an antigen, whether it is the dominance of the first exposure in "Original Antigenic Sin" or the combined effect of "Immune imprinting", is important for understanding vaccine responses. Many studies tries to distinguish between

OAS and immune imprinting as alternative hypothesis 195). In paper III, it was not our intention to find evidence for one of these hypotheses over the other, but rather to study the molecular and clonal mechanisms in secondary immune responses after different primary imprinting infections. Therefore, we also limited the studies to the secondary exposure and no further tertiary, quaternary and so on. By limiting ourselves to two exposures, one priming and one secondary, we could also determine the effect of a singular imprinting event more clearly. In addition, as is often seen in mouse studies, the serum titers generally remained constantly high after two or three influenza infections(195). This can limit the extent of which a human imprinted immune system can be replicated in mice, since you cannot wait several years in between multiple immunization.

How to study OAS and immune imprinting?

There are several different approaches for studying OAS or immune imprinting, but information regarding the involved BCRs are often missing due to it having been based on serological data historically. Here, we chose to use single cell sequencing of B cells under different influenza infections (paper III). We believe that by studying the BCR sequences in secondary immune responses you could assess whether the observed GC responses were maintained since priming, or maintained but boosted during the secondary exposure, or to which extent new GCs were formed. For example, you might expect a lower number of mutations in the BCR in B cells that have just entered the GC, and a higher numbers of mutations if the B cells have spent longer time in the GC and undergone several rounds of SHM. Here, we saw a higher number of mutations in GCs with homologous imprinting and boost, as compared to heterologous imprinting and secondary exposures. However, due to the relatively short time from primary to secondary infections (6 weeks), there are most likely still GC reactions occurring in these lymph nodes from the priming event, and we are not able to discern for certain whether these B cells had left as MBCs and then re-entered or not. The longevity of GC responses can vary between immunizations, with some diminishing after 4-5 weeks(150), while others can be maintained for months(149). Typically, live viral infections induce more

persistent GC responses, as we observe in our studies. A minimum of 12 weeks or longer is most likely needed for most of the GCs to have diminished in our set-up. The extent of GC re-entry and the role of MBCs is, in addition, highly dependent on the present epitopes. Studying the interaction between B cell and its epitope in GCs have historically been done with transgenic BCR mouse models. BCR repertoire sequencing of antigen -binding B cells in GCs allow for another approach to study at the influence of epitopes by screening By looking at the BCR sequences and their cognate epitope on the antigens you would be able to understand how certain epitope are used and back boosted over other epitopes.

Studying immune imprinting and MBCs in mice vs humans

OAS was described in humans, and understanding how it works in humans is the ultimate goal. By understanding the intricacies of OAS, vaccines and other therapeutics can be designed with this in mind, and as such steering responses towards improved protection. The problem is that OAS is very difficult to study in humans. Everyone has a different history of antigenic exposures. People of similar age might have all been exposed to a similar initial strain when they were roughly the same age as children. However, in the 15+ years after that, they would all have different exposure patterns. This makes studying the mechanistic principles in humans difficult because there are so many factors that play a role. We therefore chose mouse models as a better approach for understanding basic mechanistic principles. First, inbred mice all have the same (or at least very similar) genetic background, which eliminates the role genetics play into this. Second, mice bred under sterile conditions are naïve to influenza (or other model diseases used), so the initial and the subsequent strain exposures can be controlled. However, there are some issues with mice studies involving BCRs. One is naïve mice's reduced ability to form certain specialized CDR structures, like extra-long CDRH3 loops seen in some broadly neutralizing Abs(377): This can limit some of the transferability of the findings to humans. Several labs overcome this drawback by using transgenic mice with human BCR repertoires(345,359,378). This allows for more transferability while also providing

traceability when used in conjunction with adoptive transfer of B cells in CD45 congenic strains. By transferring B cell subsets from immunized mice into naïve recipients, and then immunizing the recipients, you can study the role of the transferred B cells in secondary reactions. Alternative approaches use the insertion of fluorescence markers or other molecular tags in transgenic mice to trace GC dynamics. Here, either by photoactivation(142) or by tamoxifen induced switches(374), GC B cells can be "fate-mapped" and the B cells or antibodies can then be traced throughout the response. By these methods, it has been shown how secondary GCs mostly contain previously naïve B cells and that the MBCs that are present usually stem from clones with high germline affinities(375). In addition, it appears that persistent GC are continuously invaded by naïve B cells, keeping the GC going(376).

Antigenic distance

Perhaps the most important aspect for whether immune imprinting takes precedence over subsequent responses is the similarity between the imprinting antigen and the subsequent ones. A highly similar antigen will be more prone to boost imprinted responses whereas a more distant antigen might not(195). For influenza viruses, it is rare to achieve B cell clones that bind both group 1 and group 2 HA antigens with traditional vaccinations or infections, while you often observe significant cross-reactivity between different subtypes in the same group(201) . In preliminary studies, we tested different HA combinations before we settled on two influenza strains within the same subtype, A/California/07/2009(H1N1) and A/Puerto Rico/8/1934(H1N1) which share around 80% of the HA amino acids. This is roughly equivalent to the antigenic distance between the H1N1 strains circulating before the 2009 pandemic and the pandemic strain, which is a good model for what a future pandemic with IAV could look like. We also expanded on this established model with more distant antigens using H5 viruses (~64% identity) as well as group 2 viruses with H3N2 (~43% identity) and H7N1 (~41% identity), but almost no effect from previous imprinting with the other subtypes were observed in these setups. The antigenic distance thus likely favored new formation as opposed to

re-activation or pre-existing responses. In the aforementioned Fate-Mapping setting, they could also observe that the amount of memory recalled serum IgG was directly correlated with the antigenic distance, and with an 80% identity they observed a small bias towards new responses(195). In general, when cross-imprinting is observed between H3N2 and H1N1, this is often accredited stem-binding clones(379).

There is still much we do not know about antigenic distance and immune responses, but understanding how distance affects imprinted responses is crucial for predicting future responses against new variable viruses.

To conclude, we are at an interesting point in time when it comes to pandemic preparedness and vaccine development. We have learned much from the two latest global pandemics, H1N1 in 2009 and SARS-CoV-2 in 2020, both the wide-spread use of new vaccine formats and a better understanding of how our immune system responds to new viruses. All of this will enable better treatments and preventative measures for any future pandemic.

Future Perspectives

It is unfortunately most likely not an “if”, but “when” the next pandemic hits. The work here has aimed to highlight where we need improvements to be better prepared for it when it occurs. We need more rational vaccine designs, where we can tailor the immune response, and we need better antivirals, whose broad activity can used to lessen the disease burden immediately and bridge the gap for vaccines.

When it comes to vaccine design, we first of all need a better theoretical understanding of how the immune system will react to a vaccine antigen. This could be predicting immunodominant epitopes, both B and T cell epitopes, or how different antigen presentations affect the response. For the vaccines in paper I, the next logical step is following up with similar studies of other antigens, both against influenza but also other respiratory viruses. Adding multiple antigens in vaccines is not something new, but we confirmed that it was viable with our format as well. Therefore, further explorations of how best to mix antigens is needed. Subunit vaccines today only contain one antigen per pathogen, i.e., only HA for influenza or only Spike for coronaviruses. However, mixing multiple antigens from the same pathogen in a more rational way than inactivated viral vaccines could improve the response, and should be explored further.

For improved broadly acting antivirals, more work is needed. Host-directed antivirals is a promising strategy for achieving broad activity, but it is still in its infancy. The examples of host-directed therapies being developed often only shown to have an effect against one or two, sometimes three viruses(380). Our efforts with LTK inhibitors are also an early exploration of this. We have showed how they display some activity, but follow-up studies should delve deeper into the mechanisms and see whether the proposed mode of action holds true. In addition, the inhibitors themselves should be improved for the antiviral purpose. Currently, the inhibitors are given orally, but modifications that could allow for direct delivery to the respiratory system could be beneficial by lowering the total dose needed to achieve the desired local concentration.

Then we have the matter of immune imprinting surrounding the whole discussion of immune responses during a pandemic. The next pandemic will most likely be caused by a highly variable virus like influenza viruses or coronaviruses, and there is much we do not know mechanistically about how imprinting will affect the responses. We do not know to which MBCs are reactivated or exactly what they do when reactivated. We do not know how differently shifted or drifted antigens impact the recall response. We do not know if certain epitopes are "doomed to fail" as a strategy and never be targeted due to other the presence of other epitopes. The list goes on…

We are now starting to have the tools required to begin answering these questions. Efficient sequencing technologies on single cell levels and clever ways to trace the fate of cells is a powerful combination that currently seems like the best way to approach this. By learning some of the "rules" for how the immune repertoire behaves when exposed to certain antigens in different scenarios can help predict some of these effects. This all requires detailed mechanistic studies that are most likely impossible to perform in humans and requires animal models. Therefore, combining detailed mechanistic animal studies and large-scale human studies where the volume of data and natural variance comes in would be powerful. However, in order to combine animal and human studies, more knowledge of how similar the immune systems actually are is needed. Mice and humans live very different lives; size, life-span, environment etc., and therefore have different challenges for the immune system to overcome(293,381). This can be seen in the species differences within the BCR V, D, and J gene loci(382), and needs to be taken into account when transferring results from one to the other.

To conclude, we are now in the early stages of a new phase of immunology. We could expect great findings in the coming decade, that change the way we view vaccines.

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VACCINES AND ANTIVIRAL AGENTS

Trimeric, APC-Targeted Subunit Vaccines Protect Mice against Seasonal and Pandemic Influenza

Elias Tjärnhage,[a] Diamond Brown,[a] Bjarne Bogen,[a,b] Tor Kristian Andersen,[a] Gunnveig Grødeland[a,b]

[a]Institute of Clinical Medicine, University of Oslo, Oslo, Norway
[b]Department of Immunology, Oslo University Hospital, Oslo, Norway

ABSTRACT Viral subunit vaccines contain the specific antigen deemed most important for development of protective immune responses. Typically, the chosen antigen is a surface protein involved in cellular entry of the virus, and neutralizing antibodies may prevent this. For influenza, hemagglutinin (HA) is thus a preferred antigen. However, the natural trimeric form of HA is often not considered during subunit vaccine development. Here, we have designed a vaccine format that maintains the trimeric HA conformation while targeting antigen toward major histocompatibility complex class II (MHCII) molecules or chemokine receptors on antigen-presenting cells (APC) for enhanced immunogenicity. Results demonstrated that a single DNA vaccination induced strong antibody and T-cell responses in mice. Importantly, a single DNA vaccination also protected mice from lethal challenges with influenza viruses H1N1 and H5N1. To further evaluate the versatility of the format, we developed MHCII-targeted HA from influenza A/California/04/2009(H1N1) as a protein vaccine and benchmarked this against Pandemrix and Flublok. These vaccine formats are different, but similar immune responses obtained with lower vaccine doses indicated that the MHCII-targeted subunit vaccine has an immunogenicity and efficacy that warrants progression to larger animals and humans.

IMPORTANCE Subunit vaccines present only selected viral proteins to the immune system and allow for safe and easy production. Here, we have developed a novel vaccine where influenza hemagglutinin is presented in the natural trimeric form and then steered toward antigen-presenting cells for increased immunogenicity. We demonstrate efficient induction of antibodies and T-cell responses, and demonstrate that the vaccine format can protect mice against influenza subtypes H1N1, H5N1, and H7N1.

KEYWORDS DNA vaccines, adaptive immunity, influenza, influenza vaccines, subunit vaccine

Hemagglutinin (HA) is the major integral surface glycoprotein of influenza viruses and extracellularly consists of a stem domain and a globular head domain. It is trimeric in its natural state and binds sialic acid (SA) moieties on host cells (1). Binding to SA leads to internalization of the virus through endocytosis, which triggers a conformational change of HA that causes fusion between viral and endosomal membranes and thereby release of the viral genome (2). The SA binding site on the highly immunogenic globular head contains important epitopes to which neutralizing antibodies can bind, potentially mediating sterilizing immunity by blocking the virus from binding host cells (3–6).

Currently used influenza vaccines base their efficacy on neutralizing antibodies against HA (7, 8). Due to antigenic drift, a prolonged production time increases the probability of mismatches between vaccine-inserted strains and the viral strains circulating to cause seasonal epidemics in the population (9–11). Subunit vaccines improve production speed compared to conventional virus-containing vaccines, but typically have a lower immunogenicity (12). Further, novel HA-based vaccines often use monomeric

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Address correspondence to Gunnveig Grødeland, Gunnveig.grodeland@medisin.uio.no.

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HA (13–17), which could result in the loss of interface conformational epitopes (18). That said, a soluble HA ectodomain has previously been stabilized by the addition of a trimerization peptide from the T4 phage fibrillin protein, also known as foldon domain (19), leading to increased immunogenicity (20, 21).

Targeting of antigens to surface markers and receptors on antigen-presenting cells (APC) has been shown to greatly enhance antigen immunogenicity (22–27), and we have previously demonstrated that DNA vaccines encoding APC-targeted HA raised protective antibody levels in mice and larger animals (13, 28–31). More specifically, we demonstrated that targeting of HA to major histocompatibility class II (MHCII) molecules was particularly efficient at raising antibody responses, whereas targeting to chemokine receptors (CCR) 1 and 5 induced a more cellular-based immune response (28). These vaccines were designed with an X-shaped structure, with two arms containing APC-specific targeting moieties and two arms containing monomeric HA (13). The resulting bivalent antigen display was likely important for cross-linking of B-cell receptors, and as such, also activation of immune responses (32). However, the bivalent display of monomeric HA did not take into consideration the possibility that important B-cell epitopes could be located at the interphases between monomers in a trimeric conformation.

Here, we have designed new APC-targeted vaccines with the aim to enable display of HA in its natural trimeric state. First, HA was linked directly to an APC-specific targeting moiety, as such omitting the previously used dimerization unit that led to bivalent display in an X-shaped structure. Second, we constructed vaccines with HA linked to either the chemokine macrophage inflammatory protein 1 alpha (MIP1α) or a single-chain variable fragment (scFv) specific for MHCII molecules, as these were previously demonstrated to be favorable for protection against influenza when comparing nine different APC-specific targeting moieties (33). Third, we designed vaccines with or without a foldon trimerization domain at the C terminus to see whether this influenced antigen conformation and immunogenicity. In sum, we designed APC-targeted vaccines where selective targeting to MHCII or chemokine receptors could improve vaccine efficacy while also retaining the native trimeric HA structure to potentially induce the wider antibody repertoire associated with live infections (34).

RESULTS

Construction and structure of APC-targeted HA proteins. The vaccines were genetically constructed by adding an APC-specific targeting domain encoding either an scFv specific for murine major histocompatibility complex class II (MHCII) molecules (I-E^d) (27), or the chemokine murine macrophage inflammatory protein 1 alpha (MIP1α) (35), to the N terminus of HA, separated by a linker (GESYAEAAAKEAAAK) (Fig. 1A). A nontargeted control vaccine was prepared by replacing the scFv specific for MHCII with a scFv against the hapten 4-hydroxy-3-iodo-5-nitrophenylacetic (NIP) (27). The vaccines were then either genetically linked or not to a C-terminal trimerization domain (19) (see Table S1 in the supplemental material). The theoretical size for the vaccine monomers composed of one HA monomer (~60 kDa) plus one scFv (~26 kDa) with linkers is about 90 kDa, and with an added 25 to 30 kDa worth of glycan moieties (Fig. S1A), yielding a total vaccine monomer size of around 120 kDa.

In order to evaluate protein expression, the constructed DNA vaccines were transiently transfected into HEK293E cells. Vaccine proteins with HA from influenza A/PR/8/1934 (H1N1) (here denoted H1) as antigen were secreted in the range of 100 to 400 ng/mL (Fig. 1B). Vaccines without the C-terminal trimerization domain were expressed at significantly higher protein levels compared to the vaccines with this domain (denoted H1F). Cells transfected with αMHCII-H1 expressed the highest levels of vaccine proteins, while MIP1α-H1 and αNIP-H1 were expressed at equal and lower levels.

The efficacy of the APC-targeted vaccines is dependent on the functionality of the APC-specific targeting moiety (13, 27, 28, 35). Thus, we evaluated binding of MHCII-targeted vaccines (I-E^d specific) to cells expressing relevant E^d or irrelevant D^d molecules.

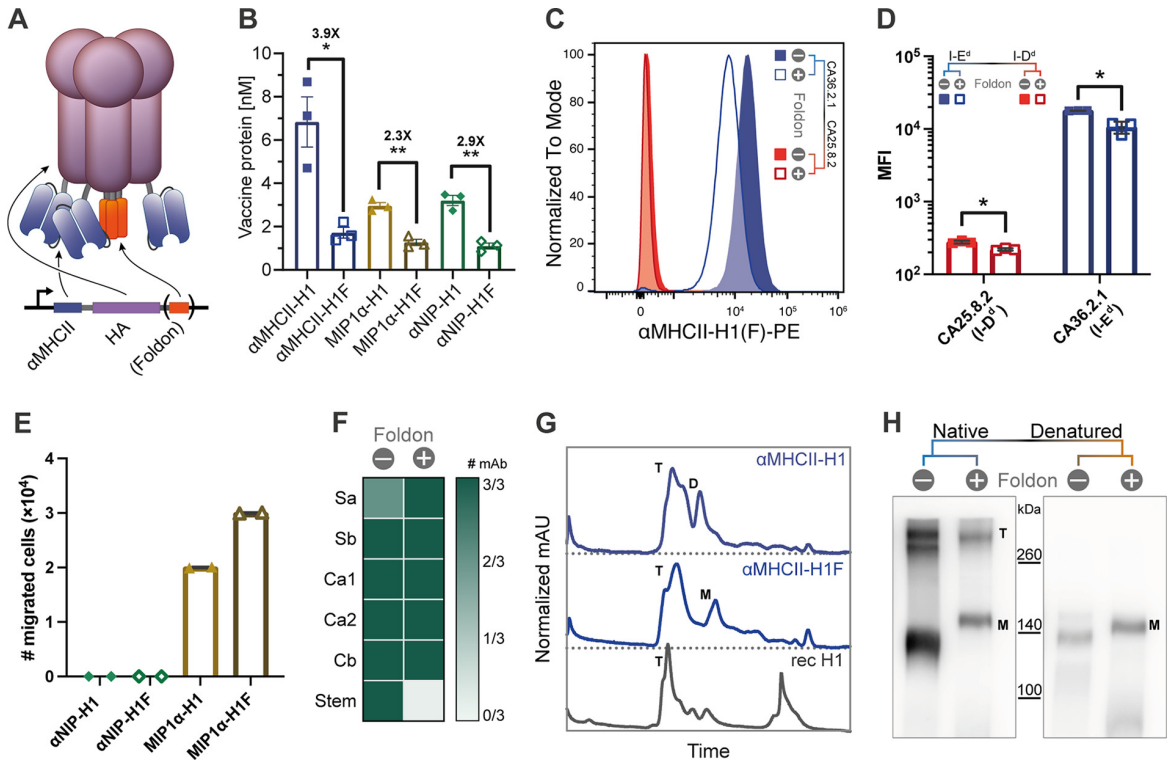

FIG 1 Vaccine protein secretion and structure. (A) Schematic representation of the genetic vaccine and the produced protein, here represented by αMHCII-H1F. (B) ELISA of supernatants from transiently transfected HEK293E cells. Bars indicate the mean ± standard error of mean (SEM) with the fold increase shown for significant increases (two-sided Mann-Whitney test). (C) Binding of MHCII-targeted vaccines to MHCII (E^d)-expressing cells (CA36.2.1), but not to MHCII (D^d)-expressing cells (CA25.8.2). (D) Mean fluorescence intensity of data from panel C (mean ± SD). (E) Chemotaxis of cells after stimulation with MIP1α-containing vaccines compared to nontargeted controls (two independent experiments, mean ± SD). (F) Conformational epitope mapping of secreted vaccine proteins by sandwich ELISA with a panel of monoclonal antibodies with known binding epitopes on HA (Sa, Sb, Ca1, Ca2, Cb, and stem). Three MAbs were used per epitope (36). (G) Size exclusion chromatography of purified vaccine protein compared to commercially available HA. Peaks corresponding to trimers, dimers, and monomers are indicated by T, D, and M, respectively. (H) Western blot analysis of purified vaccine proteins under native and denaturing conditions, detected by MAb against HA(PR8) (clone: H36-4-52). Bands corresponding to trimers, dimers, and monomers are indicated by T, D, and M, respectively.

Importantly, the vaccines efficiently bound cells expressing E^d, but not D^d (Fig. 1C and D). For vaccines targeting chemokine receptors via the chemokine MIP1α, chemotactic integrity of the targeting moiety was confirmed by evaluating cellular migration across a membrane in response to the titrated presence of vaccine proteins (Fig. 1E).

The expressed vaccine proteins were next screened against a panel of 18 monoclonal antibodies (MAbs) with known binding toward established HA epitopes in order to evaluate antigen folding to more detail (21, 36) (Fig. 1F). Importantly, 3/3 MAbs against the known immunodominant sites Sb, Ca1, Ca2, and Cb bound vaccine proteins regardless of the presence of a trimerization domain, and the Sa site was recognized by 2/3 MAbs when foldon was left out and by 3/3 when it was included. In addition, 3/3 stem binding MAbs recognized the HA stem in the vaccine without the trimerization domain, while 0/3 clones recognized the corresponding vaccine with the trimerization domain. Interestingly, looking at the fold change in signal for vaccine- over mock-transfects revealed that certain epitopes may be favored by the different vaccines. As an example, foldon vaccines displayed a higher fold change signal for the Sa site in all three MAbs, whereas the vaccines without a foldon had a higher signal for the Sb site in 2/3 MAbs (Fig. S1B).

To further evaluate the impact of a trimerization domain, analytical size exclusion and Western blotting of purified vaccine proteins were performed. When comparing the sizes of the vaccines with commercially available recombinant HA protein and

standards of known size on a Superdex 200 Increase 3.2/300 high-pressure liquid chromatography (HPLC) size exclusion column, we found that a majority of the proteins were trimeric, with some possible breakdown products at lower molecular weights. Based on areas under the peak, an estimated 80 to 85% of the protein eluted in the trimeric peak for both vaccine formats (Fig. 1G). The observation that the produced vaccine proteins were dominantly trimeric independently of a trimerization domain was confirmed also by Western blotting (Fig. 1H). Trimeric vaccines were seen under nondenaturing conditions, but not under denaturing conditions. Some monomeric vaccines were observed under nondenaturing conditions as well, indicating that the trimeric vaccines may not be very stable.

***In vivo* humoral immune responses.** To assess vaccine immunogenicity *in vivo*, BALB/c mice were vaccinated intramuscularly (i.m.) with plasmids encoding the different vaccines, followed by electroporation of the injection site to facilitate cellular DNA uptake. Antibody responses were assessed longitudinally in sera from 2 weeks after immunization, with the levels generally increasing over 10 weeks (Fig. 2). Interestingly, we did not observe any significant differences in total HA-specific serum IgG, IgG1, and IgG2a for vaccines with or without the trimerization domain (Fig. 2A to F).

Experiments were next set up to more directly compare the contribution of the different APC-specific targeting moieties in the absence or presence of a trimerization domain (Fig. 2G to L). Results demonstrated significantly higher serum levels of total IgG and IgG1 after vaccination with αMHCII-H1 compared to the nontargeted control vaccine αNIP-H1 (Fig. 2G to I). αMHCII-H1 also raised significantly higher IgG2a responses than αNIP-H1 at weeks 2 to 4 and 10 after vaccination. For MIP1α-H1 versus αNIP-H1, the difference was not significant for total IgG, IgG1, or IgG2a, even though the mean of the serum IgG responses was higher for MIP1α-H1 at all time points.

When the trimerization domain was included, many of the significant differences between targeted and nontargeted vaccines disappeared (Fig. 2J to L). However, the mean IgG and IgG1 responses after vaccination with αMHCII-H1F were consistently higher than the responses after vaccination with αNIP-H1F. When comparing MHCII-targeted to CCR1/5-targeted vaccines, we observed that MHCII-targeted vaccines both with and without a trimerization domain induced higher total IgG and IgG1 responses. This was reversed for IgG2a, where responses for MIP1α-H1/H1F and αMHCII-H1/H1F were more similar.

When summing up the four different experiments, we observed that IgG, IgG1, and IgG2a responses were higher in vaccines without a foldon, and that the APC-targeted vaccines mostly improved responses over the nontargeted control vaccine αNIP-H1/H1F (Fig. 2M). Further, a polarization toward IgG and IgG1 for MHCII-targeted vaccines and IgG2a for CCR-targeted vaccines was observed, which is in accordance with previous observations (28, 29).

***In vivo* cellular immune responses.** Similar to antibody responses, T-cell responses will contribute to the formation of protective immunity in vaccinees. Thus, we stimulated splenocytes from vaccinated mice with H1 protein and a peptide pool spanning H1, as well as the MHCI (H-2K^d) restricted HA peptide IYSTVASSL, in an ELISpot assay evaluating gamma interferon (IFN-γ) secretion. Importantly, both the APC-targeted vaccines with and without a trimerization domain could significantly raise IFN-γ secretion above that of the saline control (Fig. 3A and B), and we observed no significant influence from the presence of a trimerization domain in the APC-targeted vaccines.

Next, we set up experiments to evaluate the efficacy of APC-targeting more directly (Fig. 3C and D). Results demonstrated that αMHCII-H1, MIP1α-H1, and αNIP-H1 all significantly raised IFN-γ secretion above that of the saline control (Fig. 3C). In contrast, both the APC-targeted vaccines with a foldon significantly raised the levels of IFN-γ-producing cells compared to αNIP-H1F (Fig. 3D). In sum, we observed that APC-targeting of antigen enhanced formation of cellular immunity after vaccination in the presence of a trimerization domain, and that this effect was reduced in its absence.

APC-targeted HA protects against lethal viral challenge. Key to vaccine functionality is its ability to protect against disease. Thus, we challenged vaccinated mice with

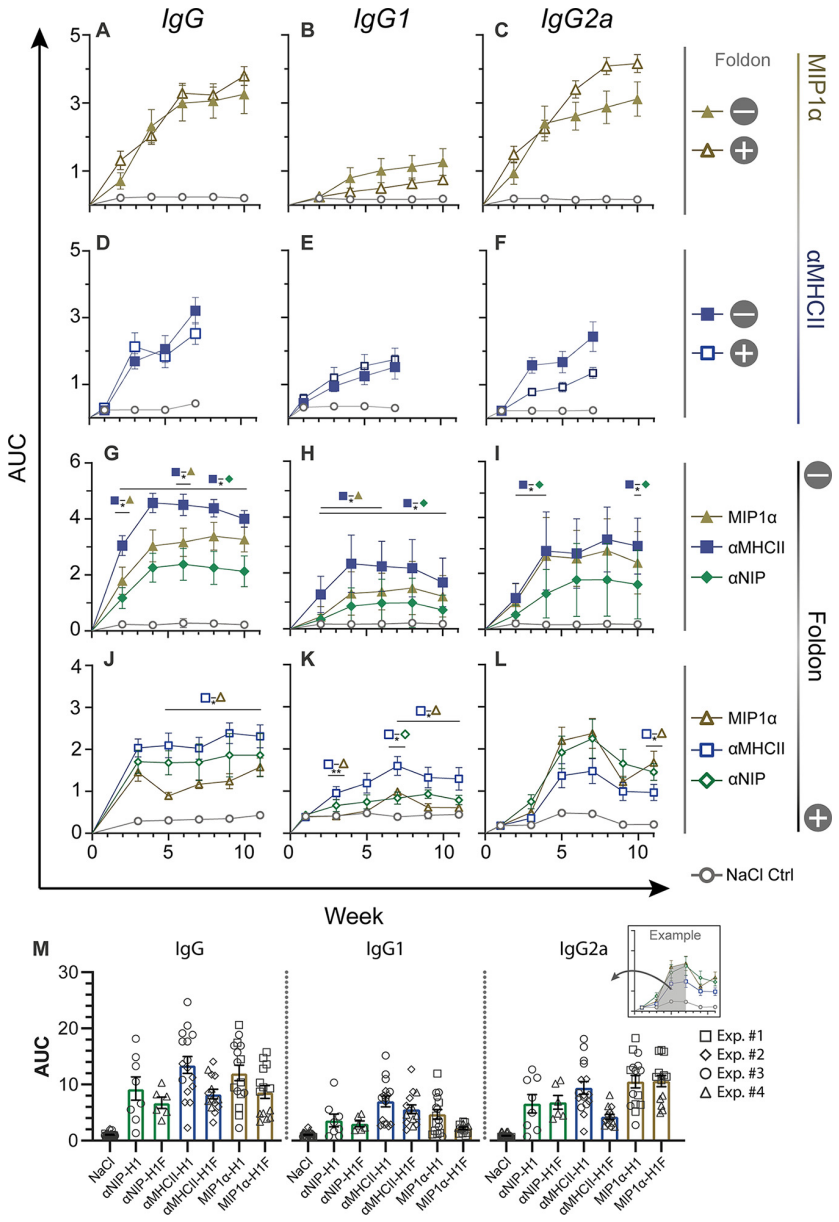

FIG 2 Efficient induction of antibodies in sera after DNA vaccination. Mice were immunized with 50 μg plasmid i.m., and blood samples were collected every 2 weeks (n = 8 mice/group for panels A to I; n = 6 mice/group for panels J to L). Serum antibody responses measured by ELISA, shown as AUC over time. (Left column) Total IgG responses. (Middle column) IgG1 responses. (Right column) IgG2a responses. (A to C) Comparison of MIP1α-H1 and MIP1α-H1F. (D to F) Comparison of αMHCII-H1 and αMHCII-H1F. (G to I) Comparison of differently targeted vaccines without trimerization domain. (J to L) Comparison of differently targeted vaccines with trimerization domain. (M) All serum IgG responses in panels A to L. Compiled responses were calculated based on the AUC from weeks 2 to 8. Different symbols indicate to which experiment the specific data points belong. Statistical analysis was performed by pairwise Mann-Whitney test at each time point. *, $P < 0.05$; **, $P < 0.005$. Data are displayed as the mean ± SEM.

a lethal dose of influenza A/Puerto Rico/8/1934(H1N1) (PR8) and monitored for weight as a marker of disease (Fig. 4). In accordance with the above-described data, we did not observe a significant difference in weight between groups receiving APC-targeted vaccines with or without the trimerization domain (Fig. 4A and B). When evaluating the effect of APC-targeting, however, we observed significant differences in weight

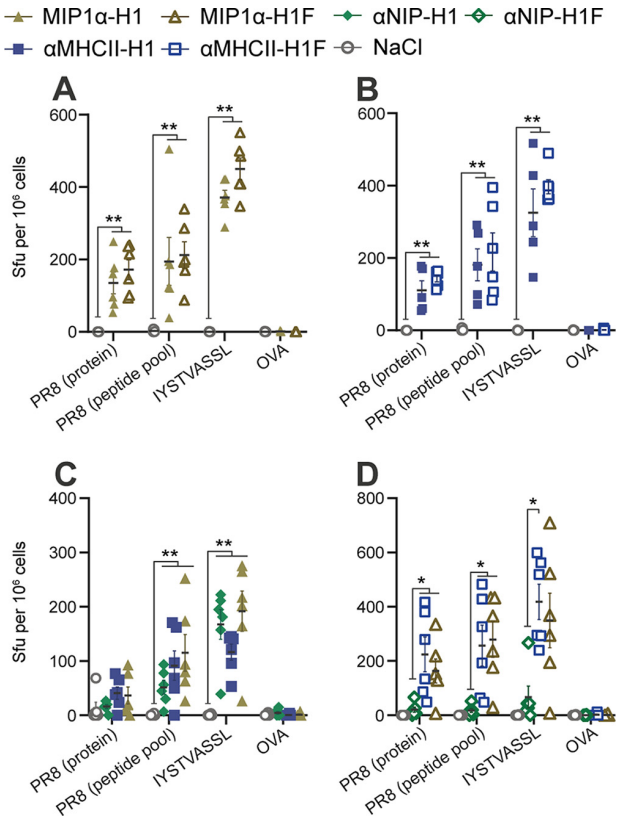

FIG 3 Vaccine-induced cellular immunity. Splenocytes from 14 days postimmunization were stimulated *in vitro* with rec. HA from influenza PR8, OVA, an HA (PR8) overlapping peptide pool, or the class I-restricted peptide IYSTVASSL, and assayed for IFN-γ secretion by ELISpot (n = 6 mice/group; each shown point is the mean of 3 technical replicates per mouse). Bars indicate the mean ± SEM. (A and B) Comparison with or without trimerization domain for (A) MIP1α vaccines and (B) MHCII-targeted vaccines. (C and D) Comparison of different APC-specific targeting moieties (C) without trimerization domain and (D) with trimerization domain. Statistical analyses were performed by pairwise comparisons of each group using two-sided Mann-Whitney tests, *, $P < 0.05$; **, $P < 0.005$. Data are displayed as the mean ± SEM.

loss between the nontargeted control vaccine αNIP-H1 and αMHCII-H1 from days 3 to 9 and between αNIP-H1 and MIP1α on days 3 to 4 (Fig. 4C). This difference was lost when the trimerization domain was included (Fig. 4D). This same trend was also observed when evaluating survival curves based on 20% weight loss as the humane endpoint (Fig. 4E to H).

Trivalent APC-targeted HA vaccination. There are 18 subtypes of influenza A that can be classified based on differences in HA. Seasonal influenza is presently caused by H1N1 or H3N2 influenza viruses. Accordingly, conventional influenza vaccines contain a selected strain from these two subtypes, selected based on the expectation that they will be relevant for preventing next season's epidemic. In addition to seasonal epidemics, there is a real potential that influenza subtypes currently circulating in birds may reassort to variants able to transmit among humans. Thus, we constructed MHCII- and CCR-targeted vaccines encoding HA from A/Vietnam/1194/2004(H5N1) (VN04) and A/Hong Kong/1073/99(H9N2) (HK99) to represent subtypes with a high potential for future zoonosis. We chose to prepare only vaccines without added trimerization domains since we did not observe significant differences between vaccines equipped with a foldon or not in the previous experiments (Fig. 2A to F, Fig. 3A and B, and Fig. 4A and B). Following transient transfection of the different vaccine plasmids in cell culture, efficient secretion of vaccine proteins with HA from H1, H5, and H9 influenza viruses was confirmed by enzyme-linked immunosorbent assay (ELISA) (Fig. S2).

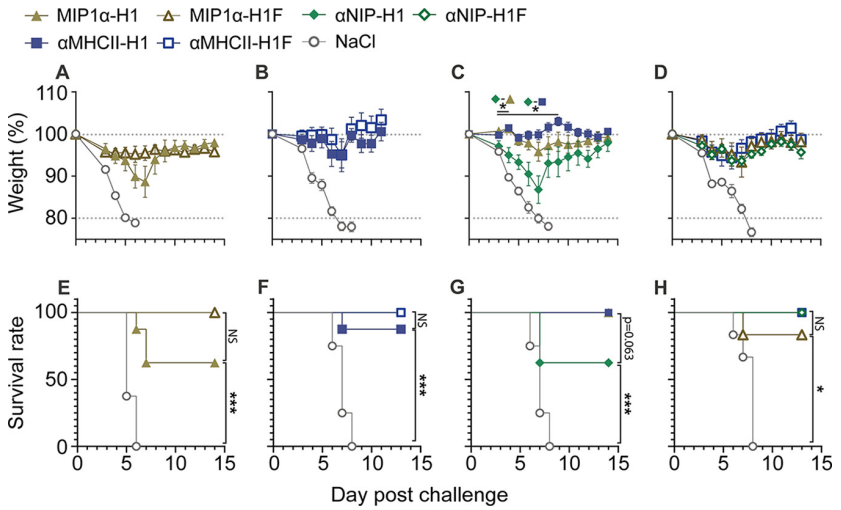

FIG 4 Vaccine effectiveness against a lethal influenza challenge. Mice were vaccinated with a single dose of the indicated vaccines and challenged 7 to −13 weeks postvaccination with a 5 × LD_{50} dose of influenza PR8 intranasally (i.n.) (n = 8 [panels A to C and E to G] or 6 [panels D and H] mice/group). Mice were monitored daily for weight loss; data are displayed as the weight mean ± SEM (panels A to D), with a humane endpoint of 20% body weight loss as basis for survival curves (panels E to H). (A and B) Comparison of vaccines with or without a trimerization domain: (A) weight after PR8 challenge 10 weeks postvaccination with MIP1α vaccines and (B) weight after challenge 7 weeks postvaccination with MHCII-targeted vaccines. (C and D) Comparison of different APC-specific targeting moieties: weight following viral challenge is shown for vaccines either (C) without a trimerization domain 10 weeks after vaccination or (D) with a trimerization domain 13 weeks after vaccination. Significant weight loss was determined by group-wise comparison using a two-sided Mann-Whitney test for each time point. Significance between vaccine groups is shown above the corresponding time point; *, $P < 0.05$; **, $P < 0.005$. (E to H) Survival curves corresponding to the above-described weight panels. Significance was calculated by log-rank (Mantel-Cox) tests. *, $P < 0.0332$; **, $P < 0.0021$; ***, $P < 0.0002$.

The MHCII-targeted vaccines encoding HA from influenza H5N1 and H9N2 were mixed with αMHCII-H1, and mice were vaccinated with either this mixture or the three vaccines independently. The total DNA concentration administered to each mouse was kept constant for the mixture and vaccination with a single vaccine. When evaluating antibody responses after vaccination, we correspondingly observed that vaccination with either αMHCII-H1, αMHCII-H5, or αMHCII-H9 significantly elevated responses above vaccination with the mixture (Fig. 5A to C). The vaccine-induced antibody responses were strain specific, except that αMHCII-H1 also raised some antibodies that cross-reacted with H5 (Fig. 5B). Vaccination with αMHCII-H1/5/9 raised significant IgG responses against HA from H1, H5, and H9 influenza viruses.

At 11 weeks after a single vaccination, mice were challenged with a lethal dose of influenza H5N1 (Fig. 5D and E). In accordance with the observed antibody responses, αMHCII-H5 offered complete protection against disease. In the group receiving the trivalent mixture, the mice initially lost weight, but 6/8 recovered from the infection. In contrast, 4/8 vaccinated with αMHCII-H9 recovered after a weight loss, and 2/8 in the saline control group recovered. All mice vaccinated with αMHCII-H1 succumbed to infection.

Next, we vaccinated mice with CCR-targeted vaccines against H1, H5, or H9 or a mixture thereof. Similar to the above-described MHCII-targeted vaccinations, DNA concentrations were kept constant for the different groups. As expected, vaccination with CCR-targeted vaccines displaying H1, H5, or H9 alone induced strong responses in an ELISA against homologous proteins (Fig. 5F). Interestingly, these responses were equaled in sera collected from mice vaccinated with the mix vaccine MIP1α-H1/H5/H9 when assayed against HA from influenza H1 and H5 viruses. Responses against H9 were significantly reduced for vaccination with MIP1a-H1/H5/H9 compared to MIP1a-H9, but still markedly present.

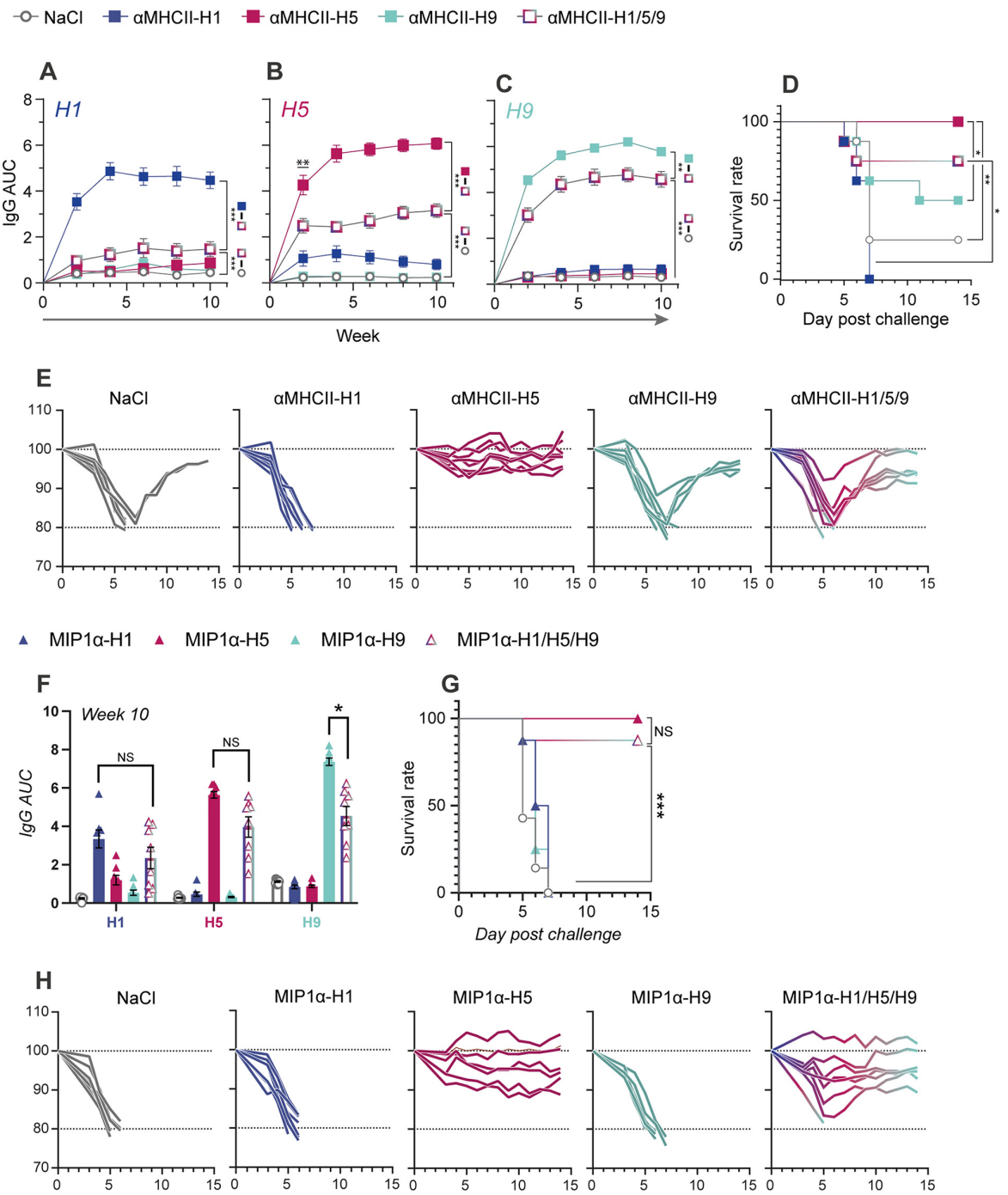

FIG 5 Efficient induction of antibodies and protection against both seasonal (H1) and potentially pandemic influenza subtypes (H5, H9). Mice were immunized i.m. with 45 μg DNA per mouse, either a mixture of 15 μg DNA per HA in trivalent mix or 45 μg of the indicated monovalent vaccines delivered independently (n = 8 per group, mean ± SEM). (A to E) MHCII-targeted monovalent and trivalent vaccines. Longitudinal serum IgG responses in ELISA against HA from influenza (A) H1 (PR8), (B) H5 (VN04), and (C) H9 (HK99), displayed as AUC. Statistical analysis was performed by a pairwise Mann-Whitney test at each time point. Shown is the statistical comparison between the trivalent and corresponding monovalent vaccine and saline control. Data are displayed as the mean ± SEM. (D) Survival rates after a viral challenge with a 5 × LD_{50} dose of influenza H5N1 virus, defined by 20% weight loss. (E) Weight of individual mice corresponding to panel D. (F to H) CCR-targeted (with MIP1α) monovalent and trivalent vaccines. (F) Serum IgG responses on week 10 after vaccination, measured in ELISA against HA from the indicated viruses. Two-sided Mann-Whitney tests between the trivalent and corresponding monovalent vaccine are shown. Data are displayed as the mean ± SEM. (G) Survival rates after viral challenge 10 weeks postvaccination with 5 × LD_{50} influenza H5N1 virus, defined by humane endpoint of 20% weight loss. (H) Weight from individual mice corresponding to panel G. Mann-Whitney test significance: *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$. Survival statistics were analyzed by log-rank (Mantel-Cox) and Gehan-Breslow-Wilcoxon tests: *, $P < 0.0332$; **, $P < 0.0021$; ***, $P < 0.0002$.

At 11 weeks postvaccination, mice were challenged with a lethal dose of influenza H5N1. In accordance with the observed antibody responses, 8/8 mice vaccinated with MIP1α-H5 survived, and 7/8 vaccinated with MIP1α-H1/5/9 survived (Fig. 5G and H).

Protein-based APC-targeted HA subunit vaccine. The first DNA vaccine for human use was recently approved for clinical use against SARS-CoV-2 in India (37), but even with the approved mRNA vaccines, there has been some public concern about the potential consequences of genomic integration. Thus, we wanted to examine how the APC-targeted HA subunit vaccine would work in a protein-based format. Here, HA from A/Puerto Rico/8/1934(H1N1) in αMHCII-H1 was replaced with HA from A/California/07/2009(H1N1) (CA07) (αMHCII-CA07). The MHCII-targeted vaccine was chosen here as our candidate vaccine since this format had proven more efficient at antibody induction (Fig. 2G and H), which is the protective mechanism for conventional vaccines. Thus, αMHCII-CA07 vaccine proteins were produced by transient transfection in 293E cells, and affinity purified for HA (Fig. S3A and B).

Mice were vaccinated with the αMHCII-CA07 vaccine proteins, with or without the adjuvant AS03. To enable benchmarking against commercially available vaccines, we also included groups vaccinated with Flublok (38) and Pandemrix (39). αMHCII-CA07 was given at 3.5 μg per mouse, whereas a dose of Flublok (quadrivalent, formulated with 2 influenza A and 2 influenza B subtypes) or Pandemrix (formulated with AS03) corresponding to 9 μg per HA was administered. Blood samples were taken at 2-week intervals for 12 weeks before a viral challenge with influenza CA07 virus.

Vaccination with the split virus vaccine Pandemrix+AS03 quickly raised antibody titers, and responses were significantly elevated above those observed for the other vaccine formats (Fig. 6A). The AS03 adjuvanted αMHCII-CA07 proteins raised total IgG responses to a similar level as Flublok initially, but significantly higher than Flublok from week 8. Nonadjuvanted αMHCII-CA07 did not induce significant levels of serum IgG above that of the saline control (Fig. 6A).

For an assessment of the protective potential of the different vaccines, mice were challenged with a lethal dose of influenza virus A/California/07/2009(H1N1). Importantly, there were no significant differences in survival observed for αMHCII-CA07+AS03, Flublok, and Pandemrix+AS03 (Fig. 6B), but a small and transient weight loss was observed at days 3 to 5 following vaccination with αMHCII-CA07+AS03 (Fig. 6C).

To complement the picture of immune formation after vaccination with the different protein or virus vaccines, we wanted to evaluate cellular immune responses. Mice were vaccinated with 9 μg HA for all vaccines, and IFN-γ production was evaluated in splenocytes collected at day 14 postvaccination (Fig. 6D). Pandemrix+AS03, Flublok, and αMHCII-CA07+AS03 raised significantly higher numbers of IFN-γ-secreting cells than nonadjuvanted αMHCII-CA07 and the saline control group when stimulated with CA07 peptides but were not significantly different from each other.

Next, we wanted to examine germinal center (GC) B cells after vaccination by fluorescence-activated cell sorter (FACS) staining of draining lymph nodes. Antigen-specific GC B cells were identified by binding of HA-streptavidin tetramer probes. Interestingly, while vaccination with Pandemrix+AS03 raised the total number of GC B cells to exceed that induced by the other vaccines, vaccination with αMHCII-CA07+AS03 significantly elevated the number of HA-reactive GC B cells compared to Pandemrix+AS03 (Fig. 6E, Fig. S4A and B). A repeat of this experiment with HA-coupled Klickmer to enable improved selection of antigen-specific B cells confirmed this result (Fig. 6F, Fig. S4C).

In sum, we demonstrated that vaccination with a low dose of the protein version of αMHCII-CA07 could raise immune responses and protection comparably to that observed after vaccination with higher protein doses of commercially available influenza vaccines.

DISCUSSION

Here, we have demonstrated that steering of trimerized HA to APC may enhance immune responses, including serum IgG (Fig. 2G to I), cellular immunity (Fig. 3D), and protection against a lethal influenza challenge (Fig. 4C and G). Interestingly, the

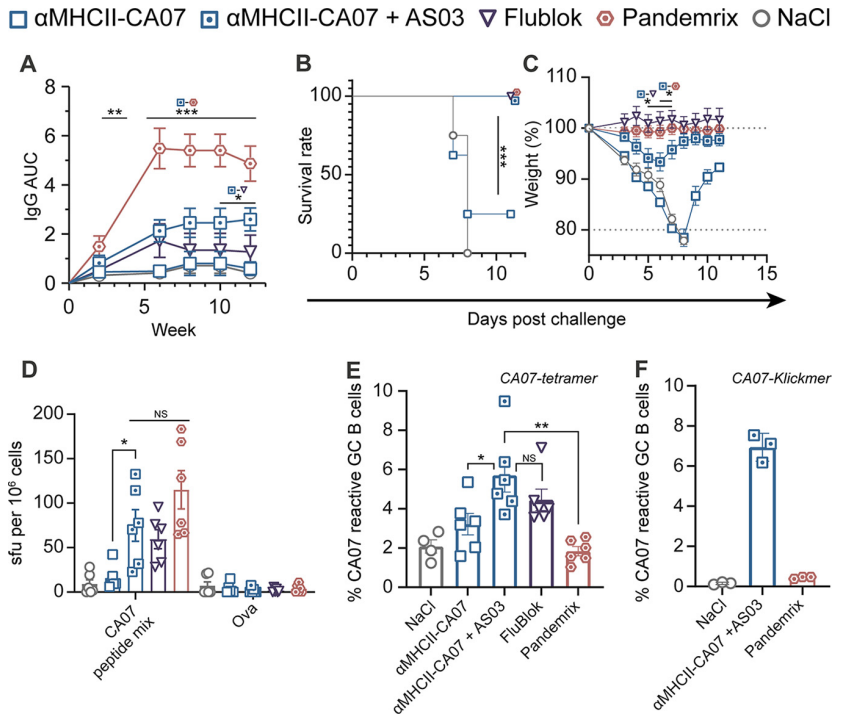

FIG 6 Protein vaccination with MHCII-CA07 induced B- and T-cell responses and protected against a lethal influenza challenge. (A to C) Mice were immunized with 9 μg HA protein per mouse for Flublok and Pandemrix or 3.5 μg of αMHCII-CA07. (A) Serum IgG levels assayed against CA07 HA in ELISA. Statistics were determined by a pairwise Mann-Whitney test at each time point. (B) Survival rate as defined by a humane endpoint of 20% weight loss; (C) weight after viral challenge with a 5 $\times$ LD50 dose of A/California/07/2009(H1N1) (Cal07) virus (n = 8 per group, mean $\pm$ SEM). The survival rate was analyzed by log-rank (Mantel-Cox) and Gehan-Breslow-Wilcoxon tests. (D to F) Mice were immunized with 9 μg CA07 HA per mouse for Pandemrix, Flublok, and αMHCII-CA07. (D) IFN-γ ELISpot of splenocytes collected on day 14 after a single immunization (n = 8 per group, mean $\pm$ SEM). (E and F) CA07-specific GC B cells on day 14 postimmunization, (E) with tetramer or (F) with Klickmer-based staining for flow cytometry. GC B cells were gated as single cells, TCRβ^{neg}, CD19^{pos}, B220^{pos}, CD38^{neg}, and GL7^{int-hi} (n = 6 per group, individual replicates $\pm$ SD). Statistical analysis by Mann-Whitney tests: *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$. Survival statistics were analyzed by log-rank (Mantel-Cox) and Gehan-Breslow-Wilcoxon tests: *, $P < 0.0332$; **, $P < 0.0021$; ***, $P < 0.0002$.

vaccine tended to be secreted as a trimer whether a trimerization unit was present or not. We showed that the vaccine can be delivered in the form of both DNA vaccine and protein.

The natural state of HA is a trimeric conformation. Even though many of the well-characterized epitopes on HA are confined to a monomer, some, such as Ca1, span two monomers (36, 40). It is therefore interesting to develop vaccines that present trimeric HA to the immune system. Previously, a stabilized trimeric HA structure has also been shown to be more immunogenic (20, 21). While the addition of a foldon in our study did not significantly influence the proportion of trimeric vaccine proteins (Fig. 1G), the presence of a trimerization domain nevertheless influenced vaccine immunogenicity. More specifically, the difference between αMHCII-H1F and the nontargeted control vaccine αNIP-H1F was reduced in its presence (Fig. 2 and 4).

Previously, we have demonstrated that targeting of antigen to APC enhanced immune responses after a single DNA vaccination in mice and larger animals (13, 27, 31). The vaccine format used for these studies were based on bivalent monomeric display of antigens that were linked to an APC-specific targeting unit via a dimerization unit that structured monomeric antigens as two flexible arms of an X (13, 27). The dual-antigen display likely enabled cross-linking of B cell receptors (BCR) in an APC-B cell synapse, and we have correspondingly demonstrated bivalent antigen display

favorable to monomeric display (32). For the present study, we omitted the previously used dimerization domain and found that HA could trimerize even in the absence of a foldon. The implication is trivalent display also of B cell epitopes, and that likely will facilitate cross-linking of BCR and efficient immune activation (41, 42).

Interestingly, we observed that the presence of a trimerization unit reduced protein secretion from transfected cells (Fig. 1B). This could be due to more rapid processing of newly produced vaccine protein in the absence of this addition, causing the potential enhanced stability not to play a key role. Protein expression *in vivo* is key for the efficacy of genetic vaccines, presenting an argument for progression toward clinical use with APC-targeted HA without a foldon. Further, the APC-specific targeting moiety is located at the N terminus of HA, which likely ends up next to the trimerization domain in the C terminus after HA folding (Fig. 1A). As such, steric interference could potentially explain both the reduced protein secretion observed and the failure of stem-specific antibodies to bind the vaccine equipped with the trimerization domain (Fig. 1F). Of note, we have previously observed that stem-binding antibodies could bind monomers (43), so binding should not be interpreted as confirmation of trimerization.

The flexibility of the subunit vaccine formats, such as DNA, mRNA, and protein, enable conscious engineering of the included antigens (44) or new combinations to produce the desired immune response. The influenza vaccines currently in use combine 3 to 4 strains or HA antigens from seasonal influenza A and B subtypes, while we here instead wanted to evaluate the combination of seasonal (H1) and potentially pandemic influenza subtypes (H5, H9). Importantly, the serum IgG responses observed against HA from influenza subtypes H5 and H9 in the trivalent mixture indicated a potential for these vaccines against potentially pandemic outbreaks of new influenza strains. At present, neither H5 nor H9 has evolved into a viral variant able to transmit between humans. However, annual zoonosis demonstrates mortality rates in the range of 50 to 60% for H5 (45, 46). It is imperative to have available vaccine strategies able to offer rapid protection against emerging H5 and H9 variants with a pandemic potential.

RNA and DNA vaccines have the advantage of enabling rapid production compared to conventional vaccines. However, no genetic vaccines were approved for use against viral infections until the recent SARS-CoV-2 pandemic, where the mRNA vaccines from Moderna (Spikevax) (47) and Pfizer-BioNTech (Comirnaty) (48) and the DNA vaccine from Zydus Cadila (ZyCoV-D) (49) were approved (37, 50, 51). While the vaccines have been demonstrated to be safe and efficient (52), the public has in some instances found it difficult to understand genetic vaccines, as they are perceived to potentially also be able to cause genomic integration (53, 54). A protein formulation of the vaccine may thus be easier to deploy in a large population, and enable more direct control over the administered dose of the antigen.

We compared a protein version of αMHCII-H1 to the commercially available vaccines Flublok and Pandemrix. Similar to αMHCII-CA07, Flublok is a recombinant HA subunit vaccine but is formulated as a quadrivalent mixture of two influenza A strains and two influenza B strains. Pandemrix is a split virion vaccine and is formulated as a monovalent vaccine since it was designed as a pandemic vaccine against the H1N1 pandemic of 2009. It is codelivered with the strong adjuvant AS03 (55). As such, a comparison of these three vaccine formats is a bit like comparing apples to pears. In addition, we used different doses of HA for the different vaccine formats; the commercial vaccines were given as 10% of the human dose, as is often done in mice, but we first used a lower dose of the MHCII-targeted protein vaccine. Nevertheless, benchmarking the responses observed after vaccination with αMHCII-CA07 to known vaccine types is of relevance. Pandemrix indeed raised stronger antibody responses than αMHCII-CA07, but the reduced responses may be favorable for a seasonal vaccine mostly aiming to update recall responses to the relevant influenza strain while retaining the ability to generate more broadly reactive antibodies without heavily imprinting the response against one strain (56).

αMHCII-CA07 induced a higher number of HA-specific GC B cells than Pandemrix.

Interestingly, even αMHCII-CA07 without AS03 raised responses comparable to those from Pandemrix (Fig. 6E). That said, Pandemrix induced more GC B cells in total than any of the other vaccines, likely due to the presence of many viral antigens in this split virion vaccine (Fig. S4B). Both Flublok and αMHCII-CA07+AS03 raised higher numbers of HA-specific GC B cells (30) (Fig. 6E), highlighting that subunit vaccines have a benefit in activating immune responses specifically against the desired antigen. In support of the latter point, Flublok could mediate equal protection from disease compared to the adjuvanted Pandemrix, and even the reduced dose of αMHCII-CA07+AS03 awarded protection, albeit with some initial weight loss (Fig. 6C). Another factor may be the interaction between vaccine antigens and specific immune cells. It is known that AS03 leads to preferential loading of monocytes over dendritic cells in draining lymph nodes (57), while we have previously seen that MHCII-targeted antigen (in DNA vaccine format) binds both macrophages and dendritic cells at roughly equal levels (13, 30).

We have here demonstrated that APC-targeted subunit vaccines can mimic the natural HA conformation and produce trimeric HA even in the absence of a trimerization domain. Importantly, the vaccines have demonstrated immunogenicity and functional efficacy against viral challenges in the forms of both DNA and protein. While the present data are from mice, the result warrants progression to larger animals as a first step toward clinical progression. The vaccines may be of use to reduce the seasonal burdens of influenza disease in the population, but our experiments also demonstrated a potential against emerging influenza subtypes with a pandemic potential.

MATERIALS AND METHODS

Cloning of vaccine constructs. A DNA sequence encoding an MHCII-specific scFv (originating from clone 14-4-4S MAb [22, 27), a short linker (GESYAEAAAKEAAAK), and HA (amino acids 18 to 541, codon optimized for mammalian protein production) from influenza A/PR/8/1934 (H1N1) (PR8, denoted H1) (13), followed by a linker (LNDIFEAQKIEWHERLVPRGS) and a foldon trimerization domain (PGSGYIPEA PRDGQAYVRKDGEWVLLSTFLG, denoted in vaccine names as H1F) was ordered for synthesis by GenScript (New Jersey, USA) (Fig. 1A). The construct was cloned into pLNOH2 (58). Vaccines encoding mammalian codon-optimized HA antigens from influenza A/Vietnam/1194/2004(H5N1) (VN04), A/Hong Kong/1073/99 (H9N2) (HK99), and A/California/07/2009(H1N1) were constructed by replacing the PR8 HA by subcloning on antigen-flanking SfiI-sites. Further, vaccines encoding the murine macrophage inflammatory protein 1 alpha (MIP1α), or a nontargeting scFv against the hapten 4-hydroxy-3-iodo-5-nitrophenylacetic acid (NIP) (originating from clone B1-8 MAb [27]) as a nontargeted control vaccine, were prepared by replacing the MHCII-specific scFv with subcloning on the BsmI/BsiWI restriction sites.

Recombinant protein vaccine production. Vaccine plasmids were amplified in TOP10 *Escherichia coli* bacteria and purified using either the Wizard Plus SV miniprep DNA purification system (catalog [cat.] no. A1460, Promega, WI, USA) for small quantities or the Qiagen plasmid mega-kit (cat. no. 12181, Qiagen, Germany) for larger quantities. For recombinant protein production, HEK293E cells were transfected with 0.25 μg plasmid per cm^2 cell tissue surface at 70% confluence with 40 μg polyethyleneimine (PEI) per μg plasmid DNA in 40 μL Opti-MEM (cat. no. 51985-026, Thermo Fisher, Massachusetts, USA) per μg plasmid DNA. Serum free Freestyle medium (cat. no. 12338018, Thermo Fisher) was used for all transfections. Cell culture medium was harvested 3 to 5 days posttransfection for small-scale production and every 5 days for large-scale transfections. For affinity purification of vaccine protein, filtered cell culture medium was applied to a protein A column loaded with either PR8-specific (clone H36-4-52, kind gift from Siegfried Weiss, Medizinische Hochschule, Hannover, Germany) or CA07-specific (clone 29E3, kind gift from Thomas Moran, Icahn School of Medicine at Mount Sinai, New York, NY, USA) monoclonal antibodies, followed by elution using 0.1 M Tris-glycine, pH 2.7. Buffer exchange was performed by dialysis with phosphate-buffered saline (PBS) using Spectra/Por dialysis membrane MWCO 12-14,000 (cat. no. 132 697, Repligen, Massachusetts, USA).

Structure characterization: MHCII ($I\text{-}E^d$) binding analysis. MHCII-transfected L-cell fibroblasts expressing $E_\beta{}^dE_\alpha{}^k$ (CA36.2.1) or D^d (CA25.8.2) (kind gift from Bernard Malissen, Centre d'Immunophénomique, Aix Marseille Université, Marseille, France) were stained with purified vaccine protein (10 μg/mL) for 30 min at 4°C, followed by incubation with biotinylated anti-HA IgG (1 μg/mL; clone: H36-4-52) and incubation with streptavidin-phycoerythrin (PE) (1 μg/mL; cat. no. S866, Thermo Fisher). Flow analysis was performed on an Attune NxT instrument (Thermo Fisher), and data analysis was performed in FlowJo (FlowJo LLC, BD, New Jersey, USA).

Structure characterization: chemotaxis. The chemotactic integrity of MIP1α-H1/H1F was assessed by quantifying Esb/MP cell migration across a 5-μm-pore polycarbonate membrane (cat. no. 3421, Corning, Inc., New York, USA) in response to the titrated presence of vaccine proteins. Results from duplicate samples (mean) after background subtraction (mean cell numbers of spontaneous cell migration, i.e., in the presence of medium alone) are presented.

Structure characterization: enzyme-linked immunosorbent assay (ELISA). High-binding 96-well microtiter plates (Costar 3590, Corning, NY, USA) were coated with 50 μL of NIP-bovine serum albumin

(BSA) (2 μg/mL) overnight at 4°C and blocked with 1% BSA-PBS for 60 min at room temperature. Next, 50 μL of supernatant from HEK293E cells transiently transfected with αNIP-H1(-F) or mock was added and incubated for 2 h at room temperature. This step was repeated for saturation. Next, titrated dilutions of the following antibody panel (kind gift from Davide Angeletti and Jonathan W. Yewdell, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD, USA) (36) were incubated in triplicates at 4°C overnight: Y8-3B3, Y8-2C6, H2-4B3, H28-E23, H36-1-1, H35-D1, H17-L2, H37-80, H18-S121, H2-4B1, H18-S413, H36-11, H17-L7, H9-A15, L2-10C1, D5, E7, 16GB, and H36-4-52. Then, plates were incubated for 2 h with biotinylated MAb specific for mouse κ-light chain (clone: 187, produced in-house) at room temperature, followed by streptavidin-alkaline phosphatase conjugate (ALP) (1:30,00; cat. no. 7105-04, SouthernBiotech, Alabama, USA) for 30 min. Detection was done following 15 min of incubation with phosphatase substrate (cat. no. P4744-10G, Merck, New Jersey, USA) at 405 nm. Antibody binding was considered positive if the area under the curve (AUC) of the dilution was higher than the AUC + 5 $\times$ standard error of the mean (SEM) of a mock transfection for that antibody. H1 PR8 vaccine secretion was measured using an influenza A H1N1 (A/Puerto Rico/8/1934) hemagglutinin/HA ELISA pair set (cat. no. SEK11684, Sino Biological, China), according to the manufacturer's instructions.

Structure characterization: Western blotting. Purified vaccine proteins were either kept native or mixed with 0.1 M dithiothreitol (DTT) and heated to 95°C for 5 min. Vaccine proteins (0.1 μg per lane) were loaded on 4 to 12% NuPAGE bis-tris gels (cat. no. NP0326BOX, Thermo Fisher, Massachusetts, USA) and separated with Bolt MOPS (morpholinepropanesulfonic acid) SDS running buffer (cat. no. B0001, Thermo Fisher). Proteins were then transferred to iBlot 2 polyvinylidene difluoride (PVDF) transfer stacks (cat. no. IB24002, Thermo Fisher), blocked in 2% skim milk in PBS with Tween 20 (PBST) at room temperature for 60 min, and incubated with biotinylated anti-HA MAb (1:3,000; clone: H36-4-52) at 4°C for 18 h. Next, the blot was incubated with streptavidin-horseradish peroxidase (HRP; 1:10,000; cat. no. 7105-05, SouthernBiotech) for 30 min at room temperature. Detection was done with WestPico chemiluminescence substrate (cat. no. 34578, Thermo Fisher). Separately, recombinant PR8 protein was deglycosylated with peptide-*N*-glycosidase F (PNGase F) according to the manufacturer's instructions (cat no. P0704S, New England Biolabs, Massachusetts, USA) to determine the size contribution of glycan moieties on HA.

Structure characterization: analytical size exclusion chromatography. Purified recombinant vaccine proteins (10 ng) or 10 ng of a commercially available HA (cat. no. 11684-V08H, Sino Biologicals) was loaded onto a Superdex 200 Increase 3.2/300 HPLC chromatography system (cat. no. 28990946, GE Healthcare, Sweden) in PBS. Molecular weights (MW) were compared against a ladder of proteins with known size (MWGF-1000-1KT, Merck, New Jersey, USA).

Animals, *in vivo* immunization, and viral challenge. All animals used in this study were female BALB/c mice (Janvier Labs, France) housed in a minimal disease unit at Oslo University Hospital. All experiments were approved by the Norwegian Animal Research Authority.

Mice were anaesthetized by an intraperitoneal (i.p.) injection of ZRF (zolazepam [3.3 mg/mL], tiletamine [3.3 mg/mL], xylazine [0.45 mg/mL], fentanyl [2.6 μg/mL]) at 10 μL/g body weight. Immunizations were performed by first shaving the hind legs of anaesthetized mice, followed by an intramuscular (i.m.) injection of 50 μL DNA (0.5 mg/mL) solution into each quadriceps, immediately followed by five-pulse electroporation of the injection site with an AgilePulse system (Harvard Apparatus BTX, Holliston, MA). Protein vaccination was performed under anesthesia by i.m. injection of 50 μL vaccine solution in each quadriceps. For commercial vaccines, 2 $\times$ 50 μL of Flublok (Sanofi Pasteur, France) or a 1:1 mixture of Pandemrix:AS03 (GSK, Belgium) was delivered i.m. Viral influenza challenges were done by infecting anaesthetized mice intranasally (i.n.) with a virus dose of 5$\times$ the 50% lethal dose (LD_{50}) in 10 μL per nostril. The LD_{50} dose was established by the Reed and Muench method and titrations of virus in mice. The viral strains used in this study were A/Puerto Rico/8/1934(H1N1), A/California/07/2009(H1N1), and RG14 [reassorted PR8 virus with H5 from A/Viet Nam/04/2005(H5N1)].

Serum Ab titer by ELISA. Blood samples were taken by puncture of the saphenous vein. Serum was obtained by centrifugation at 17,000 $\times$ *g* for 10 min, and then the supernatant was transferred to a new microcentrifuge tube and centrifuged again at 17,000 $\times$ *g* for 5 min. Sera were stored at $-$20°C.

For ELISA, 96-well microtiter plates (Costar 3590, Corning, NY, USA) were coated with one of the following recombinant influenza HA proteins: A/Puerto Rico/8/1934(H1N1) (cat. no. 11684-V08H, Sino Biological), A/Viet Nam/1194/2004(H5N1) (cat. no. 11062-V08H1, Sino Biological), or A/Hong Kong/1073/99(H9N2) (cat. no. 11229-V08H, Sino Biological) (0.5 μg/mL). Plates were blocked by 1% BSA in PBS at room temperature for 1 h. Serially diluted sera from individual mice were then incubated at 4°C for 16 to 18 h. Murine IgG was detected by incubation for 1 h with either horseradish peroxidase (HRP)-conjugated anti-mouse IgG (Fc specific) (1:5,000; cat. no. A2554, Merck, New Jersey, USA), biotinylated anti-mouse IgG1[a] (1:500; cat. no. 553500, BD Biosciences, New Jersey, USA), or biotinylated anti-mouse IgG2a[a] (1:500; cat. no. 553504, BD Biosciences). For biotinylated antibodies, a secondary incubation with HRP-conjugated streptavidin (1:5,000; cat.no. 7105-05, SouthernBiotech, Alabama, USA) at room temperature for 30 min was performed. The plates were developed by addition of 3,3′,5,5′-tetramethylbenzidine (TMB) solution (cat. no. CL07-1000ML, Merck), and the reaction was stopped after 10 min by addition of 1 M H_2SO_4. Absorbance at 450 nm was read using a Wallac EnVision 2104 Multilabel Reader (PerkinElmer, Massachusetts, USA).

Enzyme-linked immunosorbent spot (ELISpot). Spleens were harvested 14 days postimmunization. Single cell suspensions were prepared by using a gentleMACS dissociator (Miltenyi Biotec, Germany) followed by incubation in ACT (150 mM NH_4Cl, 170 mM TRIS-Base, pH 7.2) for 10 min on ice before filtration through a 70-μm nylon cell strainer (cat. no. 732-2758, VWR, Pennsylvania, USA). Splenocytes were washed twice with PBS before counting and resuspension in RPMI 1640 plus 10% FBS (cat. no. 61870036, Thermo Fisher). Cells from each mouse were then mixed with different stimuli in

triplicates and assayed in accordance with the kit protocol (cat. no. 3321-4APT, ELISpot Plus, Mabtech, Sweden) for 18 h at 37°C in a humidified atmosphere with 5% CO_2. The stimuli were recombinant (rec). HA from influenza PR8 or Cal07 (10 μg/mL; cat. no. 11684-V08H/11055-V08H, Sino Biological), ovalbumin (10 μg/mL; cat. no. vac-pova-100, InvivoGen, California, USA), IYSTVASSL peptide (7 μg/mL; ThinkPeptides, United Kingdom), PR8 HA peptide pool (15-mers with 11-amino acid [aa] overlap) (7 μg/mL; PepMix influenza A [HA/Puerto Rico/8/1934 H1N1]; cat. no. PM-INFA-HAPR, JPT Peptide Technologies, Germany), CA07 HA peptide pool (15-mers with 11-aa overlap) (7 μg/mL; PepMix influenza A [HA/California (H1N1)]; cat. no. PM-INFA-HACal, JPT Peptide Technologies), ConA (1 to 2.5 μg/mL; cat. no. inh-cona, InvivoGen), and RPMI 1640 (cat. no. 61870036, Thermo Fisher, Massachusetts, USA). The spots were counted in an ImmunoSpot device (C.T.L Cellular Technologies Limited, Ohio, USA).

Flow cytometry. Single cell suspensions were obtained from freshly harvested inguinal lymph nodes by passage through a 70-μm nylon strainer (cat. no. 732-2758, VWR). The cells were washed with PBS, and 1×10^6 cells were blocked on ice with 50% rat serum in 0.5% BSA/PBS for 30 min. The cells were then stained with a cocktail of anti-T-cell receptor β (TCRβ)-Alexa Fluor 488 (1:200; cat. no. 109201, BioLegend, California, USA), anti-CD45R/B220-PerCP/Cy5.5 (1:200; cat. no. 65-0452-U100, Tonbo Biosciences, California, USA), anti-CD19-APC/Cy7 (1:200; cat. no. 125530, BioLegend), anti-CD38-APC (1:200; cat. no. 102711, BioLegend), and anti-HU/MU GL7-Pacific Blue (1:200; cat. no.141614, BioLegend). In addition, purified recombinant HA $CA07_{Y96F}$ with an AviTag (GGGLNDIFEAQKIEWHE) was biotinylated (cat. no. BIRA500, Avidity, Colorado, USA) and then coupled at a molar ratio of 5:1 with streptavidin-PE (cat. no. 405204, BioLegend) at 4°C for 16 h or 15:1 with Klickmer-PE (cat. no. DX01K-PE, Immudex, Denmark) at room temperature for 30 min. The resulting HA complex was added to the staining cocktail at 1:100 for HA-SA-PE or at 32 nM HA-Klickmer-PE per sample. Flow analysis was performed on an Attune NxT instrument (Thermo Fisher), and data analysis was performed in FlowJo (FlowJo LLC, BD, New Jersey, USA). Cells were gated as lymphocytes, singlets, TCR_β^{neg} (non-T cells), $B220^{pos}$ $CD19^{pos}$ (B cells), $CD38^{neg}$ $GL7^{int-hi}$ (GC B cells), and HA^{pos}.

Statistical analysis. Serum antibody levels, protein secretion, and ELISpot counts were analyzed by using a pairwise two-sided Mann-Whitney test at each time point. Survival rates were analyzed by log-rank (Mantel-Cox) and Gehan-Breslow-Wilcoxon tests. All statistical analyses were performed in GraphPad Prism (California, USA).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.6 MB.

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E.T. and G.G. conceived and designed the experiments. E.T. and D.B. performed and analyzed the experiments. E.T., T.K.A., and B.B. developed and provided reagents, and B.B. also contributed to the refinement of some experiments. E.T. and G.G. wrote the paper. All authors edited and commented on the paper. Funding was obtained from the Research Council of Norway, the Horizon 2020 Framework Programme and Helse Sør-Øst.

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