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To my teachers

UNIVERSITY OF TURKU
Faculty of Medicine
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Virology
ARTTU REINHOLM: Vaccine induced adaptive immunity against SARS-CoV-2 Omicron and clade 2.3.4.4B A/H5N1 viruses
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ABSTRACT

Coronaviruses and influenza viruses are respiratory viruses with a history of causing pandemics in humans. Vaccines are one of the best ways of limiting and containing their spread. However, these viruses frequently mutate and diverge into new, emergent variants. Because of this, vaccines based on the earlier variants are often significantly less effective at inducing protective immunity against them. This necessitates the updating of vaccines with the new variants, often annually, to ensure humoral and cellular immunity are protective against circulating variants.

In the studies presented in this dissertation, we sought to analyze humoral and cell mediated immunity induced by vaccines against SARS-CoV-2 Omicron variants and avian influenza viruses. We also explored how SARS-CoV-2 breakthrough infections affect antiviral immunity. To accomplish this, we analyzed antibodies present in the serum samples taken from healthcare workers and AIV at-risk groups with ELISA and microneutralization tests. We also analyzed cell samples with activation induced marker assays combined with flow cytometry.

We show that three vaccinations with Spikevax and Comirnaty mRNA vaccines induced high titers of long-lasting S1-specific antibodies with a good neutralizing capacity against early Omicron subvariants BA.1, BA.2, and BA.5. The fourth and fifth vaccine doses based on later Omicron subvariants were shown to induce high titers of S1-specific antibodies, that declined more slowly against later Omicron subvariants BQ.1.1, XBB.1.5 and JN.1. Even though vaccine induced antibodies were considered neutralizing and thus protective, the neutralization capacity of the antibodies against the newer Omicron subvariants was substantially reduced compared to older Omicron subvariants. The combination of breakthrough infection and vaccinations induced the highest neutralization capacity. Additionally, we showed that effective CD4+ and CD8+ T-cell responses are likewise induced.

We showed that an inactivated subunit vaccine based on A/Astrakhan/3212/2020 (H5N8)-like strain induced antibodies with high neutralization capacity, and robust CD4+ T-cell activation against circulating H5N1 viruses of clade 2.3.4.4b.

The results of this dissertation show that mRNA and inactivated subunit vaccines are adaptable platforms capable of inducing efficient humoral and cellular immune responses against emergent SARS-CoV-2 variants and AIV strains.

KEYWORDS: SARS-CoV-2, Omicron, vaccine, H5N1, antibody, cell-mediated immunity

TURUN YLIOPISTO
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ARTTU REINHOLM: Rokotteiden indusoima adaptiivinen immuniteetti SARS-CoV-2 Omikron ja kladi 2.3.4.4B A/H5N1 viruksia kohtaan
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TIIVISTELMÄ

Korona- ja influenssavirukset ovat hengitystieviruksia, jotka ovat kautta historian aiheuttaneet pandemioita ihmisissä. Rokotteet ovat tehokkaita rajoittamaan virusten leviämistä. Korona- ja influenssavirukset kuitenkin muuntuvat usein, mikä johtaa uusien varianttien ilmaantumiseen. Tämän vuoksi aikaisempiin variantteihin perustuvat rokotteet ovat usein tehottomampia luomaan suojaavan immuniteetin uusia variantteja vastaan. Jotta humoraalinen ja soluvälitteinen immuniteetti pysyisivät suojaavina myös uudempia variantteja vastaan, rokotteita joudutaan päivittämään vuosittain.

Tutkimuksissamme analysoimme SARS-CoV-2- ja lintuinfluenssavirus (AIV) rokotteiden aikaansaamaa humoraalista ja soluvälitteistä immuniteettia, ja SARS-CoV-2-läpimurtotartuntojen vaikutusta immuniteettiin. Analysoimme rokotetuilta henkilöiltä saatujen seeruminäytteiden vasta-aineita ELISA- ja mikroneutralisaatiotesteillä, sekä solunäytteitä aktivaatioindusoiduilla markkeritesteillä ja virtaussytometrialla soluvälitteisen immuniteetin osoittamiseksi.

Osoitimme, että kolme rokotusta Spikevax- ja Comirnaty mRNA-rokotteilla indusoi korkeita tiittereitä pitkäkestoisia S1-spesifisiä vasta-aineita, joilla oli hyvä neutralisoiva kapasiteetti varhaisia Omicron-alavariantteja BA.1, BA.2 ja BA.5 vastaan. Neljännen ja viidennen rokoteannoksen, jotka perustuivat myöhempiin Omicron-alavariantteihin, osoitettiin indusoivan korkeatiitterisiä ja hitaammin häviäviä S1-spesifisiä vasta-aineita myöhempiä alavariantteja BQ.1.1, XBB.1.5 ja JN.1 vastaan. Vaikka vasta-aineet olivat edelleen neutralisoivia ja suojaavia, niiden neutralisaatiokapasiteetti uudempia alavariantteja vastaan oli heikompi verrattuna vanhempiin alavariantteihin. Läpimurtoinfektion ja rokotusten yhdistelmä tuotti korkeimman neutralisaatiokapasiteetin. SARS-CoV-2 rokotteet indusoivat tehokkaita CD4+- ja CD8+ T-soluvasteita. Havaitsimme, että A/Astrakhan/3212/2020 (H5N8)-kaltaiseen kantaan perustuva alayksikörokote indusoi korkean neutralisaatiokapasiteetin omaavia vasta-aineita, ja vahvan CD4+ T-soluvasteen linjaan 2.3.4.4b kuuluvia H5N1 -viruksia vastaan.

Tämän väitöskirjan tulokset osoittavat inaktivoitujen alayksikkö- ja mRNA rokotteiden olevan tehokkaita indusoimaan humoraalisia ja soluvälitteisiä immuunivasteita uudempia SARS-CoV-2-variantteja ja AIV-kantoja vastaan.

AVAINSANAT: SARS-CoV-2, Omicron, Rokotus, H5N1, vasta-aine, soluvälitteinen immuniteetti

Table of Contents

Abbreviations

ACE-2	angiotensin-converting enzyme 2
AIM	activation-induced marker assay
AIV	avian influenza virus
ARDS	acute respiratory distress syndrome
BCR	B-cell receptor
BSA	bovine serum albumin
CD	cluster of differentiation
CDR	complementarity determining region
CI	confidence interval
CLP	common lymphoid progenitor
COVID-19	coronavirus disease 2019
CTD	C-terminal domain
cTfh	circulating T follicular helper cell
CVV	candidate vaccine virus
DC	dendritic cell
DMEM	Dulbecco's modified eagle medium
DMSO	dimethyl sulfoxide
DMV	double membrane vesicle
DPP4	dipeptidyl peptidase 4
E	envelope protein (SARS-CoV-2)
EIA	enzyme immune assay unit
ELISA	enzyme-linked immunosorbent assay
EMA	European Medicines Agency
ER	endoplasmic reticulum
ERGIC	endoplasmic reticulum-Golgi intermediate compartment
Fab	fragment antigen-binding region
Fc	fragment crystallizable region
FCS	fetal calf serum
FBS	fetal bovine serum
FDC	follicular dendritic cell
Fimea	Finnish Medicines Agency

FR	framework region
GalNac	N-acetylgalactosamine
GC	germinal center
GISAID	Global Initiative on Sharing All Influenza Data
GM	geometric mean
GMT	geometric mean titer
HA	hemagglutinin
HAU	hemagglutinating units
HCoV	seasonal human coronavirus
HI	hemagglutination inhibition assay
HPAI	highly pathogenic avian influenza
hRBC	horse red blood cells
HRP	horseradish peroxidase
HSC	hematopoietic stem cell
IAV	influenza A virus
ID50	50% inhibitory dose
IFN	interferon
Ig	immunoglobulin
IL	interleukin
LNP	lipid nanoparticle
LPAI	low-pathogenic avian influenza
M	membrane protein
M1	matrix protein 1
M2	matrix protein 2
MDCK	Madin-Darby canine kidney
MERS-CoV	middle eastern respiratory syndrome coronavirus
MHC	major histocompatibility complex
MNT	microneutralization test
Mpro	main protease
mRNA	messenger RNA
N	nucleocapsid protein (SARS-CoV-2)
NA	neuraminidase
Neu5ac	N-acetylneuraminic acid
NK	natural killer cell
NKT	natural killer T-cell
NLS	nuclear localization signal
NP	nucleoprotein
Nsp	non-structural protein
NTD	N-terminal domain
OAS	original antigenic sin

OD	optical density
PA	polymerase acidic protein
PAMP	pathogen-associated molecular pattern
PB1	polymerase basic protein 1
PB2	polymerase basic protein 2
PBMC	peripheral blood mononuclear cell
PBS	phosphate-buffered saline
PLpro	papain-like protease
PRC	People's Republic of China
R-nought	basic reproduction number
RBD	receptor-binding domain
RDE	receptor destroying enzyme
RdRp	RNA-dependent RNA polymerase
RNA	ribonucleic acid
RPMI-1640	Roswell Park Memorial Institute medium 1640
S	spike protein
S1	spike subdomain 1
S2	spike subdomain 2
SARS-CoV-1	severe acute respiratory syndrome coronavirus 1
SARS-CoV-2	severe acute respiratory syndrome coronavirus 2
SHM	somatic hypermutation
SI	stimulation index
+ssRNA	positive-sense single-stranded RNA
-ssRNA	negative-sense single-stranded RNA
T_{FH}	T follicular helper cell
T_{H1}	type 1 helper T-cell
T_{H2}	type 2 helper T-cell
T_{H17}	type 17 helper T-cell
T_{REG}	regulatory T cell
TCID50	50% tissue culture infective dose
Tcm	central memory T-cell
Tem	effector memory T-cell
TEMRA	terminally differentiated effector memory T-cell
THL	Finnish Institute for Health and Welfare
TMB	3,3',5,5'-tetramethylbenzidine
TMPRSS2	transmembrane serine protease 2
TuDMM	Turku Doctoral Programme of Molecular Medicine
TYKS	Turku University Hospital
V(D)J	variable-diversity-joining recombination
VOC	variant of concern

vRNP	viral ribonucleoprotein complex
WHO	World Health Organization

List of Original Publications

This dissertation is based on the following original publications, which are referred to in the text by their Roman numerals:

I Reinholm, A., Maljanen, S., Jalkanen, P., Altan, E., Tauriainen, S., Belik, M., Skön, M., Haveri, A., Österlund, P., Iakubovskaia, A., Pasternack, A., Naves, R. A., Ritvos, O., Miettinen, S., K Häkkinen, H., Ivaska, L., Tähtinen, P. A., Lempainen, J., Kantele, A., Kakkola, L., Julkunen, I., Kolehmainen, P. (2024). Neutralizing antibodies after the third COVID-19 vaccination in healthcare workers with or without breakthrough infection. *Communications medicine*, *4*(1), 28. https://doi.org/10.1038/s43856-024-00457-3

II Belik, M., Reinholm, A., Kolehmainen, P., Heroum, J., Maljanen, S., Altan, E., Österlund, P., Laine, L., Ritvos, O., Pasternack, A., Naves, R. A., Iakubovskaia, A., Barkoff, A. M., He, Q., Lempainen, J., Tähtinen, P. A., Ivaska, L., Jalkanen, P., Julkunen, I., & Kakkola, L. (2024). Long-term COVID-19 vaccine- and Omicron infection-induced humoral and cell-mediated immunity. *Frontiers in immunology*, *15*, 1494432. https://doi.org/10.3389/fimmu.2024.1494432

III Reinholm, A., Khan, H., Laakso, T., Maljanen, S., Jalkanen, P., Gunell, M., Kallonen, T., Österlund, P., Ritvos, O., Nousiainen, A., Häkkinen, H. K., Pakkanen, S. H., Välimaa, H., Kantele, A., Lempainen, J., Julkunen, I., Kolehmainen, P., & Kakkola, L. (2025). Long-term neutralization capacity of vaccine and breakthrough infection induced SARS-CoV-2 specific antibodies against omicron subvariants BA.2, XBB.1.5, and JN.1. *Vaccine*, *68*, 127894. https://doi.org/10.1016/j.vaccine.2025.127894

IV Liedes, O., Reinholm, A., Ekström, N., Haveri, A., Solastie, A., Vara, S., Rijnink, W. F., Bestebroer, T. M., Richard, M., de Vries, R. D., Jalkanen, P., Lindh, E., Ikonen, N., Grifoni, A., Sette, A., Laaksonen, T., Holopainen, R., Kakkola, L., Lappalainen, M., Syrjänen, R. K., Kolehmainen, P., Julkunen, I., Nohynek, H., Melin, M. (2026). Influenza A(H5N8) vaccine induces humoral and cell-mediated immunity against highly pathogenic avian influenza clade 2.3.4.4b A(H5N1) viruses in at-risk individuals. *Nature microbiology*, *11*(1), 155–168. https://doi.org/10.1038/s41564-025-02183-5

1 Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) emerged in December 2019 and was the lead cause for the pandemic that infected millions of humans, causing enormous damage to the health and economy of people around the world.[1–3] Although the pandemic has been successfully contained, new emerging SARS-CoV-2 variants continue to infect people around the world and pose the risk of mutating to strains capable of reigniting the pandemic.[4,5]

Vaccinations against SARS-CoV-2 have been effective in preventing hospitalizations, healthcare burden, and serious complications from the disease caused by SARS-CoV-2, the causative agent of coronavirus disease-2019 (COVID-19).[6–9] Current vaccination programs have been proven effective against SARS-CoV-2, but the enhanced immune evasion abilities gained from rapid mutation rate of the virus remain an issue.[10–12] Evasive variants, such as XBB.1.5 and JN.1, emerging from these mutations can cause breakthrough infections, in which a vaccinated individual still gets an infection despite being vaccinated.[11,13] Newer vaccine types have been effective in combating these recent evasive variants, but their efficiency against emerging variants is unknown, especially in the long term.[5] Although the world has become more resilient and used to SARS-CoV-2, elderly or immune-deficient individuals are still at risk of getting a life-threatening disease.[14,15] Additionally, young and older individuals alike are at risk of getting long-COVID, a complication which reduces the quality of life for many months after the primary COVID-19.[16,17] Avian influenza A/H5N1 is a highly pathogenic avian influenza (HPAI) that caused several epidemics in bird populations and fur farms in coastal Finland in 2023.[18–20] These epidemics are a part of a wider pandemic that has been ongoing in wild birds since 2020.[21] Like SARS-CoV-2, A/H5N1 has the potential to cause a pandemic in humans, and its potentially devastating effects are showcased by previous pandemics inflicted by ancestral influenza strains.[22,23] To combat the rise of strains capable of infecting humans on a large scale, strategically vaccinating at risk-individuals, such as laboratory workers and fur farmers, can help prevent zoonotic spillover infections and greatly reduce the changes of adaptation to humans by A/H5N1. A/H5N8 based Seqirus vaccination has been administered to people in Finland, but to ensure that the vaccinations enhance immune protection against

A/H5N1, the responses in vaccinees must be studied at both humoral and cellular level.

In these studies, we utilized and optimized enzyme-linked immunosorbent assays, microneutralization assays, and activation induced marker assays coupled with flow cytometry to analyze the neutralizing capacities of vaccination and infection induced antibodies against SARS-CoV-2 Omicron subvariants BA.2, BA.2, BA.5, XBB.1.5, JN.1 and KP.3, and T-cell responses to SARS-CoV-2 S1 and N proteins from vaccinees up to 24 months post third- 12 months post-fourth-, and 3 months post-fifth vaccination. Avian influenza vaccine-induced immune responses were studied from the vaccinees after the first and second doses, with the analysis focusing on neutralizing capacities of antibodies against various influenza strains and T-cell responses to hemagglutinin (HA), neuraminidase (NA), and nucleoprotein (NP).

Our results obtained from these studies showed that SARS-CoV-2 Omicron variants are not efficiently neutralized by the three-dose COVID-19 vaccine regiment, while four and five dose regiments provided higher neutralizing antibody capacities that did not decline as rapidly. In the long-term, the frequency of breakthrough infections increased greatly. T-cell responses remained robust for several months post-vaccination. Individuals vaccinated with Seqirus H5N8 vaccine had an increase in T-cell responses and antibodies with high neutralization capacity against various influenza virus strains.

Overall, these results provide crucial novel information on the long-term effects of vaccines and hybrid immunity on SARS-CoV-2, and shed light in the long-term efficacy of the vaccines utilized in Finland. The results also prepare us for a potential avian influenza pandemic by providing information on the cross-reactive immune responses of H5N8 vaccine. While increasing the scientific community's basic knowledge on adaptive immunity, this project also gives valuable information for vaccination policy makers.

2 Review of the Literature

2.1 Coronaviruses

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a pleomorphic, enveloped Baltimore class IV virus capable of infecting humans, and is the causative agent for the coronavirus disease-2019 (COVID-19).[24] Like its predecessors SARS-CoV and MERS-CoV, it belongs in the genus *Betacoronavirus*, which is part of the subfamily *Orthocoronavirinae* in the *Coronaviridae* family. SARS-COV-2 first emerged in December 2019 in Wuhan, People's Republic of China (PRC), where it was isolated from a cluster of patients with a pneumonia.[2] From Wuhan, SARS-CoV-2 spread rapidly across the globe, infecting millions of humans during the course of the year, arriving to Finland in January 2020 from a Chinese national, and later from travellers coming from Spain, Italy, and Austria.[25] SARS-CoV-2 has been postulated to originate from bats, from which it spread to humans through intermediate hosts in the Wuhan Seafood Market, kickstarting the pandemic.[26,27] Candidate viruses in bats include RaTG13, which is a *betacoronavirus* with 96.1% sequence similarity to the original Wuhan variant.[28] (Figure.1)

SARS-CoV-2 primarily infects ciliated cells in the upper respiratory tract but is also capable of infecting a wide array of other cell types, such as type II alveolar cells in the lower respiratory tract, and myocardial cells in the heart.[29,30] Infection by SARS-CoV-2 causes COVID-19, which usually presents with mild symptoms in healthy adults, but can cause severe and sometimes lethal symptoms in immuno-compromised individuals, most notably in the form of acute respiratory distress syndrome (ARDS), a life-threatening condition associated with a decrease in angiotensin converting enzyme-2 (ACE-2) expression and lung damage.[31] COVID-19 can also present as a long-COVID syndrome, which can affect otherwise healthy adults.[16] SARS-CoV-2 spreads via inhalation of virions in the air, and by direct contact with fluid droplets caused by sneezing and coughing from an infected person, or indirect contact through fomites.

Seasonal human coronaviruses (HCoVs) have been circulating in humans at least since 1965 when the first one was isolated from a human patient.[32] HCoVs 229E and OC43 have been the most prevalent, with HKU3 and NL63 being discovered in the wake of severe acute respiratory syndrome coronavirus (SARS-CoV) epidemic in

2004 and 2005 respectively.[32,33] HCoVs belong to genera *Alphacoronavirus* and *Betacoronavirus*, and cause a relatively harmless disease with common cold -like symptoms, and overall the infections are much less severe compared to infections caused by variants that have emerged since SARS-CoV-1.[34]

SARS-CoV-1 and middle eastern respiratory syndrome coronavirus (MERS-CoV) were the first highly pathogenic coronaviruses to humans that emerged. Both are from the genus *betacoronavirus,* and emerged in the early 2000s. Both can cause ARDS, and have high mortality rates of 10% and 36% respectively. SARS-CoV has not been detected in humans since 2004, while MERS-CoV remains endemic in the Arabian Peninsula and causes sporadic spillover infections in humans.[35,36] SARS-CoV-1 also uses ACE-2 as its target receptor, while MERS-CoV uses dipeptidyl peptidase 4 (DPP4). Sequence similarity of SARS-CoV-2 is closer to SARS-CoV-1 than MERS-CoV, with approximately 79% similarity overall, but notably only 72% sequence similarity of the spike protein.[37] Similarly to SARS-CoV-2, SARS-CoV-1 started its spread from an animal market, and likely originated from bats.[38]

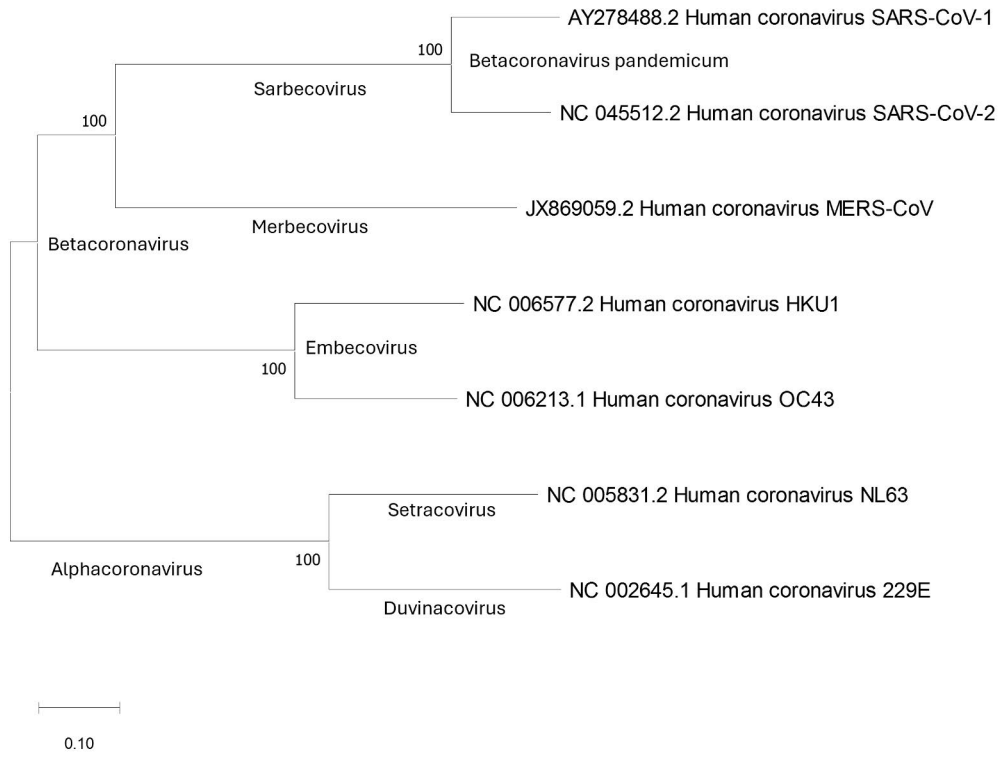

Figure 1. Phylogenetic tree of human infecting coronaviruses. Sequences were aligned with MUSCLE and phylogenetic analysis performed with GRT+G+I model. Made with MEGA 11.

2.1.1 SARS-CoV-2 Genome

SARS-CoV-2 has a non-segmented, approximately 30000 nucleotides long positive sense single stranded ribonucleic acid (+ssRNA) genome (Figure 2.). The genome is capped with a 5-prime cap structure and has a 3-prime poly-A tail. The genome encodes for spike (S), envelope (E), membrane (M), and nucleocapsid (N) proteins, which are structural proteins responsible for virion construction.[39,40] Sixteen nonstructural proteins and eleven accessory proteins are also encoded, and they play a role in viral genome replication and evasion of host immune responses.

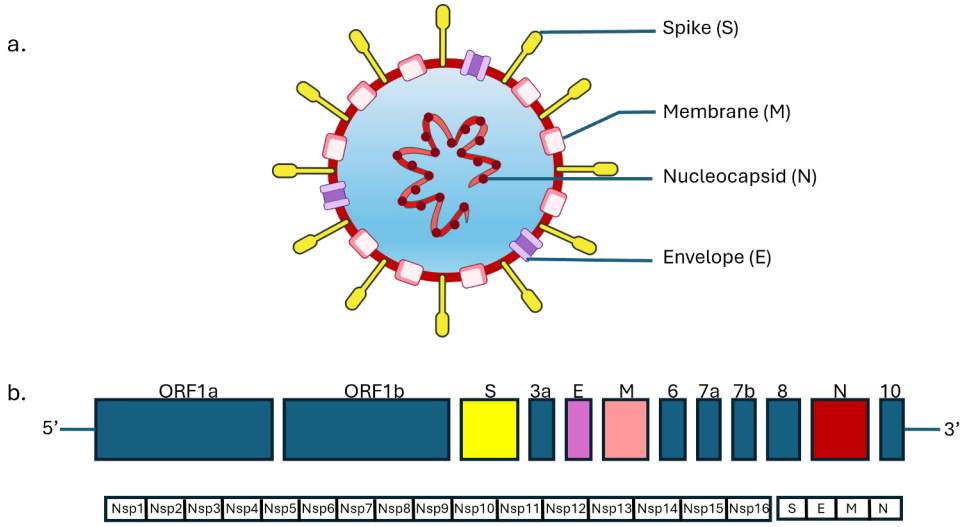

Figure 2. SARS-CoV-2 genome organization and virion structure. Made with public domain assets courtesy of NIAID.

2.1.2 Spike protein

SARS-CoV-2 S protein is approximately 1273 amino acids long type 1 membrane protein that is located as a trimer in the virion envelope.[41,42] (Figure 3[43]) The S protein consists of a signal peptide and two subunits, S1 and S2. The S1 subunit contains the receptor-binding domain (RBD), responsible for receptor recognition and binding[42], and the N-terminal domain, which regulates virus entry efficiency and contributes to the stability of the S protein.[44,45] The S2 subunit contains the fusion peptide and heptanucleotide repeats 1 and 2, which are responsible for membrane fusion, and the transmembrane domain which anchors the S protein to the virus envelope.[29]

The S protein is heavily glycosylated, which affects its antigenicity, immunogenicity, and ACE-2 receptor binding strength, which all contribute to the immune evasion capabilities of the mature virion.[46,47]

S protein contains a S1/S2 furin cleavage site typical for the type 1 viral fusion proteins, which is cleaved during its biosynthesis, destabilizing the S protein and enhancing ACE-2 binding of the S1.[48] The S protein is further cleaved during receptor attachment by transmembrane protein serine 2 (TMPRSS2) at S2' located downstream of the fusion peptide to facilitate the conformational changes of S2 necessary to expose the hydrophobic regions of the S-protein for membrane fusion and cell entry.[41,48]

The S protein is highly immunogenic, with the RBD being the primary target for antibodies.[29] Coincidentally, it is one of the main sites of mutations in the virion.

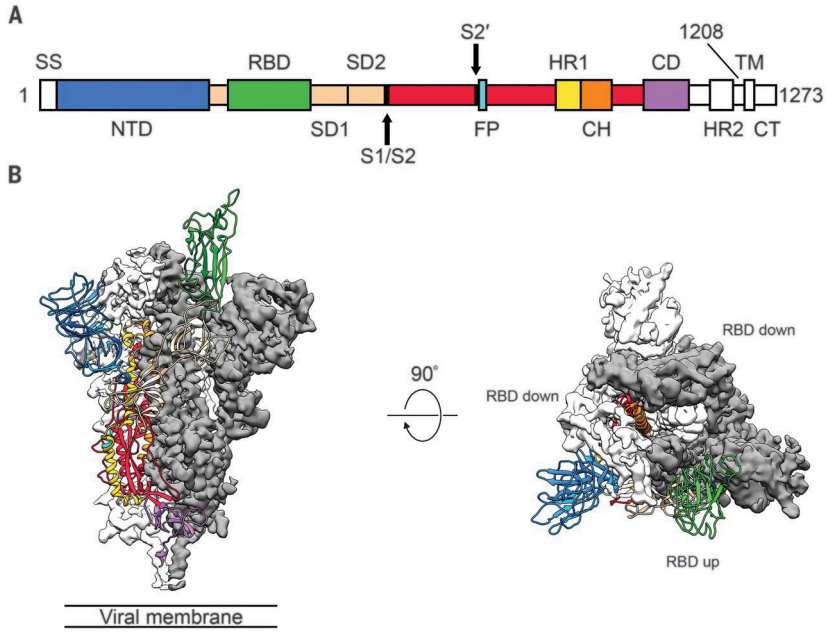

Figure 3. SARS-CoV-2 spike protein structure. A. Domains of the spike protein. SS, signal sequence; S2′, S2′ protease cleavage site; FP, fusion peptide; HR1, heptad repeat 1; CH, central helix; CD, connector domain; HR2, heptad repeat 2; TM, transmembrane domain; CT, cytoplasmic tail. Arrows denote protease cleavage sites. B. Trimeric form of the spike proteins from the side and bottom. Colors correspond to panel A. Figure from https://doi.org/10.1126/science.abb2507

2.1.3 Nucleocapsid protein

SARS-CoV-2 N protein is approximately 419 amino acids long protein responsible of binding to and wrapping the viral RNA.[49] N protein consists of three distinct domains: RNA-binding domain flanked by a disordered N-terminal domain (NTD),disordered central linker, and a dimerization domain flanked by a disordered C-terminal domain (CTD).[49–51] The disordered regions allow great steric flexibility

to the N protein, and for it to self-assemble into oligomeric filaments.[52] The CTD has been shown to bind to the membrane protein, making it important in virion assembly and its initiation.[53]Unlike the other structural proteins, N-protein is translated outside of the replication organelles formed by Nsps. N-proteins instead localize to the cytosolic side of the ROs after translation, where they are readily available for coating of viral subgenomic and genomic mRNA.[54]The coating is driven by RNA binding domains in the NTD and CTD.[51]

Like the S protein, the N proteins are highly immunogenic, inducing high titers of anti-N antibodies.[49] The N-protein is a potent activator of the complement pathway, and is linked to lung inflammation caused in ARDS in severe COVID-19 cases.[55]

Other structural proteins, nonstructural proteins (Nsp), and accessory proteins and some of their functions are listed in the table 1. In addition to the listed functions, many of the Nsps and structural proteins suppress the host immune system similarly to the accessory proteins.[56]

Table 1. SARS-CoV-2 proteins and some of their functions.

Structural protein	**Function of the protein**
E	Virion assembly and budding, viroporin[57]
M	Virion assembly and budding, most abundant structural protein in the membrane[58]
Non-structural proteins	
Nsp1	Inhibition of host mRNA translation and innate immune responses [59,60]
Nsp2	Repression of interferon production[61]
Nsp3	Participation in viral replication, part of the replication/transcription complex[62]
Nsp4	Replication organelle formation[63]
Nsp5	Cleavage of polyproteins with the Main Protease (Mpro) domain [64]
Nsp6	Replicase organelle formation[65]
Nsp7	RNA binding, part of the viral RdRp complex[66,67]
Nsp8	Primase, part of the viral RdRp complex[66,67]
Nsp9	ssRNA binding[68,69]
Nsp10	Co-factor for Nsp14 and Nsp16[70,71]
Nsp11	Unknown
Nsp12	Catalytic subunit of the RdRp[66,72]
Nsp13	Helicase and nucleoside triphosphatase[73,74]
Nsp14	Proof-reading exonuclease and methyltransferase[75,76]
Nsp15	Endoribonuclease[77]
Nsp16	Methyltransferase[78]

Accessory proteins	
ORF3a	Viroporin[79]
ORF3b	Type I interferon antagonist[80]
ORF3c	Interferon-beta antagonist[81]
ORF3d	Unknown, possibly not translated[82]
ORF6	Type I interferon antagonist[83]
ORF7a	Type I interferon antagonist by inhibiting STAT2 phosphorylation[84]
ORF7b	Apoptosis promotion, type I interferon inhibition[84,85]
ORF8	MHC-1 downregulation, type I interferon inhibition[83,86]
ORF9b	Unclear, possible contribution to immune evasion[87]
ORF9c	Unclear, possible contribution to immune evasion[87]
ORF10	Unknown

2.1.4 SARS-CoV-2 target receptors

ACE-2 is the primary cell surface receptor for the SARS-CoV-2. It is a 805 amino acids long type-1 membrane protein expressed on the surface of many cell types, particularly in the alveolar epithelial type 2 cells that are abundant in lung tissue, and nasal epithelial cells.[88,89] Its biological function is to regulate the renin-angiotensin system by cleaving AngI and AngII. Decreased ACE-2 expression has been shown to correlate with severe COVID-19 and increased lung-injury during ARDS.[90]

Transmembrane protein serine 2 (TMPRSS2) is a co-receptor for SARS-CoV-2 and is essential for the cell surface mediated entry pathway. It is an approximately 492 amino acids long type-2 transmembrane serine protease predominantly expressed in its membrane bound form.[91–93] TMPRSS2 cleaves serine residues of proteins near cell membranes. In monkey kidney cell line and human airway organoid models, Omicron subvariants have been shown to utilize TMPRSS2 to lesser extent in their cell entry when compared to previous variants such as the Delta variant.[94,95]

Neuropilin-1 and cathepsin L proteins have also been implicated in enhancing SARS-CoV-2 infectivity and cell entry, especially in endothelial and epithelial cells located in the olfactory and upper respiratory areas.[96,97]

2.1.5 SARS-CoV-2 cell entry and coronavirus replication

SARS-CoV-2 enters the host cell by binding to the ACE-2 receptor (Figure 4.).[98] The binding induces conformational changes that expose the S2’ site in the S2 domain of S protein, triggering an entry to the cell through clathrin-mediated cell surface entry pathway or endosomal pathway. In the cell surface entry pathway, the membrane fusion is assisted by the TMPRSS2 receptor, which cleaves the S2’ site,

triggering membrane fusion on the cell surface and leading to the direct internalization of the viral nucleocapsid.[29]

The endosomal route is triggered in the absence of TMPRSS2, which leaves the S2' site of the spike protein uncleaved, inhibiting membrane fusion and thus the direct release of viral nucleocapsid into the cytosol. Instead, the S protein and the virion are internalized by the host cell, enclosing the virion into an early-stage endosome. In the endosome the pH is lowered, activating cathepsin L, which cleaves the S2' site of the spike protein, triggering membrane fusion of the endosome and viral envelope.[97,99]

In both entry pathways, membrane fusion is followed by the subsequent release of the viral genome to the host cells cytosol. SARS-CoV-2 replicates in the cytosol, where the genome is immediately upon entry translated to two precursor polypeptides pp1a and pp1ab by host cell ribosomes.[100] After translation, the autocleavage of both of the polyproteins by papain-like protease (PLpro) located in Nsp3 and main protease (Mpro) located in nsp5 releases non-structural proteins 1–11.[64,100,101] These proteins shutdown host protein synthesis, inhibit host mRNA export, reshape the cell membranes into virus replication organelles, and slow down the cell's immune responses.[102,103] The synthesis of early infection Nsps is followed by the synthesis of Nsps 12-16, which are the main proteins responsible for viral RNA synthesis.[104]

After the initial polypeptide translation and cleavage upon entry, viral mRNA replication takes place in the viral replication organelles composed of double membrane vesicles (DMV) formed by Nsp3-4 protein complexes and Nsp6 interacting with host cell ER membrane and other endomembranes.[65,105,106] The replication is undertaken by the viral RNA-dependent RNA polymerase (RdRP). Although the RdRP possesses a proof-reading function, it still relatively frequently makes mistakes resulting into a relatively high mutation rate.[107] Since the DMVs where the replications takes place do not contain ribosomes, the replicated viral RNA and subgenomic mRNA are transported to the endoplasmic reticulum (ER) for translation.[106,108,109] Translated proteins consist of precursor polyproteins, as well as individual viral proteins. Of the newly translated proteins, structural S, E, and M proteins are localized on the surface of the ER membrane, which buds into vesicles coated with membrane trafficking proteins.[110,111] The vesicles are subsequently transported to the ER-Golgi intermediate compartment (ERGIC) by motor proteins along microtubules and vimentin filaments, guided by localization signals.[112–115] The vesicles fuse with the ERGIC membrane, to which the viral membrane proteins are subsequently distributed.[116] The full length viral genomes encapsulated by N-proteins, guided by packaging signals, associate with the M proteins on the surface of the ERGIC, condensating into a tight structure that buds into the lumen of an ERGIC compartment.[53,109,117–119] Finally, the mature virions egress from the cell via lysosomes, ESCRT pathway, and possibly through apoptosis of the host cell.[120–122]

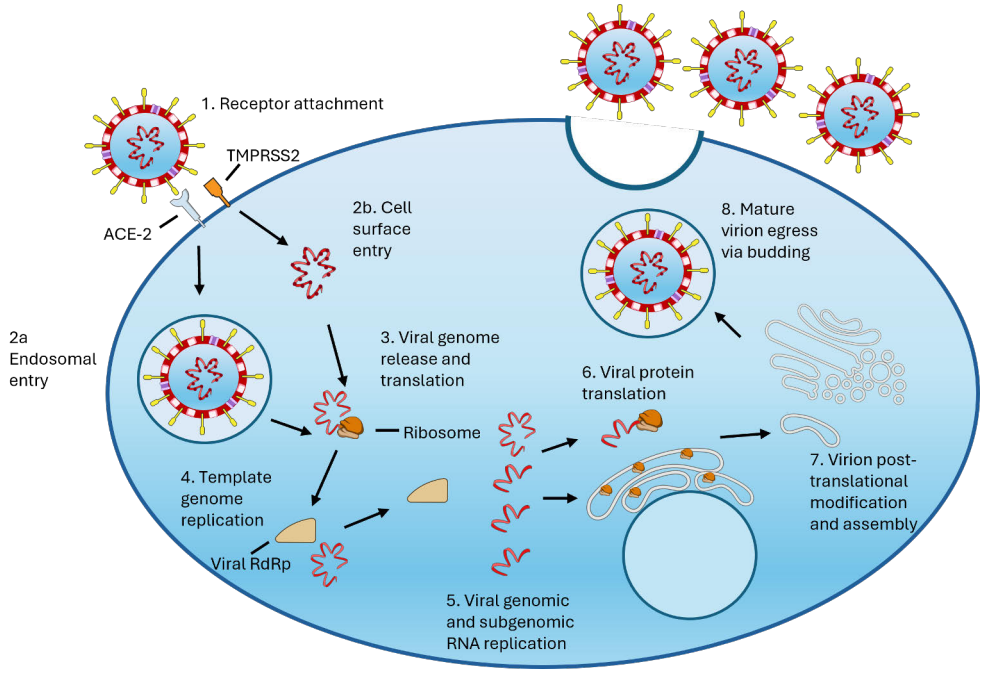

Figure 4. SARS-CoV-2 replication cycle. Made with public domain assets courtesy of NIAID.

2.1.6 SARS-CoV-2 variants

The original Wuhan variant of SARS-CoV-2 quickly sprouted to multiple new variants after its initial spread around the world (Figure 5.). The first major variant of concern (VOC) recognized by the WHO was B.1.1.7 (Alpha) variant, which was first detected in September 2020, and was rapidly followed by the B.1.351 (Beta) and B.1.617.2 (Delta) variants. All of these early variants were distinguished from the original Wuhan variant by their enhanced transmissibility and virulence, which contributed to their increased severity and infectivity.[123]

The first Omicron variant B.1.1.529 (BA.1) emerged in South Africa in November 2021. Compared to the previous SARS-CoV-2 variants, the BA.1 variant was substantially more efficient at evading the adaptive immune responses primed by previous infections and vaccinations, as well as being more readily transmitted.[124,125] It harbored 60 amino acid mutations compared to the original Wuhan strain, with about half of them being in the S-protein.[24] Most of the mutations in the S-protein were unique to Omicron, with a substantial amount of the mutations occurring in the RBD region of the S1-subdomain.[126] These S-protein mutations have been shown to increase the affinity of S-proteins towards ACE-2, which may contribute to its increased transmissibility.[127] The mutations are also a major

contributor to enhanced immune evasion capabilities and reduced pathogenicity of BA.1.[12,95]

BA.1 was followed by the emergence of subvariant B.1.1.529.2 (BA.2) in late 2021 in South Africa. BA.2 had enhanced transmissibility compared to BA.1 and spread rapidly.[126,128] It served as a common precursor for multiple recombinants and BA.2.86 sublineages. B.1.1.529.5 (BA.5) likewise emerged in late 2021 in South Africa. Despite emerging around the same time as BA.2, it rapidly outcompeted BA.2 early 2022 likely due to its enhanced immune evasion capacity, but did not completely replace it and its sublineages.[129–131] BA.5 was later outcompeted by recombinant Omicron variants and BA.2 descendants.

XBB.1.5 is a descendent of XBB, the first Omicron subvariant that emerged via recombination of Omicron BA.2 subvariants BJ.1 and BM.1.1.1.[13,132] XBB manifested increased immune evasion capacities when compared to previous variants. In addition to the mutations garnered by XBB, the XBB.1.5 subvariant harbored an additional S486P mutation in its S-protein, which enhanced its ACE2 binding affinity compared to XBB. This gave XBB.1.5 a significant growth advantage to the other circulating variants, resulting to its quick spread and dominance in the USA in early 2023.[13,133]

BA.2.86.1.1 (JN.1) is a descendant of BA.2 and first emerged in late 2023. JN.1 is a close descendant of the BA.2.86 subvariant, from which it differs by 4 amino acids, with the most notable mutation being the L455S mutation in its S-protein. The L455S mutation has been shown to aid in the immune evasion of neutralizing antibodies.[11,134] Like BA.2.86, it has increased transmissibility compared to the BA.2, BA.5, and XBB.1.5 variants, which may stem from its increased affinity to ACE-2 and its ability to utilize TMPRSS2 co-receptor more efficiently.[135,136]

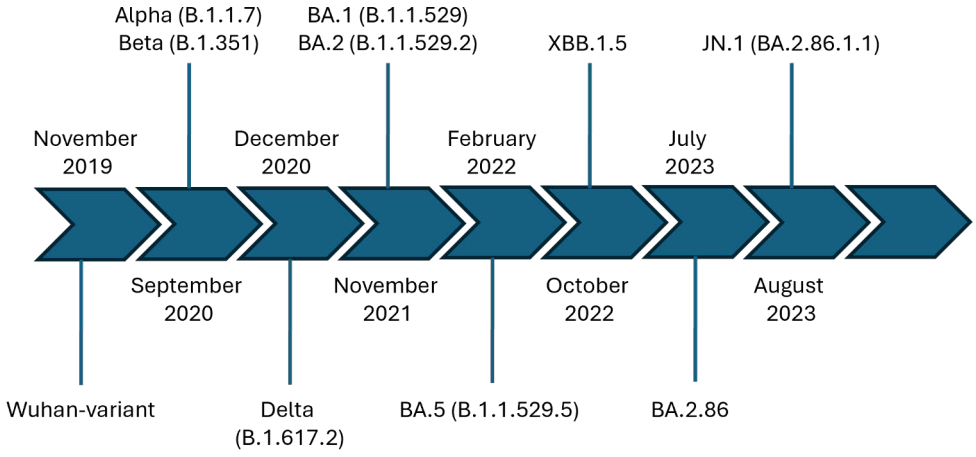

Figure 5. Timeline for the emergence of notable SARS-CoV-2 variants.

JN.1 has been followed by a plethora of subvariants carrying the FliRt mutation combination, such as LP.8.1 and NB.1.8.1 Omicron subvariants which are currently prevalent in Finland and continue to evolve[4,131]

The frequent emergence of novel SARS-CoV-2 variants has necessitated the updating of SARS-CoV-2 mRNA vaccines to utilize more recent variants as their templates to combat the ever increasing immune evasion capabilities of new variants.

2.2 Avian Influenza Virus

2.2.1 Influenza A virus genome

IAV has a segmented, approximately 13500 nucleotides long negative-sense single-stranded ribonucleic acid (-ssRNA) genome, with a total of 8 segments, each encoding for different proteins (Figure 6.). The genome encodes for structural proteins hemagglutinin (HA), neuraminidase (NA), and nucleoprotein (NP). Three polymerase forming proteins, two ion channel forming membrane bound proteins, and two non-structural proteins are also encoded.[137,138] The main functions of these proteins are displayed in table 2. In addition to the listed function, similarly to SARS-CoV-2 proteins, many of the IAV proteins inhibit host immune responses.

Each segment of the IAV genome is coated by NPs, and have a RNA-dependent RNA polymerase (RdRp) complex formed by polymerase basic proteins 1 and 2 (PB1 and PB2), and polymerase acidic protein (PA) attached to them.[139] All these components together form the viral ribonucleoprotein complex (vRNP), which is further surrounded by a capsid formed of matrix protein 1 (M1) and a membrane embedded with HA, NA, and matrix – 2 protein (M2).[140]

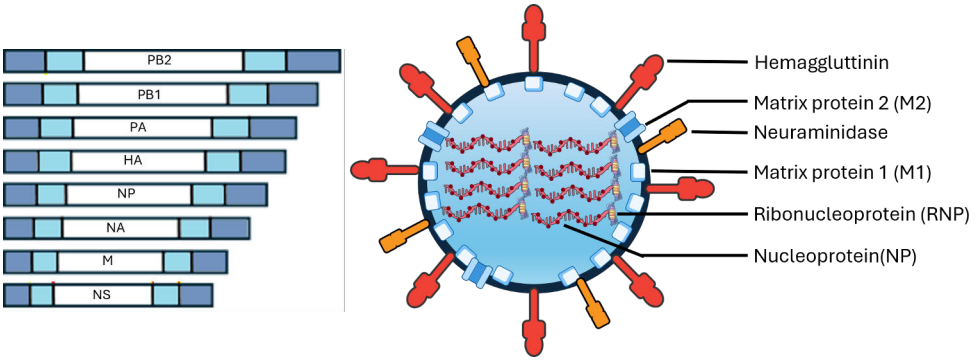

Figure 6. IAV genome organization and virion structure. Made with public domain assets courtesy of NIAID.

Table 2. IAV proteins and some of their functions.

Structural proteins	Functions of the protein
Hemagglutinin (HA)	Viral attachment, membrane fusion[141]
Neuraminidase (NA)	Clevage of silalic acid, virion release[142]
Nucleoprotein (NP)	Coating of viral mRNA, nuclear trafficking, vRNA replication[143,144]
Matrix-1 (M1)	Virion assembly, nuclear export[145]
Matrix-2 (M2)	Viroporin[146,147]
PB1	Catalytic subunit of the viral RdRp[148]
PB2	Host mRNA CAP recognition, part of the viral RdRp[149,150]
PA	Exonuclease involved in CAP snatching and virus replication, part of the viral RdRp[151]
Nonstructural proteins	
NS1	Nuclear export of viral mRNA, inhibition of host interferon responses[152,153]
NS2	Nuclear export of vRNPs, viral mRNA replication, interferon production suppression[154–156]
Accessory proteins	
PB1-F2	RdRp upregulation, innate immunity suppression, pro-apoptotic viroporin activity[157]
PB1-N40	Unknown
PA-X	Host mRNA degradation[158]
PA-N155	Viral replication factor[159]
PA-N182	Viral replication factor[159]
M42	Viroporin[160]
NS3	Host specificity factor[161]

2.2.2 Hemagglutinin

Hemagglutinin (HA) of avian influenza viruses is a homotrimeric, approximately 568 amino acids long, membrane bound glycoprotein (Figure 7.[162]). Each of the three HA subunits is composed of two glycoproteins, HA1 and HA2, and can be divided into 4 subdomains: receptor binding domain (RBD), fusion subdomain (F), membrane anchor subdomain (M), and vestigial esterase subdomain (E).[163] The HA is translated as a HA precursor in the ER, and the precursor is proteolytically cleaved by cellular proteases to yield the HA1 and HA2 glycoproteins.[162]

The HA is the protein responsible for binding to sialic acids on the surface of the host cell, which act as receptors for the virion, initiating the viral entry and membrane fusion. HA2 contains the M subdomain which anchors the HA to the surface of the virion, while HA1 has the RBD responsible for the binding of sialic acid residues on the host cells surface.[141]

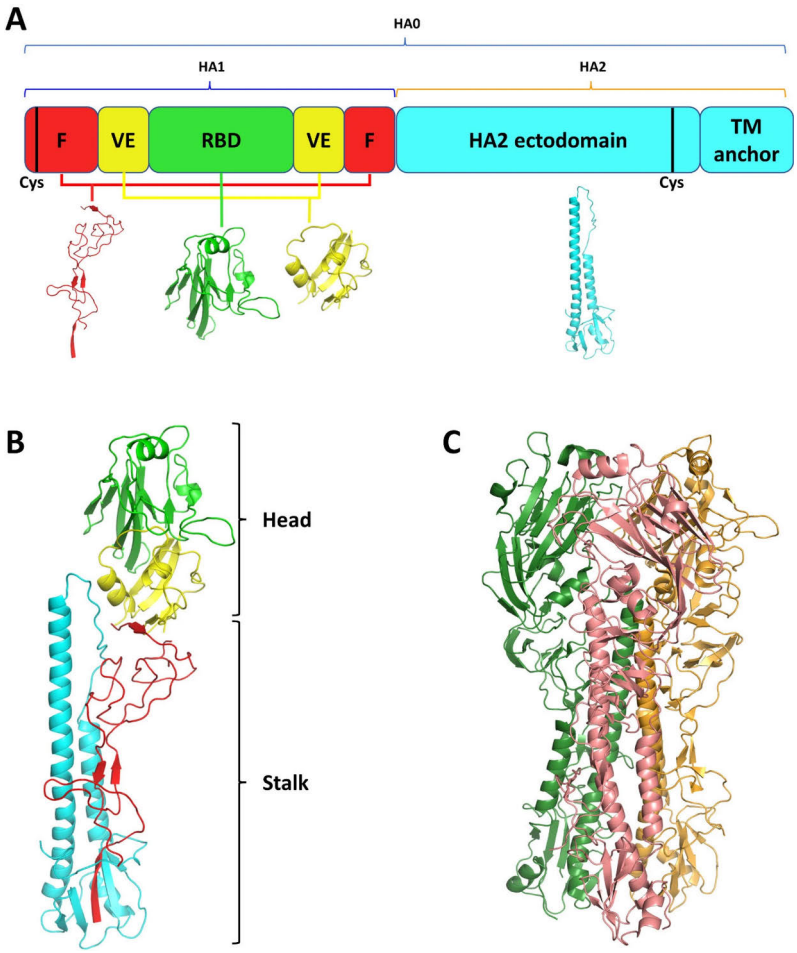

Figure 7. Hemagglutinin structure. (A) HA domains. F fusion domain (red), VE vestigial esterase domain (yellow), RBD receptor binding domain (green), TM anchor transmembrane anchor (cyan). The structure of domain is shown in corresponding color. (B) Structure of A/Denver/57/1957 HA protomer (PDB ID: 6ML8). Colors correspond to domain colors in panel (A). (C) Trimeric structure of the HA. Figure from Ghafoori et al. 2023, https://doi.org/10.1038/s41598-023-33529-w, under Creative Commons Attribution 4.0 International Licence.

In order for the virion to be infective, the HA precursor must be cleaved. In highly pathogenic strains of IAV, such as HPAIs, the HA precursor possesses a multibasic cleavage site, which is readily proteolytically cleaved to HA1 and HA2 by cellular proteases such as furin. HAs of low pathogenic IAV strains are also cleaved, but the cleavage site consists of a single arginine or lysine. Monobasic sites like this are cleaved by trypsin, which is an exogenous protease with limited expression in respiratory tissue, thus limiting infectivity of the low pathogenic IAV strains.[164,165]

IAV HA has 18 different subtypes ranging from H1 to H18, which mutate frequently due to antigenic shift and the generally high mutation rate of IAVs.[23] HPAIs are limited to H5 and H7 subtypes and although humans can be infected by them, humans are mainly infected by IAVs possessing HA subtypes H1, H2, and H3.[22,165] H9 bearing AIVs have also been known to infect humans.[166]

2.2.3 Neuramidinase

Neuraminidase (NA) is a tetrameric, approximately 499 amino acids long, membrane bound protein. Each of the four subunits consists of four domains: cytoplasmic tail, transmembrane domain, stalk, and catalytic head.[142] Main function of the NA is to cleave sialic acids linked to glycoproteins present on the cell membrane of the host cells, and cleaving of carbohydrate side chains present on the immature virions in the vicinity.[167–169] These cleaving events are essential in both the virion entry to the host cell and the egress of mature virions.[167,170,171] The cleaving activity of the NAs has also been shown to limits superinfections, as the reduced amount of sialic acids on infected cells decreases the likelihood of other IAVs to bind sialic acids to enter the cells.[172]

The length of the NA stalk region affects the tropism, infectivity, and pathogenicity of the IAV strain. In mammals, short stalk length has been shown to limit the transmission of A/H1N1, while longer stalk appears to enhance it. The longer stalk region might provide additional glycosylation sites, which can affect the stability of NA.[173,174] Notably, the currently circulating A/H5N1 clade 2.3.4.4b strains contain longer stalks. Complete absence of the stalk lead to attenuation of A/H1N1 in mice.[175]

IAV NA has 11 different subtypes ranging from N1 to N11. The subtypes N1 and N2 are the most common in human-infecting strains, with sporadic human infections by strains of subtypes N5, N7, and N9.[142]

Compared to HA, NA is less abundant on the surface of the mature virion, leading to HA skewed immune responses and immunological memory.[176]

2.2.4 Antigenic shift and drift

Due to the IAV genome being segmented and the lack of proof-reading function in the viral polymerase, IAVs undergo mutations at a high rate, with $>1 \times 10^{-3}$ mutations per site, per year.[177] The high mutation rate leads into the accumulation of point mutations, causing a phenomenon known as antigenic drift. Over time, this can lead to the emergence of IAV strains capable of evading the adaptive immune response due to the accumulation of mutations making the virion unrecognizable to the immunological memory.

Sometimes superinfection occurs when two different IAV strains infect the same host. The segmented nature of IAV genome makes the transfer and reassortment of large parts of individual segments between the IAV strains coinfecting the same cell possible.[178] This phenomenon, known as antigenic shift, can rapidly create novel strains capable of infecting humans, which can lead to pandemics due to the lack of immunity against them.[179] The rapid emergence and pandemic spread of A/H1N1pdm09 in 2009 is the prime example of this phenomenon.[180]

2.2.5 IAV receptors

Sialic acids are a diverse family of monosaccharides that are present on the surface membrane of many different cell types and are often located on the end of glycoproteins such as N-glycans.[181] They are negatively charged and hydrophilic, allowing them to act as shield to the underlying glycoproteins, preventing undesirable interactions with other cells, receptors, and enzymes. In the context of influenza viruses, they act as the primary receptor for the IAV virions.[182]

Sialic acids are linked to each other and other monosaccharides through glycosidic bonds, the type of which can have a significant impact on the infectivity and tropism of the IAVs.[183] Different linkages between the sialic acid and galactose or N-acetylgalactosamine (GalNac) greatly influence the tropism of IAV strains.[184] In humans and avians, N-acetylneuraminic acid (Neu5ac) is the main sialic acid type, and it is mostly conjugated to galactose.[182] α2-3 linkage is required for strains adapted to avian species, and α2-6 is required for strains adapted to humans. Animals possessing sialic acid bonds of both of these types, such as pigs, can be intermediate hosts capable of acting as an incubator to new reassortant viruses.[185,186]

Besides sialic acids, other co-receptors have been postulated to exist due to the ability of IAVs to infect cells even in absence of sialic acids.[187,188] For example, phosphorylated, non-sialylated glycans have been shown to be bound by a range of IAVs.[189]

2.2.6 IAV Entry and replication

IAVs enter the human host cell by binding with HAs to sialic acids on the surface of the cell (Figure 8.). Following attachment, the virion enters the cell by endocytosis via a clathrin-dependent pathway or through micropinocytosis.[190,191] The virion is internalized and is enclosed into an early-stage endosome, in which the pH is reduced from 7 to 5 by hydrogen-ion pumping V-type ATPases as the endosome matures to late stage.[192]

This reduction in the endosome's internal pH triggers conformation changes in the virion HAs, leading to partial dissociation of the HA1 glycoprotein and subsequent exposure of the HA2 glycoprotein.[193] In addition to the partial

dissociation, the conformation changes move the fusion peptide located in the HA2 closer to the endosome membrane, facilitating the attachment to the endosomal membrane and the formation of a pre-hairpin structure.[194] After attachment, the HA2 bends, bringing the endosomal and virion membranes close to each other, and form a hairpin structure which fuses the two membranes together.[195]

Before and after membrane fusion, the M2 ion channels on the virions surface uptake the H+ ions from the endosome. The subsequent acidification of the interior of the virion weakens the M1 protein capsid, rupturing it and releasing the vRNPs to the cytoplasm.[196] Once the vRNP is released to the cytoplasm, it is transported to the nucleus by host cell importin proteins. The nuclear transport is guided by the nuclear localization signal (NLS) sequences present in the NPs and polymerase proteins.[197] In the nucleus the virion undergoes further uncoating, and the viral -ssRNA is transcribed by the viral RdRp to viral mRNA.

IAVs lack a primase in their RdRp and thus initiate transcription of viral mRNA by cleaving and capturing CAP structures from host mRNAs.[198] This CAP snatching mechanism of the RdRp results in the synthesis of 5'-capped viral mRNAs, which are exported from the nucleus to the cytoplasm for translation into viral proteins by the host's protein synthesis machinery. Simultaneously, the export of the host's own mRNA molecules is inhibited to facilitate CAP snatching and to favor the translation of viral proteins.[199]

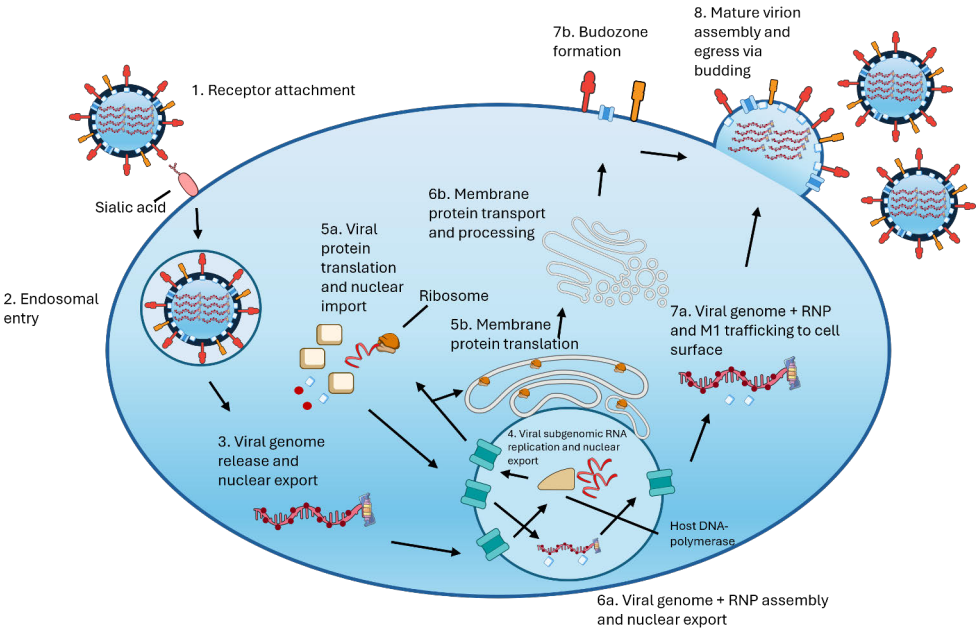

Figure 8. IAV replication cycle. Made with public domain assets courtesy of NIAID.

The transcribed viral mRNAs are trafficked to the ER or cytoplasm where they are translated by the host ribosomes.[195,200] The translated viral proteins are then transported to the Golgi for folding and post-translational modifications. Membrane proteins have already been folded and glycosylated in the ER, but attain further post-translational modifications in the Golgi.[200–202] Following translation, the protein components of the vRNP are imported to the nucleus, where the PB1, PB2, and PA form the viral RdRp, and the NPs coat the viral genome segments.[203] This is followed by the transport of vRNP and structural proteins on the cell surface membrane, where they are assembled into new virions.[204]

During assembly, the newly replicated and NP coated full length -ssRNA genome segments associated with the viral RdRp are packaged into virions. The assembly is initiated on the outer cell membrane by the clustering of HA and NA on lipid rafts enriched in cholesterol and sphingolipids, followed by recruitment of M1 to act as a scaffolding for vRNPs and M2.[205,206] Assembled virions then egress from the cell via M2 mediated budding, after which the NA proteins play a key role of cleaving SA residues to prevent the virion from being stuck on the surface of the host cell.[142,205] Egress may also happen via IAV induced apoptosis.[207]

2.2.7 HPAI clade 2.3.4.4b

An influenza clade is a taxonomical classification for a group of IAVs with similar HA sequences that are descendants from a common ancestor.[208] New clades arise as IAVs mutate through antigenic drift and antigenic shift into novel strains. The AIVs belonging to the clade 2.3.4.4 share A/H5N1/Goose/Guangdong/1/1996 as their last common ancestor, which is a HPAI A/H5N1 strain first detected in humans in 1997.[209] Prior to human infections, A/H5N1 had been detected in aquatic birds a year prior. Of the HPAIs, the A/H5N1 of clade 2.3.4.4b has been spreading around the world since 2013, when it was first detected in Eastern PRC.[210] The clade rapidly spread and diversified in the subsequent years, and in 2020-2021 it acquired mutations that lead to a significant increase in its infectivity to birds and other animals. Since 2021, outbreaks caused by A/H5N1s belonging to the clade 2.3.4.4b have been detected in birds in North America, South America, Africa, and Australia.[21,211,212] Besides birds, AIVs of clade 2.3.4.4b have been capable of infecting a wide variety of other animals, such as red foxes, sea lions, and dairy cattle.[213] Human infection have also been reported sporadically, for example in Chile, England and PRC.

In a recent outbreak in June 2023, gulls, poultry, and fur animals in Finland were infected with A/H5N1 of clade 2.3.4.4b, leading to mass deaths of the infected animals.[18,19,214] The epidemic quickly spread from fur farms and dead birds to wild

animals, infecting wild birds and mammals in Western and Southern Finland. In the infected mammals, PB2 had undergone adaptation to mammals.[19,20]

On the other side of the Atlantic, in March 2024, dairy cattle was infected with A/H5N1 of clade 2.3.4.4b in Texas and Kansas, USA, causing an outbreak that quickly spread to other states, resulting in an epidemic in 16 states by November 2024. Tropism towards the milk-secreting epithelial cells in the mammary glands of cows was especially noteworthy, given the presence of both α-2,6 and α-2,3 sialic acid linkages in the cells.[213,215] Between March 2024 and October 2024, at least 46 human spillover infections were detected in the USA, with 25 of them linked to infected dairy cows. None of the infected humans had a severe illness and mostly suffered from conjunctivitis and common influenza-like symptoms.[216] In human spillover infections, some adaptations to humans have been noticed, such as mutations leading to preferred binding of HAs with α -2,6 linked SAs. These changes are characterized by amino acid mutations in HA, leading to A134V, N182K, and E186D substitutions. However, these mutations have likely been introduced after several infection cycles in the human hosts and were not present in the vector animals.[217]

The rapid spread of A/H5N1 of clade 2.3.4.4b across the world via birds, and the adaptations leading to infections in mammals and especially humans prompted many nations, Finland included, to acquire a stockpile of AIV vaccines to vaccinate at-risk-groups.

2.3 SARS-CoV-2 and avian influenza vaccinations

2.3.1 Vaccines in general

Vaccines are based on weakened or killed pathogens, and are used to safely induce immunological memory against the disease causing live, wild type pathogen. Vaccines have greatly reduced disease burden since their inception and widespread adoption and continue to be one of the key defences against epidemics and pandemics.[218] Commonly used vaccine types against viruses utilize live attenuated virus, inactivated virus, subunits or proteins of the virus, and recently viral mRNA.[6,9,219–223]

Inactivated virus vaccines and vaccines utilizing live attenuated viruses are first vaccine types to have been developed.[224] Both the inactivation and attenuation completely eliminate or greatly reduce the ability of the pathogen to cause disease and efficiently replicate. However, the weakened pathogens still provide the adaptive immune system with the necessary epitopes for the development of a highly specific immune response in the event of an infection by the wild type pathogen the vaccine strains are related to.[223] Advances in technology allowed the development of subunit

vaccines through splitting inactivated viruses or by producing the subunits through recombinant technology.[219,222] These vaccine types use only parts of the pathogen, and are thus safer to administer since there is a minimal risk for a disease causing infection. The downside for this increased safety is the reduced immunogenicity of the vaccines, weaker cell-mediated immunity engagement, and often the need to be administer as multiple doses and accompanied by adjuvants.[219,222,223,225]

Vaccines utilizing viral mRNA are the latest vaccine type to have been employed on a large scale.[220,226] Unlike previous vaccine types, they contain no parts of the pathogen, but are instead composed of a mRNA encoding for antigenic pathogen proteins. This mRNA is encased by a lipid nanoparticle, which acts as an adjuvant and the delivery vehicle to cross the cell membranes.[227] Given their synthetic nature, they can be rapidly mass-produced and modified. Although mRNA vaccines are efficient and capable of engaging both the humoral and cell-mediated immunity, they need to be stored at temperatures as low as -80°C.[228]

The length of the immune protection induced by vaccines varies greatly, and sometimes multiple doses or annual booster doses are required to maintain protective immunity.[9,223,229,230]

2.3.2 Vaccinations in Finland

Vaccinations against SARS-CoV-2 in Finland begun in December 2020 with Comirnaty (BioNTech-Pfizer) vaccine based on the original Wuhan variant, and continued with Spikevax (Moderna), Vaxzevria (AstraZeneca), and Jcovden (Janssen Pharmaceuticals) vaccinations beginning in 2021.[9] Of all the approved vaccines in Finland, mRNA vaccines Comirnaty and Spikevax have been utilized the most. Both vaccines have been updated several times to include the circulating dominant VOCs as templates.[231,232] As of October 2025, Comirnaty LP.8.1 (BioNTech-Pfizer) is the main vaccine in use in Finland.[233]

Limited vaccinations in Finland against AIV have been conducted in the past in the years 2009, 2011, and 2018. The H5N1 epidemic in the summer of 2023 prompted the Finnish government to vaccinate at risk-individuals, such as fur farmers, veterinarians, bird ringers and laboratory workers, with the inactivated A/Astrakhan/3212/2020 (A(H5N8)) vaccine (Seqirus).[234,235]

Table 3 lists most common SARS-CoV-2 and AIV vaccines purchased or used in Finland.

Table 3. Most common SARS-CoV-2 and AIV vaccines used in Finland.

SARS-CoV-2 vaccines	Type	Template strain	Manufacturer
Comirnaty	mRNA, Spike	Various, currently SARS-CoV-2 Omicron subvariant LP.8.1	Pfizer-BioNTech
Spikevax	mRNA, Spike	Various, currently SARS-CoV-2 Omicron subvariant LP.8.1	Moderna
Vaxzevria	Adenovirus vector, Spike	SARS-CoV-2 Wuhan-Hu-1	AstraZeneca
Avian Influenza virus vaccines			
	Inactivated, split	A/H5N1/Indonesia/5/2005, clade 2.1.3.2	GlaxoSmithKline
	Inactivated, whole virus	A/H5N1/Vietnam/1203/2004-like, clade 1	Baxter
	Inactivated	A/H5N1/turkey/Turkey/1/2005 (H5N1)-like, clade 2.2.1	Novartis
	Inactivated, subunit, HA and NA	A/H5N8/Astrakhan/3212/2020-like strain (CBER-RG8A) vaccine, clade 2.3.4.4b	Seqirus Vaccines

2.3.3 Vaccines for SARS-CoV-2 and Avian Influenza

Spikevax and Comirnaty are vaccines developed early in the SARS-CoV-2 pandemic and are designed to convey protective immunity against various variants of SARS-CoV-2. They are both mRNA vaccines that utilize an mRNA segment encoding for the S-protein of SARS-CoV-2, encased in a lipid nanoparticle (LNP) as its delivery system.[231,232] The mRNA is engineered to be stable and efficiently translated with methods such as codon optimization, addition of 5'CAP structures, and modification of mRNA nucleotides.

The optimized mRNA is susceptible to degradation by ribonucleases before it is translated by host proteins synthesis machinery. To protect against this, the mRNA is packaged into a lipid nanoparticle. The LNP also assists the mRNA to pass through membranes by negating the negative charge of the enclosed mRNA.[227] The mRNA and the LNP together produce a strong enough adaptive immune response so that the use of adjuvants in vaccine is not necessary.[236]

mRNA vaccines need to be administered multiple times to confer long-lasting protective immunity, similarly to most inactivated vaccines.[222,237] mRNA vaccines induce both humoral immune responses, in the form of neutralizing antibodies, and cellular responses in the form of T-cell responses.[238,239] Both Spikevax and Comirnaty have been updated multiple times since the start of the pandemic by replacing the S-protein mRNA template of previous variant with a prevalent

circulating variant or subvariant. Spikevax contains a higher amount of template mRNA compared to the Comirnaty, with 50 ug of mRNA used as opposed to 10 and 30 ug.[231,232]

The zoonotic influenza vaccine Seqirus is a vaccine developed against H5 AIV strains. It is a subunit vaccine, which uses the HA and NA surface proteins of the inactivated A/Astrakhan/3212/2020 (H5N8)-like strain as immunogenic components.[234] Subunit vaccines build on the safety and lesser reactogenicity of inactivated vaccines, and only utilize the immunogenic parts of the inactivated virion. The virus used in them is inactivated with chemical treatment, such as with formaldehyde or beta-propiolactone, to render the virions non-infective.[222] The virion is split, and the immunogenic parts are isolated, purified, and concentrated, with rest of the virion parts discarded. Because of this, they usually are less immunogenic than inactivated whole viruses, and the addition of adjuvants such as MF59 is often essential to achieve efficient immunization[223,225]. Subunit vaccines primarily induce humoral immune responses and are weaker at inducing cell mediated immunity, especially CD8+ responses.[240,241]

Influenza vaccines are seasonally updated due to the rapid mutation rate of IAVs, and multiple vaccine strains that are annually selected by WHO are used to confer immunity against the most common circulating IAV and IBV strains.[230] Similarly, AIV vaccines utilize the most recent strains of concern as their template.

Applications route of vaccines and the vaccination timings can have significant impact on the formation of immunological memory and vaccine reactogenicity.[242] For example, long-lasting lymphocyte populations, which are strongly correlated with protective immunity and less severe disease in influenza, have been shown to be induced more broadly by intranasal vaccine based on the IAV NA compared to intramuscular injection vaccines.[243–246]

Both mRNA and inactivated virus vaccines have been efficient at preventing serious disease and reducing the healthcare burden. Nevertheless, their efficacy, safety, and reactogenicity are still undergoing constant studies to improve their efficiency.

2.4 Humoral and adaptive immune responses induced by SARS-CoV-2 and AIV vaccines and infections

Humans are under constant assault by pathogens such as bacteria, parasites, and viruses. To survive, the human body has evolved an effective defense system against these invaders. This defense is divided into innate and adaptive immunity, both of which interact and synergize with each other.[247] The innate immunity consists of physical barriers such as skin and mucosal tissue, as well as of proteins that can

quickly destroy pathogens, and cells with a broad but low specificity to antigens.[248] It is the first line of defense that both buys time for and presents the invading pathogens to the adaptive immunity, the response that takes days to develop against new pathogens. Adaptive immunity consists of cells that have high specificity towards the invading pathogens and are effective at quickly neutralizing them.[176,249,250] The cells of the adaptive immunity also form the immunological memory part of the immune system, which allows the body to mount a much faster and specific response against previously encountered pathogens during re-exposure or infection.[251,252]

Together, the innate and adaptive immunity form the immune system, the mechanism behind human immunity.

2.4.1 Antibody structure and classes

Antibodies are approximately 150–970 kDa in size, highly-specific membrane bound or secreted proteins produced by B-cells. Antibodies derive their incredible specificity for a given antigen from their immunoglobulin domains, which are 110 amino acids long β-sheet structures with a rigid scaffolding structure as a backbone complemented by regions consisting of malleable loose formations at their ends[253]. Antibodies have a distinct Y-shape, which is formed by two heavy (H) and two light (L) polypeptide chains linked to each other by disulfide bonds. The H chain region linked with the L chain is attached to the rest of the H chain by a flexible hinge region, giving the tips of the Y-shape flexibility to move and bind to epitopes. The tips have the antigen binding regions formed by approximately 110 amino acids long variable (V) regions in the H and L chains. The V regions contain highly variable complementary determining regions (CDR) and stable framework (FR) regions, which through V(D)J recombination during B-cell maturation and somatic hypermutation during antigen binding are tailored to bind to their target antigens with extremely high specificity.[254] Antibodies have neutralizing and non-neutralizing functions.[255] Fragment crystallizable region (Fc) mediates the non-neutralizing functions of the antibody, while fragment antigen-binding region (Fab) binds to the target epitope.[256]

Antibodies can be divided into 5 classes based on the H chains constant regions in their H chains (Figure 9.). Antibodies can be expressed in a membrane-bound or secreted form. IgG is the most abundant antibody class. IgG antibodies have the classic Y-shape and are secreted and expressed as 150 kDa monomers. They can be further divided into subtypes IgG1, IgG2, IgG3, and IgG4. IgG1 is the primary IgG subclass induced by antigens, while rest of the subclasses are more specifically induced for example upon allergen or parasite encounters, or cytokine stimulation.[257]

IgD does not have clearly defined role, but has been suggested to modulate B-cell selection.[258] It is one of the two antibodies expressed on the surface of maturing B-cells. IgE is secreted in response to parasitic infections and allergens. It is secreted as a monomer and weights approximately 150 kDA. IgA is prevalent in mucosal tissue and is efficient at neutralizing both viruses and bacteria, and is particularly efficient at combating toxins. It is approximately 320 kDa in its secreted, dimeric form and is, similarly to IgM, stabilized by a J chain. IgM is the largest antibody secreted in humans with size of approximately 970 kDa, and is the first one produced upon an infection. It is secreted as a pentamer stabilized by a J chain and disulfide bonds, but is also expressed in its monomer form on the surface of immature B-cells. The pentameric form allows it to bind to antigens with higher avidity, providing stabile binding despite low specificity.[254]

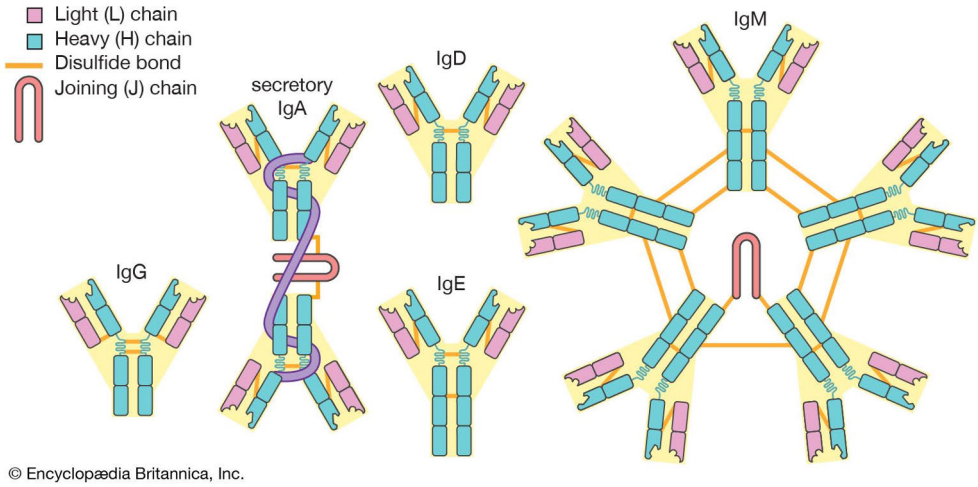

Figure 9. Antibody classes and their structure. By courtesy of Encyclopædia Britannica, Inc., copyright 2020; used with permission.

2.4.2 Cell-mediated responses

The cellular immunity arm of human immune system consists of leukocytes that are roughly divided into two cell lineages. The myeloid lineage consists of cell types that are pertinent in innate immune responses, while the lymphoid lineage is known for its role in adaptive immune responses.[259,260] Cells from both lineages have functions that extend beyond just innate or adaptive immunity, and often influence both arms of the immune system. These functions are mediated through secretion of messenger molecules called cytokines. Cytokines influence the proliferation of cells, differentiation of cells to specific cell types, and activation of cells. Most of the leukocytes secrete cytokines at a basal level but do so in substantially larger

quantities when activated by antigens and cytokines.[261] A large subfamily of cytokines called chemokines direct chemotaxis of the cells, directing them to the invading pathogens to employ their effector functions.

The myeloid lineage cells with immune effector functions consists of leukocytes, such as granulocytes, monocytes, and macrophages. They mainly engulf and destroy invading pathogens. Although these cells can present antigens to lymphocytes, with the exception of activated macrophages, they are not efficient at it. Non-leukocytic cells that stem from the myeloid lineages and are efficient at antigen presentation, while also having other important immune effector functions, include antigen-presenting dendritic cells (DC) and follicular dendritic cells (FDC).[247]

Dendritic cells express high levels of class II major histocompatibility complex (MHC) molecules on their surface and are essential in T-cell activation. They patrol for foreign antigens in blood and peripheral tissues, and are among the first leukocytes that are activated by foreign antigens. They detect pathogen associated molecular patterns (PAMP) present on the surface of the pathogens, which induce DCs to internalize them and migrate to lymphoid organs such as lymph nodes and spleen, where they present the antigen to CD4+ and CD8+ T – cells.[262] Besides T-cell activation functions, DCs secrete various costimulatory signals and chemokines, that influence the differentiation and proliferation of other leukocytes.

FDCs are distinct from DCs, and are located in lymph nodes, where they are an integral part of activated lymphoid follicles. FDCs maintain germinal center structures and present immune complexes to B-cells.[263] They also have other functions, such as cytokine secretion.

The lymphoid lineage is another subgroup within leukocytes and consists of T-lymphocytes (T-cells), B-lymphocytes (B-cells), natural killer cells (NK), and natural killer T cells (NKT). Lymphoid cells are classified based on different combinations of clusters of differentiation (CD) surface protein markers that are up- and downregulated by signaling molecules secreted by leukocytes and other cell types[264].

2.4.3 T-cells

T-cells are lymphocytes that arise from T-cell progenitor cells that stem from hematopoietic stem cells (HSCs). T-cell progenitor cells enter the thymus to undergo maturation through negative and positive selection, during which they acquire T-cell receptors that have sufficient affinity to self MHC I or self MHC II receptors that are complexed with foreign antigen. The mature T-cells are either CD4+ helper cells, or CD8+ cytotoxic cells. Upon activation by antigen presenting cells that display antigens complexed with either MHC I or MHC II receptors, they will start to exert their effector functions.[247]

As their name implies, CD4+ helper cells assist leukocytes in their activation, proliferation, and regulation by secreting guiding chemokines and cytokines. CD4+ cells differentiate to five main subtypes: T helper type 1 (T_H1), T helper type 2 (T_H2), T helper type 17 (T_H17), T follicular helper (T_{FH}), and regulatory T (T_{REG}) cells.[265] T_H1 cells are a common subtype, and secrete cytokines such as interleukin-2 (IL-2) and interferon-gamma (IFN-γ). These cytokines stimulate lymphocytes, and especially T-cells, to differentiate and proliferate. They also induce class-switching to IgG in B-cells and activate macrophages. T_H2 cells are another common subtype, and they regulate B-cell activity and proliferation via secretion of many different interleukins. T_H2 cells induce IgE class switching and inflammation among other effector functions. Both T_H1 and T_H2 cells downregulate the expression of each other, while simultaneously promoting the differentiation and proliferation of their own subtype. T_H17 cells are pro-inflammatory and they secrete IL-17A. T_{FH} cells are located in germinal center (GC) follicles where they assist B-cells by stimulating high affinity antibody production. T_{Reg} cells regulate other T-cells by suppressing their differentiation and proliferation by secreting IL-10 and other cytokines.[265]

CD8+ T-cells on the other hand interface directly with cells that display a processed antigen complexed with MHCI receptors on the plasma membrane. Based on the displayed antigen, CD8+ T-cells will either tolerate the cell or induce its apoptosis.[266]

Most CD4+ and CD8+ T-cells differentiate into effector cells upon their activation and quickly die off in the following days, but a small percentage differentiate into antigen specific memory T-cells that can persist for years in tissue and circulation, until reactivation by a secondary infection or antigen exposure.[267] Both CD4+ and CD8+ T-cells can form long-lived memory cells that circulate in the blood or take residence in tissues. These memory cells arise early on in the adaptive immune response, and they can be roughly divided into naïve, effector memory (Tem), central memory (Tcm) and terminally differentiated effector (TEMRA) subtypes.[268] The exact functions and their division among the memory cell subsets are debated but include effector functions such as cytokine secretion.[269,270] A key feature of the memory T-cells is the ability to differentiate and proliferate to effector types faster in a secondary infection by the same antigen.[250]

2.4.4 B-cells

B-cells are the antibody producing arm of the adaptive immune response. Like T-cells, they stem from HSCs that have differentiated to common lymphoid progenitors (CLP). These CLPs are located in the bone marrow, where they undergo maturation to B-cells through negative and positive selection. At the end of the maturation, B cells express B cell receptors (BCR) and low affinity antibodies on

their surface membrane and are ready to migrate to lymphoid organs. Follicular B-cells (B-2 cell) are the most common B cell type, and they express a battery of identical IgM and IgD immunoglobulins on their plasma membrane which bind with low specificity to a narrow range of antigens. B-2 cells primarily reside in lymphoid follicles in the lymph nodes and the spleen, where they scan for antigens they can bind to. They are able to bind and internalize small antigens, but require the help of antigen-presenting cells to internalize larger antigens. Besides B-2 cells, B-1 and marginal zone B cells also exist, but in smaller numbers.[271,272]

Upon binding an antigen, the B-2 cell internalizes and processes it, eventually presenting antigen-derived peptides on the surface membrane complexed with a MHC II receptor. The B-2 cell migrates near the T-cell zone in the lymph node, where the presented antigen is detected by T-cells. This leads to a T-cell dependent activation and causes the B-2 cell to migrate to a primary focus, or to form a germinal center in the lymphoid follicle. In the primary focus, B-2 cells differentiate to short-lived, low affinity IgM secreting plasmablasts. Some of these plasmablasts differentiate to plasma cells and undergo class switching to produce high amounts of IgG. This class switching is influenced by cytokines secreted by other leukocytes, which can lead to secretion of other antibody classes by the B-2 cells. Like plasmablasts, most of these plasma cells are short lived.[273]

In the germinal center, the B-2 cell undergoes somatic hypermutation (SHM) and affinity selection, a process that is assisted by T-cells. This leads to the production of plasma cells that produce large amounts of antibodies that are highly specific to the target antigen.[274]

Most of the plasmablasts and plasma cells expire after the primary infection has been quelled, but a minority of the plasma cells mature into long-lasting, non-secreting memory B cells that can reactivate to elicit strong and highly antigen specific antibody response in the event of a secondary infection by the same antigen.[252] These memory B cells reside in lymphoid organs and occasionally circulate in the blood and lymph to patrol for pathogens. Upon re-encountering the pathogen they are specific to, they rapidly differentiate to high affinity antibody producing plasmablasts, and plasma cells that undergo further SHM in GCs to acquire even greater affinity.[275]

Vaccinations aim to engage these T- and B-cell responses to protect the host from infection. The duration of the immunity post-vaccination is dependent on the vaccine type, and infection and vaccination history of the recipient. The immune memory responses induced by vaccinations and infections are an important factor when assessing the vaccine suitability.[6–8,229,245,276–278]

3 Aims

The aims of this thesis research were to assess the adaptive immune responses elicited by SARS-CoV-2 and avian influenza vaccinations. By following up the Finnish healthcare and laboratory workers receiving SARS-CoV-2 and avian influenza vaccines, the specific aims were:

1. To follow the long-term duration, cross-reactivity and neutralizing capacity of vaccine-induced antibodies (studies I–IV)
2. To reveal the longevity and subpopulation occurrence of vaccine-induced T-cells (studies II and IV)
3. To assess the effect of SARS-CoV-2 breakthrough infections on humoral and cell-mediated immunity (studies I–III)
4. To examine the adaptive immune responses elicited by the novel A/H5N8 avian influenza vaccine (study IV)

4 Materials and Methods

4.1 Study participants

For studies I-III, serum and cell samples were acquired from healthcare workers (HCW) working in Turku University Hospital (TYKS) or Helsinki University Hospital (HUS).

In study I, serum samples collected from 432 HCWs comprised of 89.1% females of 19–67 years age (median age 41 in short interval group, 46 in long interval group), were analyzed after three vaccinations.

In study II serum and cell samples collected from 111 HCWs comprised of 95.5% females of 23–66 years age, were analyzed after three (n=74, media age 43) and four (n=37, median age 60) vaccinations.

In study III serum samples collected from 225 HCWs comprised of 88.9% females of 20–67 years age, were analyzed after three (n=175, median age 44), four (n=31, median age 60), and five (n=19, median age 61) vaccinations. The vaccines used for the vaccinations are listed in table 1. The serum and cell sampling intervals are shown in Figure 10 a.

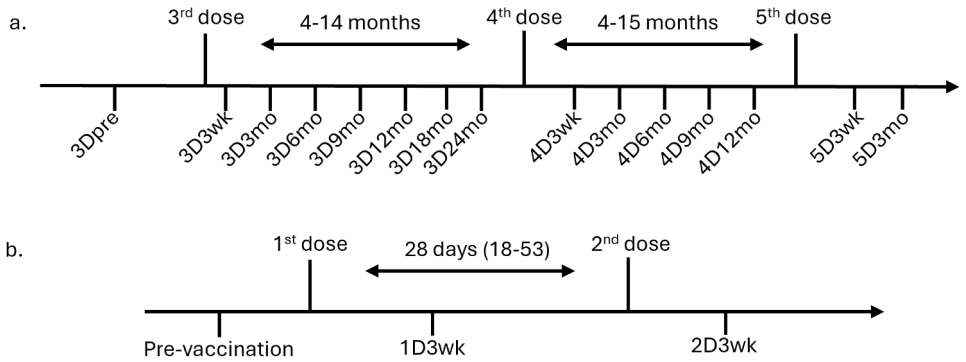

Figure 10. Timeline for vaccinations and sampling intervals. A. SARS-CoV-2 B. AIV

In study IV, samples were acquired from a cohort compromising of poultry workers (n = 3), veterinarians (n = 4), bird ringers (n = 5), and laboratory workers

(n= 27) vaccinated with Seqirus vaccine. Cohort was 82.1% female of 18–65 years age. Serum and cell samples were acquired from participants who were either previously unvaccinated against AIV (n= 30 serum, 13 cell), or had been previously vaccinated in the past with a different AIV vaccine (n= 9 serum, 5 cell). The serum and cell sampling intervals are shown in Figure 10 b.

4.2 Ethics

Studies I–III were approved by Southwest Finland health district (ETMK 19/1801/2020, EudraCT 2021–004419-14) and Helsinki-Uusimaa health district (HUS/1238/2020, EudraCT 2021–004016-26) ethical review boards. All study participants took voluntarily part in the study and signed a written informed consent before the first sampling.

Study IV was conducted in accordance with the standards of Good Clinical Practice, the Declaration of Helsinki, and local legal and regulatory requirements, and was registered in the EU Clinical Trial Information System under EU CT number 2023-509178-44-00 on 19 April 2024. The study protocol was authorized by the Finnish Medicines Agency (Fimea). Written informed consent to participate was obtained from all participants before sampling. Participation in the study was voluntary and uncompensated.

4.3 Virus variants, strains, and recombinants

In studies I-III, the D614G variant was isolated from nasopharyngeal samples and cultured in VeroE6 cells. Rest of the SARS-CoV-2 variants were isolated from nasopharyngeal samples and cultured in VeroE6-TMPRSS2-H10 cells. Culturing and passaging were done in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 2% fetal calf serum, 2 mM L-glutamine, and 2 mM penicillin–streptomycin. Fifty-percent tissue culture infective dose ($TCID_{50}$) of virus stocks was determined with endpoint dilution assay in VeroE6-TMPRSS2-H10 cells. Virus dilutions in DMEM supplemented with 2% FCS were applied onto cells plated on the previous day, and the plates were incubated for 3 days at +37 °C and 5% CO_2. Cells were fixed with 4% formaldehyde and stained with crystal violet. Virus dilution resulting in 50% cell death, deemed to approximate the $TCID_{50}$ value of the stock virus, was determined with Reed-Muench method.

In study IV, the live viruses were cultivated in MDCK cells and collected at the time of 50–75% cytopathic effect. Virus dilution resulting in 50% cell death, deemed to be approximate the $TCID_{50}$ value of the stock virus, was determined with Reed-Muench method.

In study IV, recombinant viruses were produced using an eight-plasmid rescue system[279]. Virus production after recombinant rescue was evaluated with an HA assay. Virus stocks were cultivated in MDCK cells and HA gene sequences were verified by Sanger sequencing using the 3500xL Genetic Analyzer (Applied Biosystems). SARS-CoV-2 variants and AIV strains used in studies I-IV are displayed in table 4.

Table 4. SARS-CoV-2 and AIV variants, strains, and recombinants used in dissertation studies.

SARS-CoV-2 Variant	GenBank ID	GISAID	Used in study	Type
D614G	MW717675.1	EPI_ISL_412971	I, II, III	Live
BA.1	N/A	EPI_ISL_8768822.2	I	Live
BA.2	OP199045	EPI_ISL_9695067	I, II, III	Live
BA.5	OP199047	EPI_ISL_13118918	I, II	Live
BQ.1.1	OQ411064	EPI_ISL_15762173	I	Live
XBB.1.5	OQ509907	EPI_ISL_16526646	I, II, III	Live
JN.1	N/A	EPI_ISL_19193868	III	Live
AIV strain				
A/Astrakhan/3212/2020 CVV	N/A	EPI_ISL_13655139	IV	Live
A/blue fox/UH/004/2023	N/A	EPI_ISL_18764855	IV	Live
A/Texas/37/2024	N/A	EPI_ISL_19027114	IV	Recombinant
A/Astrakhan/3212/2020	N/A	EPI_ISL_1038924	IV	Recombinant

4.4 PBMC isolation

In studies I-III, peripheral whole blood was collected into lithium-heparin tubes and PBMCs were isolated using Ficoll-Paque PLUS (GE Healthcare) density gradient centrifugation. After isolation, the PBMCs were counted, and cell viability was assessed with trypan blue dye (BioRad) with TC20 automated cell counter (BioRad). Isolated PBMCs were suspended to 5–15 –× 10^6 cells/mL in freezing medium containing 10% DMSO and 10% human AB serum (Sigma-Aldrich) and gradually frozen to -135°C until further use.

In study IV, peripheral whole blood was collected using BD Vacutainer CPT mononuclear cell preparation tubes containing buffered sodium citrate (BD 362761). A total of 48 ml of whole blood was collected from each participant. PBMCs were isolated according to manufacturer instructions and washed with Ficoll salt solution, and then counted with a Scepter 3.0 handheld automated cell counter using a 40-µm sensor. PBMCs were suspended to a concentration of 10^6 cells/ml in CryoStor CS10

medium (STEMCELL Technologies) and gradually cooled to -80 °C using a Corning CoolCell Freezing Container before being transferred to liquid nitrogen until further use.

4.5 ELISA

In studies I-III, SARS-CoV-2 S1- and N-specific antibodies in sera were detected and analyzed with an in-house enzyme immunoassay (EIA). 96-well plates (Nunc Maxisorp, Thermo Fischer Scientific) were coated with SARS-CoV-2 S1 (3.5 µg/ml) or N (2 µg/ml) proteins diluted in PBS. Serum samples were diluted 1:1000 for S1-specific EIA and 1:300 for N-specific EIA in PBS supplemented with 5 % swine serum, and were incubated for 2 h at 37°C. Bound antibodies were detected with anti-human IgG antibodies conjugated with horse radish peroxidase (Dako) diluted 1:8000 for S1-specific EIA and 1:16000 for N-specific EIA, followed by colorimetric detection with TMB (Kementec Solutions) and the measurement of the absorbance at 450 nm (Victor Nivo, Revvity).

The measured optical density (OD) values were converted to EIA units using a linear interpolation between OD-values of known positive (100 EIA units) and negative (0 EIA units) serum specimens. The thresholds to determine seropositivity were determined with standard deviations of samples that were known to be negative for anti-S and anti-N antibodies.

4.6 Microneutralization assays

In studies I–III, 50 ID_{50} of virus was added to two-fold serum sample dilution series, resulting in two-fold dilutions from 1:10 up to 1:40,960 (D614G) or 1:2560 (Omicron subvariants). Virus-serum dilution mixtures were incubated for 1 h, after which detached and counted VeroE6-TMPRSS2-H10 cells suspended in infection media (DMEM, Sigma-Aldrich) were added, followed by incubation at 37 °C at 5% CO_2 for four days. The cells were fixed with 4 % formaldehyde, stained with crystal violet, and visualized for cell death. $ID_{50} > 10$ was considered positive for neutralizing antibodies. $ID_{50} < 10$ were marked as having a titer of 5 for analyses. Serum with neutralizing antibodies, cells alone, and virus without serum were used as controls in every MNT plate.

In study IV, duplicate heat-inactivated serum samples were 2-fold serially diluted starting at 1:10 dilution in MN medium comprising OptiPro SFM medium (Gibco), supplemented with 0.2% bovine serum albumin (BSA), 1× non-essential amino acids (NEAA, Sigma-Aldrich), penicillin-streptomycin. An equal volume of virus was added to obtain 100× $TCID_{50}$ per well, and incubated for 1 h at 37 °C at 5% CO_2. 2.5×10^4 MDCK cells per well were added to 96-well flat-base tissue

culture plates (Sarstedt) and were incubated at 37 °C at 5% CO_2 for 18–20 h. Wells were fixed with ice-cold 80% acetone.

The influenza A virus in infected and fixed cells was assessed with ELISA. Cells were washed twice with washing buffer consisting of PBS containing 0.05% Tween 20 (Sigma-Aldrich). A horseradish peroxidase-labelled (HRP Conjugation Kit - Lightning-Link, Abcam) influenza A nucleoprotein-specific antibody (A7307, Medix Biochemica) was diluted 1:10,000 in PBS containing 5% milk and incubated at room temperature for 1 h. After washing six times with the washing buffer, substrate (1-Step TMB ELISA Substrate Solutions, Thermo Fischer Scientific) was added into wells and incubated at room temperature for 20 min in the dark. The reaction was stopped with 2 N sulfuric acid. Absorbances were measured within 30 min at 450 nm and 620 nm.

The neutralizing endpoint was determined by following WHO standards (*Manual for the Laboratory Diagnosis and Virological Surveillance of Influenza* (WHO, 2011)).

4.7 Hemagglutination inhibition assays

Horse red blood cells (hRBC) were washed three times with PBS for 10 min at room temperature, followed by centrifugation at 754 × *g*. Final concentrations of 2% and 10% hRBCs were made in PBS. Serum samples were absorbed with an equal volume of 10% hRBCs at 4 °C for 1 h, with mixing every 20 min to prevent non-specific agglutination. Subsequently, non-specific inhibition was avoided by incubating sera with in-house manufactured *Vibrio cholerae* filtrate comprising receptor destroying enzyme (RDE) at a 1:6 ratio (v/v) overnight at 37 °C following RDE inactivation at 56 °C for 1 h. Post RDE inactivation, 2-fold serial dilutions of sera in 0.5% BSA (Sigma-Aldrich) in PBS (0.5% BSA–PBS) were prepared in 96-well V-bottom microtitre plates (Greiner) starting at a 1:20 dilution. Viruses were adjusted to 4 haemagglutinating units (HAU) in PBS and added to each well. Plates were mixed and incubated at 37 °C for 30 min. Subsequently, 2% hRBCs was added to each well, and HI titres were determined after a 1.5-h incubation at 4 °C. The HI titres were defined as the reciprocal of the last serum dilution in which hRBC agglutination was partially or completely inhibited. The detection limit was HI titre of 10. Titers of <10 were determined as 5 for the analyses.

4.8 Activation induced marker assay and flow cytometry

In studies II and IV, cryopreserved PBMCs were thawed and resuspended in culture media (RPMI-1640, Lonza) supplemented with 10% heat-inactivated human AB

serum (Sigma), 2mM L-glutamine, and penicillin-streptomycin. After washing, cell viability was assessed with TC20 cell counter (Biorad), and 1.0×10^6 cells/well were plated in 96-well round-bottom plates (Thermo Fischer Scientific).

In study II, cells were stimulated with overlapping S peptide pools (Pepmix, JPT peptides) covering the entire S protein of the ancestral Wuhan-Hu-1 (WT) or Omicron subvariants variants BA.5 and XBB.1.5 at 0.5 µg/ml peptide per stimulation, as well as with a WT N peptide pool covering the entire N protein at 1 µg/ml peptide per stimulation (PM-WCPV-S-1, PM-SARS2-SMUT10-1, PM-SARS2-SMUT15-1, and PM-WCPV-NCAP-1, respectively). Incubation with tetanus toxoid (10 µg/ml, AJ vaccines) was used as a positive control, and incubation with an equimolar amount of DMSO was used as a negative control. Cells were cultured for 48 h at +37 °C, 5% CO_2.

In study IV, cells were stimulated with influenza H1 HA (2 µg ml^{-1}), H5 HA (2 µg/ ml), N8 NA (2 µg/ml) or PR/8 NP (2 µg/ ml; Pepmix, JPT peptides) overlapping peptide pools covering the whole proteins, after which the cells were incubated at 5% CO_2, +37 °C for 72 h. Incubation with DMSO (equimolar, Sigma-Aldrich) was used as a negative control, while incubation with tetanus toxoid (20 µg/ ml, AJ vaccines) or SARS-CoV2 JN.1 spike (1 µg/ ml; Pepmix, JPT peptides) were used as positive controls. SARS-CoV-2 JN.1 spike and PR/8 NP peptide pools were 15-mers with 11-mer overlaps commercially synthetized and pooled by JPT peptides. H1, H5 and N8 peptide pools were 15-mers with 10-mer overlaps synthetized by TC peptide Lab as crude material, which were then pooled and sequentially lyophilized.

In studies II and IV, cells were washed after stimulation with staining buffer (PBS supplemented with 2% FCS and 0.01% NaN3), and stained with Zombie Green viability dye (BioLegend, 1:1000) for 15min. Cells were washed and stained with fluorochrome-conjugated antibody panel (Table 5.)

In study II, after surface staining, cells were washed and resuspended in a staining buffer for acquisition with NovoCyte Quanteon Flow Cytometer (Agilent Technologies Inc) and analyzed with NovoExpress v1.5.0 (Agilent Technologies Inc).

In study IV, after surface staining, cells were washed and resuspended in a staining buffer for acquisition with FortessaLSR (BD Biosciences) and analyzed with FlowJo 10.10.0 (BD Biosciences)

In studies II and IV, stimulation index (SI) was calculated by dividing the frequency of CD134+CD69+CD4+ or CD137+CD69+CD8+ T cells after peptide pool stimulation by the frequency of CD134+CD69+CD4+ or CD137+CD69+CD8+ T cells after DMSO stimulation. Samples with <10,000 CD3+ T cells were excluded from all analyses and samples with <500 cTfh CD4+ T cells were excluded from the analysis of activated cTfh cells.

Table 5. Fluorochrome panel used in flow cytometry experiments.

Antibody	Fluorochrome	Manufacturer	Cat#
Anti-human CD45	APC-eFluor780	Invitrogen/Life technologies	47-0459-42
Anti-human CD3	eFluor506	Invitrogen/Life technologies	69-0038-42
Anti-human CD4	eFluor450	Invitrogen/Life technologies	48-0049-42
Anti-human CD8a	PerCP-eFluor710	Invitrogen/Life technologies	46-0087-42
Anti-human CD69	PE	BD Biosciences	555531
Anti-human CD134	PE/Cyanine7	BioLegend	350012
Anti-human CD137	APC	BioLegend	309810
Anti-human CD45RA (HI100 clone)	Brilliant Violet 785	BioLegend	304140
Anti-human CD197 (CCR7) (G043H7 clone)	PE/Dazzle 594	BioLegend	353236
Anti-human CD185 (CXCR5) (J252D4 clone)	Brilliant Violet 605	BioLegend	356930
Zombie Green Dye	FITC	BioLegend	423112

4.9 Memory B-cell analysis

In study II, PBMCs were stimulated for five days with 1 µg/ml R848 (TLR7/8 agonist; InvivoGen) and 0.01 µg/ml recombinant IL-2 (R&D systems) in RPMI-1640 medium supplemented with 10% FCS. ELISpot plates (multiscreen filtration plate, Millipore) were coated overnight at +4°C with his-tagged SARS-CoV-2 N (8 µg/ml), S1 (3.5 µg/ml), or RBD (4 µg/ml) proteins, and with mouse myostatin (8 µg/ml) tetanus toxoid (Td, 10 µg/ml; GlaxoSmithKline), and unlabeled anti-human IgG (10 µg/ml; MP Biomedicals). The plates were blocked for 1h with AIM-V medium (Thermo Fischer Scientific) supplemented with 10% FCS and 50mM of 2-mercaptoethanol. After washing the plates, antigen-specific amount of stimulated PBMCs in AIM-V+ medium was added in duplicates to the plates: 500,000 cells to SARS-CoV-N and mouse myostatin, 100,000 cells to tetanus toxoid and RBD, 50,000 cells to S1, and 10,000 cells to anti-IgG coated wells. The wells were washed with PBS/0.5% Tween-20 after incubating for 24 h, followed by lysis with distilled water. Plates were washed with PBS/0.5% Tween-20, followed by addition of alkaline phosphatase-conjugated goat anti-human IgG (Sigma-Aldrich) and incubation for 1h at +37°C. The plates were washed with PBS/0.5% Tween-20, allowed to dry, and washed thrice with PBS before adding 1-Step NBT/BCIP-substrate (Pierce) and incubating for 5 min. Washed and dried plates were imaged and analyzed with ImmunoScan C.T.L ELISpot reader. Mouse myostatin spots were regarded as nonspecific spot controls, and their number was subtracted from the

antigen-stimulated spots for each sample. Samples without specific spots for both anti-IgG and tetanus toxoid were excluded from the analysis.

4.10 Cytokines

In studies II and IV, supernatants from the stimulated PBMCs were analyzed for IFN-γ cytokine levels using the MILLIPLEX Kit HCD8MAG-15K (Millipore). Fluorescence was measured with the Luminex MAGPIX magnetic bead analyzer (Luminex Corporation). In study II, quantification was performed only for samples within the linear range. Samples below the lowest standard in the linear range were assigned a value of half of the lowest standard value, while those exceeding the highest standard in the linear range were assigned the highest standard value (5000 pg/ml). Standard samples were measured in duplicates. According to the manufacturer, samples with fewer than 35 beads per well were considered unreliable and were excluded from the analysis.

In study IV, results were expressed as SI, defined as the ratio of IFN-γ concentration after peptide pool or tetanus toxoid stimulation to the corresponding concentration after DMSO stimulation within the same sample. A stimulation response was considered positive if the IFN-γ SI value was >2.

4.11 Statistics and graphing

In studies I–IV, Graphpad Prism (versions 9 and 10) was used to perform statistics and graph the results.

In studies I-III, the statistical significances were assessed with Wilcoxon signed-rank test or Friedman test followed by Dunn's multiple comparisons for paired samples, and Kruskal–Wallis test followed by Dunn's multiple comparisons or Mann Whitney U test for unpaired samples or time points where multiple participants lacked samples. Tests were two-sided, and p-value <0.05 was considered statistically significant. The correlation between the values was analyzed using Spearman's correlation test. In study III, one phase decay analysis with 95 % confidence interval was used to analyze antibody half-lives. Data distribution was assumed to be non-normally distributed.

In study IV, the Mann–Whitney U-test was used to compare differences between different groups, while the Wilcoxon matched-pairs signed-rank test was used for within-group comparisons across different time points. As anti-IAV IgG data was normally distributed, comparisons between groups were performed with a t-test. Correlation analyses were performed using the non-parametric Spearman rank correlation coefficient. Data distribution was tested with Shapiro-Wilk normality test and determined to be non-normally distributed.

5 Results

5.1 Boosting effects of vaccine doses and different SARS-CoV-2 and influenza A (H5N8) vaccines

For a vaccine to be effective, it must elicit sufficient levels of antibodies with the capacity to neutralize the target virus, with enough longevity to remain as a barrier against reinfection for a substantial time. In our group's previous studies, we had already described how the second vaccination dose to SARS-CoV-2 boosted peak antibody levels when compared to the first vaccination dose. The efficiency of the third follow-up vaccination at inducing neutralizing antibodies needed to be assessed to determine if there were beneficial effects to the antibody responses. This question was also pertinent to the second dose of avian influenza H5N8 vaccinations. Studies I and IV investigated this with both viruses.

5.1.1 All SARS-CoV-2 vaccination combinations induced high levels of antibodies with high neutralizing levels after three vaccine doses

Participants were vaccinated and samples were collected three weeks (3D3wk), and three (3D3mo) and six months (3D6mo) after the vaccinations (Figure 11). The SARS-CoV-2 S1-specifc antibody levels were elevated in all SARS-CoV-2 vaccine combinations with both 3 week and 12 week dosing intervals, with different peak values for noninfected individuals with short- and long-interval groups (129.06 vs. 115.40 EIA units 3D3wk). The peak levels were reduced in the subsequent months with similar trend (99.58 vs 82.30 EIA units 3D3mo, 71.32 vs. 55.02 EIA units 3D6mo, 65.13 vs. 32.94 3D9mo), with similar fold changes (1.30 vs. 1.40 at 3D3mo and 1.81 vs. 2.10 3D6mo). Participants in both groups had received either Comirnaty (n = 89 short, n = 77 long) or Spikevax (n = 129 short, n = 84 long) vaccine as their third vaccination. The third vaccine for seven participants in the short group was unknown. Interestingly, the long vaccine interval group had lower peak values at all time points when compared to the equivalent time points in the short vaccine interval group.

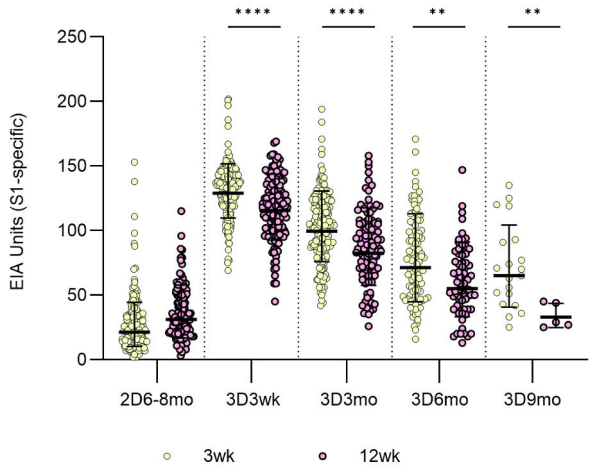

Figure 11. Level of S1-specific antibodies in serum samples from three times vaccinated participants separated based on their vaccination intervals between the first and second dose. The graphs display geometric mean indices (lines) and geometric SDs (whiskers). p-value of <0.05 was considered statistically significant and are displayed as stars (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001). Modified from study I.

Vaccinees who had received three doses of Spikevax (n=58) had higher S1-specific antibody levels when compared to three times Comirnaty dose vaccinated participants in both the short (n=98) or long (n=41) interval (D614G 126.84 vs. 123.13 vs 112.58, BA.1 95.69 vs. 92.98 vs. 85.48, BA.2 72.43 vs. 68.89 vs. 53.66) (Figure 12).

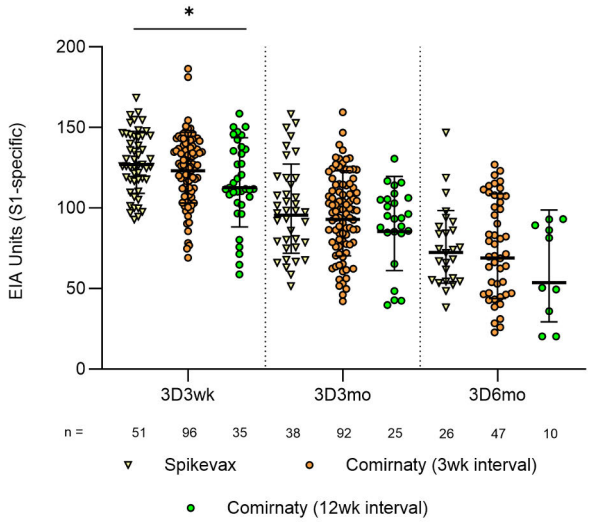

Figure 12. Level of S1-specific antibodies in serum samples from three times vaccinated uninfected participants separated based on the type of the third vaccine and the time between the first and second dose. The graphs display geometric mean indices (lines) and geometric SD (whiskers). p-value of <0.05 was considered statistically significant and are displayed as stars (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001). Modified from study I.

Microneutralization test (MNT) results correlated with the ELISA results. The short and long interval groups had antibodies with higher peak neutralization capacity against D614G (geometric mean titer (GMT) 1616 3wk vs. 910 12wk) and Omicron subvariants BA.1 (338 vs. 159), BA.2 (714 vs. 406), BA.5 (421 vs. 177), BQ.1.1 (51 vs. 34), and XBB.1.5 (39 vs 26) (Figure 13). The peak levels were reduced and were closer to equality three months later with differences between variant D614G (740 vs 370), Omicron subvariants BA.1(104 vs. 84), BA.2 (285 vs. 171), and BA.5 (179 vs. 79), with the exception of subvariants BQ.1.1 (18 vs. 16) and XBB.1.5 (16 vs. 11). Both groups received either Comirnaty (n = 21 short interval, n = 26 long interval) or Spikevax (n = 20 short interval, n = 9 long interval) vaccinations as their third vaccination.

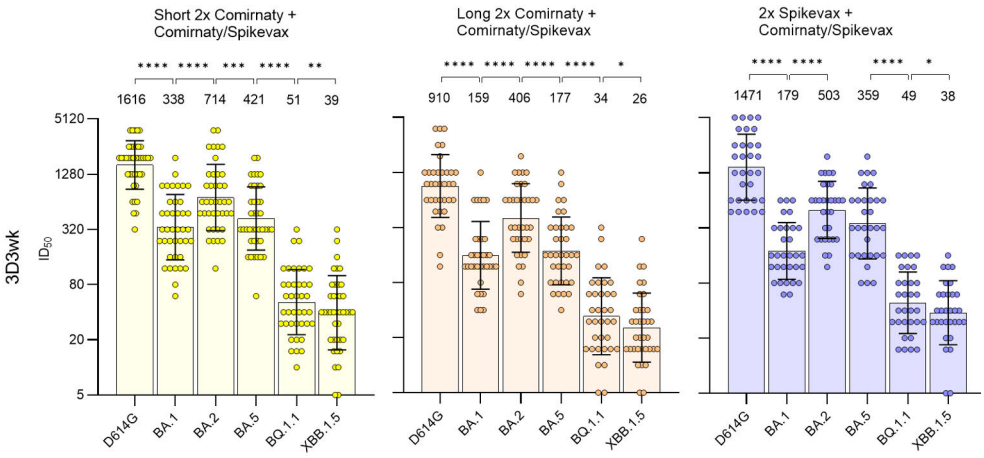

Figure 13. Neutralization capacity of antibodies against SARS-CoV-2 variant D614G and Omicron subvariants BA.1, BA.2, BA.5, BQ.1.1, and XBB.1.5 from three times vaccinated participants separated based on the vaccine combination they had received. The graphs display geometric mean indices (lines) and geometric SDs (whiskers). GMTs are displayed at the top of bars. p-value of <0.05 was considered statistically significant, and are displayed as stars (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001). Modified from study I.

Twice Spikevax vaccinated (n=31) group, of which 29 had Spikevax as their third vaccination, had antibodies with neutralization capacity equal to the short interval Comirnaty group against variant D614G (1616 vs. 1471), Omicron subvariants BA.2 (714 vs. 503), BA.5 (421 vs. 359), XBB.1.5 (51 vs. 49), and BQ.1.1 (39 vs. 38), with the exception of a lower neutralization capacity against BA.1 (338 vs. 179).

Vaccinees were separated based on what the third vaccination was to short interval Comirnaty and Spikevax, long interval Comirnaty and Spikevax, and three times Spikevax vaccinated groups (Figure 14). The short interval Spikevax group

had antibodies with the highest neutralization capacity against the variant D614G at 3D3wk (GMT Comirnaty short interval 1412.87 vs. Comirnaty long interval 877.76 vs. short interval Spikevax 1848.52 vs. long interval Spikevax 1010.07 vs. three times Spikevax 1536.23) and at 3D3mo (GMT Comirnaty short interval 659.84 vs. Comirnaty long interval 326.34 vs Spikevax short interval 835.16 vs Spikevax long interval 476.51 vs three times Spikevax 680.16). This was also the case against Omicron subvariants BA.1 and BA.2.

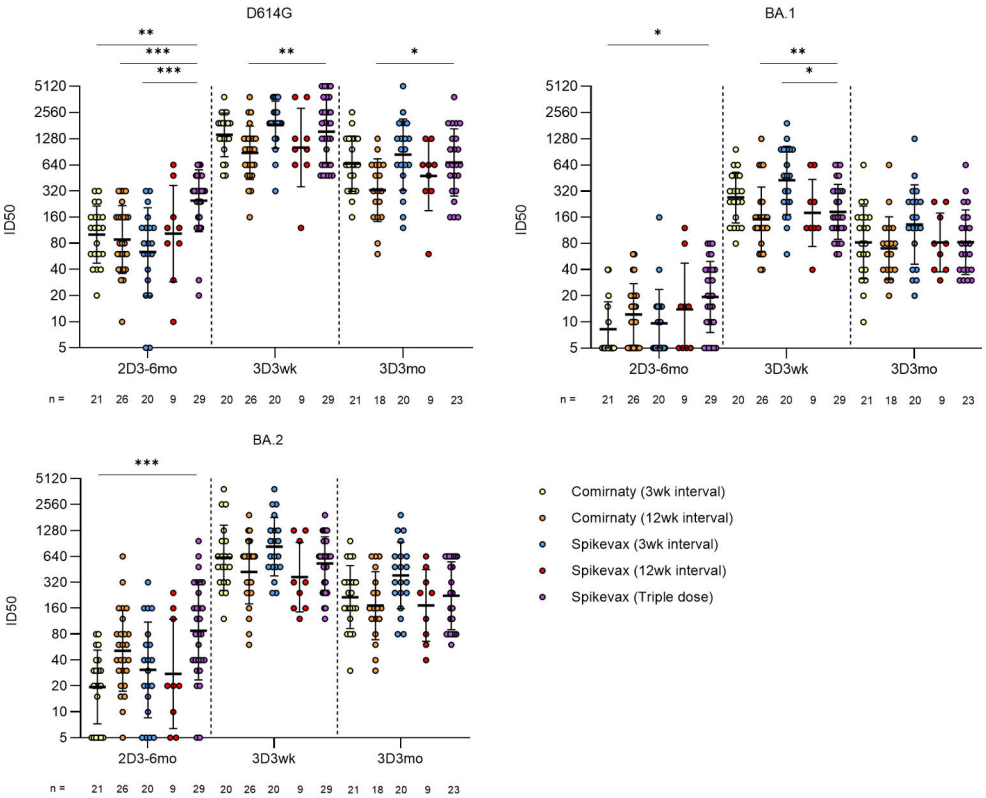

Figure 14. Neutralizing antibody capacities of antibodies in serum samples obtained from uninfected vaccinees separated based on their third vaccine dose and time interval between the first and second dose. The graphs display geometric mean indices (lines) and geometric SDs (whiskers). p-value of <0.05 was considered statistically significant and are displayed as stars (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001). Modified from study I.

In conclusion, Comirnaty and Spikevax vaccines induced high levels of S1 specific antibodies with robust neutralization capacity against the D614 variant and Omicron subvariants BA.1, BA.2, BA.5, BQ.1.1, and XBB.1.5 regardless of the interval between the first and second dose, and the vaccine type of the third dose.

5.1.2 Inactivated influenza A(H5N8) vaccine boosts neutralizing capacity of antibodies in naïve and previously vaccinated participants

The boosting effects are not limited to SARS-CoV-2 mRNA vaccines, but also apply to the inactivated influenza A(H5N8) vaccine. In study IV, the neutralization capacity of the antibodies was assessed with microneutralization assays (MNT) and hemagglutinin inhibition (HI) assays.

Naïve (n=30) and previously with avian influenza vaccine vaccinated participants (n=9) received two doses of Seqirus inactivated influenza A(H5N8) vaccine. The first vaccination induced high neutralizing antibody titers for both groups, with noticeably higher increase in the previously vaccinated group three weeks post-first vaccine dose (1D3wk) when compared to the naïve group (Figure 14). The neutralization titers were similar in both groups at 1D3wk sampling against A/Astrakhan/3212/2020 (GMT 15 vs 252) and A/blue fox/UH/004/2023 (GMT 34 vs 229). Of the naïve participants, 63% (19/30) and 97% (29/30) had antibodies with high enough neutralization capacity to cross the positivity threshold of GMT of 10 for A/Astrakhan/3212/2020 and A/blue fox/UH/004/2023, respectively (Figure 15).

The second dose increased the geometric mean neutralization titers further for the naïve vaccinees for both A/Astrakhan/3212/2020 and A/blue fox/UH/004/2023 (3.13- and 2.06-fold respectively), but had no boosting effect in the previously vaccinated group, with a GMT decrease of 1.80- and 1.17-fold observed instead.

In both groups, for A/Astrakhan/3212/2020 the HI results correlated strongly with the MNT results. In the naïve group, peak GMTs were 42 three weeks after the first vaccination and increased to 97 three weeks after the second vaccination, a fold increase of 2.31. In the previously vaccinated group, peak GMTs were 273 three weeks after the first vaccination and decreased to 236 three weeks after the second vaccination, a fold decrease of 1.16. The same trends were observed when tested with A/Texas/ 37/2024, with peak GMTs of 54 and 113 three weeks post first and second vaccination for naïve group, respectively, and 408 and 401 three weeks post first and second vaccination in previously vaccinated group, respectively.

To summarize, the two dose regimen induced antibodies with high neutralizing capacity in both groups, with just one dose elevating the neutralizing capacity in previously vaccinated participants.

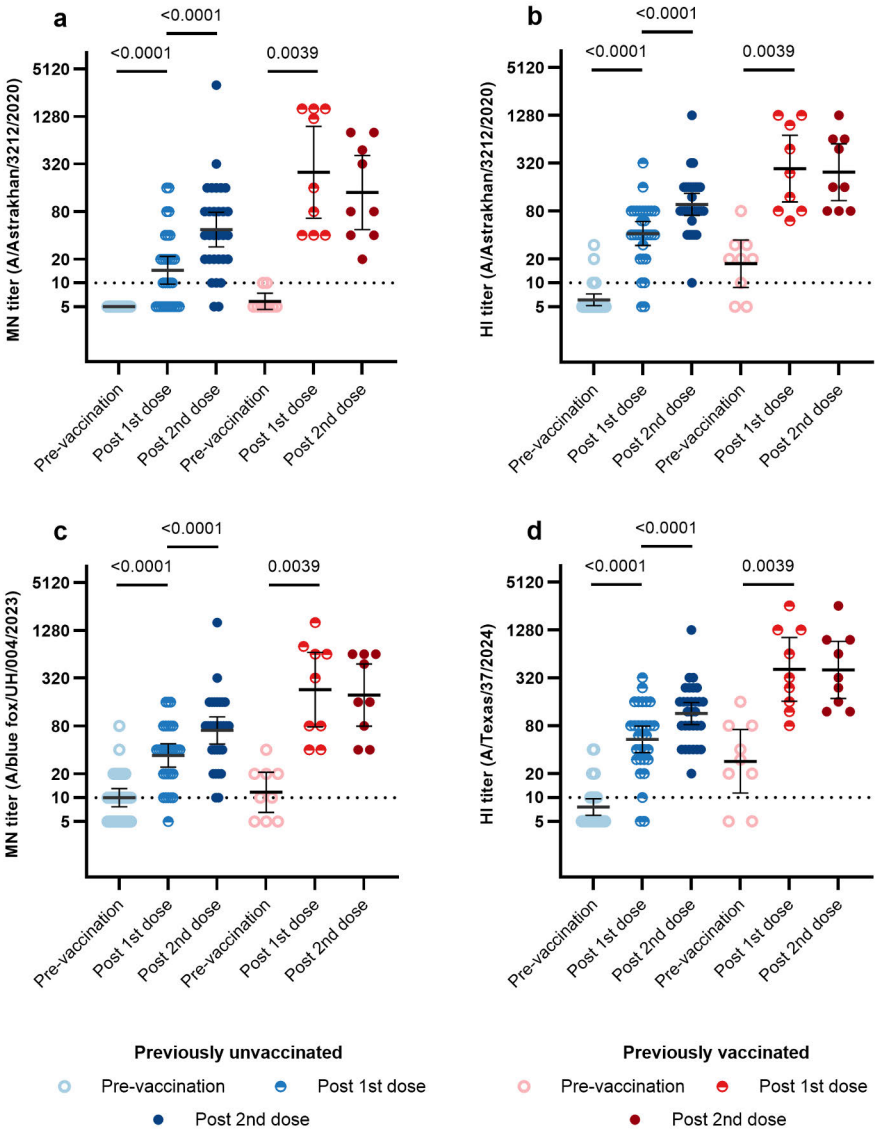

Figure 15. Neutralizing antibody capacities of antibodies in serum samples obtained from previously avian influenza unvaccinated and vaccinated participants against A. A/Astrakhan/3212/2020, B A/Astrakhan/3212/2020 (HI) C. A/blue fox/UH/004/2023 D. A/Texas/37/2024. The graphs display geometric mean indices (lines) and 95% CIs (whiskers). p-value of <0.05 was considered statistically significant. Dashed line represents the threshold for positivity. Modified from study IV.

5.2 Boosting effects of the fourth and fifth SARS-CoV-2 vaccine doses and breakthrough infections

Possible exhaustion of B- and T-cells resulting from frequent vaccinations and breakthrough infections necessitated the determination of the effects of booster doses on the levels of antigen specific antibodies and T-cell responses. Studies II and III aimed to analyse this.

5.2.1 No reduction of peak S1- or N-specific antibody levels after repeated booster doses with Covid-19 vaccines or SARS-CoV-2 breakthrough infections

Breakthrough infections with SARS-CoV-2 induced high levels of S1-specific antibodies, peaking at the similar levels after one (107 EIA), two (98 EIA), or three (107 EIA) breakthrough infections (Figure 16 a). The N-specific antibody levels were also increased by the first breakthrough infection (16 EIA), with two (36 EIA), or three (43 EIA breakthrough infections increasing the levels even further (Figure 16 b). The antibody levels were significantly elevated compared to the uninfected individuals. In 32/225 (15%) individuals, the N-specific antibody levels did not rise despite one or more breakthrough infections.

The S1-specific antibody peaks were comparable with the peak levels induced by the first three SARS-CoV-2 doses (104 EIA) and subsequent the fourth and fifth booster doses (106 EIA and 107 EIA). S1-specific antibody levels of infected vaccinees consistently remained at higher levels in all time points compared to the uninfected vaccinees (Figure 17). Breakthrough infection groups had the geometric mean of EIA units in all time points at similar levels compared to the peak levels in uninfected three times vaccinated individuals (geometric mean EIA of 107 vs 119 at 3D3wk, 114 at 3D3mo,108 at 3D6mo, 112 at 3D9mo,103 at 3D12mo, and 89 at 3D24mo) (Figure 17).

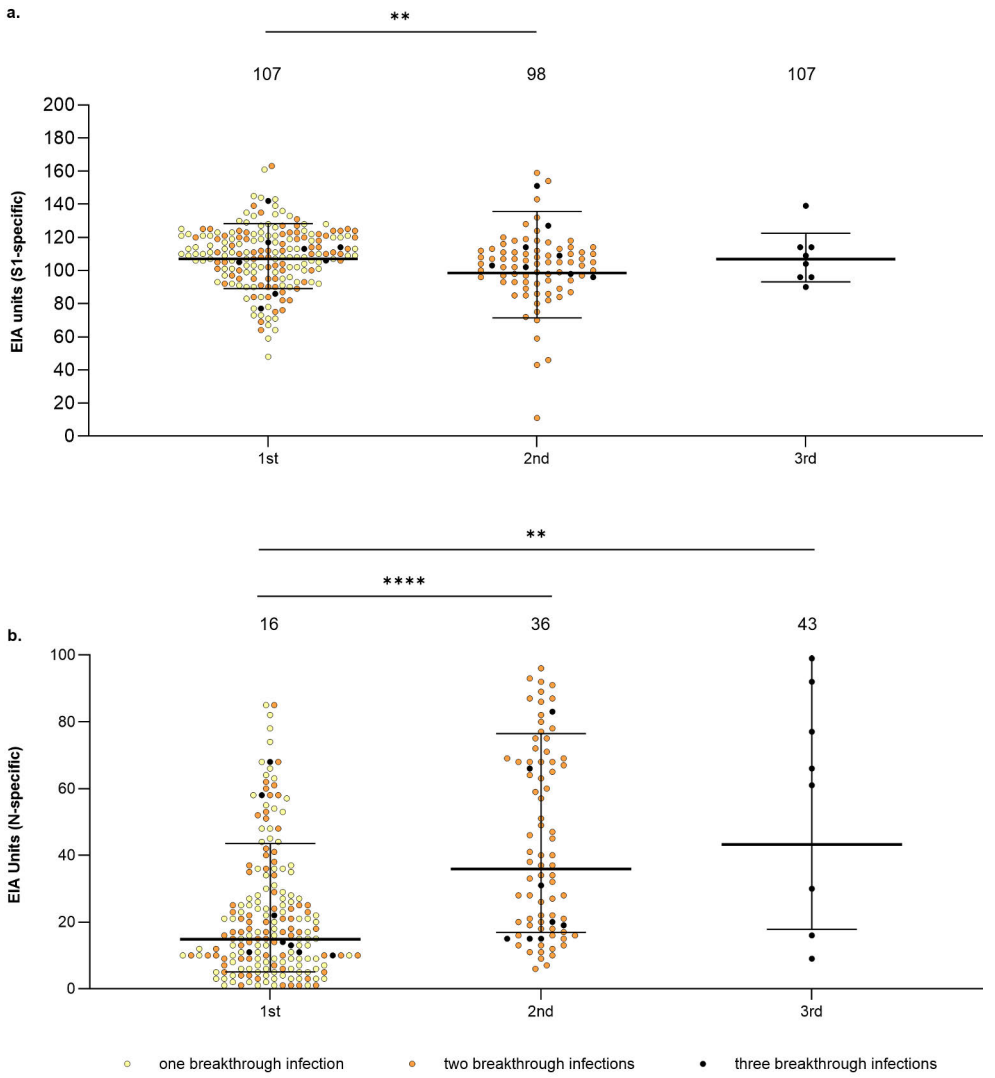

Figure 16. Level of antibodies in serum samples obtained from three times vaccinated and SARS-CoV-2 infected participants. A) S1-specific IgG antibody levels B) N-specific IgG antibody levels. The graphs display geometric mean indices (lines) and geometric SDs (whiskers). Geometric mean levels are shown on top of the groups. p-value of <0.05 was considered statistically significant and are displayed as stars (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001). Modified from study III.

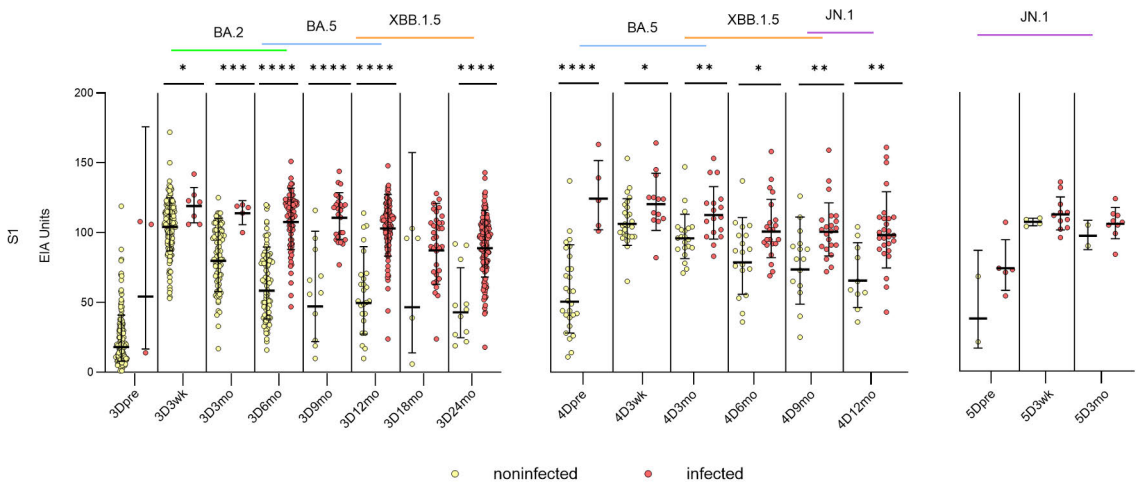

Figure 17. Level of S1-specific antibodies in serum samples obtained from vaccinated uninfected and infected participants. The vaccinees had received three, four or five vaccinations. The graphs display geometric mean indices (lines) and geometric SDs (whiskers). The dominant SARS-CoV-2 subvariant at the time of sampling is displayed on top of the groups. p-value of <0.05 was considered statistically significant and are displayed as stars (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001). Modified from study III.

5.2.2 Peak neutralization capacity against Omicron subvariants after multiple vaccinations

Neutralization capacity of antibodies in sera obtained from noninfected vaccinees (n=44) after the third, fourth, and fifth vaccinations induced escalating neutralization capacity peaks against variant D614G (538 at 3D3wk, 624 at 4D3wk, 867 at 5D3wk), and Omicron subvariants BA.2 (126 at 3D3wk, 169 at 4D3wk, 501 at 5D3wk), XBB.1.5 (16 at 3D3wk, 41 at 4D3wk, 211 at 5D3wk), and JN.1 (25 at 3D3wk, 35 at 4D3wk, 77 at 5D3wk) (Figure 18).

Neutralization capacity peaks were not reduced after any subsequent vaccination or breakthrough infections post-third vaccination, indicating that repeated, relatively frequent exposures to SARS-CoV-2 antigens did not cause a decline in antibody levels or their neutralizing capacities.

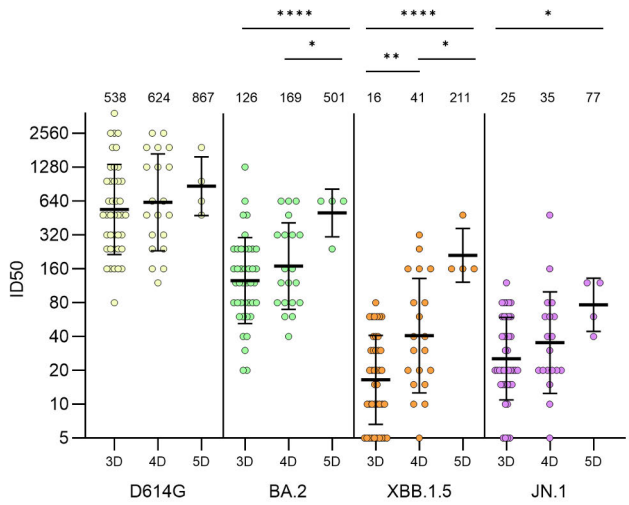

Figure 18. Neutralization capacity of antibodies in serum samples obtained from vaccinated uninfected participants with three, four, or five vaccinations. The graphs display geometric mean indices (lines) and geometric SDs (whiskers). Geometric mean levels are displayed on top of the groups. p-value of <0.05 was considered statistically significant and are displayed as stars (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001). Modified from study III

5.3 Waning antibodies on a long-time frame

An important question when considering the efficiency of vaccinations is the rate of decay in the levels of antigen-specific antibodies, and subsequently the neutralization capacity of the remaining antibodies. Studies II and III aimed to analyze this question.

5.3.1 COVID-19 vaccine-induced humoral immunity up to 24 months post three vaccine doses

S1-specific antibodies induced by three doses of SARS-CoV-2 vaccinations declined during 24 month follow-up period at time points 3D3wk (104 EIA), 3D3mo (80 EIA), 3D6mo (58 EIA), 3D9mo (48 EIA), 3D12mo (50 EIA), 3D18mo (46 EIA) and 3D24mo (43 EIA) in uninfected individuals (Figure 18a). Antibodies from uninfected participants with three or more samples reached a plateau at 9 months post-vaccination with an average of 46.75 EIA units (range 35.86–55.30 with 95% CI), with a half-life of the antibodies being on average 84.66 (range 55.20–130.4 with 95% CI) (Figure 18b).

5.3.2 Booster dose induced humoral immunity up to 12 months post fourth dose

S1-specific antibodies induced by four doses of SARS-CoV-2 vaccinations declined during the 12 month follow-up at time points 4D3wk (106 EIA), 4D3mo (96 EIA), 4D6mo (79 EIA), 4D9mo (73 EIA), and 4D12mo (66 EIA) in uninfected vaccinees (Figure 19 a).

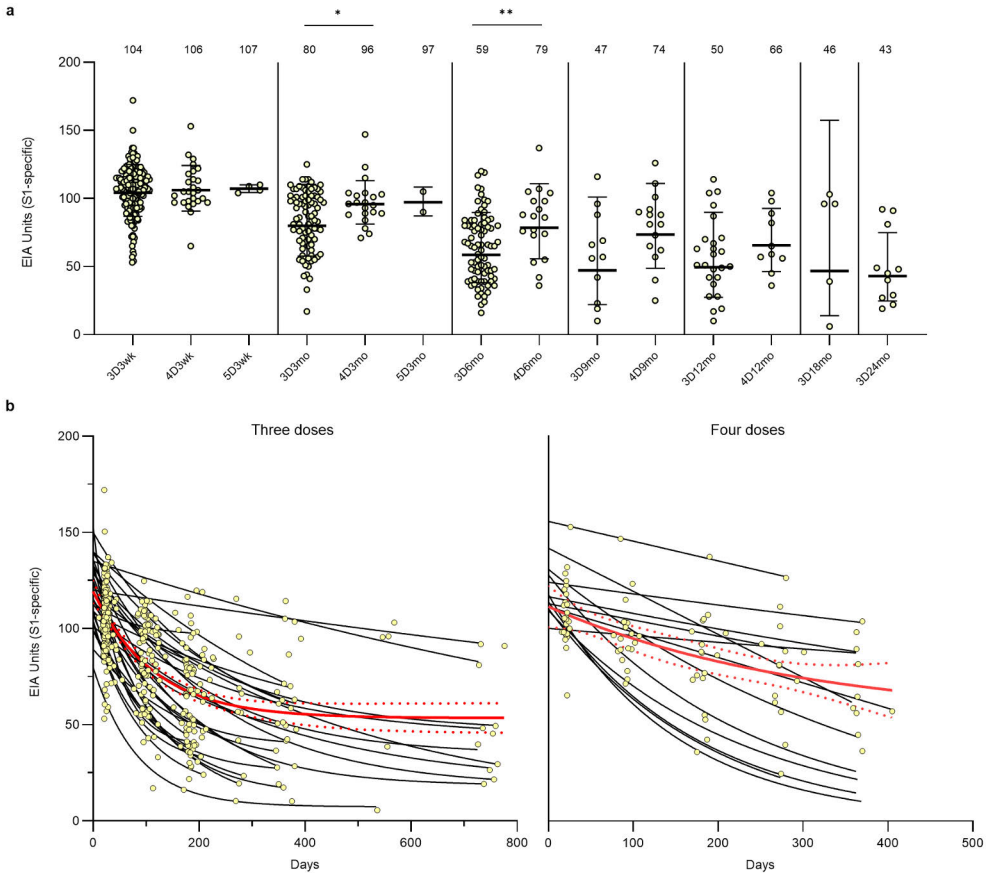

Figure 19. Levels of S1-specific antibodies in serum samples obtained from vaccinated uninfected participants. Vaccinees received three, four, or five vaccinations. A. S1-specific antibody levels at various time points post vaccinations. B. Decline of antibodies following the third and fourth vaccination. The graphs display geometric mean indices (lines) and geometric SDs (whiskers). Geometric mean levels is displayed on top of the groups. Red curves represent the average decay of S1-specific antibodies, while black curves represent decline in antibody levels. Dotted red curves represent 95% CI. p-value of <0.05 was considered statistically significant and are displayed as stars (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001). Modified from study III

Antibodies in uninfected participants with three or more samples vaccine doses reached a plateau at 12 months post-vaccination with an average of 26.28 EIA units (range -infinity – 76.04 with 95% CI), with the half-life of the antibodies being on average 344.9 (range 66.39–732.3 with 95% CI) (Figure 19 b).

S1-specific antibody levels declined significantly after both third and fourth vaccination, but plateaued well above the positivity threshold. The decline was slower after the fourth dose.

5.4 Neutralization capacity of antibodies against Omicron subvariants induced by combinations of infections and vaccinations

Even if a vaccine combination can elicit long lasting antibodies at high levels and capable of neutralizing the target virus, their efficiency against emergent variants and subvariants is not guaranteed. Studies II, III, and IV investigated cross-reactivity of vaccine induced antibodies with different SARS-CoV-2 Omicron variants and avian influenza strains, as well as the immune evasion effects of the Omicron subvariants on the induced antibodies.

5.4.1 Hybrid immunity and subvariant evasion

In study III, neutralization capacity of vaccine-induced antibodies was significantly lower against the Omicron subvariants BA.2, XBB.1.5 and JN.1 compared to the D614G variant at all time points up to 24 months post third dose. Vaccine-induced antibodies had very low neutralization capacity particularly against XBB.15 and JN.1 after three vaccine doses (GMT 16, 25 at 3D3wk; 9, 12 at 3D3mo; 6, 10 at 3D6mo, 6, 7 at 3D12mo; 5, 5 at 3D24mo) (Figure 20).

Infected vaccinees followed the same trend as uninfected vaccinees, but with a key difference being that infections consistently inducing in thrice vaccinated participants higher peak neutralizing capacities against D614G variant (GMT of 538 in uninfected vs 960 in infected at 3D3wk, 480 at 3D3mo, 1580 at 3D6mo, 1158 at 3D12mo, and 595 at 3D24mo), and Omicron subvariants BA.2 (GMT of 126 in uninfected vs 1280 in infected at 3D3wk, 480 at 3D3mo, 421 at 3D6mo, 499 at 3D12mo, and 267 at 3D24mo), XBB.1.5 (GMT of 16 uninfected vs 40 in infected at 3D3wk, 10 at 3D3mo, 63 at 3D6mo, 69 at 3D12mo, and 40 at 3D24mo), and JN.1 (GMT of 25 in uninfected vs 160 in infected at 3D3wk, 40 at 3D3mo, 29 at 3D6mo, 32 at 3D12mo, and 33 at 3D24mo) in all time points.

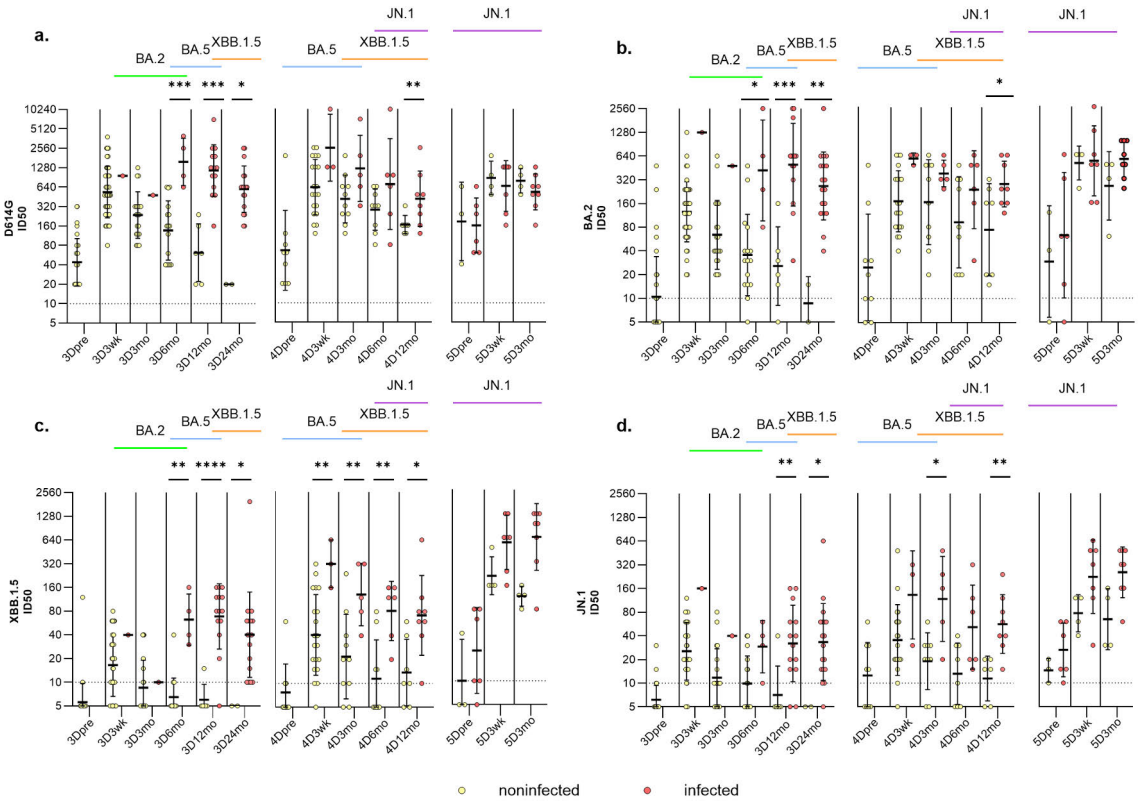

Figure 20. Neutralizing antibody levels in serum samples obtained from uninfected and infected vaccinees with three, four, and five vaccination doses. Neutralization capacities are displayed against A. D614G B BA.2 C. XBB.1.5 and D. JN.1 viruses. The graphs display geometric mean indices (lines) and geometric SD (whiskers). Dominant variant at the time of sampling is displayed on top of the groups. Dotted line represents the positivity threshold. p-value of <0.05 was considered statistically significant and are displayed as stars (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001). Modified from study III.

Same trends were evident after the fourth and fifth vaccine doses, albeit to a lesser extent. Compared to equivalent timepoints after the third dose, in uninfected vaccinees the fourth dose induced slightly higher neutralization capacity against the Omicron subvariants BA.2, XBB.1.5, and JN.1 (GMT 126, 16, 25 at 3D3wk vs. 169, 41, 35 at 4D3wk; 64, 9, 12 at 3D3mo vs. 164, 22, 19 at 4D3mo; 36, 6, 10 at 3D6mo vs. 92, 11, 13 at 4D6mo; 26, 6, 7 at 3D12mo vs. 74, 14, 11 at 4D12mo). Notably, when compared to equivalent time points after three and four doses, the fifth booster dose increased the neutralization capacity significantly against XBB.1.5 and JN.1 subvariants in uninfected vaccinees in all time points (GMT 211 and 223 at 5D3wk, 117 and 254 at 5D3mo, respectively).

Compared to uninfected vaccinees, after the fourth dose infected vaccinees still had a significantly higher neutralization capacity peaks against D614G variant

(GMT of 624 in uninfected vs 2552 in infected at 4D3wk, 1219 at 4D3mo, 697 at 4D6mo, 412 at 4D12mo) and Omicron subvariants BA.2 (GMT of 169 in uninfected vs 581 in infected at 4D3wk, 376 at 4D3mo, 235 at 4D6mo, 277 at 4D12mo), XBB.15 (GMT of 41 in uninfected vs 320 in infected at 4D3wk, 132 at 4D3mo, 82 at 4D6mo, 72 at 4D12mo) and JN.1 (GMT of 35 in uninfected vs 132 in infected at 4D3wk, 117 at 4D3mo, 51 at 4D6mo, 56 at 4D12mo) at most timepoints.

Notably, in uninfected vaccinees the fifth COVID-19 vaccine dose boosted the neutralizing capacity of the antibodies to be on par with the peak neutralization capacities of the infected vaccinees against the D614G variant (GMT 867 in uninfected vs. 651 in infected 5D3wk) and Omicron subvariant BA.2 (GMT of 501 in uninfected vs 534 in infected at 5D3wk), raising the peak capacity. The peak neutralization capacities against XBB.1.5 (GMT of 211 in uninfected vs 556 in infected at 5D3wk), and JN.1 (GMT of 77 in uninfected vs 223 in infected at 5D3wk) still remained significantly higher in infected vaccinees.

Emergence of Omicron and its subvariants coincided with an increased number of infected individuals from the 3D6mo time point onwards (86 infected participants at 3D6mo, 137 at 3D12mo, and 161 at 3D24mo).

In summary, these results show a clear trend of higher neutralization capacity in vaccinees with breakthrough infections. However, updated fourth and fifth booster vaccines induced high neutralizing capacities against more recent Omicron subvariants XBB.1.5 and JN.1.

5.4.2 Mono- and bivalent vaccines based on Omicron subvariants BA.1, BA.2, and BA.5

In study II, the monovalent Comirnaty (Pfizer) or Spikevax (Moderna) (n=8) vaccinated and monovalent BA.1 or bivalent BA.4/5 (n=6) vaccinated participants had no significant differences in their neutralizing antibody capacities against Omicron subvariants BA.2, BA.5, and XBB.1.5 (Figure 21).

The fifth booster dose based on the Omicron subvariant XBB.1.5 induced significantly higher peak neutralization capacities against subvariants XBB.1.5 (GMT 211) and JN.1 (GMT 77) compared to the peak capacities induced against XBB.1.5 by previous mono- or bivalent vaccines (GMTs 53 and 31, respectively).

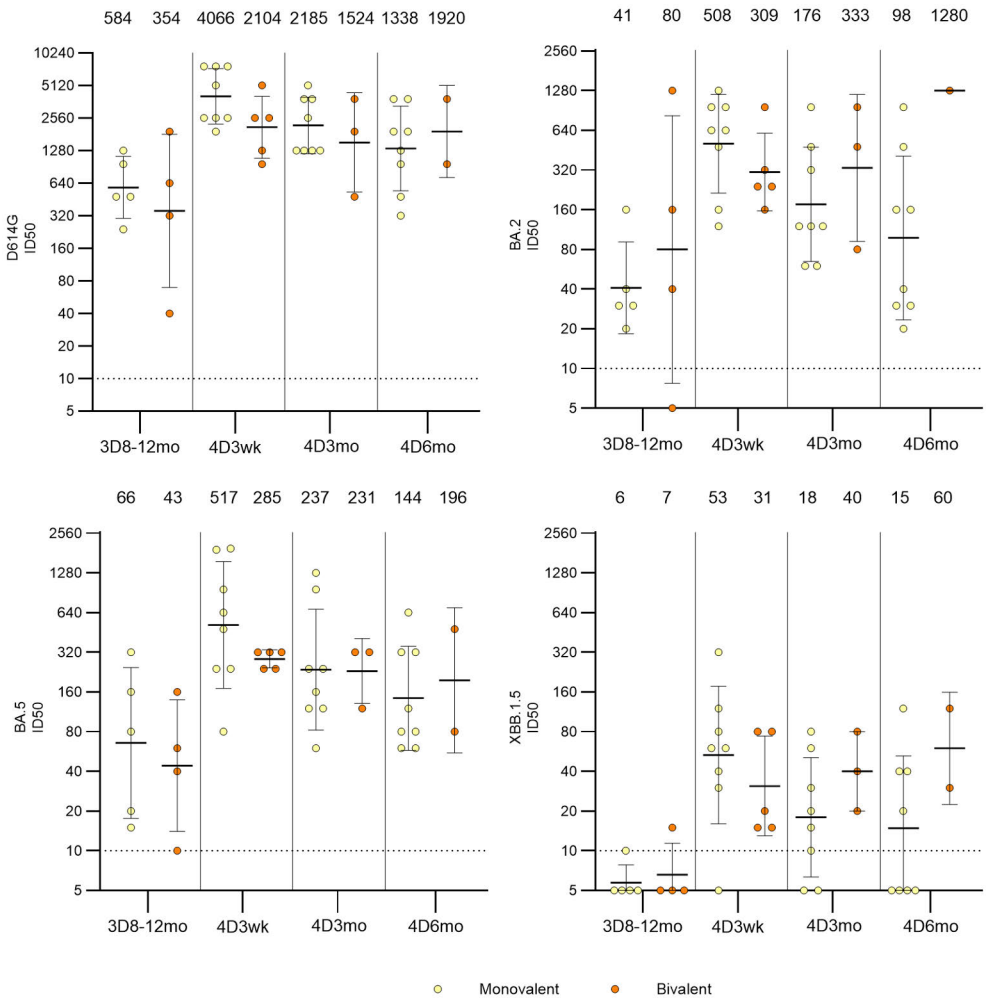

Figure 21. Neutralization capacity of antibodies against SARS-CoV-2 D614G variant and Omicron subvariants BA2, BA5, and XBB.1.5 from participants vaccinated with monovalent or bivalent vaccines. The graphs display geometric mean indices (lines) and geometric SDs (whiskers). Dotted line represents the positivity threshold. Geometric mean titers are displayed on top of the groups. p-value of <0.05 was considered statistically significant and are displayed as stars (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001). Modified from study I and II.

5.4.3 Avian influenza virus vaccine induced neutralizing antibody cross-reactivity

In study IV, the cross-reactivity of vaccine-induced neutralizing antibodies after the first avian influenza vaccine dose was determined against A/Astrakhan/3212/2020 and A/blue fox/UH/004/2023 viruses with MNT and against A/Astrakhan/3212/2020 and A/Texas/37/2024 viruses with hemagglutinin inhibition

assay. The cross-reactivity of AIV vaccine-induced antibodies was evident in the previously unvaccinated participants (n=30) in both the MNT (GMT 15 and 34) and HI assays (GMT 42 and 53.6). The previously vaccinated participants (n=9) showed a similar trend of cross-reactive antibodies in both the MNT (GMT 252 and 229) and HI (GMT 273 and 408) assays.

After the second avian influenza vaccine dose, the cross-reactivity persisted in previously unvaccinated participants , with a significant increase in the neutralization capacity against all AIV strains. In the previously vaccinated group the neutralization was maintained approximately at the same level as after the first vaccine dose.

5.5 T-cell immunity and cytokines

Long-term adaptive immunity is dependent on not just the antibody responses, but also on the T-cells activation post-vaccination, especially when facing emerging variants or subvariants. Studies II and IV concentrated on analyzing COVID-19 and avian influenza vaccine-induced T-cell immunity.

5.5.1 SARS-CoV-2 T-cell activation by Omicron subvariants on a long timeframe

In study II, CD4+ T-cells were activated post the third and fourth vaccine doses by stimulating with SARS-CoV-2 Omicron subvariant BA.5 or XBB.1.5 S-protein and N-protein peptide pools (Figure 22 a). The activation induced with the Omicron subvariant BA.5 and XBB.1.5 peptide pools remained comparable in both the thrice vaccinated uninfected (SI 10.20 and 11.08 at 3D3w, 6.55 and 8.36 at 3D3mo, 9.00 and 9.09 at 3D6mo, 6.58 vs 5.70 at 3D8-12mo) and infected participants (SI 17.06 and 15.28 at 3D3mo, 23.04 and 12.49 at 3D6mo, 11.17 and 11.62 at 3D8-12mo). The SI induced with Omicron subvariant peptide pools did not significantly differ from those induced with the wt-S peptide pool.

Post the fourth vaccine dose, T-cells obtained from uninfected and infected vaccinees followed the same activation trends after stimulation with Omicron subvariants BA.5 and XBB.15 in all time points (SI 8.13 and 5.96 at 4D3wk, 4.54 and 5.08 at 4D3mo, and 5.66 and 9.17 at 4D6mo). The activation with both subvariants was comparable to the activation induced by S-wt.

CD8+ T-cells stimulated with SARS-CoV-2 Omicron subvariant BA.5 or XBB.1.5 S-protein and N-protein peptide pools were very weakly activated post the third and fourth vaccine dose (Figure 22 b). In the thrice vaccinated participants, the uninfected group showed lower activation in all time points when compared to infected participants with both the BA.5 (SI 1.49 vs 2.07 at 3D3mo, 1.32 vs 2.41 at

3D6mo, 1.08 vs 1.53 at 3D8-12mo) and XBB.1.5 (SI 1.27 vs 3.17 at 3D3mo, 1.41 vs 1.59 at 3D6mo, 0.85 vs 1.93 at 3D8-12mo) peptide pool stimulation. In summary, the vaccination elicited robust CD4+ T-cell responses that persisted months after vaccinations and breakthrough infections against Omicron subvariants BA.5 and XBB.1.5. CD8+ T-cell responses were considerably weaker.

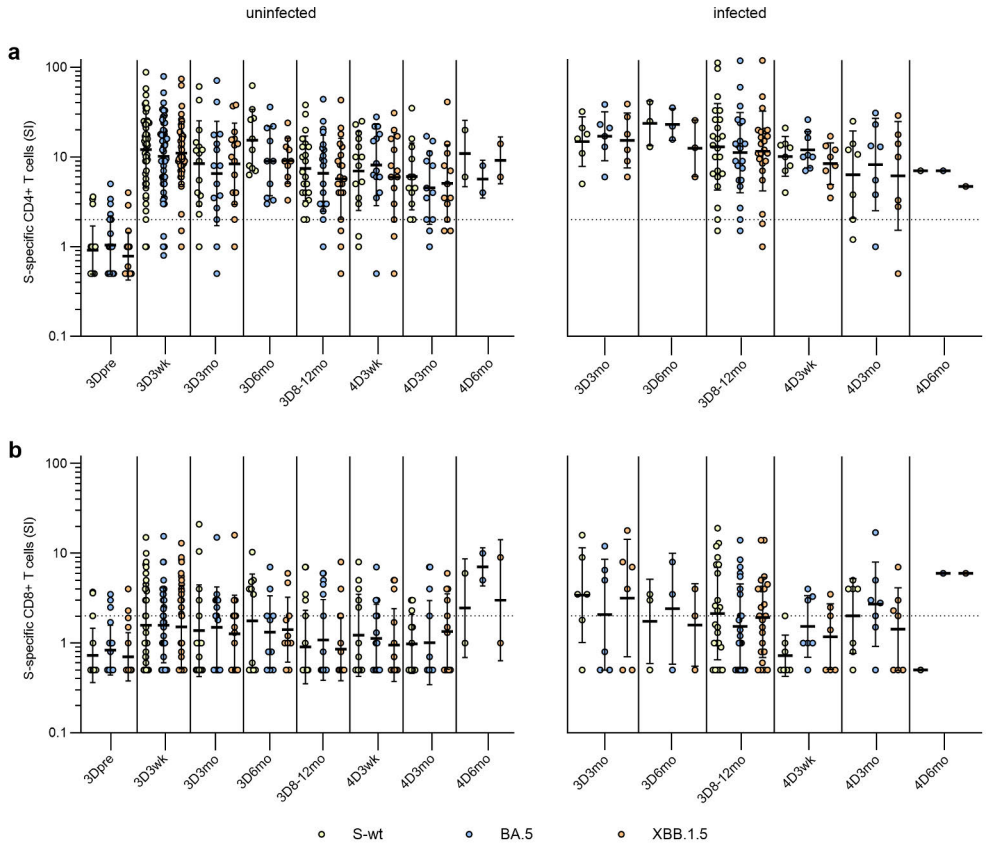

Figure 22. Stimulation of T-cells with wt, BA.5 or XBB.1.5 S-protein peptide pools. A. CD4+ and B. CD8+ T-cell activation from cells acquired from uninfected participants with three or four vaccine doses. Dotted line represents the cut-off for positive activation. The graphs display geometric mean indices (lines) and geometric SD (whiskers). p-value of <0.05 was considered statistically significant. Comparisons within a group between two time points were conducted using two-sided Wilcoxon matched-pairs signed-rank test. Modified from study II.

5.5.2 No T-cell exhaustion was observed after the third and fourth doses

In study II, T-cells collected from uninfected (n = 20) and infected (n = 29) vaccinees were stimulated with DMSO, tetanus toxoid, or peptide pools covering the SARS-CoV-2 S-protein and N-protein of variant Wuhan Hu-1.

When stimulated with the Wuhan Hu-1 variant S peptide pool, S-specific CD4+ T-cell stimulation index was elevated in three times vaccinated (SI 12.1 at 3D3wk, 8.4 at 3D3mo, 13.0 at 3D6mo and 8.0 at 3D8-12mo) compared to the stimulation index of 0.9 at pre-vaccination time point. In infected vaccinees, the stimulation indices were somewhat higher compared to the uninfected vaccinees (SI 14.8 at 3D3mo, 23.7 at 3D6mo, and 11.0 at 3D8-12mo).

Post fourth vaccine dose, the S-specific CD4+ T-cells stimulated with the Wuhan Hu-1 variant S peptide pool also showed an elevated stimulation index when compared to the pre-vaccination time point, but lower than at corresponding time points post third vaccine dose (SI 7.0 at 4D3wk, 6.1 at 4D3mo, and 11.0 at 4D6mo). In infected vaccinees, the stimulation indices were at similar levels compared to the uninfected vaccinees (SI 10.2 at 4D3wk and 5.1 at 4D3mo)

N-protein peptide pool stimulation activated antigen specific T-cells only in infected participants, and to a lesser extent than activation induced by S-protein peptide pools.

5.5.3 Cytokine secretion in SARS-CoV-2 stimulated PBMCs collected from COID-19 vaccinated participants

IFN-γ secretion from PBMCs isolated from SARS-CoV-2 vaccinated individuals post third and fourth vaccine doses was increased compared to the PBMCs collected pre-vaccination when the cells were stimulated with SARS-CoV-2 wild type and Omicron subvariant XBB.1.5 S peptide pools (Figure 23). The wild type N protein peptide pool did not significantly increase the IFN-γ secretion in PBMCs collected post-third and fourth vaccine doses in neither uninfected and infected vaccinees.

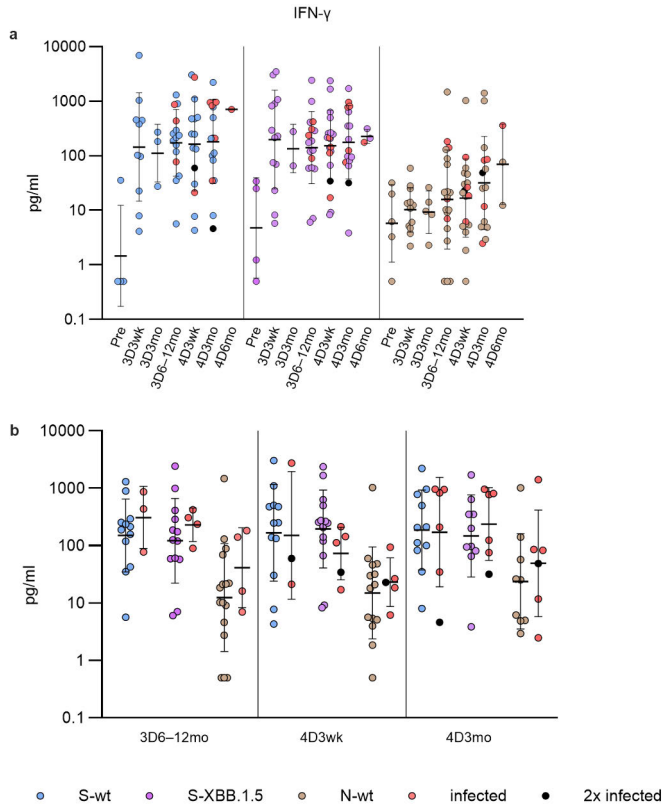

Figure 23. Interferon-gamma secretion from PBMCs of COVID-19 vaccinated participants stimulated with SARS-CoV-2 peptide pools. A. Uninfected and infected showed in same groups B. Uninfected and infected separated. Peptide pools used in the stimulation experiment are indicated in the figure as different colors. The graphs display geometric mean indices (lines) and geometric SDs (whiskers). p-value of <0.05 was considered statistically significant. Modified from study II.

5.5.4 Memory B-cells following three and four COVID-19 vaccine doses

The circulating memory cells differentiated into antibody secreting cells when stimulated *in vitro* with SARS-CoV-2 variant Wuhan Hu-1 S1-domain, receptor binding domain (RBD), or whole N-protein (Figure 24). In uninfected vaccinees, S1 and RBD induced an increase in the differentiation of B-cells to antibody secreting cells after both the third (GM 21.37 and 14.87) and fourth (GM 48.80 and 14.51) vaccine doses when compared to naïve individuals (GM 3.87 and 3.04 cells). No such increase was observed with the N-protein stimulation of cells obtained from uninfected participants. In infected vaccinees, all antigens induced an increase in the differentiation to antibody secreting cells.

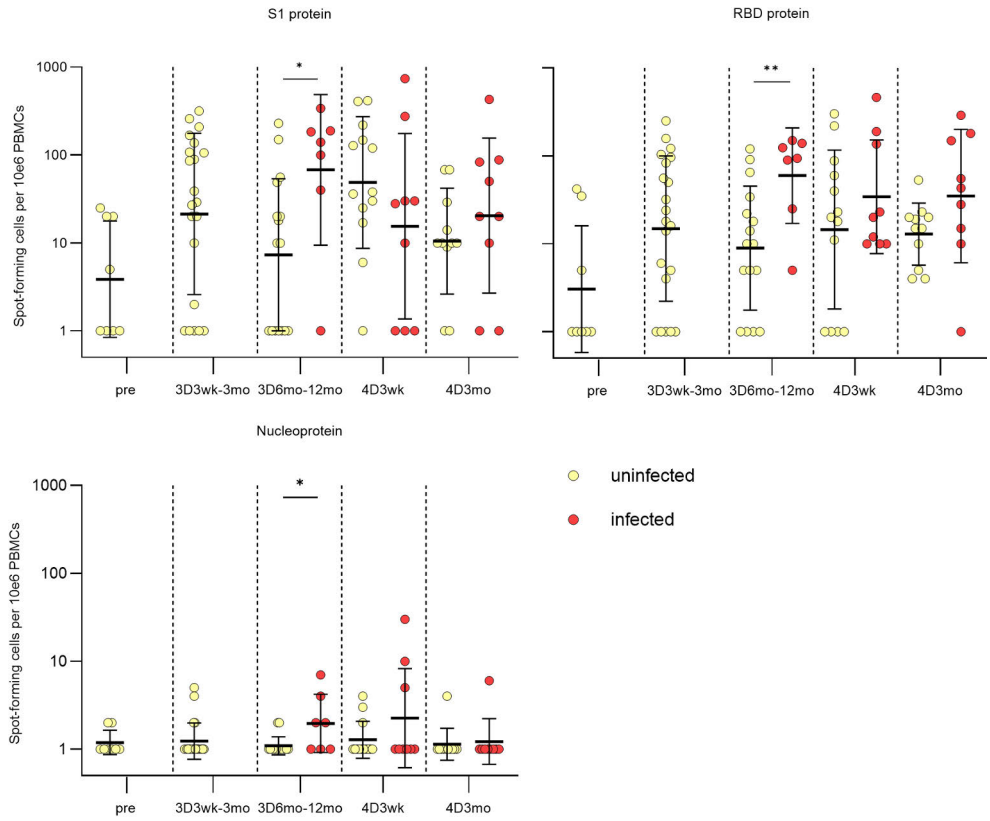

Figure 24. Spot-formation in S1, RBD or N protein stimulated B-cells collected from uninfected and infected vaccinees with three or four vaccinations . The graphs display geometric mean indices (lines) and geometric SDs (whiskers). p-value of <0.05 was considered statistically significant and are displayed as stars (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001). Modified from study II.

5.5.5 Avian influenza vaccine-induced T-cell activation

Avian influenza vaccinations induce significant increases in the SIs of CD4+ T-cells stimulated with influenza virus H1, H5, N8, and NP peptide pools in both the previously unvaccinated and previously vaccinated participants after two vaccine doses (Figure 25).

In the previously unvaccinated participants (n = 13), the second vaccine dose resulted on an average 3.1-fold increase in CD4+ T-cell activation induced by H5 peptide pool stimulation compared to the pre-vaccination samples. In previously vaccinated participants (n = 5) the increase in activation induced by H5 peptide pool was slightly higher, with an average fold increase of 4.0. These increases in CD4+ T cell activations were also observed on the induction with H1, N8 and PR8 NP peptide pools.

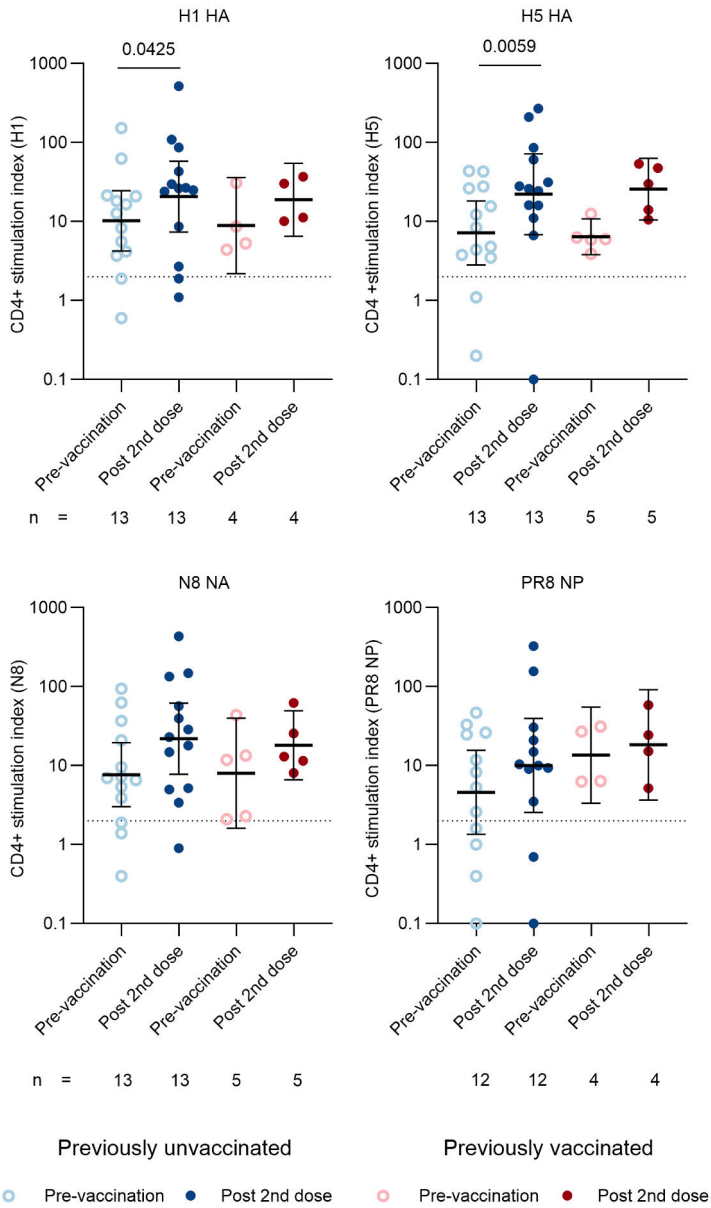

Figure 25. CD4+ T-cell activation after stimulation with H5, H1, N8, or NP peptide pools in participants vaccinated with an avian H5N8 influenza vaccine. Light blue and dark blue dots represent vaccinees with no previous vaccinations, while light red and dark red dot represent vaccinees with previous vaccinations. Dotted line represents the cut-off for positive activation. The graphs display geometric mean indices (lines) and 95% CIs (whiskers). p-value of <0.05 was considered statistically significant. Comparisons within a group between two time points were conducted using two-sided Wilcoxon matched-pairs signed-rank test. Modified from study IV.

5.5.6 Cytokine secretion in influenza peptide stimulated PBMCs collected from avian influenza vaccinated participants

Interferon-gamma (IFN-γ) secretion from PBMCs isolated from avian influenza vaccinated participants was increased when stimulated with influenza virus H1, H5, N8, or NP peptide pools (Figure 26). Previously unvaccinated participants had an increased IFN-γ secretion after two AIV vaccinations when stimulated with H5 or N8 (average fold increase of 5.9 and 5.3, respectively). Previously vaccinated participants similarly had an increased IFN-γ secretion after stimulation with H5 or N8 (average fold increase of 5.0 and 3.7).

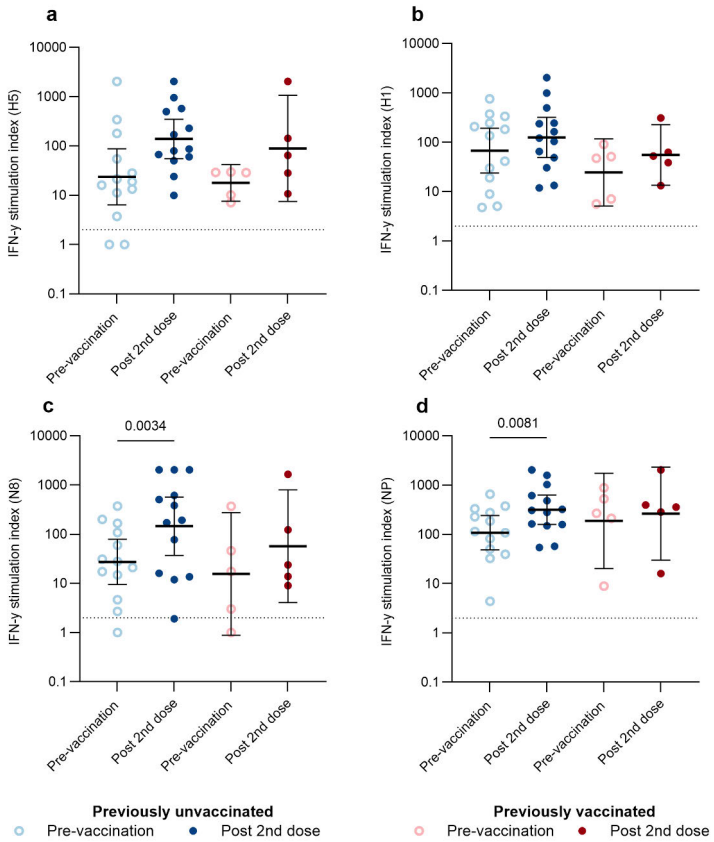

Figure 26. Interferon gamma secretion after stimulation with A. H5 B.H1 C. N8 and D. NP peptide pools. Light blue and dark blue dots represent participants with no previous avian influenza vaccinations, while light red and dark red dot represent participants with previous avian influenza vaccinations. Dotted line represents the cut-off for positive activation. The graphs display geometric mean indices (lines) and 95% CIs (whiskers). p-value of <0.05 was considered statistically significant. Modified from study IV.

6 Discussion

SARS-CoV-2 and AIV share many similarities, such as their zoonotic origins, mode of transmission, and rapid mutation rate giving rise to emergent immune-evasive variants and strains.[22,23,26,134] While influenza viruses and to some extent AIV have been firmly established in the immunological memory of human populations around the globe, SARS-CoV-2 encountered a largely naïve population on the onset of its pandemic spread. As such, from the perspective of adaptive immunity these viruses have existed for very different lengths of time, and may provide insight into how adaptive immune responses are initially formed, and how the immunological memory evolves as new variants emerge to compete with the circulating variants. These virological similarities and epidemiological differences make them an informative pair to contrast each other. The vaccines employed against them differ from each other significantly, with the newer mRNA vaccines having been largely utilized against SARS-CoV-2, and older inactivated subunit vaccines against AIV. The research work presented in this dissertation sought to determine if neutralizing antibodies induced against both of these viruses were long-lasting, and if they were capable of cross-neutralization. In addition, the cellular immune responses were assessed. In the discussion part of this dissertation, these results are inspected in the broader context with other studies and the virology field in general.

6.1 Inactivated viruses and mRNA as vaccine platforms

Vaccines have undergone significant developments in the past decade, both with the improvements on previous concepts such as inactivated virus vaccines, and large-scale implementation of newer technology utilizing mRNA and nanoparticles.[219,220,227,228]

Inactivated virus vaccines, such as the vast majority of influenza vaccines, are one of the earliest mass-produced vaccine types.[224] However, the production of inactivated influenza vaccines is slow, virus strains propagated are prone to mutations, and impurities may accumulate during the manufacturing process.[220] These factors can have drastic consequences, such as loss of specificity or choosing

mismatching strains for the annual IAV vaccinations, hampering the efficiency of the vaccines.[280] In addition, there is also the concern of production safety when highly pathogenic viruses are produced. With some vaccines, improper inactivation could lead to a symptomatic disease after vaccination, as has happened for example with inactivated polio vaccines in the 1950s.[281] To circumvent the problems associated with inactivation, parts of the inactivated virus, such as the surface antigens in the case of Seqirus AIV vaccine, rather than the whole virus, have been deployed.[234] This refinement of inactivated virions to subunits does not come without downsides, as for example the immunogenicity of the subunit vaccines is usually considerably weakened.

Another common problem with most vaccines utilizing inactivated viruses, especially subunit vaccines utilizing only parts of the inactivated virus, is their relatively modest immunogenicity.[223] This could be due to a failure to activate cellular immunity leading to insufficient induction of a durable, long-lasting immunity , and may necessitate the application of multiple vaccine doses.[219,222,277] The use of adjuvants can alleviate this, and for example an adjuvant MF59 is included in the Seqirus AIV vaccine. Besides changes in the formulation, the administration route of the vaccine can have a significant effect, as can be seen in the better efficiency of intranasal route versus the intramuscular route in the case of different inactivated influenza virus vaccines.[229,276,282]

The issues with inactivated virus and subunit vaccines makes their replacement or supplementation with mRNA vaccines highly desirable. mRNA vaccines and the technologies surrounding them have been the subject of interest for several decades, with the key ideas being studied as early as 1977.[226,283] Even though in theory mRNA vaccines are easier and safer to mass-produce and can have mRNA encoding for several antigens from one or multiple viruses, their delivery and stability have been the main issues preventing their widespread use, especially in areas where reliable freezing storage infrastructure is unreliable.[7,227,228] The global pandemic caused by SARS-CoV-2 accelerated the research on mRNA vaccines, and COVID-19 vaccine was the first large scale application of mRNA vaccine technology, and as such a true trial by fire to test mRNA vaccine viability in a rapidly evolving pandemic. Multiple mRNA vaccine formulas with varying mRNA dosages and delivery vectors have been used, with the Pfizer's Comirnaty and Moderna's Spikevax being the most commonly and widely used in Europe, including Finland.[9] Given that almost all participants in out studies received full vaccine series, the mass-production abroad, and delivery and storage of mRNA vaccines in Finland were a resounding success.

The vaccinees studied in this dissertation had received mRNA vaccines against SARS-CoV-2 or inactivated virus subunit vaccine against avian influenza virus.

6.1.1 The effect of dosage and variant-specific SARS-CoV-2 mRNA vaccines

As mRNA vaccines are relatively new, the effects of dosage and the choice of antigen templates is important to evaluate, these phenomena are also studied in this dissertation. The amount of mRNA in the vaccines has an impact on immune responses. Our results imply that the levels of neutralizing antibodies are higher in response to immunization with 50 µg mRNA-containing Spikevax compared to the 30 µg mRNA-containing Comirnaty. This is a common outcome with large mRNA dosage not only on neutralizing antibodies but also on T-cells.[278] However, given that higher mRNA dosage can induce adverse effects more often, and lower dosage is sufficient to induce high levels of neutralizing antibodies, especially when coupled with optimal administration route and control of vaccine release rate, the relatively minor increase in the strength of the immune response is likely not worth the health risks and increased manufacturing costs.[276,282,284,285]

Since the introduction of first COVID-19 mRNA vaccines, these vaccines have been updated to match the circulating SARS-CoV-2 variants. These variant-specific vaccines have been successfully and rapidly adapted and produced on a large scale without significant delays. Some of the vaccinees in the cohorts used in our studies received variant-specific vaccines as booster doses. Both the original and the updated variant-specific vaccines induced high levels of antibodies with robust neutralization capacities against the original Wuhan strain and early Omicron subvariants, as has been also shown in other studies.[286–288] Our vaccinee cohorts were too small to separate vaccinees to variant-specific vaccine groups, however, other studies have shown that these variant-specific vaccines have remained effective against subsequent Omicron variants, even in immunocompromised individuals.[287,289]

6.2 Immune evasion of SARS-CoV-2 and vaccine-induced cross-reactivity against SARS-CoV-2 Omicron subvariants and avian influenza viruses

The rapid evolution of SARS-CoV-2 variants has made it challenging to have up-to-date vaccinations against the circulating variants. Notably, the emergence of immune evasive subvariants has made it necessary to update the vaccine similarly to seasonal influenza vaccines in order to maintain the protective immunity especially in at-risk-groups, such as the immunocompromised and elderly. The Omicron BA.1 subvariant of SARS-CoV-2 especially marked the start of an increasing immune evasion capacity.[12,125]

6.2.1 Cross-neutralization of Omicron subvariants by early and updated COVID-19 vaccines

The enhanced immune evasion by Omicron subvariants is evident in our results showing the greatly diminished neutralization capacity of antibodies induced by the three dose vaccine regimen and/or infections by early (BA.1, BA.2 and BA.5) variants against XBB.1.5 and JN.1 subvariants compared to the neutralizing antibodies against D614G and early Omicron subvariants. Infections by the early Omicron subvariants and variant-specific vaccines based on them seem to offer very little cross-protection against both XBB.1.5 and JN.1, as was also shown by others[10]. Given that neutralizing antibodies strongly correlate with protective immunity[290],this highlights the need for annual updates for variant-specific vaccines, a situation where mRNA vaccines seem to be useful due to their rapid modification and production characteristics. In many countries, Finland included, healthcare institutions have been recommending the updating of SARS-CoV-2 vaccines on a regular basis, and this has become the case in many national vaccination programs.[5]

The benefits of COVID-19 vaccine updates are evident. The fifth Covid-19 vaccine booster doses in Finland were based on the XBB.1.5 S gene, and we show significantly increased neutralization capacities of vaccine-induced antibodies against not only XBB.1.5, but against JN.1 as well. This has also been shown by other studies[6,8] , indicating that some cross-reactivity exists when vaccine variants are phylogenetically closely related. This is also reflected by the neutralization capacity of antibodies in participants infected around the time XBB.1.5 was considered dominant, as antibodies in their sera were still capable of neutralizing JN.1 to similar extent compared to XBB.1.5. This phenomenon is not entirely new in the case of SARS-CoV-2, as this had been detected also with previous variants, such as with Delta variant and Omicron BA.1 subvariant being cross-neutralized to some degree by antibodies induced by the early variant infections and by the three dose vaccine regimen given early during the pandemic.[9] The lack of cross-neutralization against subvariants that are phylogenetically distant from the vaccine variant can be seen in our and other studies in the equally low neutralizing antibody capacity against XBB.1.5 and JN.1 after the third and fourth COVID-19 vaccine doses, which are based on the Wuhan and BA.1/2 variants, respectively.[291,292]

Importantly, despite the lower neutralization capacity the vaccines still reduce the number of hospitalizations and deaths in breakthrough infections that have been common since the emergence of Omicron subvariants.[293,294] The milder disease could possibly act as form of a booster dose by helping to establish hybrid immunity, which provides the best cross-neutralization potential according to our studies.

6.2.2 Lower neutralizing capacity of antibodies against emerging Omicron variants

Even though the updated variant-specific vaccines elicited strong neutralization capacities, they did so to a lesser extent when compared to the neutralization capacities induced against the pre-Omicron variants by the original Wuhan-based COVID-19 vaccinations.

The broader memory B cell population in combination with immune-evasion properties of the XBB.1.5 and JN.1 subvariants may explain why the neutralization peaks after vaccinations and infections do not reach as high levels as they did with earlier variants. Preference to memory B cells produced by earlier vaccinations and infections combined with lower overall detection of the XBB.1.5 and JN.1 subvariants may have hindered subvariant-specific antibody production in our cohorts. The lower neutralization peaks against XBB.1.5 and JN.1 subvariant compared to previous variants could also indicate that the subvariant-induced generation of new plasma cells from the memory B-cell population induced by the primary infection may be hampered. This may have resulted in unspecific, yet still somewhat neutralizing antibodies to be produced by the established memory B cells.

Original antigenic sin (OAS) is a well-known phenomenon with other viruses, such as influenza and dengue, and is the result of immune system's preference toward pre-existing memory B cells, at the expense of more variant-specific new B-cells being produced upon challenge by a slightly mutated variant of the pathogen that caused the primary infection.[295] In our cohorts, SARS-CoV-2 S1-specifc antibodies are abundant and at same levels after each vaccination despite the lower variant-specific neutralization capacity, indicating that the subsequent vaccinations stimulate memory B cells to produce antibodies, but with lower neutralization or effector function specificity to the newer variants compared to older variants. This same trend, albeit to a lesser extent compared to antibodies in noninfected participants, could be observed following breakthrough infections, which are known to induce antibodies with strong neutralization capacity based on our and others previous research.[296] These observations would support the OAS hypothesis, as has also been reported in several other studies.[297,298]

Besides OAS, an interesting option regarding the decline in the variant-specific neutralization peaks is the possibility of antibodies shifting from neutralizing to non-neutralizing in COVID-19 vaccinated and Omicron-infected individuals. Epitopes outside the receptor-binding domain do not mutate as extensively and could possibly be detected by antibodies induced by previous variants.[299] Non-neutralizing antibodies have been shown to be able to bind wide range of S-proteins and still confer protective immunity in animal models, which could explain why COVID-19 caused by emerging variants is not as severe.[300] However, the diminished severity can also be caused by a weaker fusogenicity of the Omicron variants, or the increased

preference towards upper respiratory epithelial cells that are rich in ACE-2 receptors. Preference towards the production of N-protein neutralizing antibodies due to complement hyperactivation being attenuated by anti-N antibodies may have also skewed the production towards non-S1 neutralizing antibodies.[55,301]

To conclude, the neutralizing capacity of antibodies induced by COVID-19 vaccines based on early variants is greatly reduced against new Omicron subvariants and this is likely due to the combination of enhanced immune evasion and OAS. The neutralization capacity is greatly, but not completely, restored by vaccines based on more recent Omicron subvariants.

6.2.3 Avian influenza vaccine-induced neutralizing antibodies and their cross-reactivity

The changes in neutralization capacity of vaccine-induced antibodies against SARS-CoV-2 are not new to the scientific community, as the vaccines against IAVs have had the same challenges for decades.

Like SARS-CoV-2, influenza A viruses undergo mutations that produce a plethora of new virus strains every year, which greatly contributes to annual IAV infections despite vaccination efforts.[302] Compared to SARS-CoV-2, the mutation rate of IAV is even faster due to the combination of antigenic drift, antigenic shift, and the lack of proofreading function in the RdRp.[107] The widespread AIV infections in birds and mammals, the severity of an AIV infection in humans, and the possibility of a readily human-to-human transmitting AIV strain arising due to accumulating mutations raised the urgent need for a vaccine able to broadly neutralize HPAIVs. Because of this, the European Union jointly acquired a stockpile of A/Astrakhan/3212/2020 (A(H5N8), clade 2.3.4.4b) strain-based vaccines in the hopes of them to elicit cross-neutralizing antibodies against the circulating clade 2.3.4.4b AIVs.

Finland was the first country to initiate AIV vaccinations in 2025, and together with collaborators in Finnish Institute of Health and Welfare we followed-up the immunity induced by AIV vaccines. We showed that the two-dose vaccination regimen with the inactivated virus subunit vaccine induced neutralizing antibodies against three recently circulated A(H5N1) clade 2.3.4.4b HPAI viruses that had been isolated from animals.[19,303] The neutralization capacity of the vaccine-induced antibodies against the A/Texas/37/2024 strain recombinant, which was a recombinant based on the virus isolated from an AIV infected dairy worker, was especially important, given that this strain is capable of infecting humans and resulted in numerous AIV infections in humans in North America.[304] Likewise, since many fur farm workers were likely exposed to the A/blue fox/UH/004/2023 strain and strains close to it, the vaccine-induced cross-neutralizing antibodies against this

AIV strain is an encouraging finding. In a study previously conducted in Finland, cross-reactivity was seen against strains heterologous to the vaccine strain.[305]

Even though based on our studies the neutralizing capacity of the AIV vaccine-induced antibodies seems to be strong, it remains to be seen how long the antibodies last and how well the antibodies neutralize emerging AIV strains.

6.2.4 Challenges in antibody analyses in a pandemic situation

The rapid evolution of both SARS-CoV-2 and influenza A viruses has made their study frustratingly slow at times, given the frequency of the emergence of new subvariants. Acquisition and culturing of the variant virus stocks is a time-consuming and mutation prone endeavor. In some studies, this has been the reason for the use of pseudo-typed viruses instead of the live viruses in neutralization assays. There is strong evidence that the results garnered from experiments utilizing pseudoviruses correlate with the ones obtained from live virus assays, and with the benefit of being easier, safer, and faster.[306–308] However, assays with pseudoviruses do not account for the staggering number of interactions that arise from the viral proteins interacting with each other and with cellular proteins, or post-translation modification occurring during replication, or the effects of multiple replication cycles. In addition, pseudovirus-based assays require an established pipeline for the recombinant virus production. Ideally, the assays with live viruses and with pseudoviruses should be used in combination and standardized to gain the benefits of both.

The use of pseudovirus and live virus assays would greatly benefit from calibration to an international standard to ensure that their results are comparable not only between the methods, but between different laboratories as well. Given that the definitive neutralization capacity required for protective immunity is not clearly established, it is difficult to foresee if the neutralization detected in our experiments would be sufficient to protect against a serious disease. The lack of standardization is a problem in many SARS-CoV-2 studies despite the existence of an international WHO standard, and hinders the meta-analysis of neutralization capacity and its link to protective immunity in SARS-CoV-2 literature. With AIVs, many standardization efforts and general guidelines by WHO for IAV MNTs have been undertaken and adopted by laboratories, leading to much more comparable and repeatable results when looking at AIV research as a whole.

Nevertheless, we can still get an estimate of the effects of vaccinations on protective immunity from our studies, since even a modest increase in neutralizing capacity of antibodies has been linked to protective immunity.[309,310]

6.3 T-cell activation and lack of exhaustion following multiple vaccinations

In addition to antibodies, virus vaccines and infections elicit cell-mediated immunity. T-cells form the core of this cellular immunity: CD4+ T-cells assist B-cells and CD8+ T-cells kill virus-infected cells. Previous infections and vaccinations result in memory T-cells. Memory cell clones formed by these initial exposures are long-lasting, and they can persist for many years.[250,251,311] Like B-cells, T-cell responses favour the clones formed by the initial exposure to the antigen.[312] This effect can be directly observed in the faster activation and proliferation of memory CD8+ cells compared to naïve CD8+ cells, and indirectly in the reduction of the activation of naïve CD4+ cells.[313] Despite this preference, some clonal selection of CD8+ cells for the proliferation and memory T-cell formation has been observed for new vaccine variants.[314,315]

The initial CD4+ and CD8+ memory T-cell formation against SARS-CoV-2 was induced by a SARS-CoV-2 infection or COVID-19 vaccinations. Although most humans have likely had a seasonal human coronavirus (HCoV) infection in the past, the immunological memory established by those viruses did not have a significant cross-protective effect to SARS-CoV-2 on either the humoral or cellular immunity, although some antibody cross-reactivity has been detected.[316] Phylogenetically more closely related SARS-CoV and MERS-CoV on the other hand have not infected many people, and thereby the immunological memory against them is not prevalent in the general population, nor in our cohorts. Influenza A viruses have been circulating in humans for many decades and annual vaccinations have been administered for a long time, resulting in immunological memory for seasonal influenza A viruses. As such, humans have pre-existing and often cross-reactive antibodies against various HA and NA subtypes.

6.3.1 CD4+ and CD8+ T-cell activation after COVID-19 and AIV vaccinations

The ability of a vaccine to activate T-cells is crucial for the immune response. Consistent with other studies[317,318], we observed a strong CD4+ activation and memory cell formation after three COVID-19 vaccine doses, and subsequent booster doses elicited activation and proliferation of CD4+ cells. We also concluded that two AIV vaccine doses were able to elicit moderate to strong CD4+ responses in both AIV naïve and previously vaccinated participants, the latter suggesting that the existing memory T cell populations did not interfere with the activation of T cells with a new specificity.

As in previous studies[319], in our study the CD8+ T-cell activation and establishment of CD8+ memory cells was not efficient with inactivated virus subunit

influenza virus vaccine. Killing or attenuating viruses weakens their antigenicity, and thus the ability to induce strong CD8+ cell responses. The use of only the immunogenic proteins of the inactivated virus further weakens the antigenicity, which likely influenced the CD8+ responses elicited by the AIV vaccines in our studies as well.[320,321] On the other hand, as shown by others as well[277,322], CD4+ cells seem to be more readily activated, as is evident by the rise in the HA and NA specific CD4+ cells in our vaccinees. As an interesting point of comparison, similarly to inactivated and subunit influenza vaccines, inactivated SARS-CoV-2 vaccines predominantly induce CD4+ responses.[323] Interestingly, COVID-19 mRNA vaccinations resulted in CD8+ cell activation already after one dose. This is a surprising feature, given that similarly to inactivated subunits, mRNA is expected to activate cell immunity poorly, and in our and other studies antibody levels peaked only after three COVID-19 vaccine doses.[278] However, since the vaccine mRNA is delivered by the LNP into the cell and the encoded protein is synthesized inside of the cell similarly to the viral mRNA instead of being externally picked up for processing, this may have an impact on the antigen presentation by MHC I receptors and therefore elicit stronger CD8+ responses.[324]

The inability of inactivated vaccines to activate CD8+ cells however is not universal. A study with vaccinia virus demonstrated that by utilizing cross-presentation of the virion by dendritic cells to CD8+ T-cells, inactivated vaccinia virus vaccines could induce strong CD8+ responses.[321,325,326] Whether this is a property of the whole inactivated vaccinia virus only or if this extends to possible subunit vaccines is an interesting avenue of study.

It remains to be seen if the subsequent SARS-CoV-2 variants induce existing memory T-cells, or if the antigenic drift produces a sufficiently different variant that will lead to a large scale proliferation of new variant-specific memory T-cells. With AIV vaccinated individuals it remains to be seen if long-lasting memory T cell population is established.[327] Even though strong CD4+ T-cell-specific responses are induced by both vaccine types, the CD8+ responses could be enhanced, possibly with adjuvants and optimized delivery, to achieve a more robust and broader T-cell memory.

6.3.2 Lack of T-cell exhaustion following multiple vaccinations

In addition to the induction of effector and memory T-cells, the possibility of vaccine-induced T-cell exhaustion was one of the main worries after it became apparent that repeated vaccinations against SARS-CoV-2 in a relatively short time were necessary for upholding protective immunity.[328]

T-cell exhaustion is a detrimental condition of T-cells that sometimes arises after continuous exposure to the same antigen through vaccinations and infections, resulting into an attenuated response to the antigen. This can weaken or alter the adaptive immune response to the antigen upon further challenge.[329,330] This is especially a concern for individuals who have had serious COVID-19, but also to otherwise healthy individuals who might then develop a long-COVID syndrome.[17,331]

Studies by us and others indicate that T-cell exhaustion has not taken place since the neutralizing antibodies are induced against emerging SARS-CoV-2 variants, the T-cell responses do not decline even after a year post the third vaccine dose, and T-cell responses are elicited after fourth and fifth vaccine doses.[328] In the case of AIV, given that CD4+ T-cell responses in our vaccinees were elevated in previously vaccinated individuals after both vaccine doses it is also unlikely that T-cell exhaustion is taking place. This will likely continue to be the case for both SARS-CoV-2 and AIV, as they do not establish chronic infection like for example HIV and hepatitis B virus, which more readily lead to T-cell exhaustion.[332]

6.4 B-cell activation, lack of exhaustion, and antibody kinetics

Much of our research on SARS-CoV-2 was focused on the neutralizing antibodies secreted by plasmablasts and long-lived plasma cells, but we also wanted to assess the capacity of memory B cells to differentiate and proliferate when stimulated with viral antigens derived from various SARS-CoV-2 variants. Given that memory B cells are prone to OAS when there are only minor structural differences between the antigen responsible for the primary infection and the antigen causing the secondary infection[295], it was important to validate if the cells were capable of differentiating when challenged by altered variants of the original Wuhan variant.

6.4.1 Establishment and durability of long-lived plasma cells

Establishment and maintenance of memory B-cell capable of starting rapid production of neutralizing antibodies is one of the main goals of a successful vaccination.

Studies have shown that the memory B-cell population is not restricted to be specific only for early variants but has the capacity to develop new plasma cells upon repeated exposure to novel variants.[333,334] We and others have shown that booster vaccine doses increase both the half-life and the neutralization capacity of antibodies against early and new variants[335]. As such, booster doses are likely required to update the memory B-cell and plasma cell populations for renewal of protective neutralizing

antibody levels to combat antigenic drift and emerging variants with both SARS-CoV-2 and AIV.[336,337]

The longevity of plasma cells was indirectly established in our SARS-CoV-2 experiments, showing both the neutralizing capacity and the level of S1-specific antibodies plateauing above the threshold after approximately 7 months post-vaccination following three or four vaccine doses in noninfected vaccinees. The kinetics of declining antibody levels are partially explained by the natural decline in antibody production in the absence of infecting pathogen or the cessation of vaccine mRNA translation, followed by apoptosis of plasmablasts. The antibodies produced at later time points are likely produced by long-lasting plasma cells residing in the bone marrow.[338] Neutralizing antibody capacities and overall S1-specific antibody levels stabilized well above the neutralization threshold at least against earlier variants. This has also been confirmed with similar cohorts in other studies.[337,339]

Curiously in the case of AIV, in our neutralization assays a strong immune response could be observed in the previously vaccinated cohort as a rapid production of neutralizing antibodies against all AIV strains, already after the first vaccination. These vaccinees had received their previous vaccinations against AIV in 2018 with a vaccine containing antigens derived from a clade 2.2.1 A/H5N1 strain, and still after six years the vaccinees could mount a rapid and strong neutralizing antibody responses against phylogenetically distinct strains in the clade 2.3.3.4b.[340]

The strong increase in the capacity of neutralizing antibodies could indicate that long-lived memory B-cells were successfully established by the previous vaccinations. These memory B-cells could undergo somatic hypermutation upon a challenge by a new AIV antigen, leading to a rapid production of high-affinity neutralizing antibodies against the new clade 2.3.3.4b AIV strains. Additionally, the memory B-cells may rapidly differentiate to plasmablasts that produce antibodies capable of cross-neutralizing the new clade 2.3.3.4b AIV strains. However, the phylogenetical distance between clade 2.3.3.4b to the previous vaccine strains belonging to clades 2.2.1, 2.1.3.2, and 1 indicates that the AIV vaccine-induced antibodies would not be cross-neutralizing.[340] On the other hand, given the rapid rise in the neutralization capacity, it is unlikely that the plasma cells produced from the non-neutralizing memory B-cells would have had enough time to acquire enhanced affinity towards the clade 2.3.3.4.b AIV strains. Therefore, it could be that some cross-reactivity from previous vaccinations has existed, enabling antibody affinity maturation and the production of highly specific and neutralizing antibodies. This sort of priming effect was seen in a previous study in Finland, where previously administered seasonal influenza vaccines induced cross-reactive antibodies against A/H1N1 viruses.[221] However, a A/H1N1 group virus was used as a template in the seasonal IAV vaccine, thus being more closely related to other seasonal IAVs,

whereas in the previous AIV vaccines a more phylogenetically distant template virus was used.

Taken together, SARS-CoV-2 and AIV vaccines seem to be capable of inducing a cross-reactive and long-lasting memory B-cell population.

6.4.2 Lack of B-cell exhaustion following multiple SARS-CoV-2 vaccinations

B-cell exhaustion can manifest after multiple vaccinations or infections as poor B-cell proliferation capacity upon antigen challenge.[329] In addition, due to ageing, immunosenescence can also affect B-cell proliferation.[249,341] In our ELIspot experiments, circulating memory B-cells isolated from participants with up to four COVID-19 vaccine doses, and in some cases SARS-CoV-2 breakthrough infections, were still capable of differentiating to antibody secreting cells.

Both the longevity of the antibody responses, and the broadening of memory B-cell population and their capability to proliferate after several vaccinations and Omicron subvariant infections is encouraging. This indicates that B-cell exhaustion and OAS are unlikely to affect the robustness of the immunological memory. Given that the high mutation rate of SARS-CoV-2 necessitates annual vaccinations for at-risk individuals, this finding supports to continue to update vaccines based on emerging variants.

6.5 Limitations of the study

6.5.1 Female dominated sample sets

The SARS-CoV-2 study cohort, while extensive, comprised of healthcare workers, a sector dominated mostly by adult women. This may have skewed the results towards one sex and somewhat narrow age range. The avian influenza cohort similarly composed mostly of females. It was also limited by its small size and its lack of fur farm workers, a crucial at-risk group to which the vaccination was mainly advocated for.

6.5.2 Large amount of breakthrough infections at later time points, cross-reactivity to previous vaccines or infections

The large number of breakthrough infections following the emergence of Omicron BA.2 made it challenging to find uninfected vaccinees with samples from all time points up to 24 months post third dose, limiting the size of our uninfected cohort at

later time points. Also, later time points in three and four COVID-19 vaccine dose cohorts, and the fifth dose cohort in general, had small sample set sizes, which may have impacted the significance of the kinetics analyses in MNT assays.

6.5.3 Small sample sizes limit T-cell analysis

Due to the limited amount of Seqirus AIV vaccinations given, the cohort for T-cell analyses was limited. This impacted the statistical significance of our findings, especially with the previously vaccinated group. In the case of follicular T helper cell (cTfh) analyses, an insufficient number of cells (<500 cTfh) in some samples decreased the size of the cohort further. Activated memory T-cell populations were also small, which may have impacted the accuracy of subset distribution analysis.

7 Conclusions

In this dissertation, it was shown that SARS-CoV-2 Comirnaty and Spikevax mRNA vaccines induce high levels of S-specific antibodies, which have a strong neutralization capacity against SARS-CoV-2 Omicron subvariants. The fourth and fifth booster doses induced higher or as high S-specific antibodies, which neutralized emerging Omicron subvariants more efficiently and declined more slowly. To effectively cross-neutralize newer emerging subvariants, the vaccines must be updated with the dominant circulating variant templates to maintain a strong neutralization capacity. This is highlighted by the large number of breakthrough infections in vaccinees. Breakthrough infections lead to the formation of strongly neutralizing antibodies. Given that the combination of breakthrough infections and updated vaccinations resulted to the highest neutralization capacity after booster vaccinations, it remains imperative that at-risk-individuals continue to be vaccinated to protect them from severe COVID-19. In the case of individuals who are not at-risk, the neutralization capacity of antibodies plateauing well above the positive threshold should confer a degree of protection against severe COVID-19 caused by older variants. In both cases, the vaccinations could attenuate a disease-causing variant to act as a booster against more recent variants, and as such help to establish a robust hybrid immunity.

It is also shown that robust S-antigen specific CD4+ and CD8+ T-cell, as well as B-cell activation is induced by vaccinations and infections alike, and that no T-cell or B-cell exhaustion seemed to take place even after several vaccinations or breakthrough infections. This highlights the safety of the current mRNA vaccines against SARS-CoV-2, underscores their efficiency in inducing cell-mediated immune responses, and should encourage policy makers to continue current booster vaccination programs.

With avian influenza virus, it was shown that two vaccine doses of inactivated subunit A/Astrakhan/3212/2020 (H5N8)-like strain based vaccine induced HPAI 2.3.4.4B cross-neutralizing antibodies in previously AIV unvaccinated and vaccinated participants. Notably, the vaccinations induced antibodies with high neutralization capacity after just one dose when administered to a vaccinee who had been previously vaccinated with a vaccine based on a phylogenetically unrelated

AIV strain. Additionally, we showed that stimulation with H1, H5, and N8 peptide pools induced robust CD4+ T-cell activation in both groups. The surprisingly strong cross-neutralization capacity against various AIV strains highlights the importance of prophylactic vaccination of at-risk-individuals, as the protection offered by the relatively cheap vaccinations may be crucial in slowing down the spread of a potential human adapted AIV, limiting the damage they cause to societies.

To conclude, both mRNA and inactivated subunit virus vaccines have their benefits and short-comings, but they are still highly effective at preventing serious disease. Both are relatively inexpensive, and the mRNA vaccine platforms boast high adaptability. In the studies of this dissertation, both vaccine types resulted in strongly induced long-lasting neutralizing antibody and cell-mediated immune responses after multiple doses, both of which are correlated with protective immunity. In the case of the Seqirus AIV vaccine, one dose was enough to engage memory B-cells induced by previous vaccinations, which could be observed in the rapid production of neutralizing and cross-reactive antibodies. This was also evident with the SARS-CoV-2 mRNA booster doses engaging the memory B cells to produce neutralizing antibodies. The mRNA vaccines especially have proven to be efficient, cost-effective, and easily mass-produced, and in the geographical locations with sufficient logistical and storage networks should be adopted as a major vaccine platform in the future not only for SARS-CoV-2, but other viruses as well.

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