Weaving the Threads of Memory: How Pre-existing Immunity Shapes B Cell Responses to Influenza A Virus

Department of Microbiology and Immunology

Institute of Biomedicine

Sahlgrenska Academy, University of Gothenburg

UNIVERSITY OF GOTHENBURG

Gothenburg 2025

Cover illustration: Embroidered, weaved and designed by Laura Reusch with BioRender.

© Laura Reusch 2025
laura.reusch@gu.se

ISBN 978-91-8115-078-0 (PRINT)
ISBN 978-91-8115-079-7 (PDF)

Printed in Borås, Sweden 2025
Printed by Stema Specialtryck AB

“You have to be a champion of failure.”

Katalin Karikó

For everyone who does not believe in themselves.

Weaving the Threads of Memory: How Pre-existing Immunity Shapes B Cell Responses to Influenza A Virus

Laura Reusch

Department of Microbiology and Immunology, Institute of Biomedicine
Sahlgrenska Academy, University of Gothenburg
Gothenburg, Sweden

ABSTRACT

Influenza A virus (IAV) poses a persistent global health threat due to its ability to evolve rapidly, requiring annual updates to seasonal vaccines. Despite significant advancements, a universal vaccine capable of providing long-term and broad protection has not been developed yet. It was investigated here, how pre-existing immune components—antibodies (Abs), memory B cells (MBCs), and CD4 T cells—shape B cell responses to drifted IAV haemagglutinin (HA). We also explored innovative immunogen design strategies, targeting a conserved epitope to overcome immunodominance (ID) and enhance vaccine efficacy.

While pre-existing CD4 T cells accelerated Ab and GC responses, pre-existing Abs were shown to mask epitopes and exhibit feedback mechanisms, thereby reshaping ID patterns of B cell responses. The valency of antigens used for vaccination influenced the extent of Ab-mediated modulation, with multivalent antigens showing greater effects compared to monovalent counterparts. Whereas MBC rapidly differentiated into antibody-secreting cells (ASC) rather than re-entering secondary germinal center reactions (GCs), this localized Ab secretion contributed to secondary responses rather than the presence of serum-Abs. Finally, MBC and naïve B cells were regulated differently after vaccination with a multivalent particle.

To address challenges posed by antigenic variability and to induce broad immunity against IAV, a computationally designed HA stem mimetic was developed. This immunogen selectively engaged MBCs of IAV-experienced individuals and induced cross-reactive Ab responses against both group 1 and group 2 IAV strains after vaccination in mice.

By combining insights into ID, the effect of pre-existing immunity on secondary B cell responses and rational antigen design, this work highlights key mechanisms driving protective and broad B cell responses to IAV, thereby providing valuable insights to inform the development of a universal IAV vaccine. These findings also offer broader implications for combating other highly variable pathogens, including severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and human immunodeficiency virus (HIV).

Keywords: Influenza A virus, haemagglutinin, universal vaccine, memory B cells, immunodominance, computational vaccine design, antibodies, CD4 T cells

ISBN 978-91-8115-078-0 (PRINT)
ISBN 978-91-8115-079-7 (PDF)

SAMMANFATTNING PÅ SVENSKA

Influensavirus fortsätter att vara ett stort globalt hälsoproblem. I Sverige testade 16.460 personer positivt för influensa under säsongen 2023-2024 varav 335 behövde intensivvård och 610 avled inom 30 dagar efter diagnos. De flesta av dessa var över 65 eller under 4 år. Eftersom influensaviruset snabbt kan förändra sig genom mindre mutationer och utbyte av sina gener krävs årliga uppdateringar av säsongsvacciner. En del av viruset som ofta muterar är proteinet hemagglutinin. Ett flertal områden på hemagglutinin attackeras av immunsystemet i en bestämd hierarki, ett fenomen som kallas immunodominans. Trots stora framsteg finns ännu inget universellt influensavaccin som ger långvarigt och brett skydd.

I denna studie undersökte vi hur befintliga immunkomponenter påverkar B-cellernas svar mot muterat influensavirus hemagglutinin.
De aktuella immunkomponenterna var:
- antikroppar, som kan binda och oskadliggöra viruset eller varna immunsystemet för det,
- minnes-B-celler, som antingen kan snabbt utsöndrar nya antikroppar eller anpassa sig till eventuella mutationer på viruset,
- och CD4 T-celler, som hjälper B celler att utveckla ett effektivt immunförsvar

Vi utforskade också datorbaserade vaccindesigntrategier för att kringgå immunodominans och förbättra vaccinets effektivitet mot flera olika influensastammar.

Våra resultat visade att redan existerande CD4 T-celler påskyndar antikroppssvaret och förbättrar B-cellernas förmåga att anpassa sig till nya virusvarianter. Samtidigt kan befintliga antikroppar blockera vissa delar av hemagglutinin och därmed omforma hur immunsystemet riktar in sig, det vill säga immunodominansmönstren. Hur många hemagglutinin-molekyler varje vaccinpartikel bar på påverkade också i vilken grad antikropparna kunde omforma B-cellssvaret. Minnes-B-celler föredrog dessutom att snabbt producera antikroppar jämfört med att anpassa sig vidare mot viruset. Detta innebär att lokalt producerade antikroppar vid injektionsstället påverkar B-cellernas svar mer än antikroppar som redan cirkulerar i blodet. Slutligen uppförde sig

naiva B-celler, dvs de som inte tidigare stött på influensa, och minnes-B-celler olika under upprepade exponeringar för viruset.

För att hantera utmaningar med hemagglutininets variation och skapa bred immunitet mot olika influensastammar tog vi fram ett datorutformat hemagglutininliknande protein. Det innehöll en mindre, mer koncentrerad del av hemagglutinin som förekommer hos flera virustyper. Detta nya antigen engagerade minnes-B-celler från individer som tidigare vaccinerats med ett godkänt vaccin. Dessutom utlöste det antikroppssvar mot många olika influensaavirusstammar efter vaccination hos möss.

Genom att kombinera insikter om immunodominans, effekten av befintlig immunitet på sekundära B-cellssvar och datorstödd proteindesign, belyser denna avhandling centrala mekanismer som driver skyddande och breda B-cellssvar mot influensavirus. Detta ger värdefulla insikter till utvecklingen av universella influensavacciner och som dessutom är användbara mot andra snabbt muterande patogener såsom SARS-CoV-2 och HIV.

LIST OF PAPERS

This thesis is based on the following studies.

I. Danica F Besavilla, Laura Reusch*, Josue Enriquez*, Karin Schön, Davide Angeletti. **Pre-existing CD4 T cell help boosts antibody responses but has limited impact on germinal center, antigen-specific B cell frequencies after influenza infection.** Front Immunol. 2023 Aug 30:14:1243164.

II. Laura Reusch, Nimitha R Mathew, Karin Schön, Danica F Besavilla, Ivan Kosik, James S Gibbs, Madeleine C Mankowski, Jonathan W Yewdell, Mats Bemark, Davide Angeletti. **The impact of pre-existing antibodies and memory B cells upon vaccination with influenza A virus depends on their antigenic site specificity and antigen valency.** Manuscript

III. Sarah Wehrle*, Andreas Scheck*, Laura Reusch*, Flavio Matassoli, Sandrine Georgeon, Karla M Castro, Johannes Cramer, Wayne Harshbarger, Stéphane Rosset, Sarah Andrews, Karin Schön, Badiaa Bouzya, Ronan Rouxel, Normand Blais, Enrico Malito, Adrian McDermott, Thomas Krey, Corey P Mallett, Ventzislav Vassilev, Davide Angeletti**, Bruno E Correia**. **Computationally designed stem-epitope mimetics elicit broadly reactive antibodies.** Manuscript

CONTENTS

Abbreviations iv
1 Introduction 1
1.1 Influenza A virus 1
1.1.1 Structure of Influenza A virus and its lifecycle 1
1.1.2 Influenza A virus Haemagglutinin 3
1.1.3 Antigenic shift and drift 6
1.1.4 Current seasonal influenza vaccines and future vaccine development 8
1.2 B cell Immunity 10
1.2.1 B cell memory 10
1.2.2 T cell help to B cells 17
1.2.3 The effect of pre-existing immune components on B cell repsonses 21
2 Aims 24
2.1 Paper I and II 24
2.2 Paper III 24
3 Methods, methodological limitations and ethical considerations 25
3.1 Viruses and proteins 25
3.1.1 Virus 25
3.1.2 Proteins 26
3.2 Infection and vaccination 28
3.2.1 Infection 28
3.2.2 Vaccination 28
3.3 Mouse models 29
3.3.1 Cell fate tracking model 29
3.3.2 Cell transfer model 30
3.4 Adoptive transfer models 31
3.4.1 Antibodies 31
3.4.2 Memory B cells 32

3.4.3 CD4 T cells 33
3.5 Multicolour and spectral flow cytometry 34
3.6 Functional assays to evaluate antibody function and specificity 35
3.6.1 ELISA 35
3.6.2 Binding to infected cells 35
3.6.3 ADCC 36
3.6.4 Neutralization assays 36
3.7 Ethical considerations 37
4 Results and discussion 38
4.1 Paper I 38
4.2 Paper II 42
4.3 Paper III 49
5 Summarizing Conclusions and future perspectives 53
Acknowledgements 56
References 62

ABBREVIATIONS

3R	replace-reduce-refine
Ab/Abs	antibody/antibodies
APC	antigen presenting cell
ADCC	antibody-dependent cellular cytotoxicity
all Δ4	Ca1Δ4, Ca2Δ4, CbΔ4, SaΔ4, SbΔ4
ASC	antibody secreting cell
Bcl6	B cell lymphoma 6
BCR	B cell receptor
BM	bone marrow
Bmax	maximum binding capacity
bnAbs	broadly neutralizing Abs
BRM	resident MBC
BSA	Bovine serum albumin
CD40L	CD40 ligand
CDR	complementary determining region
CXCL13	C-X-C chemokine ligand 13
CXCR5	C-X-C chemokine receptor 5
ΔCb	escape in Cb site
dpi	days post infection
dpv	days post vaccination

ELISA	enzyme-linked immunosorbent assay
eMBC	early memory B cells
ERT2cre	cre recombinase-estrogen receptor 2 fusion protein
Fc	fragment-crystallizable
FcγRIV	Fc gamma receptor IV
FDC	follicular dendritic cell
GC	germinal center
gp	glycoprotein
HA/H1	hemagglutinin/hemagglutin in group 1
HAU	hemagglutination units
HBSS	Hanks' Balanced Salt Solution
HIV	human immunodeficiency virus
IAV	influenza A virus
IBV	influenza B virus
ID	immunodominance
IFN-γ	interferon gamma
Ig	immunoglobulin
IL	interleukin
i.m.	intramuscular
i.n.	intranasal
i.p.	intraperitoneal

kDA	kilodalton
LN	lymph node
llPC	long lived plasma cell
M	matrix protein
M2	matrix-2 protein
mAb	monoclonal antibody
MBC	memory B cell
MDCK	Madin-Darby canine kidney
MHC(I/II)	major histocompatibility complex (class I/class II)
medLN	mediastinal lymph node
MOI	multiplicity of infection
mRNA	messenger ribonucleic acid
NA	neuraminidase
NP	nucleoprotein
NS	nonstructural protein
OAS	original antigenic sin
others	Ca1, Ca2 and Sa antigenic sites
OVA	ovalbumin
PA	polymerase acidic
PB	plasmablast
PB1	polymerase basic 1
PB2	polymerase basic 2
PC	plasma cell
PD-L2	programmed cell death ligand 2
pIgG	polyclonal IgG
pLN	Popliteal lymph node
PR8	A/Puerto Rico/8/34 virus
r	recombinant
RBS	receptor-binding site
RNA	ribonucleic acid
S1P	sphingosine-1-phosphate
S1PR2	sphingosine-1-phosphate receptor 2
SA	streptavidin
SARS-CoV2	Severe acute respiratory syndrome coronavirus 2
s.c.	subcutaneous
SHM	Somatic hypermutation
SPR	surface plasmon resonance
$TCID_{50}$	median tissue culture infective dose
TCR	T cell receptor
T_{fh}	T follicular helper cell
T_H	T-helper
T_{reg}	regulatory T cells
UV	ultraviolet
V(D)J	variable-diversity-joining
V_H	heavy variable region
vRNP	viral ribonucleoprotein

wt	wildtype

1 INTRODUCTION

1.1 INFLUENZA A VIRUS

With three pandemics during the 20th century and already one pandemic during the 21st century, we have identified influenza as a dangerous pathogen and are able to characterize the infective agent down to the molecular level. But reports of influenza-like diseases have been recorded for millennia. As early as 410A.D., Hippocrates described a disease that could be interpreted as influenza; including coughs, fever and a seasonal re-occurrence [1, 2]. Although it is debatable whether or not this disease was in fact caused by influenza, several more accounts of similar diseases have been recorded all throughout European history through the middle ages to modern times [3]. The first virological confirmed influenza pandemic with identified virus subtype was the devastating “Spanish Flu” (A H1N1), with almost half of the world population infected and 40-50 million fatalities [4]. After 3 more pandemics, the most recent one being the “Swine Flu” (A H1N1) in 2009 claimed comparably less deaths, 18,449 [5], still the threat of a new pandemic is high and influenza returns seasonally, representing a high global burden [6]. It becomes evident that although humans have been in contact with this virus for centuries and potentially millennia, we have not developed sufficient immune responses to keep the disease in check. Neither have we developed a universal vaccine but rather rely on seasonal vaccines that need to be updated yearly. A universal vaccine confers broad protection against multiple seasonal and potential pandemic IAV strains for many seasons, ideally for a lifetime [7].

1.1.1 STRUCTURE OF INFLUENZA A VIRUS AND ITS LIFECYCLE

The negative-sense ribonucleic acid (RNA) orthomyxovirus influenza has four different types, of which A and B are regularly infecting humans and causing epidemics and pandemics [8]. Until recently, there were two lineages of Influenza B virus (IBV) called “Victoria” and “Yamagata” [9]. Although, the “Yamagata” line potentially became extinct after the COVID-19 pandemic, probably due to social distancing

practices and evolutionary features of Yamagata IBV [10]. Influenza A virus (IAV) can be classified by the expression of surface proteins haemagglutinin (HA) and neuraminidase (NA), of which 18 and 11 variants exist respectively. There are at least 17 more, total 19 proteins encoded by eight gene segments of IAV [11]. Another one of them being the nucleoprotein (NP) that plays an important role during replication as it is a part of the viral ribonucleoprotein (vRNP) [12]. The surface proteins HA and NA exert two critical and opposing functions in the lifetime of the virus, attachment and release, respectively. The trimeric HA binds to sialic acids via the so-called receptor-binding site (RBS) and is either endocytosed into the cell or released by the enzymatic activity of NA, cleaving from the sialic acids again [11, 13] (Figure 1, right). Once a virion enters a host cell, often in the respiratory tract after contact with virion-containing aerosol, the vRNPs, containing NP, are released from the endosome and enter the nucleus of the host cell. Here, the viral genome is replicated, and messenger RNA (mRNA) is transcribed, exported into the cytoplasm and translated there into IAV proteins. Surface components are translated in the endoplasmic reticulum and internal proteins in cytoplasmic ribosomes. Together with newly replicated vRNPs, the proteins are assembled to virions, with HA, NA and matrix-2 (M2) on the cell surface, they are released via budding from the host cell [14], this is facilitated by NA [11, 13]. Now a new infection cycle can begin (Figure 1, right).

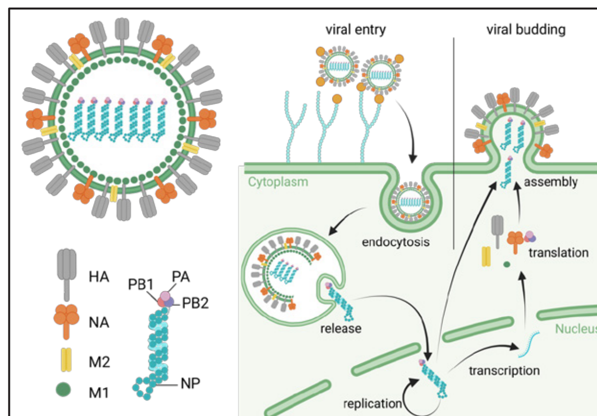


Figure 1: *Structure (left) and Lifecycle (right) of Influenza A virus. Created with BioRender.*

1.1.2 INFLUENZA A VIRUS HAEMAGGLUTININ

HA is a surface glycoprotein of IAV and forms trimers, of which each monomer consist of a globular head, a stem and a short transmembrane domain [15]. Facilitating fusion of virions to host cells, it is indispensable for the virus' lifecycle. In addition to its localization as a surface protein, this might explain the predominance of HA as the most abundant surface protein of IAV [16] and the most common target for Ab (antibody) responses, followed by NA and NP [17](Figure 2, far-left panel). This phenomenon of hierarchical immune responses to pathogenic components is called immunodominance (ID) and includes targeting of proteins, antigenic sites and even peptides by the immune system [17-20]. HA proteins of IAV can be phylogenetically divided into 2 groups, the common HA 1 (H1) belonging to group 1 and HA 3 (H3) to group 2.Within HA, the head domain is preferentially targeted by antibodies (Abs) while anti-stem Abs are rarer [18, 21-23](Figure 2, mid-left panel).The head undergoes minor mutations readily and in comparison, the stem is conserved and mutates slower [24]. Universal vaccine design often focuses on the induction of anti-stem Abs to induce broadly neutralizing Abs (bnAbs) [7].

1.1.2.1 THE HA STEM AND CROSS-PROTECTIVE ANTIBODIES

In the past decades, several Abs have been isolated from human subjects that target group 1 and group 2 IAV HAs and are classified thereby as bnAbs. The first of those, namely C179, was isolated by Okuno et al in 1993 and had neutralizing activity against both group 1 and group 2 IAVs. This Ab has been shown to target the more conserved stem region of the HA protein and is therefore considered a bnAb. [25] Since then, many HA stem binding Abs have been isolated from infected and or vaccinated human subjects [26-31]. Still, bnAbs are still rare among those stem-specific Abs. The most famous and potent HA group cross-reactive bnAb is FI6, isolated by Corti et al in 2011 that could bind all 16 IAV HA subtypes and neutralized several viruses of HA group 1 and 2 [26] (Figure 2, mid-right panel). The CR9114 Ab offers the broadest protection, as shown in mice after infection with IAV group 1 and 2 viruses as well as B/Victoria and B/Yamagata strains [32, 33].

Stem directed bnAbs offer broader protection than head-directed Abs, partially due to loss of viral fitness after mutations in the stem domain

[34, 35]. The HA stem contains a conserved antigenic site (= area that is often targeted by Abs with overlapping epitopes) around a hydrophobic pocket that is frequently targeted by these bnAbs [36], such as FI6 [26] (Figure 2, mid-right panel) and CR6261 [37]. Development of vaccines and/or vaccination regimens to induce HA group 1 and 2 cross-reactive bnAbs by targeting the stem and in particular this conserved site is crucial for universal vaccine development and to overcome seasonal drifting HA heads that escape pre-existing immunity.

In humans, Abs to the stem are observed regularly and they present sometimes neutralizing but mostly other protective effector functions [38-40]. Anti stem bnAbs in mice are very rare but anti stem Abs mount other protective effector functions of steric or fragment-crystallizable region (Fc-region)-mediated nature [41-43]. Those can include antibody-dependent cellular cytotoxicity (ADCC), complement activation, opsonization for phagocytosis, Ab mediated neutrophil activation, improved antigenic uptake for processing or capture by follicular dendritic cells (FDC) [44].

1.1.2.2 THE HA HEAD AND ITS IMMUNODOMINANCE PATTERNS

The plasticity and seasonal mutations on the immunodominant HA head can not only be explained by the error-prone polymerase but its ID (targeting by Abs) conveys evolutionary pressure and therefore only escaped variants can successfully infect an individual with pre-existing immunity. Interestingly, there are also pre-defined ID patterns among antigenic sites on the HA head. For H3 these are Site A-E [45-47] and H1 presents antigenic sites Ca1, Ca2, Cb, Sa and Sb (Figure 2, far-right panel) [48, 49], of which Site A, B and D for H3 and Sa, Sb, Ca2 for H1 partially overlap with the RBS [50, 51]. The S (strain specific) sites Sa and Sb sites are located towards the top of the head domain; therefore, the immune system is readily able to recognize those sites on the virion surface. C (cross-reactive) sites Ca1, Ca2 and Cb sites are situated more towards the middle or the bottom part of the head domain, therefore seemingly more hidden.

ID among the H1 antigenic sites has been well defined and studied in recent time in C57BL/6 mice after intranasal (i.n.) infection, UV-inactivated virus vaccination intramuscular (i.m.) and intraperitoneal

(i.p.) of the mouse-adapted A/Puerto Rico/8/34 virus (PR8) [18]. These ID patterns are plastic across time and different immunization methods (Figure 2, far-right panel). Interestingly, even temperature differences can affect the folding of HA and therefore hide or expose different antigenic sites, an example for this are the pre- and post-fusion states of HA trimers [52].

Other factors to consider when determining B cell ID is also the genetic background of the model used, as germline complementary-determining regions (CDRs) will probably be the first ones recognizing their specific epitopes and those antigenic sites therefore are going to be dominantly targeted, at least during the early phase of the response. For example, C57BL/6 and BALB/c mouse models show differences in B cell ID patterns to the five main antigenic sites on PR8 HA head [18]. Importantly, Abs close to germline immunoglobulin (Ig) specific for the Cb antigenic site [53] are most abundant in early extrafollicular and follicular B cell responses [54], during which little to no affinity maturation occurs and mainly germline or close-germline Ig expressing B cells can be found [55, 56]. The Sb antigenic site is dominant at later timepoints in serum and the germinal center (GC) of mice [18] and the main dominant site targeted in humans [57-59]. We can only speculate why this site, alongside with spatially close Sa, are the main dominant antigenic sites in several species. For once, they are situated on the top end of the HA protein, making them easily accessible when HA is embedded in a lipid membrane, such as on whole virus particles or infected cells [14]. Additionally, due to its proximity to the RBS [50, 51], the antigenic site presents a vulnerable target to block virus attachment to host cells. Both theories are supported by the fact that Sa and Ca2 antigenic site, which are situated close to Sb and the RBS [50], are presenting relative high dominance as well in humans [57-59].

Studying responses against close to germline antigenic sites as well as generally dominant and vulnerable antigenic sites, such as Cb and Sb respectively, will help gain insights into how and why B cells respond to certain structures. Considering genetics, location of antigenic sites and accessibility. By using H1 proteins and appreciating the well-defined ID patterns of its antigenic sites, we can learn how to direct responses to more subdominant epitopes and therefore provide valuable insights

into the development of broader and longer lasting or even universal vaccines against IAV.

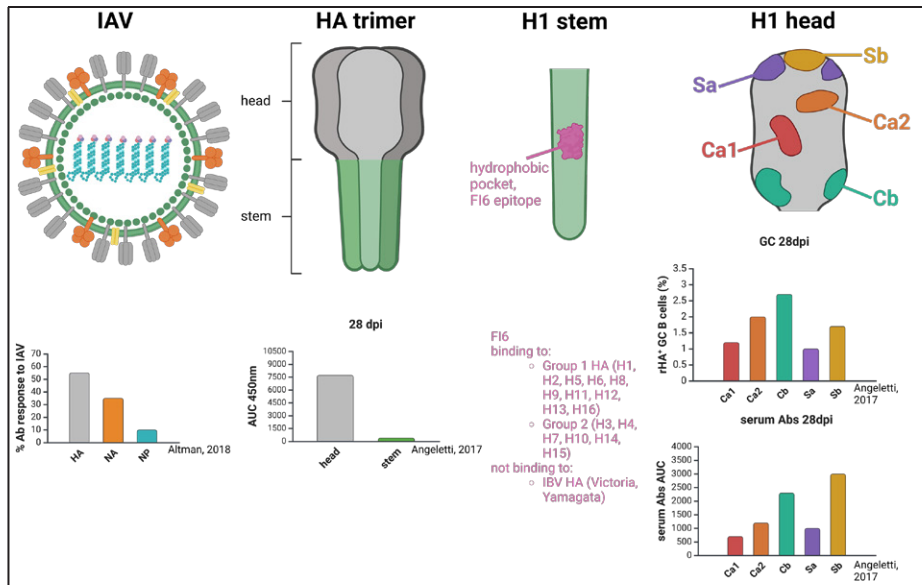


Figure 2: *IAV and H1 HA with their ID characteristics. From left to right: IAV and serum Abs ID among different proteins (shown are the 3 main targets). HA trimer and serum Abs ID between head and stem at 28 days post infection (dpi). H1 stem with hydrophobic, mainly targeted pocket indicated with list of HA proteins targeted by FI6 monoclonal Ab (mAb). H1 head with the 5 mainly targeted antigenic sites, with ID patterns of serum Abs as well as in the GC at 28 dpi. Created with BioRender.*

1.1.3 ANTIGENIC SHIFT AND DRIFT

IAV is highly plastic and poses a great pandemic risk due to antigenic shift and drift. Antigenic shift describes the exchange of gene segments while a host cell is infected by two viral strains. This explains how new strains containing never observed combinations of HA and NA genes emerge. These new rearrangements are responsible for several pandemics [3]. Therefore, understanding as well as monitoring of antigenic shift is crucial for pandemic preparedness. Antigenic drift on the other hand is caused by minor mutations, supported by an error-prone viral polymerase [60]. Seasonal IAV outbreaks are an outcome of antigenic drift but do only pose a low risk for pandemic outbreaks [61].

Nevertheless, understanding antigenic drift and targeting more vulnerable epitopes is needed for universal vaccine development.

1.1.3.1 ANTIGENIC SHIFT: THE EXCHANGE OF GENES

The H1N1 pandemic of 1918, the Spanish Flu is thought to be a product of an antigenic shift event in which a pig was infected with an avian virus and then later was transmitted to humans [62]. Viral reassortment is an antigenic shift event in which several viruses infect the same host cell, and this results in the output of viral particles containing gene segments from different strains, therefore producing a completely new strain if viable. The H1N1 pandemic of 2009, the Swine Flu, is a quadruple reassortment virus. It contains genes from polymerase acidic (PA) and polymerase basic 2 (PB2) from avian lineages, polymerase basic 1 (PB1) from human lineages, HA, NP and non-structural (NS) from swine lineages and NA and matrix (M) from an avian-like swine lineage, which is an H1N1 [63]. As demonstrated, antigenic shift therefore poses a high pandemic risk. Preparation efforts contain monitoring of emergent strains in animal reservoirs [64, 65], development of antivirals [66] and inducing immunity against various IAV proteins and conserved sites of IAV proteins across multiple strains.

1.1.3.2 ANTIGENIC DRIFT: INTRODUCTION OF MINOR MUTATIONS.

Antigenic drift represents a key mechanism of IAV evolution and is characterized by the accumulation of minor mutations in viral proteins until the virus escapes pre-existing immunity. It represents therefore a more gradual change in comparison to antigenic shift. This phenomenon has been observed for several viruses and proteins, including IAV HA and NA [18, 67], SARS-CoV2 spike protein and HIV envelope glycoprotein (gp) [68-72], but seems to be completely absent and even detrimental in others, such as measles [73].

For IAV HA, the low fidelity of the RNA polymerase is often attributed the causal effect of antigenic drift and seasonal recurrence of IAVs. It was although shown to exhibit comparably low mutational rates (2.5x 10^{-5} substitutions per nucleotide per cell infection [74]) while still being able to introduce a few minor aa substitutions [75]. To fully recapitulate seasonal IAV outbreaks, the host immune system needs to be considered as well, as escaped variants have a higher potential for

further dissemination [69]. Abs and CD8 T cells apply evolutionary pressure to IAV and support the development of antigenic variants [69, 76].

It is crucial for the development of a universal vaccine against IAV to understand viral drift and to develop strategies bypassing this escape mechanism [77]. Again, inducing immune responses against more conserved proteins and epitopes could circumvent viral escapes. Even if such universal vaccine is ultimately not achievable, if selective pressure is inducing IAV to mutate even in conserved sites, valuable lessons can still be learned to redirect immune responses.

1.1.4 CURRENT SEASONAL INFLUENZA VACCINES AND FUTURE VACCINE DEVELOPMENT

The first “vaccines”, or variolation, were already used somewhere between the 11th and 17th century in parts of Asia [78]. Since then, we have successfully developed vaccines against many fatal and high morbidity-causing diseases such as IAV [79-82], polio [83], measles [84], cholera [85], rabies [86], SARS-CoV2 [87, 88] and many more. Various approaches were used for this, and formulations range from inactivated [89] or live attenuated [83] whole pathogens to recombinant (r) protein [90] and even mRNA technologies [87, 88].

Several of these approaches have also been applied to IAV vaccines, though those offer only seasonal protection and rarely are cross-protective between different IAV strains. Considering the long history humans have with IAV, mortality and morbidity caused, and efforts put into vaccine development, the story of influenza vaccines is not a successful one. Even the first approach to develop a vaccine against influenza failed miserably. In the late 19th century, Richard Pfeiffer was able to isolate a pathogen from nasal tissue of infected patients an thought it was the cause of the influenza disease [91]. During the H1N1 epidemic of 1918, patients with this pathogen were sought after to generate antisera and develop a vaccine. This pathogen, *Haemophilius influenzae*, a bacterium, was later determined to be inducing secondary disease rather than being the cause of influenza [92, 93]. In the early twenties after filtration experiments it was proven, that a bacterium could not cause the influenza, [93]. After successfully developing a method to grow viruses in embryonated chicken eggs, the United States

approved the first IAV vaccine in the 1940s. It was produced by chemically inactivated whole virus particles from eggs [82]. IAV vaccine production in chicken eggs continued to be the gold-standard until shortly after the 2009 H1N1 pandemic, when several novel vaccine approaches were approved: virus grown in mammalian or insect cell culture, recombinant protein (rHA) and the formulation of multivalent vaccines (containing more than one stain of IAV and also IBV) [94].

Today in the European Union, many different formulations of seasonal IAV are available for the 2024/2025 flu season. Including multivalent, attenuated nasal vaccines [79], mainly used in children from 2-18 years of age. Also inactivated, adjuvanted vaccines with surface antigen are available [80], which in contrast are being used in the elderly (from 50 years of age). An example for use of influenza vaccine in healthy adults is a quadrivalent, recombinant protein vaccine [81].

Many more strategies are currently in development to not only improve vaccine efficiency, reduce adverse effects, generate longer lasting immunity, but also to generate protection against multiple IAV and IBV strains and ideally create a universal vaccine, that confers protection against all known and unknown IAV and IBV strains for a lifetime. These strategies include mRNA vaccines [95-97], targeting vulnerable epitopes with computationally designed immunogens [98-101], viral vectored vaccines [102-104] and even DNA vaccines [105, 106].

The need for a universal influenza vaccine is high [7] and especially HA seems to be a valuable target for such a development due to the conserved stem domain among influenza strains [24] and the in detail defined B cell responses to the head [18] that can be leveraged to direct immune responses to vulnerable targets. Notable advances in the direction of a universal flu vaccine were made within the last decade, including the support of subdominant and cross-reactive B cell responses by an HA mosaic nanoparticle [101], better protection via anti stem Abs to different IAV strains after immunization with an HA that presents a hyperglycosylated head [107], induction of anti-stem bnAbs by a so called “mini HA” in mice and non-human primates that could outcompete human anti stem bnAbs [108], a structure-based stem immunogen on nanoparticles that conferred protection to a group 1 IAV [109] and an HA head-stem chimera approach using repeated

vaccinations with the same stem but different heads that conferred immunity to IAV via Abs even when CD8 T cells were not present [110, 111]. Harnessing the knowledge about ID patterns against the HA head and identifying determinants to reshape B cell responses to produce long-lasting and protective Abs can guide the development of universal vaccine design. The road to such a vaccine might be very long, but even small advancements can inform vaccine design to produce immunity that protects longer than only one season.

1.2 B CELL IMMUNITY

Our immune system consists of two main arms, adaptive and innate immunity. While the innate immune system is indispensable for survival of the infected organism, does not require attuning for protection and is fast-acting, it is often responsible for pathology during disease and not very specific due to receptors that recognize pathogenic and danger patterns rather than responding to a unique molecule. The adaptive immune system on the other hand requires a first contact with the pathogen, to achieve full functionality. Once immune memory is generated, it can react fast and protect with high specificity due to unique antigen receptors of the two memory lymphocytes, B and T cells [112]. While the T cell receptor (TCR) recognizes peptides loaded onto major histocompatibility complex (MHC) molecules presented by antigen presenting cells (APC) that previously processed proteins intracellularly into those peptides, the B cell receptor (BCR) recognizes epitopes on whole proteins [113]. Uniquely, the BCR also has a secreted form, Abs. Since protection against many pathogens, including IAV, is mostly mediated by Abs, B cells and B cell memory are essential components of the immune system for combating these diseases [17].

1.2.1 B CELL MEMORY

1.2.1.1 INDUCTION OF MEMORY B CELLS DURING A PRIMARY INFECTION OR IMMUNIZATION

Naïve B cells circulate between secondary lymphoid organs get into contact with antigens to as fast as possible [114]. Those could stem from pathogens directly, cellular debris or a vaccination. Once a B cell

gets in contact with an antigen that binds to its BCR and is internalized, the primary immune reaction begins. The activated naïve B cell will be attracted to the border of the T cell zone when recognizing a T-dependent antigen (protein) [56] or directly start to clonally expand if the recognized antigen induces a T-independent immune reaction; those are mostly polysaccharide antigens or other molecules with repeating patterns capable of crosslinking BCRs [115, 116]. At the T cell zone border, B cells encounter a specialized type of T helper cell, the T follicular helper cell (T_{fh}). They in turn have previously been activated by their specific peptide presented on APCs or are activated by the antigen, previously internalized via BCR binding, presented on B cells [117, 118]. This interaction of TCR with MHC + peptide is not sufficient to fully activate the B cell, several co-stimulatory signals are required as well. Those include CD40/CD40 ligand (CD40L) interactions [119-121], cytokine secretion (for example interleukin (IL)-21) [122-124], CD80/86 and CD28 interactions and many more [125-127]. Once a naive B cell at the border of the T cell zone receives a sufficient amount of stimulatory signals, it is fully activated (Figure 3, right) [117, 118]. Famously, anti CD40 or CD40L Abs are being used to disrupt B cell responses in experimental settings or even in the clinic [119, 128].

To initially and quickly combat pathogens, activated B cells with higher affinity to the antigen will directly differentiate into early and short-lived antibody secreting cells (ASC), namely plasma blasts (PB), outside the B cell follicle (Figure 3, right) [18, 121, 129, 130]. When only limited amounts of antigen are present, activated B cells prefer differentiation into early memory B cells (eMBC), which mainly do not class switch, acquire no somatic hypermutation (SHM) and can be found already 2.5 days after contact with antigen. They are transcriptionally different from their GC-progeny counterpart [131] and contribute a high diversity to the memory repertoire and thereby allowing broader protection [132]. MBC fate of activated B cells is governed by for example by haematopoietically expressed homeobox transcription factors [133].

The third fate an activated naïve B cell at the T cell zone border can undergo is the formation or entry into a GC (Figure 3, left). Before a B cell enters into the GC, it will most likely undergo class-switching to IgG, IgA or IgE first [55], GC progeny therefore rarely expresses isotypes IgM or IgD [134]. For the initiation of a GC response, the expression of the

transcription factor B-cell lymphoma 6 (Bcl6) is needed in both B cells and T cells [118, 135, 136]. Within the two zones of the GC, B cells proliferate but also undergo SHM and selection. In the dark zone, the B cells expand and therefore express many genes required for mitosis [137-140] and SHM [140, 141]. The expanded and hypermutated GC B cells are then selected in the light zone. This happens based on an intricate interplay between the capture of limited antigen presented on FDCs and subsequent antigen presentation for T_{fh} to survival signals. Therefore, BCR activation as well as CD40-CD40L interactions are needed [137, 140, 142-144]. GC B cells that can't capture or have only low affinity to antigen on FDCs and do not receive T_{fh}-derived survival signal, will go into apoptosis. Apoptosis of GC B cells occurs to a very high degree and therefore, efficient mechanisms are needed to remove dead cells, such as down-regulating the Decay Accelerating Factor to engage the complement for phagocytosis [145] and influx of macrophages into the GC that take up B cells in a highly efficient manner, the tingible body macrophages [146]. Receiving an appropriate amount of T cell help can either lead into another entry into the dark zone and the cycle begins again, or B cells are selected for GC output as a memory B cell (MBC) or plasma cell (PC) (Figure 3, left). Recently, several groups have shown that not only high affinity clones are selected for MBC and PC differentiation. Elegant fate mapping and antibody characterization experiments by Sprumont et al [147] revealed the generation of both high- and low-affinity PC clones from GCs after IAV infection and HA immunization, showcasing the evolutionary advantage of diversified serum responses. If PCs receive the appropriate signals, they can become long lived PCs (llPC) and reside in the bone marrow (BM), secreting Abs over prolonged periods of time, ideally for a lifetime [148]. MBCs are also selected similarly and contribute to a broad immune memory [149]. A recent study used single cell BCR sequencing and transcriptional signatures to delineate the temporal and spatial output of MBCs from lymphoid organs after IAV infection [150]. It was illustrated how MBC are exported from GCs all throughout the immune response and disseminate into various organs. This in accordance with the fact that GCs after IAV infection can persist for prolonged amounts of time and therefore antigen specific MBC are generated even months after IAV infection [151]. It is yet to be fully defined which signals are required for the differentiation to MCB or PC

from the GC and which ones determine the cell to enter another cycle in the dark zone.

In summary, the generation of MBC occurs at different time points and from different microenvironments in secondary lymphoid organs, they express several BCR isotypes and display various affinities to antigen. The circumstances of differentiation are dictated by antigen availability, BCR affinity and survival/differentiation signals received by T_{fh}.

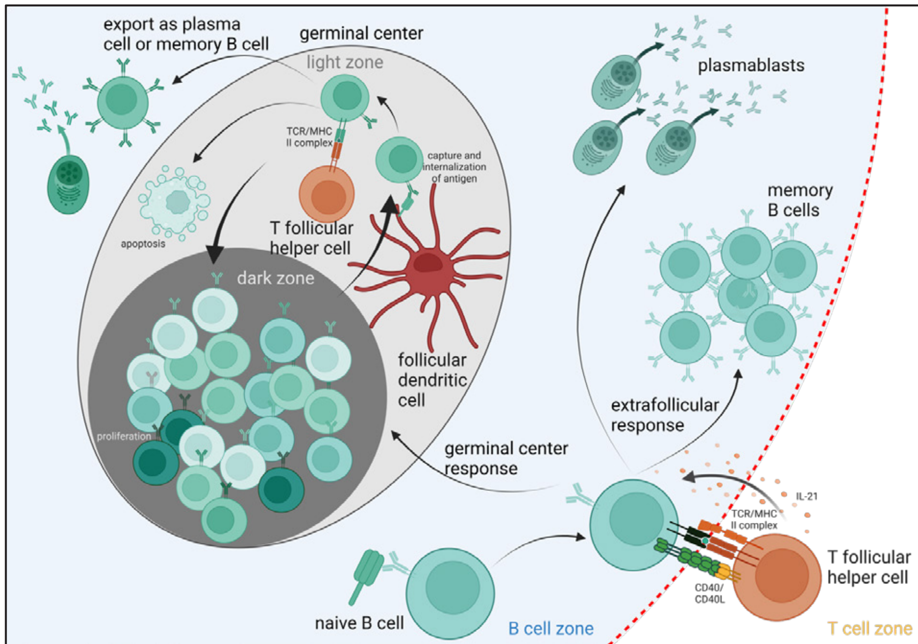


Figure 3: *Generation of MBC in secondary lymphoid organs. B cells in green, T cells in orange. GC response (left) and extrafollicular response (right). Created with BioRender.*

1.2.1.2 MEMORY B CELL REACTIVATION

MBCs mostly circulate between blood and in secondary lymphoid tissues [152], recently also resident MBC (BRM) in various mucosal tissues as a first layer of defense to reinfection have been characterized and their reactivation studied in both mice and humans [150, 153-164]. Generally, BRM are poised to differentiate into ASC rather than to participate in secondary GC reactions [154, 159, 160]. This fate decision is not as clearly delineated for peripheral lymphoid

tissue resident MBC as discussed in this work and by many others [125, 141, 157, 165-180].

When an MBC recognizes its cognate antigen via the surface expressed BCR and receives help from T_{fh}, they are reactivated, requiring less stimuli than naïve B cells for activation [181]. An immediate differentiation into ASC is the preferred fate and is reasonable in regard to immediate protection and represents a key role of B cell immune memory: Quick supply of massive amounts of Ab upon contact with antigen [154, 155, 158-160, 177, 182-185]. Although the re-entry of MBC into a secondary GC, the other main function of B cell immune memory, is indispensable to protect from newly emerging antigenic variants and to provide diversification of the immune memory repertoire while maintaining high affinity and replenishing the MBC pool [141, 186]. But re-entry in secondary GCs of MBC is also very rare, only up to 10% of B cells in secondary GCs have previously been in contact with antigen after re-challenge with the same antigen [170, 172].

In the last decades, most studies aiming to identify determinants of MBC fate decisions (ASC differentiation or GC re-entry) have utilized the expression of surface molecules to predict differentiation outcomes. Those include canonical MBC surface markers: the co-stimulatory receptor CD80, CD73 (producing extracellular adenosine and therefore down-regulating inflammatory responses [187]), and immune-checkpoint receptor ligand programmed cell death-ligand 2 (PD-L2) [125, 175, 180, 188]. Zuccarino-Catania et al have shown a decade ago that double negative MBC of CD80 and PD-L2, that have less SHM and are therefore considered closer to naïve B cells, often re-enter secondary GCs. Whereas Double positive MBC of CD80 and PD-L2 have higher SHM and are considered more mature, they were shown to differentiate into ASC directly. Also, Ig isotype expressed was shown in various works to be highly influential on MBC fate decisions [125]. Adoptive transfers and re-vaccination with an antigen identical to the priming antigen have shown that IgM^+ MBC tend to re-enter secondary GCs, while switched Ig^+ MBC directly contribute to Ab secretion by differentiation into ASC [173, 174, 177, 178]. Contradictory, another study conducted by McHeyzer-Williams et al showed using a cell fat tracking approach, that switched Ig^+ MBC do re-enter secondary GCs and undergo further rounds of SHM [141]. These determinants and their

correlated outcomes are highly debated as they are contradictory; also different animal and antigen exposure models have been used that complicate interpretations [157]. It is especially important to consider that those studies used very simple and biologically irrelevant antigens such as nitrophenol or ovalbumin (OVA). Recently, a series of works dedicated to the question why only very few MBC re-enter secondary GCs and which factors could influence this fate-decision apart from surface molecule expression patterns have been conducted using IAV HA as model antigen and elegant fate-mapping experiments of both B cells and Abs [170-172]. They as well as other works have considered B cell intrinsic factors to affect MBC fate decisions, such as SHM amount/affinity [125, 176, 189] or even the initial variable–diversity–joining (V(D)J) region germline arrangement [170]. Using sequential immunization with recombinant IAV HA protein, Mesin et al have shown that MBCs re-entering a secondary GC show a wide spread of SHM amount, but often no mutations in the heavy variable region (V_H); their common unmutated ancestors had higher affinity to antigen than not re-entering MBC clones. This led to the conclusion that not SHM amount, but initial V(D)J germline arrangement was a main factor in determining MBC fate decisions [170]. In contrast to this, a very recent study using HIV gp140 recombinant protein for sequential immunizations demonstrated that 1.) a boost with antigen of higher affinity increases GC re-entry of pre-existing MBC, 2.) a boost with antigen of lower affinity recruits pre-existing MBC with higher SHM amount to secondary GCs and 3.) refueled GCs contain more activated B cells with lower SHM. This indicates, that in the context of HIV gp140, MBC re-entry into secondary GCs is indeed affinity dependent. [189]

As B cell intrinsic factors did not seem to sufficiently define hallmarks or even just predictors of MBC fate decisions, more attention has been pointed towards B cell extrinsic factors, mainly pre-existing immune components. In section 1.2.3, we will discuss their impact on MBC fate decisions and review insights from recent studies.

A clear B cell extrinsic factor affecting MBC fate decisions is localization. As previously mentioned, BRM at mucosal surfaces are transcriptionally programmed to differentiate rapidly into ASC. In lymphoid tissue, if boosting is conducted at the priming site, MBC re-

enter secondary GCs at a higher fraction than during re-challenge at a distal site [169].

All works mentioned above were conducted using laboratory mouse models. Cell fate tracking and adoptive transfer experiments cannot be conducted in humans, therefore recent studies with infected or vaccinated patients relied on BCR sequencing techniques and their lineage tracing. Humans have a more complex history of antigen exposure than mice as they live for much longer and are exposed to pathogens in the environment constantly. They can only be sampled from easily accessible or peripheral sites, such as the blood or lymph nodes (LNs) close to the skin.

B cell intrinsic marker CD27 has recently been identified to differentiate between MBC developmental stages, a $CD27^{bright}$ and more mature population that is replenished by a $CD27^{dull}$ population that acquires more SHMs and therefore likely re-enters GCs [190]. In the gut, $CD45RB^{-}$ $CD69^{-}$ MBC subsets, identified in samples from organ donors, was shown to be predisposed to re-enter GCs while also expressing genes associated with ASC differentiation.

During the SARS-CoV2 pandemic, several studies have probed vaccination-draining LNs after SARS-CoV2 [191-193] and IAV [194, 195] vaccination. They all showed that MBC readily re-enter GCs induced by vaccination in harsh contrast to what is observed in mice. This was true for both, SARS-CoV2 that humans were comparably antigenically inexperienced to [196, 197], and for IAV, that humans tend to have a complex antigenic history with [198]. When only blood was sampled and B cells in the periphery were observed, increases in SHM of MBCs after antigen contact was shown and therefore their re-entry into GCs indicated by proxy [72, 199].

Factors that make an MBC re-enter a GC reaction or differentiate into ASC remain to be elucidated and the contradiction between very few MBC re-entering GCs in mice after PR8 HA vaccination [170, 172] and more and more improved BCR affinities of MBC after IAV vaccination in humans [194, 195] requires to be resolved as well.

1.2.2 T CELL HELP TO B CELLS

Many antigens, such as IAV HA and other proteins, require T cell help to fully activate B cells. Certain antigens exist, mainly carbohydrate or other repetitive molecules, do not require T cell help. These antigens bypass this requirement by crosslinking BCRs and prolonged signaling [115, 116, 200]. Nevertheless, T cell help is crucial in mounting successful B cell responses against most pathogens, also IAV [201] by providing activating signals, supporting the selection process in the GC and modulating the immune environment [202].

1.2.2.1 T CELL GENERATION AND ACTIVATION

Same as all other leukocytes, T cells stem from a pluripotent hematopoietic progenitor in the BM but mature in the thymus [203]. Here, a specialized environment in the thymic cortex facilitates and drives the commitment of thymocytes to various types of mature T cells. Thymocytes are then selected via their specificity to self-peptides expressed on thymic epithelial cells, thymic dendritic cells and thymic B cells and mature into either CD4 or CD8 single-positive lineages. T cells that have strong interactions to self-peptides in the thymus medulla are deleted as they pose a risk for autoimmunity. [204, 205]

Mature, naive T cells patrol various tissues, but mainly the lymphatic system [206] and the blood. Hereby sampling peptides presented on the MHC of APCs via their TCR. With a high probability, they will encounter their antigens from pathogens that have infected the host [207]. Through their TCR co-receptor, conventional T cells are divided into two subsets: the CD8 and CD4 T cells. CD8 T cells are generally considered as cytotoxic, responsible for killing infected host cells and pathogens directly [208]. They recognize peptides presented on MHC class I (MHC I) molecules and can be activated through various distinct pathways to differentiate into effector CD8 T cells and might therefore even require CD4 T cell help [209, 210]. CD4 T cells on the other hand are generally considered as helper T cells, they recognize peptides presented on MHC class II (MHC II) molecules [211]. CD4 effector cells can be classified into numerous subsets: T helper 1 (T_H1), T_H2, T_H17, T_{fh} and regulatory T (T_{reg}) cells [212].

Survival and differentiation co-stimulatory signal are needed from APCs for final activation of naïve T cells to effector differentiation, then

they can react to their cognate antigen without co-stimulation. These are delivered in the form of for example via binding of CD28 on T cells to B7 molecules on APCs [126] and cytokines such as IL-6 [213] or IL-23 [214, 215]. There are many other co-stimulatory signals at play between APCs and T cells that fine-tune the immune response. CD4 effector T cell fate decisions and effector functions have been well defined in the past decades [212]. Critical CD4 T cell responses include the differentiation into T_H1 effectors. They are induced by interferon gamma (IFN-γ) and IL-12, secrete IFN-γ and IL-2 and are thereby take part mainly in responses to infections of intracellular pathogens, such as viruses or mycobacteria, but also in autoimmune disease [212, 216]. CD4 T cells can also differentiate into T_{fh} cells, mentioned previously in 1.2.1.1 and 1.2.1.2. This is induced by IL-6 and T_{fh} cells secrete IL-21, IL-10 and IL-4 [118]. More details about T_{fh} cell differentiation, effector functions and memory responses are summarized in the following section.

1.2.2.2 T FOLLICULAR HELPER CELLS

As T_{fh} cells are specialized to provide help for B cells and ensure a functional humoral response to T cell dependent antigens. Within the GC, they play a key role in the selection of B cells in the light zone [137, 144].

To migrate into B cell follicles and GCs, activated T_{fh} cells start to upregulate Bcl6, which is required for differentiation of naïve CD4 T cells to T_{fh} cells, and thereafter express C-X-C chemokine receptor 5 (CXCR5), which binds to its ligand C-X-C chemokine ligand 13 (CXCL13 [117]. This in turn is initially secreted by FDCs and stromal cells in the reticular network to attract not only activated B cells but also T_{fh} cells to induce a GC reaction [217]. This happens not only in lymphoid tissue but also at mucosal sites [218]. T_{fh} cells later are the main producers of CXCL13 themselves [117].

Direct interactions of T_{fh} cells with B cells are via the MHC-peptide-TCR complex, CD40 and CD40L interactions and secretion of cytokines such as IL-21 and IL-4. CD40 activation promotes B cell survival, inducing CSR and SHM and facilitating B cell proliferation as well as differentiation into MBC or ASC. In T_{fh} cells, CD40L signaling will lead to GC formation, T_{fh} cell induction and maintenance as helping the T

cell to select B cells with highest affinities [120]. IL-21 and IL-4 secreted from T_{fh} cells bind to IL-21R and IL-4R on B cells respectively and have synergistic effects to the CD40 signaling [123, 124, 219]. This enables T_{fh} cells to fulfill their two main functions: Initiating full naïve and memory B cells activation and selecting GC B cells according to their affinity in the light zone. Dysregulation of T_{fh} cells can lead at excessive activity to autoimmune diseases or at lower/absent activity impaired humoral responses [118].

Therefore, it is critical for future vaccine design to also consider T_{fh} cells and to understand their mechanism of help delivered to B cells and optimizing immune responses in their breadth, duration and quality. Insights need to be gained into the complex interactions between T_{fh} and B cells as findings from recent indicate that global or bystander help of T_{fh} cells that enhance the magnitude of GC and Ab responses [220-222] and induce a quicker onset of GC reactions [54]. This represents a contrast to the classical dogma of restricted help only to B cells that recognize and present the same antigen [223-225].

IAV can be used as a model pathogen to study global versus antigen-specific T_{fh} cell help to B cells. B cells usually recognize surface antigens, such as IAV HA, while T cells recognize peptides that originate from all proteins processed by the proteasome and loaded onto MHC molecules, like the internal IAV NP [113]. According to the classical dogma, HA specific B cells could only receive help from HA specific T cells and vice versa for NP (Figure 4) [223-225]. Non-cognate versus cognate help can also be described as intra-molecular (help only to cell of specificity to same molecule) versus inter molecular help (help to cells of specificity to other molecules). In the case of inter-molecular or non-cognate help during IAV infection or whole virus particle vaccination, an HA specific B cells could take up a whole virion, process and present all antigens on MHC II and therefore receive help from NP specific T_{fh} cells.

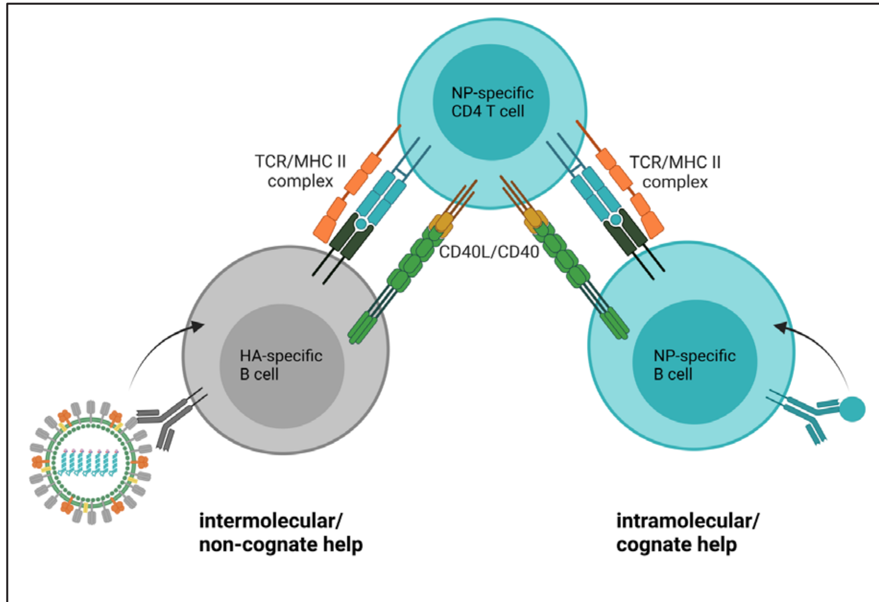


Figure 4: *Graphical representation of intermolecular/non-cognate help (left) and intramolecular/cognate help (right) delivered by CD4 T cells to B cells. Created with BioRender.*

1.2.3 THE EFFECT OF PRE-EXISTING IMMUNE COMPONENTS ON B CELL REPSONSES

In recent years, more attention has been given to pre-existing immune components when dissecting MBC fate decisions and secondary B cell responses in general, including naïve B cells. Presence of Abs could potentially block epitopes or mediate feedback, similar for MCB that rapidly differentiate into ASC and secrete high amounts of Ab. And lastly, helper T cells will be able to provide activation signals to MBC that accelerate and amplify recall responses.

1.2.3.1 ANTIBODIES

Interestingly, Abs seem to have great effects on B cell fate decisions, while being also the agent secreted by those cells. This would represent a certain degree of autologous regulation by secretion of Abs. Several recent studies have investigated Ab-mediated effects on B cell reactivation, facilitated by the ease of Ab production and administration into the murine host [18, 171, 172, 189, 226-253].

Generally, pre-existing Abs were able to modulate B cell responses mainly via epitope masking or feedback. They reduced GC formation and naïve B cell activation by masking of immunodominant epitopes and thereby reshaped responses towards subdominant and variant-specific epitopes and increasing general breadth of secondary B cell responses while not interacting with secondary serum Ab responses, as shown for SARS-CoV2, HIV and IAV antigens [72, 77, 189, 226, 229, 235, 245, 248]. Abs with high affinity suppressed B cell responses via clearing antigen and Fc-receptor signaling [230, 236].

It has been observed that repeated exposures to similar antigens reduce the response to novel antigenic sites, a phenomenon termed the “Original Antigenic Sin” (OAS) [254, 255]. As MBC tend to dominate secondary Ab responses when priming and challenging antigen are not too distant from each other, the majority of Abs secreted after secondary antigenic contact are directed against previously encountered antigen. OAS was recently further investigated after immunizations with the same antigen and was in these works called “primary addiction” by Schiepers et al [171, 172]. OAS or “primary addiction” has been observed not only in the context of its classical

association with IAV [172, 230, 254, 255] but also SARS-CoV2, which is a fairly new pathogen in the human population [72, 243].

While a direct effect of Abs on MBC re-entry in GC is yet to be determined [172], it has been observed that Ab presence, concentration, affinity and antigenic site specificity affect the recruitment of naïve B cells into GCs both *in silico* and *in vivo* [228-230, 236, 248]. But Abs can via feedback mechanisms also enhance a diversification of the B cell response and therefore promote the generation of bnAbs against conserved pathogenic epitopes [77, 228, 233, 248].

Pre-existing Abs need to be considered for future, successful vaccine design, especially when designing universal vaccines. By simply designing vaccines that elicit bnAbs targeting conserved or subdominant epitopes, issues of ID and imprinting can be circumvented [233, 236]. Additionally, sequential fine-tuned immunizations can be used to generate broadly neutralizing responses [243, 248] or Ab-treatment used while vaccinating to guide patient-responses to desired epitopes [228].

1.2.3.2 MEMORY B CELLS

Most Abs during a secondary response are secreted by MBCs differentiated into ASCs, especially after heterologous challenges [171, 172, 256], which is supported by the notion that they are generally poised to differentiate towards Ab secretion rather than GC re-entry [177, 182-185]. Therefore, local Ab secretion at site of antigen encounter is very high [159, 160, 257, 258] and would probably locally inhibit B cell responses to a whole antigen or a single epitope during secondary responses. MBC are also able to remove antigen from the environment via BCR internalization, this way MBC are activated and can present antigen processed to peptides to T_{fh} [259].

Previous studies, mentioned in 1.2.3.1, have focused mainly on the effect of serum Abs on secondary B cell responses rather than the effect quickly differentiating an Ab secreting MBCs have. In most of the system observed, MBCs were present, although their contribution was not sufficiently delineated.

1.2.3.3 CD4 T CELLS

Cognate help provided by T cells is indispensable for the development of a B cell response against protein antigens and are required for the final activation of MBC during second antigen encounter [117, 185, 259]. Several studies have provided an insight into the support of naïve, memory and rare B cell responses by pre-existing T_{fh} against various pathogens and antigens such as IAV and HA [54, 165, 172, 220, 221, 260-263]. Using cell fate mapping as well as Ab time stamping experiments, Schiepers et al have shown how while pre-existing Abs inhibit secondary GC responses to immunization with the same or a similar antigen, while pre-existing CD4 T cells against the antigen promoted GC entry of MBCs [172]. The effect of pre-existing CD4 T cell specificity has so far not been addressed in this regard.

2 AIMS

The overarching aim of this thesis is to suggest strategies to refocus ID and B cell responses to IAV HA vulnerable antigenic sites and to delineate the influence of pre-existing immune components on secondary B cell responses, in particular MBC responses. Of particular interest are secondary responses to drifted virus or protein as they can help to determine factors influencing bnAb development.

2.1 PAPER I AND II

The aim of the first two papers is to dissect the effect of pre-existing T helper cells (Paper I) as well as pre-existing Abs and MBC (Paper II) on secondary naïve and memory B cell responses to a drifted/different antigen. We make use or prime-challenge experiments and adoptive transfer models to achieve this aim.

2.2 PAPER III

In Paper III, we aimed to induce bnAbs by developing an HA stem epitope mimetic design, detect antigen specific MBC from IAV immunized donors and to in detail define cross-reactive humoral responses to various IAV strains after immunization with the particle.

3 METHODS, METHODOLOGICAL LIMITATIONS AND ETHICAL CONSIDERATIONS

To be able to induce B cell responses against IAV and its proteins while being able to fate map cells and supply the immune system with different pre-existing immune components as well as harvesting secondary lymphoid organs, we made use of murine experimental models. IAV ID patterns are well defined for the murine C57BL/6 strain, which was used for the majority of Paper I and Paper II; all other strains used in these studies are congenic. In Paper III, BALB/c mice were used. As for further immune response enhancement, the T cell epitope from the IAV M2e was fused to the stem design proteins developed in Paper III, the use of this mouse strain was necessary. Recognition of the M2e peptide is restricted to the H-2^b MHCII, which is expressed by BALB/c but not C57BL/6 mice [264].

It is important to consider that murine and human immune systems are differing to a high degree, and we cannot recapitulate the history of previous IAV infections/immunizations in adult humans with its diverse memory components. Nevertheless, the murine system provides us with important advantages in comparison to the human system. We therefore conducted all experiments in mice. In the future it will be important to adapt the models also to human studies.

3.1 VIRUSES AND PROTEINS

3.1.1 VIRUS

All viruses (including the Δ virus panel mutants expressing only one of the five main antigenic sites of the HA head Ca1Δ4, Ca2Δ4, CbΔ4, SaΔ4, SbΔ4. And the mutant expressing all of the main five antigenic sites of the HA head except Cb, ΔCb.) were grown in embryonated chicken eggs or Madin-Darby canine kidney (MDCK) cells *in vitro* culture. It is important to keep in mind that those culture systems will introduce host proteins into the viral stock, such as for example OVA. Therefore, appropriate controls were used in all experiments. If needed, virus was

concentrated on a sucrose gradient as described previously [22]. Subsequently, viral titer was determined by median Tissue Culture Infectious Dose ($TCID_{50}$). For immunization and whole virus ELISA, virus was ultraviolet (UV)-inactivated for 20-30min in ice. The content of HA was estimated by hemagglutination assay as described previously [18].

3.1.2 PROTEINS

All proteins used were recombinant and from PR8 wt virus, with exception of the computationally designed stem-particle and H1 from A/California/07/2009 virus as well as H3 from A/Brisbane/10/07 virus used in Paper III, to ensure genetic similarity to viruses used for infection and to exclude impurities from other viral proteins.

HA proteins (Figure 5, top; PR8 wildtype (wt) HA, S12 HA, Ca1Δ4 HA, Ca2Δ4 HA, CbΔ4 HA, SaΔ4 HA, SbΔ4 HA, ΔCb HA, H1 from A/California/07/2009 virus, H3 from A/Brisbane/10/07 virus) were produced in mammalian cell culture and purified by Protein Production Sweden, a national core facility. HA proteins were designed with an Avi-Tag to be biotinylated and subsequently tetramerized via streptavidin (SA) to a fluorochrome for antigen specific staining of B cells. For biotinylation, the Biotin Protein Ligase standard reaction kit (Avidity) was used.

NP protein was obtained from Sino Biologoical and has been used in other studies [153].

NA protein was produced in-house. In short, Expi293F™ cells (Thermo Fisher) were transfected with a plasmid expressing the NA construct via Expifectamine 293 (Thermo Fisher) and processed according to the manufacturer's instructions. NA was purified via a HisTrap Excel column (Cytvia) and stored in a 1:1 mix with glycerol at -20°C.

All recombinant PR8 wt proteins were purified and concentrated using 30 kilodalton (kDa) molecular weight cutoff protein concentrators (Thermo Fisher) if necessary.

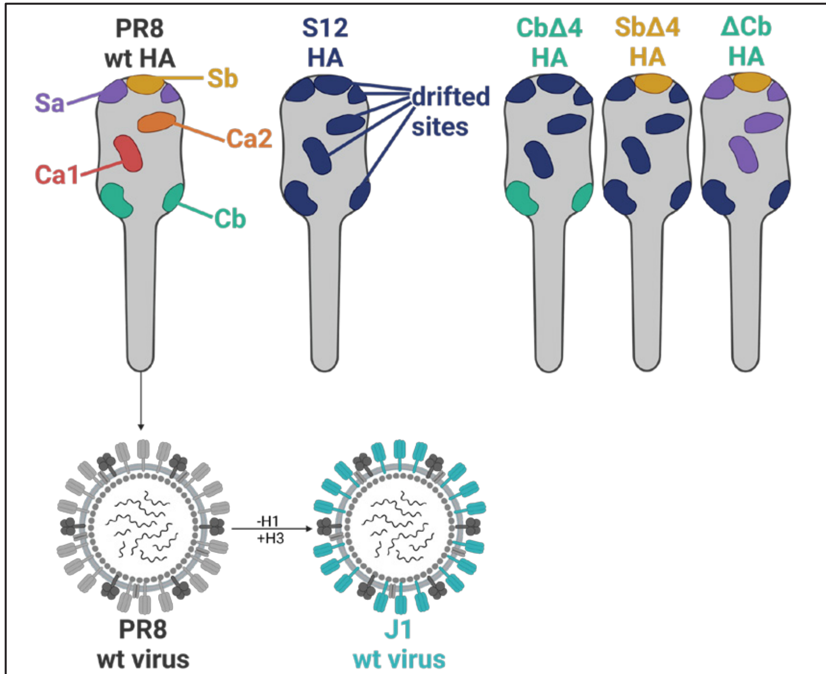


Figure 5: *IAV viruses (bottom) and rHA proteins (top) used for infections and vaccinations in Paper II. Antigenic sites: Ca1 (red), Ca2 (orange), Cb (green), Sa (purple), Sb (yellow). Drifted antigenic sites in dark blue. Ca1, Ca2 and Sa are summarized as "other" (purple) on ΔCb HA. H1 in grey, H3 in blue. Created with BioRender.*

3.1.2.1 COMPUTATIONAL DESIGN AND PRODUCTION OF STEM-EPITOPE MIMETIC

A protein scaffold containing a similar α-helix to the one contained in the FI6 epitope on the HA stem was identified and selected for structural similarity as well as accessibility of the FI6 antibody. Finally, the scaffold selected for the design was the murine apolipoprotein E. To finally mimic the FI6 epitope, sidechains from it were grafted onto the α-helix and mutated if steric hindrance occurred. Epitope mimicry was not evaluated by sequence but by surface similarity, hydrophobic residues were preferred to mimic the hydrophobic pocket of the FI6 epitope more closely. Stability was also considered during the design. The recombinant design was expressed in *Escherichia coli* cell culture and purified using Ni-NTA affinity chromatography and lastly size exclusion chromatography (Cytvia).

3.2 INFECTION AND VACCINATION

We applied a standardized method of infection and various vaccination models to induce B cell responses to IAV in mice. This enabled us to use immunogens of various valencies and antigen contents as well as infectious versus non-infectious agents.

3.2.1 INFECTION

PR8 virus and its Δ4 mutants or S12 were used for infection. Virus was stored at -80°C to retain infectivity and thawed on ice only immediately before infection. Animals were infected as described previously [265]. Briefly, mice were put to sleep in a chamber with continuous isoflurane flow and 25-500 $TCID_{50}$ virus per 25ul Hanks' Balanced Salt Solution (HBSS) + bovine serum albumin (BSA) was injected intranasally with a pipette. Mice were observed for subsequent bubbling of the solution and to let them recover in prone position. Bubbling might indicate that virus did not reach the lungs. This i.n. infection method ensures virus to reach the lung and induces potent B cell responses that can be observed in lungs, the lung-draining mediastinal lymph node (medLN) and spleen. Generally, virus is cleared from the lungs of mice by latest 9dpi [266].

3.2.2 VACCINATION

We applied several vaccination methods to apply different mixtures of antigen, varying valency of antigen particles and to induce immune memory in only the T cell compartment.

The latter is achieved by peptide vaccination. Generally, B cells recognize in the case of T cell dependent antigens three dimensional structures on proteins and rarely show specificity to linear peptide epitopes [267]. T cells on the other hand, recognize linear peptides presented on MHC molecules [268]. To induce T cell memory and therefore pre-existing T_{fh} to provide help to B cells, we used peptide vaccinations [269].

With increasing valency, we used monovalent rHA and UV-inactivated virus. HA and SA-HA were used for subcutaneous (s.c.) vaccinations into the foot hock with an MF59®-like squalene-based oil-in-water adjuvant AddaVax (Invivogen) that induces humoral and cellular

immune responses; MF59® is used for IAV vaccination in humans [270]. For monovalent protein, 10ug rHA was used. UV-inactivated virus provides a high valency of 300-400 HA trimers per virion [271] and introduces also other IAV antigens into the immune system. UV-inactivated virus was injected at 2000x hemagglutination units (HAU) s.c. in the foot hock or i.p. and does not require an adjuvant [272].

For vaccination with the stem mimetic, adjuvant AddaS03 was used. This is also an oil-in-water emulsion and AS03, the equivalent formulation used in humans, is used in influenza vaccines regularly [273]. Here, also both T_H1 and T_H2 immune responses are induced [274].

3.3 MOUSE MODELS

3.3.1 CELL FATE TRACKING MODEL

When activated B cells enter a GC, they start to express the G-coupled receptor sphingosine-1-phosphate receptor 2 (S1PR2) on their surface which binds to sphingosine-1-phosphate (S1P) secreted in the GC and retains the B cells there [275]. S1PR2 overexpression alone caused B cell clustering in LN follicles even without antigenic challenge [276]. To make use of this transient expression of S1PR2 by all B cells entering a GC reaction, Shinnakasu et al created a transgenic mouse model with a tamoxifen-inducible Cre recombinase–estrogen receptor 2 fusion protein (ERT2cre) in the second exon of the *S1pr2* gene [277]. This model also contained a transgene in which tdTomato is flanked by a stop cassette controlled by the *Rosa26* promotor. In these mice, when tamoxifen is provided, all GC B cells (expressing S1PR2) will also start to express tdTomato and can be differentiated from non-GC B cells. Additionally, due to the constitutively expressed *Rosa26*, those cells and all their PC and MCB progeny will be marked with tdToamto. (Figure 6, top right)

We made use of this *S1pr2*-ERT2cre-tdTomato mouse model for our cell fate tracking experiments and provided mice with tamoxifen during a primary immune response to an IAV infection until 26dpi, ensuring the tdTomato marking of as many GC-derived MBC as possible. At 28dpi, animals are vaccinated with rHA as in the foot hock. We used various

Δ4 HA mutants to evaluate the effect of antigenic site-specific immune components on the secondary response. 7 days post vaccination (dpv), the animals are sacrificed and primary infection-draining medLN as well as secondary vaccination-draining popliteal lymph node (pLN) are taken in addition to serum. (Figure 6, middle). tdTomato-expression is required to differentiate the secondary GC re-entering MBC from naïve B cells entering a GC for the first time (Figure 6, bottom). With this approach, we can't account for MBC generated during an extrafollicular response. This problem can be overcome using cell-transfer models as described in 3.4.2.

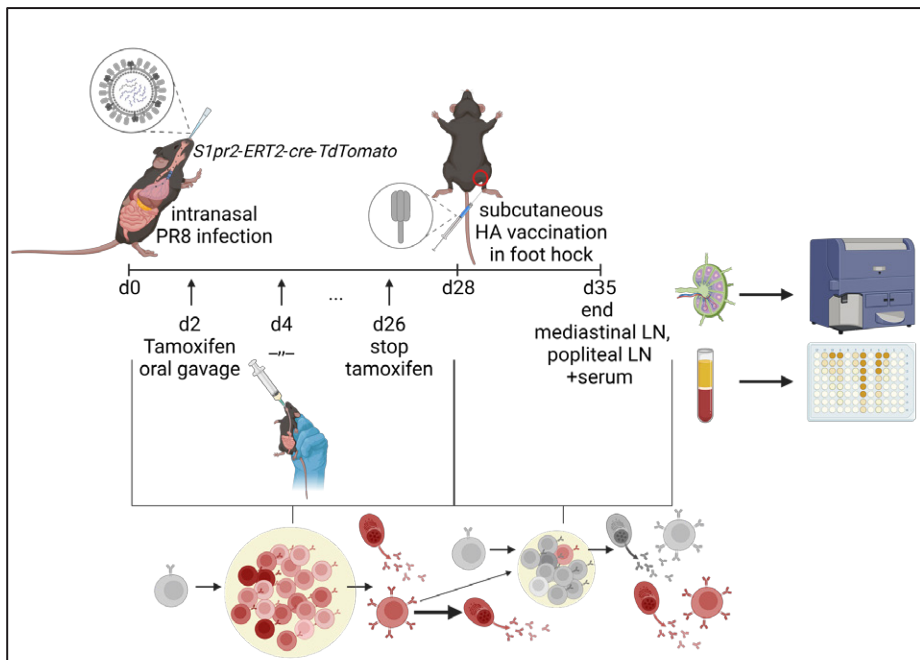


Figure 6: *Graphic representation of the cell fate tracking mouse model. Induction of tdTomato expression by tamoxifen (top right), experimental scheme (middle) and following fate-mapped B cells (bottom). Created with BioRender.*

3.3.2 CELL TRANSFER MODEL

Donor mice were C57BL6 mice, expressing the CD45 isoform CD45.2 on all leukocytes [278] and encode the Ig heavy (Igh)[b] allotype [279]. To be able to differentiate not only cells but also Ab secreted of donor

and recipient, we bred a C57BL/6 congenic mouse strain. *B6.SJLPtprcaPepcb/BoyJ*, expressing CD45.1 isoform, and *B6.CgGpi1aThy1a Igha/J*, expressing the Igh[a] allotype, (both Jackson) were crossed to generate the PepBoyIgha strain expressing both CD45.1 and Igh[a]. B cells and T cells of donor and recipient can therefore be differentiated via their CD45 isoform expression and Abs in serum of recipient can be differentiated using enzyme-linked immunosorbent assay (ELISA) secondary antibodies specific for either Igh[a] or Igh[b] allotype.

3.4 ADOPTIVE TRANSFER MODELS

To recreate the presence of pre-existing immune components of defined specificity, we resorted to adoptive transfer models of Abs as well as B and T cells. Consequently, we were able to define the specificity of Abs, CD4 T cells and MBC and in the recipient. Adoptive transfer models overcame many problems and more accurately differentiate the origin of such immune components (primary versus secondary immune reaction). Congenic models were used for adoptive transfers to ensure MHC-compatibility and engraftment of the components into the recipient.

3.4.1 ANTIBODIES

Abs used in adoptive transfers were either monoclonal antibodies (mAbs) or polyclonal IgG (pIgG) isolated from infected C57BL/6 mice.

mAbs were purified from hybridoma by BioXCell and are suitable for *in vivo* application.

To generate pIgG, C57BL/6 mice were infected with PR8 virus, one of its Δ4 mutants or S12. At 28dpi, animals were sacrificed and terminally bled under ketamine anesthesia. IgG was isolated from the obtained serum using a MelonGel column (Thermo Fisher) on a chromatography system. MelonGel removes other Ig isotypes (IgM, IgA, IgE) and does not require elution, therefore being very gentle on the pIgG purified and ensuring a very quick workflow. Following this, the pIgG is biotinylated to be identifiable in recipient serum and so it can be removed from serum mixtures of recipients that received biotinylated pIgG. Finally, biotinylated pIgG is titrated using a standardized ELISA protocol to

determine maximum binding capacity (Bmax). To determine eventual differences in pIgG avidity, an ELISA- based avidity assay using Urea as chaotropic agent was applied. Using Bmax as a measure for Ab amount instead of mass ensures the same amount of HA bound by a defined Bmax of pIgG as serum often contains various affinities of Abs [147]. The same titration technique is also applied to mAbs.

Abs are diluted in PBS and transferred into the recipient i.p.. Four hours later, the animals are challenged using one of the two vaccination techniques described in 3.2.2 PR8 wt and the ΔCb escape mutant viruses or PR8 wt HA are used for vaccination. Animals are sacrificed 14 dpv after Ab transfer only to ensure a mature GC reaction to develop. When combined with a MBC transfer, animals are sacrificed at 7dpv.. Organs taken for flow cytometric analysis were spleen (after i.p. vaccination) or pLN (after s.c. foot hock vaccination). Recipient serum taken at 14dpv is treated with SA-gel bead for 30min to remove biotinylated donor pIgG.

Adoptive Abs transfers can in theory be combined with any cell transfer desired.

3.4.2 MEMORY B CELLS

As the *S1pr2*-ERT2cre-tdTomato mouse model did not allow for the differentiation between progeny of MBCs generated during an extrafollicular response and GC progeny, and the Ab transfer model could not recapitulate the high concentration of Abs caused by local Ab secretion, we setup an adoptive MBC transfer accompanied with adoptive pIgG transfer. This allows for the precise definition of antigenic site specificity of pre-existing immune components and local Ab secretion by MBC-derived PC.

Donor animals were infected with PR8 wt and left to develop MBC for 28d. The recipient is primed using the PR8 reassortant J1, expressing an H3 instead of the PR8 wt H1 [151, 280]. This ensured the presence of an immune response environment in the recipient and in particular CD4 T cells that can support a donor MBC response. It was not possible to successfully engraft and re-activate HA specific donor MCB without previous J1 priming. We chose J1 for priming instead of PR8 wt to circumvent competition with recipient MBC specific for PR8 wt HA.

MBCs from the donor are isolated using a B cell negative selection kit commercially available (Stemcell) and adding an anti-IgD and anti-GL7 biotinylated antibodies to remove naïve and GC B cells from the picture. This likely leaves only IgM or switched MBC after isolation. Cells are transferred at 1-3x 10^6cells/recipient parallel to transfer of 1xBmax of pIgG. The recipients were left to rest overnight and were vaccinated the next day i.p. with 2000x HAU UV-inactivated virus. Animals were bled from the vena saphena at 3dpv and sacrificed at 7dpv. MedLN, spleen and serum were taken from the recipient. While organs were processed for flow cytometric assessment, serum was used for ELISA. (Figure 7)

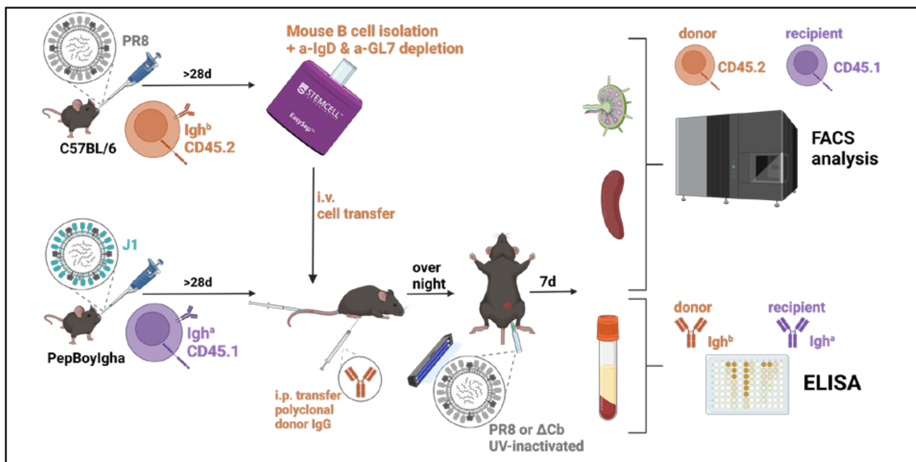


Figure 7: *Graphic representation of the MBC + pIgG transfer. Donor immune components shown in orange and recipient immune components in purple.* Created with BioRender.

3.4.3 CD4 T CELLS

To evaluate the effect of pre-existing CD4 T cells and their help to B cells and their fate decisions, in particular to delineate whether B cells prefer cognate to non-cognate help, we also conducted CD4 T cell transfers.

C57BL/6, expressing CD45.2, donor mice were immunized s.c. in the foot hock with peptides from PR8 NP and OVA as control. CD4 T cells were isolated via the Mouse CD4 T cell Isolation kit (Stemcell) by negative selection from spleen and pLN of recipients. 2 x 10^6 isolated $CD4^+$ T cells were i.v. transferred into the recipients, PepBoyIgha,

expressing CD45.1. Then, the recipient was left to rest overnight and infected i.n. the next with PR8 virus. At 14dpi medLN was collected and donor versus recipient T_{fh} were analyzed.

3.5 MULTICOLOUR AND SPECTRAL FLOW CYTOMETRY

The main method to analyze samples in this work was flow cytometry. It enabled the detection of surface- and intracellular-expressed proteins as well as the detection of antigen specific B cells using fluorescently labelled antigen. Additionally, it is also used for neutralization assays, where IAV NP expressed by infected cells is stained with a fluorescently labelled antibody.

While staining for surface markers for B cell phenotyping has been used for decades and staining panels are established in the community, we applied a novel method to not only stain for antigen but even antigenic site specificity. To achieve this, we used PR8 wt HA and Δ4 HA mutants labelled with different fluorochromes and defined antigenic site specificity with a multi-step gating strategy (Figure 8). This strategy was modified to the proteins used for vaccination and can be more or less stringent, prioritized based on the experimental contribution to key findings. Especially spectral flow cytometry can be applied when staining for multiple antigenic mutants as one can add potentially up to 30 differently labelled antigens.

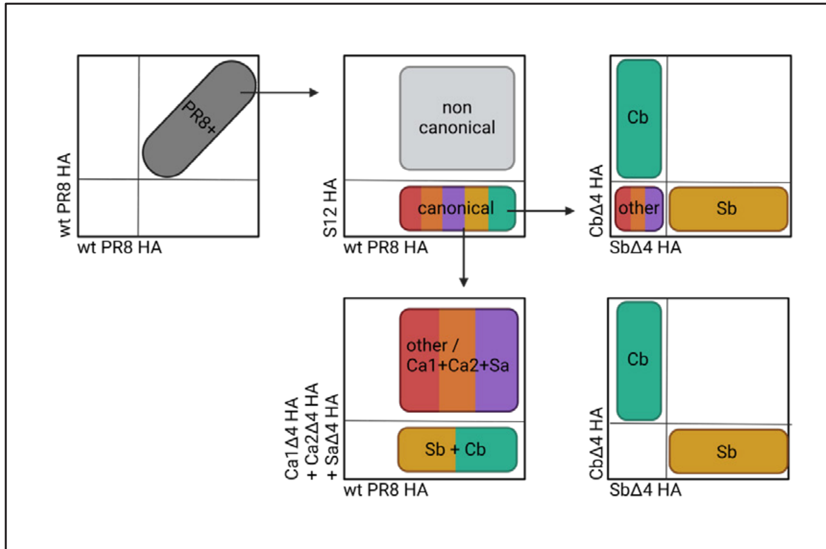


Figure 8: *Gating strategy to determine antigenic site-specificity of B cells to PR8 wt HA. Used for cell fate tracking (top) and MBC + pIgG transfers (bottom). Colours indicate antigenic site specificity as defined in Figure 5. Created with BioRender.*

3.6 FUNCTIONAL ASSAYS TO EVALUATE ANTIBODY FUNCTION AND SPECIFICITY

3.6.1 ELISA

The ELISA assays conducted in this work were standardized to ensure comparability between different experiments. We used either recombinant protein or whole, UV-inactivated virus for coating. For Paper III, whole virus was used for ELISA out of the necessity that not all rHA proteins were available and that stem specific Abs bind more effectively to HA when the protein is in its natural conformation [281].

3.6.2 BINDING TO INFECTED CELLS

When MDCK cells are infected with any IAV in vitro, they will start to express the viral proteins HA, NP and M2e on their surface prior to budding of viral particles [11]. We used this fact to stain infected cells with serum derived from vaccinated animals to determine binding of

Abs to HA protein in its natural conformation in infected cells. The assay was conducted as previously described [282]. Briefly, MDCK cells were infected at multiplicity of infection (MOI) = 3 for 5h. After trypsinization, the cells were incubated with serum for 60min at 37°C and after that with a fluorescently labelled anti-mouse kappa light chain antibody to detect binding of serum Abs to the infected cells. To determine whether a cell was infected or not, finally the MDCK cells were permeabilized and fixed and stained with an anti NP antibody. The samples were then acquired on a flow cytometer, binding of serum Abs was only analyzed on virus infected cells.

3.6.3 ADCC

As anti-stem Abs in mice rarely show neutralizing effects on IAV, we sought out to determine whether they showed other Fc-functionalities, such as ADCC [42]. The assay was performed as previously described [283]. Briefly, MDCK cells were infected with virus at MOI = 3 and after 6h, mouse serum was combined with ADCC effector cells expressing luciferase controlled by the nuclear factor of activated T cells (NFAT) response element and transiently expressing Fc-gamma receptor IV (FcγRIV) (Promega). This effector cell-serum mixture was added onto the infected MDCK cells and left over night. Finally, cells were lysed, and luciferase substrate was provided. Luminescence was measured within 5min, and fold induction was calculated over a negative control.

3.6.4 NEUTRALIZATION ASSAYS

Neutralization is considered the holy grail of protective Ab-activity, providing potentially sterile immunity [44]. We used neutralization assays to determine protection from Abs induced by PR8 wt virus and ∆4 vaccination to another H1 IAV strain to show cross-protective activity of those antibodies. Specifically, induction of Abs binding to more conserved antigenic sites through ∆4 HA vaccination. Briefly, virus was incubated with serial dilutions of sera for 1h at 37°C. Then, MDCK cells were infected with the mixture at MOI = 0.1. 18h after infection, cells were harvested and stained for intracellular NP expression, indicating infection. The samples were then run on a flow cytometer.

3.7 ETHICAL CONSIDERATIONS

All studies conducted in this work were approved by an ethical committee, the Swedish Board or Agriculture (Jordbruksverket). Ethical permits for work with mice used were 1666/19, 2230/21, 28/23 and for breeding, ethical permit 3307/20 was used. With all experiments, we adhered to the 3R (replace-reduce-refine) principles as closely as possible.

When conducting infection and vaccination studies on mice, it is mainly important to consider the severity of disease on the animals. Therefore, it is crucial to titrate virus thoroughly before use in experiments to ensure the development of a strong response while reducing the suffering of animals as much as possible. Vaccination in general does not induce severe pathogenicity, although we selected comparably less painful vaccinations routes.

For this work, many strains were bred in-house and the ration of male to female progeny is always 50:50. This means by only using female animals for experiments, one would have to sacrifice half of the pups without them serving a purpose. Therefore, in this work, a mix of male and female mice were used in almost all experiments. The ration was kept at ~50:50 while still age-matching all animals.

In general, we could not avoid a certain amount of suffering, disease and stress during our studies with mice. Although we applied our best efforts to reduce them as much as possible. These studies cannot be conducted in human participants and *ex vivo* approaches, such as organs on chips, have not yet been developed to an extent that recreates immune response in a living organism to a satisfying degree. Nevertheless, certain aspects of this work will require validation in humans to finally give us insight into universal IAV vaccine development in humans.

4 RESULTS AND DISCUSSION

4.1 PAPER I

In this study, we investigated the role of pre-existing T cell help on GC responses and how they shape humoral responses during IAV infection and/or vaccination. Our focus was especially on the questions if pre-existing T cells influenced naïve B cell responses and whether non-cognate T cells help could enhance B cells responses (Figure 9). We used the two-protein system of NP (intra-virion) and HA (on surface of virion) of IAV PR8 to achieve our goals (Figure 4).

Induction of pre-existing CD4 T cells by peptide vaccination. Functional CD4 T cells were induced by NP peptide vaccination, they were of T_{fh} and T_H1 phenotypes (Figure 9, A), as shown by cytokine expression profiles and antigen-specific TCR staining. After i.n. infection, they exhibited similar ID patterns to peptides as described in previous works [284] (Figure 9, B).

Dissection of inter- and intramolecular antigen-specific T cell help. We showed that help from CD4 T cells was mainly intramolecular or cognate, supporting responses only to the priming antigen. For example, pre-existing NP-specific CD4 T cells enhanced Ab responses to NP but failed to enhance early HA-specific B cell responses, even when the proteins were used in a mixture for vaccination (Figure 9, C). This result showed that non-cognate help is not a significant occurrence during IAV infection, a finding consistent with several previous studies [220, 261, 285]. Although a study by Tarke et al has recently shown that non-cognate T cell help is a common occurrence in SARS-CoV2 memory responses [286]. With these results, we highlight the need to explore strategies that leverage conserved CD4 T cell epitopes for broader protection against IAV variants.

Accelerated GC formation but no increased antigen-specific GC B cell frequencies.

Pre-existing CD4 T cells supported earlier formation of GCs after IAV infection, as shown by increased GC size at 7dpi. This acceleration, however, did not result in a higher frequency of antigen-specific GC B cells (Figure 9, d). The presence of primed CD4 T cells also did not

change the frequency of switched MBC (Figure 9, E), suggesting that their primary role is to facilitate early Ab production rather than improving selection in the GC. A favoring of ASC fate by pre-existing T_H1 cells has been shown before [287] and highlights a decoupling of Ab quantity and GC B cell dynamics.

Quality of serum Ab responses to NP and HA evaluated by avidity. We reasoned, that pre-existing CD4 T cells could potentially improve the quality of an Ab response as well and therefore assayed Abs from serum for avidity via an ELISA assay. We did not observe any notable changes in Ab avidity to NP in primed versus non-primed animals, suggesting that the stronger responses in ELISA assay for titer determination were likely due to more efficient secretion or increased numbers of ASC (Figure 9, F). Interestingly, early NP-specific Abs had higher baseline avidity compared to HA-specific Abs (Figure 9, F), which caught up later during the response, reflecting differences in the affinity maturation of NP- and HA-specific B cells. This further highlights the challenge of achieving robust and early Ab responses to highly variable antigens such as HA. Likely, NP-responses were generated outside of the GC in an extrafollicular response, while HA responses matured in the GC and specific, high affinity ASC were exported later.

Implications for (universal) IAV Vaccine Design.

The findings underscore the potential of targeting conserved CD4 T cell epitopes, such as those on NP, to enhance vaccine efficacy. While NP-specific Abs were not highly protective, their immunogenicity and conservation between different IAV strains [14, 288] make NP a desirable target to elicit durable T cell responses. The lack of enhancement in HA-specific responses by NP-specific CD4 T cells revealed a need for strategies that directly prime HA-specific T cells to optimize humoral immunity. To include both, intra-virion proteins as well as surface proteins in vaccines, inactivated or attenuated whole virus particles could be used, or vaccines could be formulated as a mix of peptide- and protein components.

Limitations

We showed that presence of CD4 T cells improved anti IAV serum Ab

responses, but we were not able to elucidate whether this was due to improved GC entry of improved GC selection. To answer this question, cell fate tracking approaches of both B and T cells as well as a higher resolution of timepoints could be beneficial. Additionally, later timepoints should also be tested to determine the longevity of this increased Ab response and to use challenge-studies to show improve protection by T cell priming. In this study, we also focused on two separate proteins whereas it would also be interesting to determine the effect of antigenic site specificity on T cell help, especially in regard to drifted virus mutants. Unfortunately, we were unable to prime CD4 T cells against HA and therefore unable to show an enhancing effect of HA specific CD4 T cells to B cell responses against HA. Additionally, different vaccination approaches should be tested, as adjuvants have polarizing effects in T cell differentiation [289, 290] and generation of HA specific CD4 T cells by peptide prime could be achieved by for example an increased dose.

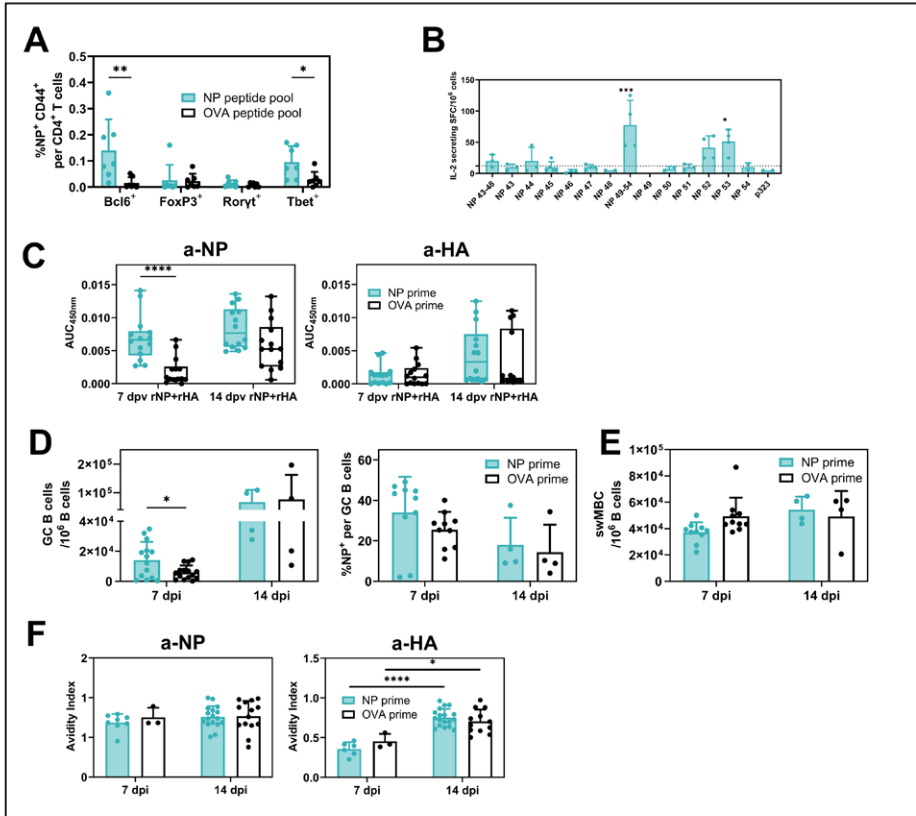


Figure 9: *Pre-existing CD4 T cells deliver only cognate help during IAV infection to naïve B cells, enhance Ab responses and increase early GC size.* ***A:*** *Frequency of NP peptide-specific CD44+ T cells among four CD4 T cell subsets defined by transcription factor expression (Bcl6 – T_{fh}, FoxP3 – T_{reg}, Rorγt – T_H17, Tbet – T_H1) in the pLN after immunization with NP (blue) and OVA (black/white) peptide pools.* ***B:*** *IL-2 secreting SCF as determined by ELISpot in spleen after PR8 wt infection and stimulation with NP peptides.* ***C:*** *AUC450nm of sera obtained from mice primed with NP (blue) and OVA (black/white) peptides and vaccinated with an rNP and rHA mixture at 7 and 14dpv. Specificity determined against NP (left) and HA (right).* ***D:*** *GC B cells/10^6 B cells (left) and %NP+ per GC B cells (right) after NP (blue) and OVA (black/white) peptide prime and PR8 wt infection at 7 and 14dpi in medLN.* ***E:*** *switched MBC/10^6 B cells after NP (blue) and OVA (black/white) peptide prime and PR8 wt infection at 7 and 14dpi in medLN.* ***F:*** *Avidity index of sera obtained from mice primed with NP (blue) and OVA (black/white) peptides and infected with PR8 wt at 7 and 14dpi. Specificity determined against NP (left) and HA (right).*
*Significance determined with one-way ANOVA with multiple comparisons to negative control peptide using Dunnett's correction at an alpha of 0.05 (B) and Student's t-test (A, C, D, E, F). * = p≤0.05; ** = p ≤0.01; *** = p≤0.001; *** = p≤0.0001. For bars, mean +SD and for box plots median +/- interquartile range are indicated.*

4.2 PAPER II

In Paper II, we explored the dynamic interplay between pre-existing Abs, MBCs, and naive B cells in response to drifted IAV antigens. We took advantage of cell fate tracking and adoptive transfer models to delineate secondary B cell response determinants and thereby used vaccination methods of different valency to visualize how Abs and B cells "see" antigen. (Figure 10)

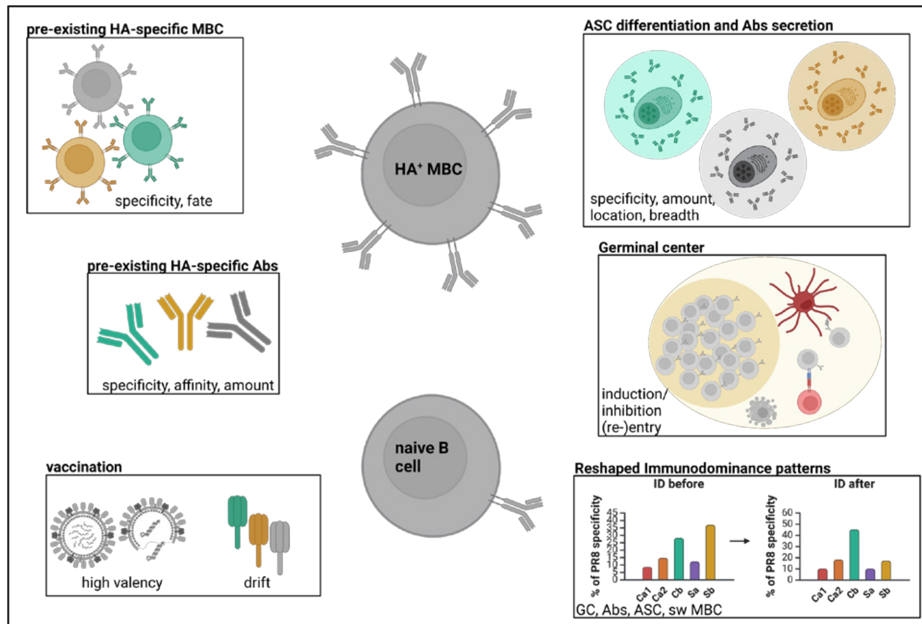


Figure 10: *Effect of pre-existing Abs and MBC on vaccination with drifted antigens and antigens of variant valency reshapes MBC as well as naïve B cell responses. Outcomes can range from Ab secretion, GC reaction induction and entry to reshaped ID patterns of GC reactions and serum Ab. Created with BioRender.*

MBC responses to mutated and conserved antigenic sites on a drifted antigen.

Utilizing fate-tracking models and antigenic site-specific escape mutants of rHA protein (namely CbΔ4, SbΔ4 and S12, Figure 5) for secondary vaccination, we demonstrated that drifted vaccination reshapes secondary GC responses by promoting naive B cell entry whilst favoring MBC differentiation to ASCs as previously shown [170, 172]. Not all drifted antigens induced robust de novo responses to

novel epitopes (Figure 11, A), indicating that some antigenic sites might have a higher potential for OAS/primary addiction. Slight changes in the vaccination antigen showed expanded GC sizes compared to homologous vaccination (Figure 11, B). We also observed an increase of serum Abs specific for PR8 wt HA at 7dpv in especially the homologous challenge (Figure 11, C), in which the ASC fate was preferred most by MCB compared to all other vaccinations. ID composition of GC and ASC after vaccination with drifted antigen was guided by the present canonical antigenic site (Figure 11, D). Sb-specific MBCs showed greater reactivation potential in comparison to Cb-specific MBCs, likely due to differences in epitope exposure as well as quality of pre-existing immune components. And finally, non-canonical antigenic sites were preferably targeted by secondary GCs and ASC-responses, likely leveraged by blocking of canonical antigenic sites via pre-existing Abs (Figure 11, D).

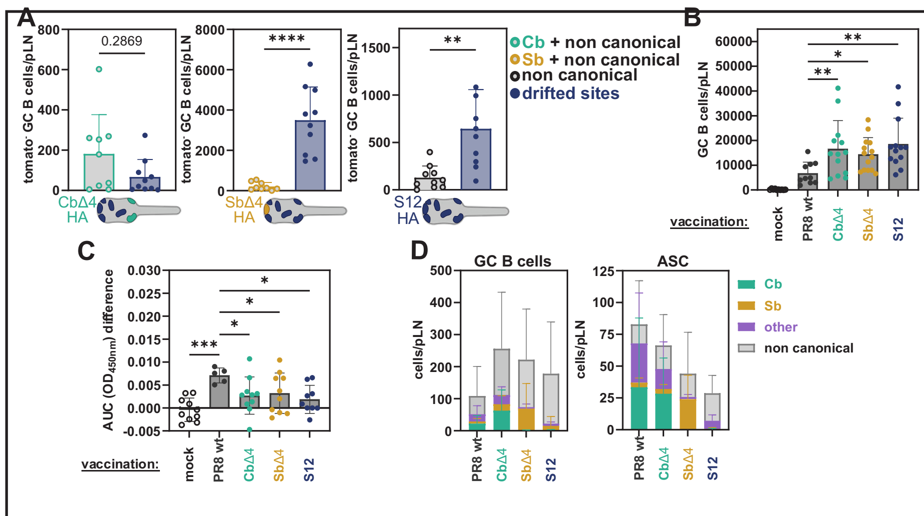


Figure 11: *Vaccination with a drifted antigen reshapes secondary B cell responses.* ***A:*** *Total number of tomato*$^{-}$ *GC B cells in vaccination-draining pLN after Cb∆4 rHA (left), Sb∆4 rHA (middle) and S12 rHA (right) vaccination at 7dpv.* ***B:*** *Total number of GC B cells in vaccination-draining pLN at 7dpv of rHA vaccination.* ***C:*** *AUC difference between -1dpv and 7dpv after rHA challenge of PR8 wt rHA-specific IgG.* ***D:*** *Total number and ID patterns of PR8 wt HA*$^{+}$ *GC B cells (left) and ASC (right) in pLN after rHA vaccination at 7dpv. Cb (green), Sb (yellow), other (purple), non-canonical (grey). Significance determined with a paired t-test (A) and one-way ANOVA (B, C). Lowest p-value indicated in A to show non-significance. * = p≤0.05; ** = p ≤0.01; *** = p≤0.001; *** = p≤0.0001. For bars, mean +SD is indicated. All plots contain data from ≤ 2 experiments.*

Integrating antigen valency into antibody feedback and epitope masking.

A major discovery of Paper II was the dependence of Ab-mediated suppression to vaccinating agents on antigen valency. Multivalent antigens facilitated strong suppression of naïve B cell responses in the presence of adoptively transferred Abs while no blocking or enhanced B cell responses were detectable after transfer of Abs and vaccination with monovalent PR8 wt rHA (Figure 12, A). This distinction highlights the importance of antigen presentation format in modulating immune responses but cannot explain the secondary B cell responses to drifted antigens in the cell fate tracking experiments.

Polyclonal IgG from infected mice versus monoclonal antibody transfers.

To dissect the effects of pre-existing Abs, we performed adoptive transfers of both, mAbs and pIgG. Most previous works have relied on mAb transfers or near-monoclonal mouse models [189, 228, 230, 232, 234-236], which do not recapitulate a real life *in vivo* scenario with various antigenic site specificities and affinities of Abs [18, 147]. Other studies have been using whole serum, which is a poorly defined mix of Abs and hard to dose, additionally those studies have been using mainly monovalent antigen and used different infection models [229, 231]. Our results showed that high affinity mAbs inhibited naïve B cell responses to some degree also to whole HA or even other viral proteins when multivalent antigens were used. Interestingly, Sb-specific mAbs had a greater suppressive effect, potentially due to differences in Fc receptor engagement. (Figure 12, B left). pIgG transfers mimicked physiological scenarios better but still reduced naïve B cell entry into GCs while reshaping ID patterns, favoring responses to antigenic sites not targeted by the pIgG transferred. Unlike mAbs, pIgG exhibited more nuanced suppression, likely due to varying affinities, specificities and IgG subclasses present. (Figure 12, B right).

Local antibody secretion at vaccination draining lymph nodes by ASC explains the varied secondary B cell responses to monovalent vaccine.

Differences in immune responses to drifted monovalent antigen as observed during cell fate tracking experiments could not be explained by the presence of Abs, we sought out to determine how pre-existing Abs could still affect secondary immune responses. Rapid

differentiation of MBCs into ASCs at vaccination sites will lead to localized Ab secretion in draining lymph nodes [171, 172, 189, 257, 258]. These local Abs likely suppress responses and mediate Ab feedback, shaping the specificity and magnitude of GC responses. Interestingly, even the distant prime-draining medLN showed reactivation, suggesting systemic modulation of immune responses by Abs or other immune components (Figure 12, C).

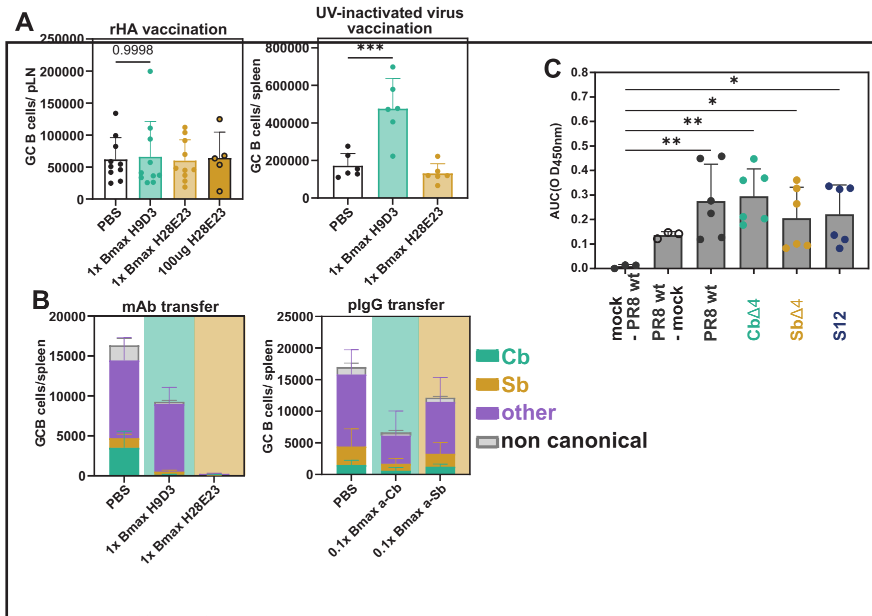


Figure 12: *Effect of pre-existing Abs on B cell responses rely on multivalency or local Ab secretion, they reshape ID patterns.* ***A:*** *Total number of GC B cells after mAb transfer and rHA (left) vaccination in pLN and UV-inactivated PR8 wt virus vaccination (right) in spleen. mAb H9D3 (a-Cb, green) and mAb H28-E23 (a-Sb, yellow) were used.* ***B:*** *Total number and ID patterns of PR8 wt HA⁺ GC B cells after mAb transfer (left) and pIgG transfer (right) and UV-inactivated PR8 wt virus vaccination. Specificity of Abs transferred indicated in background colour (a-Cb = green; a-Sb = yellow).* ***C:*** *PR8 wt rHA specific AUC of IgG in medLN supernatant at 7dpv after rHA vaccination in foot hock.*
*Significance determined by one-way ANOVA (A, C). Lowest p-value indicated in A (left) to show non-significance. * = p≤0.05; ** = p ≤0.01; *** = p≤0.001; *** = p≤0.0001. For bars, mean +SD is indicated. All plots contain data from ≤2 experiments.*

Adoptive co-transfers of polyclonal IgG and memory B cells reveal differential regulation of naïve B cells and memory B cells by pre-existing antibody and the requirement of CD4 T cell help for engraftment.

To overcome limitations of both cell fate tracking and Ab transfer experiments, we conducted adoptive transfers of MBC accompanied with pIgG transfers. We mix- and matched pIgG specificities and drifted vaccines to investigate the scenario of an escape in the Cb antigenic site (ΔCb) of PR8 wt HA. Priming of the recipient animals with an IAV expressing a different HA than PR8 wt was necessary to induce recall responses by 7dpv and guarantee engraftment or donor MBC. A clear difference in engraftment of donor cells can be observed when comparing total numbers of PR8 wt HA^+ B cells in the spleen of primed and naïve recipient (Figure 13, A). This is likely due to the induction of CD4 T cells specific to other viral antigens. Results from Paper I showed that non-cognate help from CD4 T cells did not support naïve B cell responses (Figure 9, C). Whereas early GC formation was supported (Figure 9, D), which might have delivered the required survival signals to the donor MBC to initiate a secondary response. Pre-existing CD4 T cells that offer at least non-cognate help therefore seemed to be required for a recall MBC response. Transferred MBC rapidly differentiated into ASC and Ab secreted by them dominated the serum response at 7dpv in comparison to recipient Ab (Figure 13, B). When very high amounts and diverse specificities of Abs were present, such as the “all Δ4” transferred group, in which pIgG against Ca1Δ4, Ca2Δ4, CbΔ4, SaΔ4 and SbΔ4 was transferred, Ab secretion was very low from not just recipient but also host cells. Interestingly, presence of CbΔ4 pIgG enhanced serum responses to the Cb-escaped PR8, ΔCb, likely due to cross-reactive Abs being present in the transferred pIgG that still recognized the *in vitro* drifted antigenic site. While pre-existing Abs and MBC reshaped total GC and ASC responses, they also clearly reshaped ID patterns. Particularly CbΔ4 specific pIgG during vaccination with the Cb antigenic site present on HA reshaped recall ASC responses towards the Sb antigenic site while enhancing responses of naïve-progeny ASC towards the Cb epitope (Figure 13, C). We showed thereby that MBC and naïve B cell responses are differentially regulated by pre-existing Ab that are also able to refocus site-specific responses in both populations.

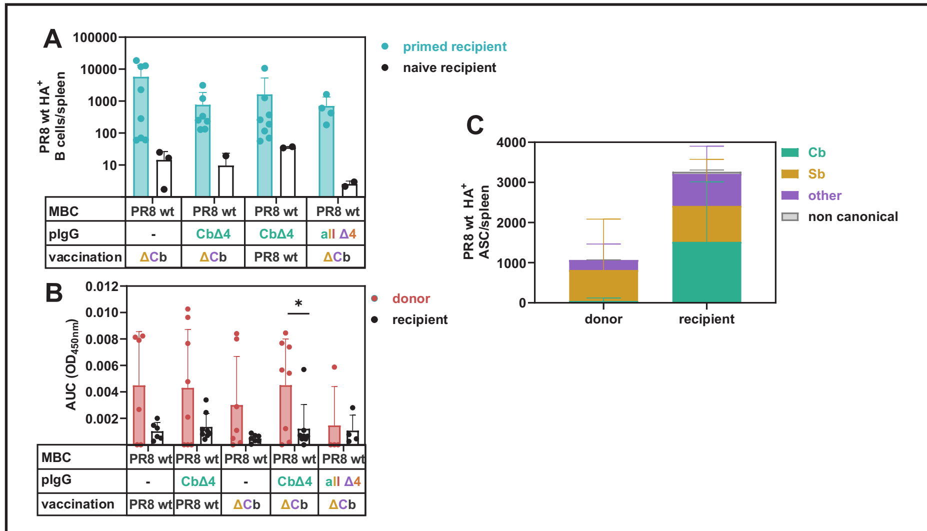


__Figure 13:__ Transfer of MBC requires priming of the donor, while engrafted MBC dominate Ab responses and are regulated differently to naïve B cells by pre-existing Abs. ***A:*** *PR8 wt HA⁺ donor B cells (GC B cells + ASC + switched MBC + IgM MBC) in spleen of recipient at 7dpv after MBC and pIgG transfer. Blue = primed recipient, black/white = naïve recipient.* ***B:*** *AUC of PR8 wt rHA specific IgG2a at 7dpv after MBC and pIgG transfer. Donor IgG2a = red; recipient IgG2a = black/white.* ***C:*** *Total number and ID patterns of PR8 wt HA⁺ ASC in spleen of recipient at 7dpv after PR8 wt MBC and CbΔ4 pIgG transfer and PR8 wt UV-inactivated virus vaccination. Significance determined by one-way ANOVA (B). * = p≤0.05; ** = p ≤0.01; *** = p≤0.001; *** = p≤0.0001. For bars, mean +SD is indicated. All plots contain data from ≤2 experiments.*

Impact on (universal) IAV vaccine design.

The findings of Paper II provide key insights for IAV vaccine design:

a. Multivalent vaccines: While multivalent antigens elicit robust immune responses, they are more susceptible to Ab-mediated suppression. Depending on whether B cell responses to vaccines are supposed to be fully or partially blocked or only inhibited to a small degree, valency needs to be considered and could be optimized to meet specific qualifications.
b. Sequential vaccinations: Secondary challenge with drifted antigens enhances naïve B cell activation and reduces ID to canonical sites, broadening the immune repertoire. Sequential vaccinations could be considered to guide responses to conserved antigenic sites.
c. Targeting conserved epitopes: Vaccines that preserve subdominant conserved epitopes, such as the HA stem, could elicit

cross-reactive responses and mitigate drifted vaccination. A direction explored in Paper III.

d. Combining Ab transfer and vaccination: Using Vaccines after transfer of a defined mixture of Abs could help guide B cells responses to desired epitopes and mask dominant, non-protective antigenic sites.
e. Considering pre-existing MBC: We have shown that pre-existing Abs alone cannot sufficiently explain impaired secondary GC responses, this was caused by pre-existing MCB differentiating into ASC and secreting large amounts of Abs. Therefore, when testing patients for pre-existing immunity, MBC should be examined as well.

Limitations

By using mAb and pIgG transfers, we were able to compare our findings from previous studies using monoclonal models [228, 230, 236] as well as recapitulate real life scenarios [76, 291, 292]. Although we were not able to in detail define the characteristics of the way Abs modulate B cells responses as other studies have done [171, 172, 228, 230, 233, 236]. More timepoints after MBC transfer and subsequent vaccination need to be assayed to provide a deeper insight into MBC dynamics upon reactivation and their differentiation into ASC to observe how local Ab secretion reshapes B cell responses. Later timepoints are also of high interest as it is important to determine whether those responses persist. Humans have a complex history with IAV antigens that was not fully recapitulated by the two exposures that we used in our models. Therefore, studies in humans or repeated antigen exposures in mice should be conducted. Finally, our vaccination approaches as well as mAb transfer models should be revised and standardized. The UV-inactivated virus particles contain other IAV proteins as well that could influence B cell responses and therefore vaccinations with a multivalent particle only including HA protein should be studied as well. The mAbs we used for our transfer experiments were of different IgG subclass that can engage different Fc receptor responses. Using the same IgG subclass therefore could prevent this limitation.

4.3 PAPER III

In Paper III we highlight the potential of rationally designed epitope-focused immunogens to induce the production of IAV HA specific bnAbs. Two computationally designed immunogens, an FI6-focused and a stem-mimetic construct, were assessed for their ability to induce bnAbs in vaccination responses. (Figure 14)

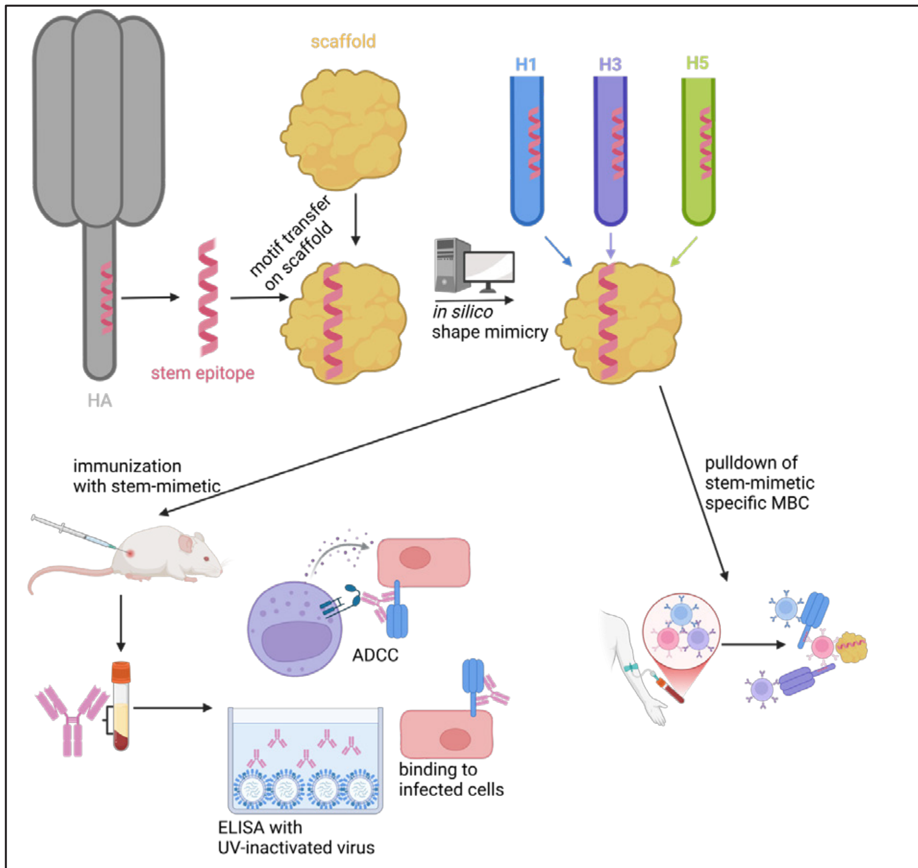


Figure 14: *Graphical representation of Paper III from design of the stem-mimetic (top) to pull-down of stem-mimetic binding MBC from human subjects (bottom right) and immunization of mice with stem-mimetic followed by characterization of induced Ab response (bottom left). Created with BioRender.*

Computational design of a particle mimicking the surface of a conserved epitope on the stem of influenza A virus haemagglutinin. An IAV stem epitope commonly targeted by bnAbs [26-29] was grafted onto a scaffold to mimic only the surface of the epitope. The scaffold selected was similar in structure and sufficient space for epitope presentation and manipulation. Surface mimicry was further improved by computational design and strong binding to prominent bnAbs was shown. To engage T cells by vaccination with the stem mimetic, an IAV M2e epitope was fused to the design that is a known target by T cells in BALB/C mice [264]. A further improvement of immunogenicity and binding to several bnAbs was achieved by fusion to a ferritin nanoparticle, producing a highly multivalent antigen. The structure of the stem-epitope mimetic was characterized by X-ray crystallography while bound to the FI6 mAb and binding to several bnAbs was confirmed using surface plasmon resonance (SPR).

Computationally designed stem mimetic and FI6-focused design engage memory B cells of influenza A virus vaccinated individuals. The FI6 focused design, which induced weaker HA stem responses in mice in comparison to the stem mimetic, was used in addition to the stem mimetic to sort human B cells from vaccinated donors. Both bound to MBCs expressing V_H1-69 and V_H3-23, which are connected to many stem-reactive bnAbs [31, 293]. MBCs that bound the stem-mimetic and H1 only were mainly expressing V_H1-69, while MBCs binding to the stem mimetic and H1 and H3 were mainly expressing V_H3-23. This highlights the antigen's ability to interact with a diverse repertoire of human IAV-experienced MBC and potentially engage them in a response to refine even more towards cross-reactivity. (Figure 15, A)

Haemagglutinin cross-reactive antibody responses induced by vaccination of mice with the computationally designed stem mimetic. We vaccinated mice with three doses of the stem mimetic design to determine its potential to induce HA cross-reactive immune responses.

Sera from mice vaccinated with the stem design were able to bind to an H1 recombinant protein and to several different group 1 and group 1 whole virus particles in an ELISA assay (Figure 15, B). We could even detect binding to an H7 IAV that is currently not circulating in the human

population [65]. In general, combining sequential HA and stem-mimetic vaccinations improves cross-reactivity in comparison to HA or stem-mimetic vaccination alone. This was also mirrored by a binding assay of serum Abs to virus infected cells. Importantly, Abs induced by stem-mimetic vaccination were not neutralizing. It is known that anti-stem Abs often require other Fc effector functions [42], we therefore conducted ADCC functional assays with sera from stem-mimetic vaccinated mice. The stem-epitope robustly induced Abs that exhibited ADCC reactivity against H1 and H3 viruses, but again a combination of HA and stem-mimetic vaccinations improved ADCC activity even more (Figure 15, C).

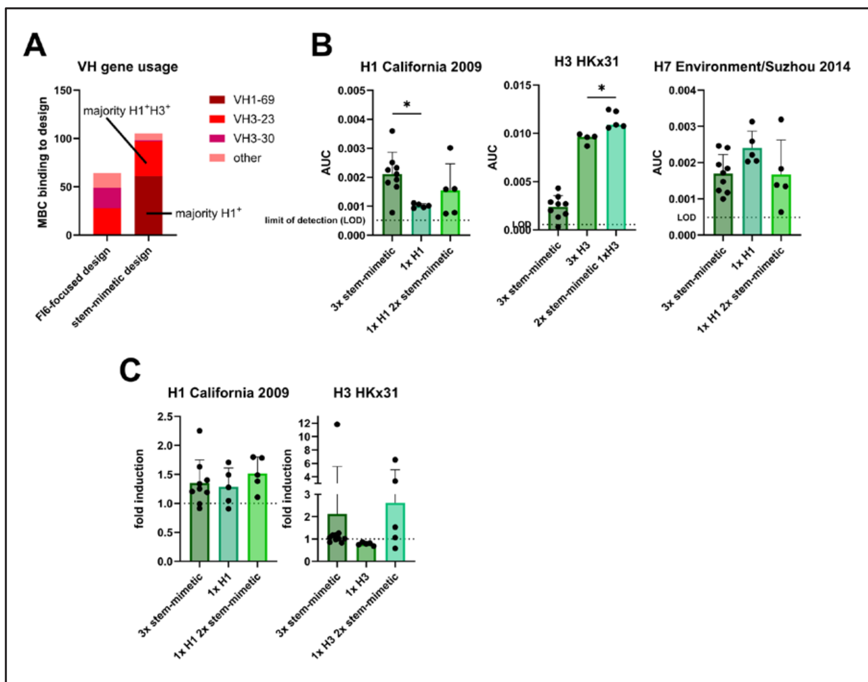


Figure 15: *Stem-mimetic design engages with MBC from IAV vaccination study subjects and induced IAV HA cross-reactive responses after immunization of mice.* ***A:*** *V_H gene expression of MBC binding to FI6 focused design and stem mimetic-design, that also bind H1 or H3 or both.* ***B:*** *Whole virus (as indicated) ELISA IgG AUC of sera from animals immunized with stem mimetic design and HA as indicated.* ***C:*** *ADCC fold induction of sera after immunization of mice with stem design and HA as indicated. Fold induction was calculated over serum control from mice vaccinated with irrelevant serum.*

Implications for (universal) IAV vaccine design.

The ability of stem-mimetic immunogens to selectively reactivate bnAb-producing MBCs and generate cross-reactive Abs offers valuable insights for universal IAV vaccine development:

a. Broad protection: The engagement of MBCs targeting conserved epitopes ensures sustained immunity against antigenically drifted or zoonotic strains.
b. Effector Functions: By boosting Fc-mediated responses despite missing neutralizing capacities, these immunogens provide an important layer of protection.
c. Translation of design process: The computational approach to designing immunogens eliciting cross-protective responses and bnAbs is a universal concept that could also be applied to other pathogens, such as SARS-CoV2, HIV or *P. falciparum*.
d. Engagement of pre-existing MBCs: By re-activating pre-existing MBC of humans, stem-mimetic designs could be leveraged to improve pre-existing immune repertoires without the need to vaccinate against various IAV strains.

Limitations.

The FI6 focused design only showed binding to FI6 mAb but not to other bnAbs, showing the lack of breadth of this application, that was though overcome by using the stem mimetic design, which bound to several bnAbs. While the stem mimetic was able to induce HA cross-reactive responses after vaccination in mice, the results are not translatable to the pull-down of human MBCs with the design as mice do not express the V_H1-69 gene in contrast to humans. This gene encodes for most bnAbs specific to the HA stem [31, 293]. Vaccination of human subjects with the stem mimetic would only be possible after further characterization and validation of safety in pre-clinical studies. Although vaccination with the stem mimetic induced Abs binding to both group 1 and 2 IAVs and exhibiting functional properties, such as ADCC, protection in challenge experiments and neutralization assays was not conferred by these Abs (data not shown). Therefore, further characterization of those Abs or vaccination studies in humanized mice containing the V_H1-69 gene are needed.

5 SUMMARIZING CONCLUSIONS AND FUTURE PERSPECTIVES

With Paper I and II we have dissected the effect of pre-existing immune components and vaccination strategies on IAV directed B cell responses. Some of those approaches and insights are used in Paper III to design an immunogen that can induce IAV group 1 and 2 cross-reactive Ab responses. All three studies provide strategies to combat challenges posed by antigenic drift and shift in IAV strains. Together, they emphasize the interplay between computationally designed immunogens, pre-existing immunity, and the generation of broadly protective Ab responses as a framework for future (universal) IAV vaccine development.

By integrating fate-mapping, adoptive transfer, and drifted antigen vaccination models, Paper II uncovers key regulatory mechanisms in secondary B cell responses. The findings highlight the complexity of Ab feedback and its dependence on antigen valency as it plays a pivotal role in the efficiency of engagement by pre-existing Abs. These insights pave the way for designing vaccines that optimize memory and naive responses, while addressing challenges posed by antigenic drift. This was applied in Paper III, where the stem-mimetic was presented multivalently on a nanoparticle, which also engaged CD4 T cells with the M2e epitope attached. These findings underscore the importance of presenting conserved epitopes in an optimized structural and valency context to maximize immunogenicity and protection.

Computationally designed immunogens targeting conserved stem epitopes of the HA stem bypass the ID of the highly variable HA head. Additionally, we have also shown how combination of pre-existing Abs and MBC can redirect B cell responses to desired antigenic sites on the HA head while producing high amounts of Ab. It remains to combine both approaches and to test e.g. computationally designed HA head mimetics expressing only a certain antigenic site or to direct immune responses by using stem-specific Abs in an HA vaccination context. Some Abs have shown enhancing effects on HA head responses and these approaches could also be explored to direct responses towards

the stem. This poses for certain challenges though as the HA stem is not as easily accessible as the HA head.

These studies collectively exemplify how a nuanced understanding of B cell memory dynamics and rational vaccine design, can redefine vaccine development for a broad spectrum of infectious diseases.

Notably, vaccination with the stem-mimetic was shown bypass ID of HA by inducing the production of broadly reactive Abs and binding to broadly reactive MBC in IAV-experienced individuals. To evaluate if broad immunity is maintained or ideally even improved, it could be used as a prime during cell fate tracking and adoptive stem-mimetic induced Ab transfer experiments. By vaccinating young children with an immunogen to induce broad immunity and being able to maintain this over a lifetime even after contacts with IAV could be considered a universal vaccine. Other computationally designed immunogens could be used to vaccinate against other IAV proteins, such as M2e, NP or NA and multi-epitope cocktails, eventually even presented on the same scaffold. This might induce even more cross-protective responses by making epitopes on inaccessible proteins available to B cells and pre-existing Abs. Use of computationally designed immunogens requires clinical studies for determination of safety and efficiency.

The latter can also be used, if clearly defined, to modulate immune responses to future vaccines. As many individuals have varied IAV exposure histories [77], vaccine designs should aim to modulate pre-existing immunity to favor subdominant, conserved epitopes. Tailored vaccination strategies, including heterologous and sequential prime-boost regimens [189], combination of vaccination with Ab transfer and computationally designed immunogens inducing cross-reactive responses could efficiently reactivate bnAb-producing MBCs while maintaining robust naive B cell recruitment. Here, valency of antigens should also be considered and tailored to the desired immune response outcome. And finally, there is a great need to define ID patterns of human Abs and MBC and to incorporate individual vaccination and infection histories.

Of course, all vaccination strategies used here and definition of the effect or pre-existing immune components to IAV could also be applied

to the development for other universal vaccines. This is especially desirable for highly variable pathogens such as SARS-CoV2 [294-296], HIV [189, 297-299], *P. falciparum* [300-302] or also RSV [303-305]. Combinatory approaches, targeting of various proteins and integrating Ab feedback as well as individual antigen exposure history will also be beneficial here.

The convergence of structural and computational vaccine design, and insights into the immune memory has unlocked new potential for combating antigenic drift in IAV and beyond. By targeting conserved epitopes and leveraging memory responses, these approaches address long-standing challenges in vaccinology, paving the way for universal vaccines capable of offering broad and durable protection against drifted pathogens.

ACKNOWLEDGEMENTS

I know that this is the first page you went to, don't try to hide that! But I promise you, I've put extra much love and effort in this one and the following pages:

A PhD project is nothing without a good supervisor. And I was lucky that I came across a world-class one, **Davide**. Or maybe I knew it from the beginning. Evidence: I wanted to work with B cell responses to viruses, in combination with vaccines (not a very broad interest) – then struggled to find groups that did interesting stuff – but finally found you … luckily, I asked you to hire me just after you had received funding for a position. Funny business aside, I would not be where I am today without you. The perfect mixture of being left alone (one could also say it was trust and some sort of pushing me outside of my comfort zone) and offering a helping hand at all times (whenever I came by and asked if you had time…you made time for me) produced someone who feels like they can do anything they have set their mind to. From guiding me through my stubborn plan to make the MBC transfers work to honestly telling me when to stop with an experiment. From making us sit in the weekly meeting and presenting as much as possible to practice to inviting us for lunch and dinner and making sure that we at least had one major bonding experience per year. From telling me everything will be fine to making me grow 100m tall and challenging me just enough to finally start believing in myself. Thank you, for all of this and everything else. What an honor to be your first PhD student defending their thesis!

And as if one of those first-class supervisors wasn't enough, I was blessed with a second one. My dearest **Mats**, you can't even imagine how much you have helped me through those almost 5 years here! Of course, scientifically you have contributed so much, sat through 3-hour long meetings discussing the most complicated of all experiments and later, digging yourself through the chaotic manuscript. Your ideas were always the best and with your beautiful brain you have made this work at least 500% better! But that is not enough, you took me by your hand and made me feel so much secure in everything I did, from Rehovot to Galway. You are kind and funny and just brilliant. And I wish you all the best success in Lund!

And even if I did not have nearly enough time with him, I also want to thank my other co-supervisor, **Nils**. Who already pushed me in my scientific career when I had not even started my position (while I came to visit for my interview here, I sat on his blue couch for 30min where he asked me very seriously: "Why do you want to be a scientist?"). Lastly, even if it wasn't always the most comfortable situation presenting a JC in front of him, it made us learn and grow. So, thank you Nils for making all of us better scientists!

Not all PhD students are fortunate enough to have a lab technician by their side. But I can even proudly say that I didn't just have any, I've had you, **Karin**. Through all the neutralization assays, ELISAs, virus productions and titrations and all the intravenous injections could never weigh up against your wonderful nature! The true star of our lab

did not just win uncountable marathons on the street, she also won the one to my heart <3

My gratitude to **Nimitha** does not only extend far into the past but also into the future. As the person who first started optimizing my experiments and the one might even take over what is left of it, you contributed a heavy part to this project but in the end that is far from everything. With your kind spirit, calm mind and lovely character you have somehow made all situations easier to handle and lifted my spirits. I wish you all the best for the future and hope to see your name soon under the list of labs at the University!

If our group was a family, then **Hannes** would be my 10-minute older twin brother. We have shared so many of these PhD-experiences at the same time and came out on the other end whole. And that's probably because we've also had so much to laugh about and were just the greatest support for each other. I'm so very grateful to have been your PhD-sibling through those years! Keep going strong, Papa-bear!

Danica, our favorite master student that we somehow convinced to stay. How lucky we were that you did…your playful nature and just made my every day! There is no one in this world that understands my lab-related pains the same way you do…and letting them out together and sharing experiences made all chaos and daily grind seem less heavy. Of course, all the cookies and flan were also helpful. In German we say: "Love goes through the stomach." And I think that's beautiful.

And our "petit dernier" **Romain**…so small and already spread his wings with a halftime done and a paper to be published! And there is much more to adore about you. To me you were (even if some might disagree with those statements 😉) always helpful, always warm and always respectful. I'm sure, one day you'll win 2 Nobel prizes: one for Medicine (the boring one) and one for being awesome (which is the actual achievement).

And our dearest **Zhaoshan**, I wish you all the best for your future and hope you find an awesome job here in Sweden! Congratulations to your defense and wonderful publication! It was so nice having you with us with your calm nature and cute smile. Keep the cat warm, it fits perfectly into your sweater :D

And also, my best wishes to **Janarthan** as he continues his PhD journey. It has been a great experience sharing the lab with you, and I wish you every success in your future endeavors. Of all of us, you are probably the best at teaching and future generations of students will definitely benefit from that.

No Lab in Sweden is complete without at least 2 people speaking German. And my counterpart was our dearest **Lydia**. It was probably for the better that I had someone I could practice with, otherwise I would have completely forgotten… I hope you'll find an awesome position soon and that you'll continue lifting heavy!

After years in Sweden, I'm hoping **Josue** found some strategies to get all the hot chiles he needs for his awesome salsas! You brightened up our everyday and always had the best stories to tell (and the best Mezcal…) … wherever you will end up in this world next, you will be missed!

And of course, also our long-gone Postdocs, **Katerina** (also poop-talk contributor) and **Ilya**, who are the most talented and hard-working scientists I have ever met! My time with you guys was too short and I would've loved to learn so much more from you. I wish you all the best for your future and will miss you a lot!

Thanks also to mine and Hannes' "shared" MSc student **Lina**. You were a part of this for almost a year and in that time, I learned so much about supervision, plasma cells and responsibility. You always brought fun and laughter into our lab/office! May you continue to thrive in science and may ASMR continue to keep you sane!

I wouldn't have been able to do something like 10% of the work I have done if it wasn't for you, **Pernilla**! You are the best of animal technicians! Having practically lived at EBM for some intense times, I was always so happy to see you smile and to know that the mice were cared for outstandingly well!

Greatest of thank also to my **Collaborators in Switzerland/Lausanne (Bruno, Sarah, Sandrine, Karla, Andreas)**! They were super patient with me when I was having troubles (which happened a lot). Together, we have been through issues with FedEx, antibodies that somehow did not bind and a paper that should have been finished by 2021. Thank you all so much for this experience!

Of course, also thanks to the lab who started all of this at NIH and continued to support Davide and me on this journey. Thanks to **Ivan** for sending mAbs and answering all my questions. To **Madeleine and James** for optimizing the Δ4 mutants and of course to **Jon** for valuable input, a very stimulating and insightful scientific discussion and of course: for Davide!

To everyone whom it may concern: Poop office is love, poop office is life! In particular to add:

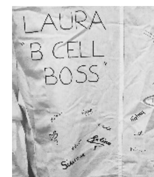
- My dearest **Nikita** (who always claimed she never took part of the poop-talk, but secretly she was enjoying it), you are a beacon in the dark and a shoulder to lean on! You were there in the hardest times (rainy summers and dark winters included) and were able to put a smile on my face by taking me out to concerts and movies, to dinners and our little photo-shoots. You truly are a great woman and an awesome scientist … I am happy to be in this world with you. Stay savage!
- And the true poop-doctor, **Andrew**. The kindest guy the world has even seen with the darkest humor. Best combo in my opinion, you always give me the laughs every time I greet you with a "herro". You might feel a bit too warm most of the time, but that probably only means that you radiate the warmth within to all people that come close to you. Thank you for letting me close, I like it warm!

- Not to forget: **Li-Ching**, the wisest and craziest of the bunch! You were our favorite office-neighbor and I'm just so happy that I inherited your big FACS-monitor 😉 All the best for your future!

Of course, there are so many more dear people in our department that deserve some thanks from me! My talented, tea-spilling and just funniest colleagues from the department, including **Maria S** (the most unhinged at parties and AWs), **Maria Ö** (a super talented photographer and scientist), **Najmeh** (hardest working and always so kind), **Bani** (the warmest of all and luckily stayed with us for a long time), **Tsvetanka** (the Belgian beer-sommelier that really lets loose after a few of them), **Alex** (being the best party planner and most generous person ever), **Frida N** (the actual original savage who's presence I miss so much), **Cristina** (who I'm hoping to see much more now that I am moving to Stockholm), **Anneli** (the very best technician, baker and lunch-companion ever who I so much hope to work together with again soon), **Patrik** (the actual gem who is the best support when it comes to taking care of flow cytometers…and from time to time shoots out the best comments), **Aylin** (always brings happiness and laughter into all situations, **Roland** (the best gameshow-judge and an awesome teaching-companion), **Manuela** (who is always up for a wonderful elevator-conversation), **Linn** (who I hope has an awesome PhD-experience and is the best at organizing gifts), **Inta** (who actually wasn't there anymore but who I connected with a lot via reminiscing about this department)…and of course there are **so many more** that I unfortunately forgot but please don't take it the wrong way (I have thesis-brain and my time with you was still awesome) ♥

Of course, also all the spouses/partners of my colleagues can't be forgotten as they were also definitely part of this journey! **Moa**, who always hosted us so warmly at her home and I wish you all the best for your PhD-journey…keep strong, Mama-bear! The fun-guy (sorry, had to add this one 😊) **Cole** who always saves our DnD adventures with his craftiness and magic. And lastly **Caitlyn**, the Swedish-not Swedish mom-friend that just seems to be able to take care of everyone in any situation imaginable!

Of course, the past can't be ignored while acknowledging people that greatly shaped my path. In this case and with much gratitude and some nostalgia, I want to thank the **BMIA** team at CureVac too. For teaching me flow cytometry, for valuable life-lessons and wonderful social interactions … but mainly for making me the "B cell Boss" that I am today. Thank you, **Sven**, **Katell**, **Fatma**, **Henoch**, **Marina**, **Kathrin**, **Simone**, **Galina**, **Alexandra** and so many more!



Auch wenn 1500km entfernt, hat meine Familie trotzdem den größten Einfluss mein Leben hinterlassen! **Mama&Papa**, ihr habt es mir ermöglich hier zu stehen, wo ich heute bin. Finanzielle Unterstützung hat da nur den kleinsten Teil ausgemacht, denn ihr habt mir auch vorgelebt, wie man hart arbeitet und alles erreichen kann, wenn man es sich nur fest genug in den Kopf setzt. Mit viel Liebe habt ihr mir das Beste von euch gegeben und da ist es nur fair, wenn ich auch mein Bestes gebe!

Es gibt niemand anderen auf der Welt, der meiner Vergangenheit so nahesteht, wie mein kleiner-großer Bruder **Andi**. Von dem kleinen verspielten Jungen zu dem erwachsenen, intelligenten Mann durfte ich Teil deines und du Teil meines Wachstums sein. Das ist eine einzigartige Sache in diesem Leben und ich werde sie für immer wertschätzen!

Ein großes Danke geht aus an **Jürgen**, der sich kühn meiner Mama angenommen hat als ich das Land verlassen habe und einfach nur der alleralleraller beste und liebevollste Ehemann für sie ist! Keep on rocking!

Und natürlich auch ein riesiges Dankeschön an den Rest der Familie (im Schwabenländle és Balmazújvárosban) für eine wunderschöne Kindheit gefüllt mit Liebe und Leben! **Jochen&Elke, Mimi, Marcel, Alex&Nicole mit Sophia und Jannik, Oma und Opa Reusch, Zsike&József, Jocó&Éva Áronnal, Andika&Péter Marcikával, Enikő&Gergő Kirával és Fridával.**

Carina, Frida, Stefan, och vart Hollywood-Drottningen **Ulla**, som saknas så mycket! Tack för att ni blev min svensk-familj, jag kunde hade önskad för en bättre! Tack for flytt-hjälpen och bil-poolen, tack för solnedgångar över Vättern och havet och tack för tre nya hem (Huskvarna-Jönköping-Haverdal). Varje år med er var full av kärlek och skratt, hoppas att vi kan har mycket mer!

A biggest of thanks, for year after year after year of support to my sisters at heart in Germany:
Mädels, ich kann es kaum glauben…aber wir haben quasi ein Jahrzehnt miteinander verbracht. Liebe **Alex**, liebe **Chrissy**, liebe **Miri** (+ Niko 😉) … ich kann nur für mich selbst sprechen, aber ihr seid einfach die besten Freundinnen, die man sich nur wünschen kann! Wir haben uns gegenseitig begleitet durch die ersten Studium-Erfahrungen, über harte Trennungen und schwere Uni-Zeiten hinweg bis heute mit wöchentlichen Videoanrufen. Und schaut nur, wo wir heute sind: vier Frauen, die einander durch dick und dünn unterstützen. Ich liebe euch alle so sehr!

But my life didn't start at university, of course I've had wonderful friends at my side even before, and still!
Seit der sechsten Klasse (das sind mehr als 19 Jahre) kann ich mich glücklich schätzen, **Katrin** an meiner Seite zu haben! Über Theater-AG und Latein bis hin zu unserer Zeit in Tübingen warst du immer für mich da. Danke für deine langjährige Freundschaft und ich hoffe auf viele mehr!
Und mein "Kitzelbär" **Annika**, so viele wunderschöne Erinnerungen (von Freizeiten bis zu den besten Mahlzeiten und einfach nur den random besten Zeiten) teile ich mit kaum jemand anderem! Du bist einfach und wirst immer ein fester Teil meines Lebens bleiben!

And as I don't want this to become the longest chapter of the thesis, I will summarise everyone else in Sweden who welcomed me so wholeheartedly and made my time here 1000x more beautiful (including **Oscar and Andrea, Carl, Stefan, Greta, Melis…and so many more!**)

Ett stort tack också till finaste hunden och bästa nervsystem-lugnare **Luna** (som vore inte i mitt liv utan **Helene, Annika + Göran**).

And when I already started talking about animals, all of this would have never been possible without all of those (I don't want to say how many) **mice**. I am immensely grateful that I was able to do science using life animals and even more aware of the fact, that they potentially suffered throughout their time with me. I am so sorry, but I also hope that your sacrifice will propel us forward in our fight against the flu and other diseases!

And the last person to thank in this list is to me like the last author of a scientific publication, **Fred**. You witnessed all my highs and lows from a unique perspective and treated me in a way no one else ever did—the best way possible. You were there when I came home in every human state imaginable, and you gave them the grace and space they deserved. I wouldn't have made it through to this side of the story whole without you and your cubbles. You are the best place in the world. You are my home. You are my peace.

REFERENCES

1. Barberis, I., et al., *History and evolution of influenza control through vaccination: from the first monovalent vaccine to universal vaccines.* J Prev Med Hyg, 2016. **57**(3): p. E115-E120.
2. Martin, P.M. and E. Martin-Granel, *2,500-year evolution of the term epidemic.* Emerg Infect Dis, 2006. **12**(6): p. 976-80.
3. Raoult, D. and M. Drancourt, *Paleomicrobiology : past human infections*. 2008, Berlin: Springer. xiii, 226 p.
4. Johnson, N.P. and J. Mueller, *Updating the accounts: global mortality of the 1918-1920 "Spanish" influenza pandemic.* Bull Hist Med, 2002. **76**(1): p. 105-15.
5. (WHO), W.H.O. *Pandemic (H1N1) 2009*. [Archived webpage] 2010 27. 03 2011 [cited 2024 11/05].
6. Paget, J., et al., *Global mortality associated with seasonal influenza epidemics: New burden estimates and predictors from the GLaMOR Project.* J Glob Health, 2019. **9**(2): p. 020421.
7. Mathew, N.R. and D. Angeletti, *Recombinant Influenza Vaccines: Saviors to Overcome Immunodominance.* Front Immunol, 2019. **10**: p. 2997.
8. Paules, C. and K. Subbarao, *Influenza.* Lancet, 2017. **390**(10095): p. 697-708.
9. Hutchinson, E.C., *Influenza Virus.* Trends Microbiol, 2018. **26**(9): p. 809-810.
10. Koutsakos, M., et al., *Influenza lineage extinction during the COVID-19 pandemic?* Nat Rev Microbiol, 2021. **19**(12): p. 741-742.
11. Kosik, I. and J.W. Yewdell, *Influenza Hemagglutinin and Neuraminidase: Yin(-)Yang Proteins Coevolving to Thwart Immunity.* Viruses, 2019. **11**(4).
12. Hu, Y., et al., *Influenza A Virus Nucleoprotein: A Highly Conserved Multi-Functional Viral Protein as a Hot Antiviral Drug Target.* Curr Top Med Chem, 2017. **17**(20): p. 2271-2285.
13. Gaymard, A., et al., *Functional balance between neuraminidase and haemagglutinin in influenza viruses.* Clin Microbiol Infect, 2016. **22**(12): p. 975-983.
14. Krammer, F., et al., *Influenza.* Nat Rev Dis Primers, 2018. **4**(1): p. 3.
15. Wilson, I.A., J.J. Skehel, and D.C. Wiley, *Structure of the haemagglutinin membrane glycoprotein of influenza virus at 3 A resolution.* Nature, 1981. **289**(5796): p. 366-73.
16. Wasilewski, S., et al., *Distribution of surface glycoproteins on influenza A virus determined by electron cryotomography.* Vaccine, 2012. **30**(51): p. 7368-73.
17. Altman, M.O., D. Angeletti, and J.W. Yewdell, *Antibody Immunodominance: The Key to Understanding Influenza Virus Antigenic Drift.* Viral Immunol, 2018. **31**(2): p. 142-149.
18. Angeletti, D., et al., *Defining B cell immunodominance to viruses.* Nat Immunol, 2017. **18**(4): p. 456-463.
19. Yewdell, J.W., *Confronting complexity: real-world immunodominance in antiviral CD8+ T cell responses.* Immunity, 2006. **25**(4): p. 533-43.
20. Cassotta, A., et al., *Deciphering and predicting CD4+ T cell immunodominance of influenza virus hemagglutinin.* J Exp Med, 2020. **217**(10).
21. Jegaskanda, S., et al., *Antibody-dependent cellular cytotoxicity is associated with control of pandemic H1N1 influenza virus infection of macaques.* J Virol, 2013. **87**(10): p. 5512-22.
22. Altman, M.O., et al., *Lamprey VLRB response to influenza virus supports universal rules of immunogenicity and antigenicity.* Elife, 2015. **4**.
23. Tan, H.X., et al., *Subdominance and poor intrinsic immunogenicity limit humoral immunity targeting influenza HA stem.* J Clin Invest, 2019. **129**(2): p. 850-862.
24. Kirkpatrick, E., et al., *The influenza virus hemagglutinin head evolves faster than the stalk domain.* Sci Rep, 2018. **8**(1): p. 10432.
25. Okuno, Y., et al., *A common neutralizing epitope conserved between the hemagglutinins of influenza A virus H1 and H2 strains.* J Virol, 1993. **67**(5): p. 2552-8.
26. Corti, D., et al., *A neutralizing antibody selected from plasma cells that binds to group 1 and group 2 influenza A hemagglutinins.* Science, 2011. **333**(6044): p. 850-6.
27. Dreyfus, C., et al., *Highly conserved protective epitopes on influenza B viruses.* Science, 2012. **337**(6100): p. 1343-8.

28. Nakamura, G., et al., *An in vivo human-plasmablast enrichment technique allows rapid identification of therapeutic influenza A antibodies.* Cell Host Microbe, 2013. **14**(1): p. 93-103.
29. Kallewaard, N.L., et al., *Structure and Function Analysis of an Antibody Recognizing All Influenza A Subtypes.* Cell, 2016. **166**(3): p. 596-608.
30. Wu, Y., et al., *A potent broad-spectrum protective human monoclonal antibody crosslinking two haemagglutinin monomers of influenza A virus.* Nat Commun, 2015. **6**: p. 7708.
31. Joyce, M.G., et al., *Vaccine-Induced Antibodies that Neutralize Group 1 and Group 2 Influenza A Viruses.* Cell, 2016. **166**(3): p. 609-623.
32. Sutton, T.C., et al., *In Vitro Neutralization Is Not Predictive of Prophylactic Efficacy of Broadly Neutralizing Monoclonal Antibodies CR6261 and CR9114 against Lethal H2 Influenza Virus Challenge in Mice.* J Virol, 2017. **91**(24).
33. Wang, X., et al., *Adenovirus delivery of encoded monoclonal antibody protects against different types of influenza virus infection.* NPJ Vaccines, 2020. **5**(1): p. 57.
34. Doud, M.B. and J.D. Bloom, *Accurate Measurement of the Effects of All Amino-Acid Mutations on Influenza Hemagglutinin.* Viruses, 2016. **8**(6).
35. Heaton, N.S., et al., *Genome-wide mutagenesis of influenza virus reveals unique plasticity of the hemagglutinin and NS1 proteins.* Proc Natl Acad Sci U S A, 2013. **110**(50): p. 20248-53.
36. Ekiert, D.C., et al., *Antibody recognition of a highly conserved influenza virus epitope.* Science, 2009. **324**(5924): p. 246-51.
37. Throsby, M., et al., *Heterosubtypic neutralizing monoclonal antibodies cross-protective against H5N1 and H1N1 recovered from human IgM+ memory B cells.* PLoS One, 2008. **3**(12): p. e3942.
38. Wrammert, J., et al., *Broadly cross-reactive antibodies dominate the human B cell response against 2009 pandemic H1N1 influenza virus infection.* J Exp Med, 2011. **208**(1): p. 181-93.
39. Tonouchi, K., et al., *Structural basis for cross-group recognition of an influenza virus hemagglutinin antibody that targets postfusion stabilized epitope.* PLoS Pathog, 2023. **19**(8): p. e1011554.
40. Corti, D. and A. Lanzavecchia, *Broadly neutralizing antiviral antibodies.* Annu Rev Immunol, 2013. **31**: p. 705-42.
41. Chen, Y.Q., et al., *Hemagglutinin Stalk-Reactive Antibodies Interfere with Influenza Virus Neuraminidase Activity by Steric Hindrance.* J Virol, 2019. **93**(4).
42. DiLillo, D.J., et al., *Broadly neutralizing hemagglutinin stalk-specific antibodies require FcgammaR interactions for protection against influenza virus in vivo.* Nat Med, 2014. **20**(2): p. 143-51.
43. Mullarkey, C.E., et al., *Broadly Neutralizing Hemagglutinin Stalk-Specific Antibodies Induce Potent Phagocytosis of Immune Complexes by Neutrophils in an Fc-Dependent Manner.* mBio, 2016. **7**(5).
44. Lu, L.L., et al., *Beyond binding: antibody effector functions in infectious diseases.* Nat Rev Immunol, 2018. **18**(1): p. 46-61.
45. Wiley, D.C., I.A. Wilson, and J.J. Skehel, *Structural identification of the antibody-binding sites of Hong Kong influenza haemagglutinin and their involvement in antigenic variation.* Nature, 1981. **289**(5796): p. 373-8.
46. Wiley, D.C. and J.J. Skehel, *The structure and function of the hemagglutinin membrane glycoprotein of influenza virus.* Annu Rev Biochem, 1987. **56**: p. 365-94.
47. Skehel, J.J., et al., *A carbohydrate side chain on hemagglutinins of Hong Kong influenza viruses inhibits recognition by a monoclonal antibody.* Proc Natl Acad Sci U S A, 1984. **81**(6): p. 1779-83.
48. Caton, A.J., et al., *The antigenic structure of the influenza virus A/PR/8/34 hemagglutinin (H1 subtype).* Cell, 1982. **31**(2 Pt 1): p. 417-27.
49. Gerhard, W., et al., *Antigenic structure of influenza virus haemagglutinin defined by hybridoma antibodies.* Nature, 1981. **290**(5808): p. 713-7.
50. Hensley, S.E., et al., *Hemagglutinin receptor binding avidity drives influenza A virus antigenic drift.* Science, 2009. **326**(5953): p. 734-6.

51. Li, Y., et al., *Single hemagglutinin mutations that alter both antigenicity and receptor binding avidity influence influenza virus antigenic clustering.* J Virol, 2013. **87**(17): p. 9904-10.
52. Wu, N.C. and I.A. Wilson, *Structural Biology of Influenza Hemagglutinin: An Amaranthine Adventure.* Viruses, 2020. **12**(9).
53. Kavaler, J., et al., *A set of closely related antibodies dominates the primary antibody response to the antigenic site CB of the A/PR/8/34 influenza virus hemagglutinin.* J Immunol, 1990. **145**(7): p. 2312-21.
54. Rothaeusler, K. and N. Baumgarth, *B-cell fate decisions following influenza virus infection.* Eur J Immunol, 2010. **40**(2): p. 366-77.
55. Roco, J.A., et al., *Class-Switch Recombination Occurs Infrequently in Germinal Centers.* Immunity, 2019. **51**(2): p. 337-350 e7.
56. Lam, J.H. and N. Baumgarth, *The Multifaceted B Cell Response to Influenza Virus.* J Immunol, 2019. **202**(2): p. 351-359.
57. Luo, Z., et al., *Vaccination with antigenically complex hemagglutinin mixtures confers broad protection from influenza disease.* Sci Transl Med, 2024. **16**(745): p. eadj4685.
58. Paparoditis, P.C.G., et al., *Site-specific serology unveils cross-reactive monoclonal antibodies targeting influenza A hemagglutinin epitopes.* Eur J Immunol, 2024. **54**(10): p. e2451045.
59. Liu, S.T.H., et al., *Antigenic sites in influenza H1 hemagglutinin display species-specific immunodominance.* J Clin Invest, 2018. **128**(11): p. 4992-4996.
60. Cheung, P.P., et al., *Comparative mutational analyses of influenza A viruses.* RNA, 2015. **21**(1): p. 36-47.
61. Ferguson, N.M., A.P. Galvani, and R.M. Bush, *Ecological and immunological determinants of influenza evolution.* Nature, 2003. **422**(6930): p. 428-33.
62. dos Reis, M., A.J. Hay, and R.A. Goldstein, *Using non-homogeneous models of nucleotide substitution to identify host shift events: application to the origin of the 1918 'Spanish' influenza pandemic virus.* J Mol Evol, 2009. **69**(4): p. 333-45.
63. Smith, G.J., et al., *Origins and evolutionary genomics of the 2009 swine-origin H1N1 influenza A epidemic.* Nature, 2009. **459**(7250): p. 1122-5.
64. Blagodatski, A., et al., *Avian Influenza in Wild Birds and Poultry: Dissemination Pathways, Monitoring Methods, and Virus Ecology.* Pathogens, 2021. **10**(5).
65. Zeng, Y., et al., *The PB1 protein of influenza A virus inhibits the innate immune response by targeting MAVS for NBR1-mediated selective autophagic degradation.* PLoS Pathog, 2021. **17**(2): p. e1009300.
66. Jones, J.C., et al., *Influenza antivirals and their role in pandemic preparedness.* Antiviral Res, 2023. **210**: p. 105499.
67. Hufnagel, D.E., et al., *Characterizing a century of genetic diversity and contemporary antigenic diversity of N1 neuraminidase in influenza A virus from North American swine.* Virus Evol, 2023. **9**(1): p. vead015.
68. Fischer, W., et al., *HIV-1 and SARS-CoV-2: Patterns in the evolution of two pandemic pathogens.* Cell Host Microbe, 2021. **29**(7): p. 1093-1110.
69. Yewdell, J.W., *Antigenic drift: Understanding COVID-19.* Immunity, 2021. **54**(12): p. 2681-2687.
70. Bouvin-Pley, M., et al., *Drift of the HIV-1 envelope glycoprotein gp120 toward increased neutralization resistance over the course of the epidemic: a comprehensive study using the most potent and broadly neutralizing monoclonal antibodies.* J Virol, 2014. **88**(23): p. 13910-7.
71. Tan, T.J.C., et al., *Evidence of antigenic drift in the fusion machinery core of SARS-CoV-2 spike.* Proc Natl Acad Sci U S A, 2024. **121**(15): p. e2317222121.
72. Kaku, C.I., et al., *Recall of preexisting cross-reactive B cell memory after Omicron BA.1 breakthrough infection.* Sci Immunol, 2022. **7**(73): p. eabq3511.
73. Fulton, B.O., et al., *Mutational Analysis of Measles Virus Suggests Constraints on Antigenic Variation of the Glycoproteins.* Cell Rep, 2015. **11**(9): p. 1331-8.
74. Sanjuan, R. and P. Domingo-Calap, *Mechanisms of viral mutation.* Cell Mol Life Sci, 2016. **73**(23): p. 4433-4448.
75. Aggarwal, S., et al., *Biochemical characterization of enzyme fidelity of influenza A virus RNA polymerase complex.* PLoS One, 2010. **5**(4): p. e10372.

76. Huang, K.Y., et al., *Focused antibody response to influenza linked to antigenic drift.* J Clin Invest, 2015. **125**(7): p. 2631-45.
77. Auladell, M., et al., *Influenza virus infection history shapes antibody responses to influenza vaccination.* Nat Med, 2022. **28**(2): p. 363-372.
78. Plotkin, S.A., *Vaccines: past, present and future.* Nat Med, 2005. **11**(4 Suppl): p. S5-11.
79. EMA. *Fluenz, influenza vaccine (live attenuated, nasal)*. 2024 20.11.2024 [cited 2024 12.12.2024]; Available from: https://www.ema.europa.eu/en/medicines/human/EPAR/fluenz.
80. EMA. *Fluad, influenza vaccine (surface antigen, inactivated, adjuvanted)*. 2024 03.12.2024 [cited 2024 12.12.2024]; Available from: https://www.ema.europa.eu/en/medicines/human/EPAR/fluad.
81. EMA. *Supemtek, Quadrivalent influenza vaccine (recombinant, prepared in cell culture)*. 2024 05.12.2024 [cited 2024 12.12.2024]; Available from: https://www.ema.europa.eu/en/medicines/human/EPAR/supemtek.
82. Francis, T.M., T. P., *Vaccination of Human Subjects with Virus of Human Influenza*. Proc Soc Exp Biol Med, 1936. **33**: p. 604-606.
83. Koprowski, H., *Immunization of man against poliomyelitis with attenuated preparations of living virus.* Ann N Y Acad Sci, 1955. **61**(4): p. 1039-49.
84. Katz, S.L., J.F. Enders, and A. Holloway, *The development and evaluation of an attenuated measles virus vaccine.* Am J Public Health Nations Health, 1962. **52(2)Suppl**(Suppl 2): p. 5-10.
85. Holmgren, J., *Modern History of Cholera Vaccines and the Pivotal Role of icddr,b.* J Infect Dis, 2021. **224**(12 Suppl 2): p. S742-S748.
86. Scheiermann, N., et al., *Reactogenicity and immunogenicity of the newly developed purified chick embryo cell (PCEC)-rabies vaccine in man.* Zentralbl Bakteriol Mikrobiol Hyg A, 1987. **265**(3-4): p. 439-50.
87. Baden, L.R., et al., *Efficacy and Safety of the mRNA-1273 SARS-CoV-2 Vaccine.* N Engl J Med, 2021. **384**(5): p. 403-416.
88. Polack, F.P., et al., *Safety and Efficacy of the BNT162b2 mRNA Covid-19 Vaccine.* N Engl J Med, 2020. **383**(27): p. 2603-2615.
89. Gresset-Bourgeois, V., et al., *Quadrivalent inactivated influenza vaccine (VaxigripTetra).* Expert Rev Vaccines, 2018. **17**(1): p. 1-11.
90. van den Ende, C., et al., *The immunogenicity of GSK's recombinant hepatitis B vaccine in children: a systematic review of 30 years of experience.* Expert Rev Vaccines, 2017. **16**(8): p. 789-809.
91. Pfeiffer, R., *Die Aetiologie der Influenza.* Zeitschr. f. Hygiene, 1893. **13**: p. 357-386.
92. Wollstein, M., *Pfeiffer's Bacillus and Influenza : A Serological Study.* J Exp Med, 1919. **30**(6): p. 555-68.
93. Olitsky, P.K. and F.L. Gates, *Experimental Studies of the Nasopharyngeal Secretions from Influenza Patients : X. The Immunizing Effects in Rabbits of Subcutaneous Injections of Killed Cultures of Bacterium Pneumosintes.* J Exp Med, 1922. **36**(6): p. 685-96.
94. Milian, E. and A.A. Kamen, *Current and emerging cell culture manufacturing technologies for influenza vaccines.* Biomed Res Int, 2015. **2015**: p. 504831.
95. Leonard, R.A., et al., *Improved influenza vaccine responses after expression of multiple viral glycoproteins from a single mRNA.* Nat Commun, 2024. **15**(1): p. 8712.
96. Hauguel, T., et al., *Preclinical immunogenicity and safety of hemagglutinin-encoding modRNA influenza vaccines.* NPJ Vaccines, 2024. **9**(1): p. 183.
97. Wang, Y., et al., *A decavalent composite mRNA vaccine against both influenza and COVID-19.* mBio, 2024. **15**(9): p. e0066824.
98. Sautto, G.A., et al., *A Computationally Optimized Broadly Reactive Antigen Subtype-Specific Influenza Vaccine Strategy Elicits Unique Potent Broadly Neutralizing Antibodies against Hemagglutinin.* J Immunol, 2020. **204**(2): p. 375-385.
99. Bullard, B.L., et al., *An epitope-optimized human H3N2 influenza vaccine induces broadly protective immunity in mice and ferrets.* NPJ Vaccines, 2022. **7**(1): p. 65.
100. Yang, L., et al., *Mechanisms that promote the evolution of cross-reactive antibodies upon vaccination with designed influenza immunogens.* Cell Rep, 2023. **42**(3): p. 112160.
101. Kanekiyo, M., et al., *Mosaic nanoparticle display of diverse influenza virus hemagglutinins elicits broad B cell responses.* Nat Immunol, 2019. **20**(3): p. 362-372.

102. Sayedahmed, E.E., et al., *Adenoviral Vector-Based Vaccine Platforms for Developing the Next Generation of Influenza Vaccines*. Vaccines (Basel), 2020. **8**(4).
103. Kumari, S., et al., *Assessment of Immune Responses to a Trivalent Pichinde Virus-Vectored Vaccine Expressing Hemagglutinin Genes from Three Co-Circulating Influenza A Virus Subtypes in Pigs*. Vaccines (Basel), 2023. **11**(12).
104. Liu, L., et al., *Recombinant turkey herpesvirus expressing H9 hemagglutinin providing protection against H9N2 avian influenza*. Virology, 2019. **529**: p. 7-15.
105. Bazhan, S., et al., *Immunogenicity and Protective Efficacy of Influenza A DNA Vaccines Encoding Artificial Antigens Based on Conservative Hemagglutinin Stem Region and M2 Protein in Mice*. Vaccines (Basel), 2020. **8**(3).
106. Kalenik, B.M., et al., *Response to a DNA vaccine against the H5N1 virus depending on the chicken line and number of doses*. Virol J, 2020. **17**(1): p. 66.
107. Eggink, D., P.H. Goff, and P. Palese, *Guiding the immune response against influenza virus hemagglutinin toward the conserved stalk domain by hyperglycosylation of the globular head domain*. J Virol, 2014. **88**(1): p. 699-704.
108. Impagliazzo, A., et al., *A stable trimeric influenza hemagglutinin stem as a broadly protective immunogen*. Science, 2015. **349**(6254): p. 1301-6.
109. Yassine, H.M., et al., *Hemagglutinin-stem nanoparticles generate heterosubtypic influenza protection*. Nat Med, 2015. **21**(9): p. 1065-70.
110. Krammer, F., et al., *H3 stalk-based chimeric hemagglutinin influenza virus constructs protect mice from H7N9 challenge*. J Virol, 2014. **88**(4): p. 2340-3.
111. Krammer, F., et al., *Chimeric hemagglutinin influenza virus vaccine constructs elicit broadly protective stalk-specific antibodies*. J Virol, 2013. **87**(12): p. 6542-50.
112. Murphy, K. and C. Weaver, *Janeway's immunobiology*. 9th edition. ed. 2016, New York, NY: Garland Science/Taylor & Francis Group, LLC. xx, 904 pages.
113. Heesters, B.A., et al., *Antigen Presentation to B Cells*. Trends Immunol, 2016. **37**(12): p. 844-854.
114. Okada, T. and J.G. Cyster, *B cell migration and interactions in the early phase of antibody responses*. Curr Opin Immunol, 2006. **18**(3): p. 278-85.
115. Mond, J.J., A. Lees, and C.M. Snapper, *T cell-independent antigens type 2*. Annu Rev Immunol, 1995. **13**: p. 655-92.
116. Vos, Q., et al., *B-cell activation by T-cell-independent type 2 antigens as an integral part of the humoral immune response to pathogenic microorganisms*. Immunol Rev, 2000. **176**: p. 154-70.
117. Crotty, S., *A brief history of T cell help to B cells*. Nat Rev Immunol, 2015. **15**(3): p. 185-9.
118. Crotty, S., *T Follicular Helper Cell Biology: A Decade of Discovery and Diseases*. Immunity, 2019. **50**(5): p. 1132-1148.
119. Han, S., et al., *Cellular interaction in germinal centers. Roles of CD40 ligand and B7-2 in established germinal centers*. J Immunol, 1995. **155**(2): p. 556-67.
120. Luo, W., F. Weisel, and M.J. Shlomchik, *B Cell Receptor and CD40 Signaling Are Rewired for Synergistic Induction of the c-Myc Transcription Factor in Germinal Center B Cells*. Immunity, 2018. **48**(2): p. 313-326 e5.
121. Marsman, C., et al., *Termination of CD40L co-stimulation promotes human B cell differentiation into antibody-secreting cells*. Eur J Immunol, 2022. **52**(10): p. 1662-1675.
122. Dvorscek, A.R., et al., *IL-21 has a critical role in establishing germinal centers by amplifying early B cell proliferation*. EMBO Rep, 2022. **23**(9): p. e54677.
123. Bryant, V.L., et al., *Cytokine-mediated regulation of human B cell differentiation into Ig-secreting cells: predominant role of IL-21 produced by CXCR5+ T follicular helper cells*. J Immunol, 2007. **179**(12): p. 8180-90.
124. Good, K.L., V.L. Bryant, and S.G. Tangye, *Kinetics of human B cell behavior and amplification of proliferative responses following stimulation with IL-21*. J Immunol, 2006. **177**(8): p. 5236-47.
125. Zuccarino-Catania, G.V., et al., *CD80 and PD-L2 define functionally distinct memory B cell subsets that are independent of antibody isotype*. Nat Immunol, 2014. **15**(7): p. 631-7.
126. Esensten, J.H., et al., *CD28 Costimulation: From Mechanism to Therapy*. Immunity, 2016. **44**(5): p. 973-88.

127. Good-Jacobson, K.L., et al., *CD80 expression on B cells regulates murine T follicular helper development, germinal center B cell survival, and plasma cell generation.* J Immunol, 2012. **188**(9): p. 4217-25.
128. Vonderheide, R.H., *CD40 Agonist Antibodies in Cancer Immunotherapy.* Annu Rev Med, 2020. **71**: p. 47-58.
129. Pape, K.A., et al., *Naive B Cells with High-Avidity Germline-Encoded Antigen Receptors Produce Persistent IgM(+) and Transient IgG(+) Memory B Cells.* Immunity, 2018. **48**(6): p. 1135-1143 e4.
130. Paus, D., et al., *Antigen recognition strength regulates the choice between extrafollicular plasma cell and germinal center B cell differentiation.* J Exp Med, 2006. **203**(4): p. 1081-91.
131. Glaros, V., et al., *Limited access to antigen drives generation of early B cell memory while restraining the plasmablast response.* Immunity, 2021. **54**(9): p. 2005-2023 e10.
132. Good-Jacobson, K.L., *Engraving our first B cell memories into the repertoire.* Trends Immunol, 2021. **42**(11): p. 951-952.
133. Laidlaw, B.J., et al., *The transcription factor Hhex cooperates with the corepressor Tle3 to promote memory B cell development.* Nat Immunol, 2020. **21**(9): p. 1082-1093.
134. Sundling, C., et al., *Positive selection of IgG(+) over IgM(+) B cells in the germinal center reaction.* Immunity, 2021. **54**(5): p. 988-1001 e5.
135. Good-Jacobson, K.L., *B cells turn on, tune in with LSD1.* Nat Immunol, 2019. **20**(1): p. 3-5.
136. Kealy, L., et al., *The Histone Methyltransferase DOT1L Is Essential for Humoral Immune Responses.* Cell Rep, 2020. **33**(11): p. 108504.
137. Victora, G.D., et al., *Germinal center dynamics revealed by multiphoton microscopy with a photoactivatable fluorescent reporter.* Cell, 2010. **143**(4): p. 592-605.
138. Gitlin, A.D., Z. Shulman, and M.C. Nussenzweig, *Clonal selection in the germinal centre by regulated proliferation and hypermutation.* Nature, 2014. **509**(7502): p. 637-40.
139. Pae, J., et al., *Cyclin D3 drives inertial cell cycling in dark zone germinal center B cells.* J Exp Med, 2021. **218**(4).
140. Victora, G.D., et al., *Identification of human germinal center light and dark zone cells and their relationship to human B-cell lymphomas.* Blood, 2012. **120**(11): p. 2240-8.
141. McHeyzer-Williams, L.J., et al., *Class-switched memory B cells remodel BCRs within secondary germinal centers.* Nat Immunol, 2015. **16**(3): p. 296-305.
142. Ersching, J., et al., *Germinal Center Selection and Affinity Maturation Require Dynamic Regulation of mTORC1 Kinase.* Immunity, 2017. **46**(6): p. 1045-1058 e6.
143. Dominguez-Sola, D., et al., *The proto-oncogene MYC is required for selection in the germinal center and cyclic reentry.* Nat Immunol, 2012. **13**(11): p. 1083-91.
144. Finkin, S., et al., *Protein Amounts of the MYC Transcription Factor Determine Germinal Center B Cell Division Capacity.* Immunity, 2019. **51**(2): p. 324-336 e5.
145. Dernstedt, A., et al., *Regulation of Decay Accelerating Factor Primes Human Germinal Center B Cells for Phagocytosis.* Front Immunol, 2020. **11**: p. 599647.
146. Gurwicz, N., et al., *Tingible body macrophages arise from lymph node-resident precursors and uptake B cells by dendrites.* J Exp Med, 2023. **220**(4).
147. Sprumont, A., et al., *Germinal centers output clonally diverse plasma cell populations expressing high- and low-affinity antibodies.* Cell, 2023. **186**(25): p. 5486-5499 e13.
148. Robinson, M.J., et al., *Long-lived plasma cells accumulate in the bone marrow at a constant rate from early in an immune response.* Sci Immunol, 2022. **7**(76): p. eabm8389.
149. Nakagawa, R., et al., *Permissive selection followed by affinity-based proliferation of GC light zone B cells dictates cell fate and ensures clonal breadth.* Proc Natl Acad Sci U S A, 2021. **118**(2).
150. Mathew, N.R., et al., *Single-cell BCR and transcriptome analysis after influenza infection reveals spatiotemporal dynamics of antigen-specific B cells.* Cell Rep, 2021. **35**(12): p. 109286.
151. Yewdell, W.T., et al., *Temporal dynamics of persistent germinal centers and memory B cell differentiation following respiratory virus infection.* Cell Rep, 2021. **37**(6): p. 109961.
152. Aiba, Y., et al., *Preferential localization of IgG memory B cells adjacent to contracted germinal centers.* Proc Natl Acad Sci U S A, 2010. **107**(27): p. 12192-7.

153. Tan, H.X., et al., *Lung-resident memory B cells established after pulmonary influenza infection display distinct transcriptional and phenotypic profiles.* Sci Immunol, 2022. **7**(67): p. eabf5314.
154. Onodera, T., et al., *Memory B cells in the lung participate in protective humoral immune responses to pulmonary influenza virus reinfection.* Proc Natl Acad Sci U S A, 2012. **109**(7): p. 2485-90.
155. Allie, S.R., et al., *The establishment of resident memory B cells in the lung requires local antigen encounter.* Nat Immunol, 2019. **20**(1): p. 97-108.
156. Weisel, N.M., et al., *Comprehensive analyses of B-cell compartments across the human body reveal novel subsets and a gut-resident memory phenotype.* Blood, 2020. **136**(24): p. 2774-2785.
157. Reusch, L. and D. Angeletti, *Memory B-cell diversity: From early generation to tissue residency and reactivation.* Eur J Immunol, 2023. **53**(4): p. e2250085.
158. Gregoire, C., et al., *Viral infection engenders bona fide and bystander subsets of lung-resident memory B cells through a permissive mechanism.* Immunity, 2022. **55**(7): p. 1216-1233 e9.
159. MacLean, A.J., et al., *Secondary influenza challenge triggers resident memory B cell migration and rapid relocation to boost antibody secretion at infected sites.* Immunity, 2022. **55**(4): p. 718-733 e8.
160. MacLean, A.J., et al., *Regulation of pulmonary plasma cell responses during secondary infection with influenza virus.* J Exp Med, 2024. **221**(7).
161. Oh, J.E., et al., *Migrant memory B cells secrete luminal antibody in the vagina.* Nature, 2019. **571**(7763): p. 122-126.
162. Oh, J.E., et al., *Intranasal priming induces local lung-resident B cell populations that secrete protective mucosal antiviral IgA.* Sci Immunol, 2021. **6**(66): p. eabj5129.
163. Joo, H.M., Y. He, and M.Y. Sangster, *Broad dispersion and lung localization of virus-specific memory B cells induced by influenza pneumonia.* Proc Natl Acad Sci U S A, 2008. **105**(9): p. 3485-90.
164. Barker, K.A., et al., *Lung-resident memory B cells protect against bacterial pneumonia.* J Clin Invest, 2021. **131**(11).
165. McHeyzer-Williams, L.J., C. Dufaud, and M.G. McHeyzer-Williams, *Do Memory B Cells Form Secondary Germinal Centers? Impact of Antibody Class and Quality of Memory T-Cell Help at Recall.* Cold Spring Harb Perspect Biol, 2018. **10**(1).
166. Shlomchik, M.J., *Do Memory B Cells Form Secondary Germinal Centers? Yes and No.* Cold Spring Harb Perspect Biol, 2018. **10**(1).
167. Pape, K.A. and M.K. Jenkins, *Do Memory B Cells Form Secondary Germinal Centers? It Depends.* Cold Spring Harb Perspect Biol, 2018. **10**(1).
168. Kuraoka, M., et al., *Complex Antigens Drive Permissive Clonal Selection in Germinal Centers.* Immunity, 2016. **44**(3): p. 542-552.
169. Kuraoka, M., et al., *Recall of B cell memory depends on relative locations of prime and boost immunization.* Sci Immunol, 2022. **7**(71): p. eabn5311.
170. Mesin, L., et al., *Restricted Clonality and Limited Germinal Center Reentry Characterize Memory B Cell Reactivation by Boosting.* Cell, 2020. **180**(1): p. 92-106 e11.
171. Schiepers, A., et al., *Molecular fate-mapping of serum antibody responses to repeat immunization.* Nature, 2023. **615**(7952): p. 482-489.
172. Schiepers, A., et al., *Opposing effects of pre-existing antibody and memory T cell help on the dynamics of recall germinal centers.* Immunity, 2024. **57**(7): p. 1618-1628 e4.
173. Lutz, J., et al., *Reactivation of IgG-switched memory B cells by BCR-intrinsic signal amplification promotes IgG antibody production.* Nat Commun, 2015. **6**: p. 8575.
174. Pape, K.A., et al., *Different B cell populations mediate early and late memory during an endogenous immune response.* Science, 2011. **331**(6021): p. 1203-7.
175. Anderson, S.M., et al., *New markers for murine memory B cells that define mutated and unmutated subsets.* J Exp Med, 2007. **204**(9): p. 2103-14.
176. Krishnamurty, A.T., et al., *Somatically Hypermutated Plasmodium-Specific IgM(+) Memory B Cells Are Rapid, Plastic, Early Responders upon Malaria Rechallenge.* Immunity, 2016. **45**(2): p. 402-14.
177. Dogan, I., et al., *Multiple layers of B cell memory with different effector functions.* Nat Immunol, 2009. **10**(12): p. 1292-9.

178. Gitlin, A.D., et al., *Independent Roles of Switching and Hypermutation in the Development and Persistence of B Lymphocyte Memory.* Immunity, 2016. **44**(4): p. 769-81.
179. Foster, W.S., et al., *Tfh cells and the germinal center are required for memory B cell formation & humoral immunity after ChAdOx1 nCoV-19 vaccination.* Cell Rep Med, 2022: p. 100845.
180. Tomayko, M.M., et al., *Cutting edge: Hierarchy of maturity of murine memory B cell subsets.* J Immunol, 2010. **185**(12): p. 7146-50.
181. Good, K.L., D.T. Avery, and S.G. Tangye, *Resting human memory B cells are intrinsically programmed for enhanced survival and responsiveness to diverse stimuli compared to naive B cells.* J Immunol, 2009. **182**(2): p. 890-901.
182. Arpin, C., J. Banchereau, and Y.J. Liu, *Memory B cells are biased towards terminal differentiation: a strategy that may prevent repertoire freezing.* J Exp Med, 1997. **186**(6): p. 931-40.
183. Kometani, K., et al., *Repression of the transcription factor Bach2 contributes to predisposition of IgG1 memory B cells toward plasma cell differentiation.* Immunity, 2013. **39**(1): p. 136-47.
184. Zabel, F., et al., *Viral particles drive rapid differentiation of memory B cells into secondary plasma cells producing increased levels of antibodies.* J Immunol, 2014. **192**(12): p. 5499-508.
185. Inoue, T. and T. Kurosaki, *Memory B cells.* Nat Rev Immunol, 2024. **24**(1): p. 5-17.
186. Auladell, M., et al., *Recalling the Future: Immunological Memory Toward Unpredictable Influenza Viruses.* Front Immunol, 2019. **10**: p. 1400.
187. Zhang, N., et al., *Adenosine A2a receptors induce heterologous desensitization of chemokine receptors.* Blood, 2006. **108**(1): p. 38-44.
188. Sharma, P., et al., *Immune checkpoint therapy-current perspectives and future directions.* Cell, 2023. **186**(8): p. 1652-1669.
189. Termote, M., et al., *Antigen affinity and site of immunization dictate B cell recall responses.* Cell Rep, 2025. **44**(1): p. 115221.
190. Grimsholm, O., et al., *The Interplay between CD27(dull) and CD27(bright) B Cells Ensures the Flexibility, Stability, and Resilience of Human B Cell Memory.* Cell Rep, 2020. **30**(9): p. 2963-2977 e6.
191. Turner, J.S., et al., *SARS-CoV-2 mRNA vaccines induce persistent human germinal centre responses.* Nature, 2021. **596**(7870): p. 109-113.
192. Alsoussi, W.B., et al., *SARS-CoV-2 Omicron boosting induces de novo B cell response in humans.* Nature, 2023. **617**(7961): p. 592-598.
193. Kim, W., et al., *Germinal centre-driven maturation of B cell response to mRNA vaccination.* Nature, 2022. **604**(7904): p. 141-145.
194. Turner, J.S., et al., *Human germinal centres engage memory and naive B cells after influenza vaccination.* Nature, 2020. **586**(7827): p. 127-132.
195. McIntire, K.M., et al., *Maturation of germinal center B cells after influenza virus vaccination in humans.* J Exp Med, 2024. **221**(8).
196. Nguyen, T.H.O., et al., *CD8(+) T cells specific for an immunodominant SARS-CoV-2 nucleocapsid epitope display high naive precursor frequency and TCR promiscuity.* Immunity, 2021. **54**(5): p. 1066-1082 e5.
197. Anderson, E.M., et al., *Seasonal human coronavirus antibodies are boosted upon SARS-CoV-2 infection but not associated with protection.* Cell, 2021. **184**(7): p. 1858-1864 e10.
198. Krammer, F., *The human antibody response to influenza A virus infection and vaccination.* Nat Rev Immunol, 2019. **19**(6): p. 383-397.
199. Scharf, L., et al., *Longitudinal single-cell analysis of SARS-CoV-2-reactive B cells uncovers persistence of early-formed, antigen-specific clones.* JCI Insight, 2023. **8**(1).
200. Williams, G.S., et al., *CD4+ T cell responses in mice lacking MHC class II molecules specifically on B cells.* Eur J Immunol, 1998. **28**(11): p. 3763-72.
201. La Gruta, N.L. and S.J. Turner, *T cell mediated immunity to influenza: mechanisms of viral control.* Trends Immunol, 2014. **35**(8): p. 396-402.
202. Finn, C.M. and K.K. McKinstry, *Ex Pluribus Unum: The CD4 T Cell Response against Influenza A Virus.* Cells, 2024. **13**(7).
203. Staal, F.J., et al., *The functional relationship between hematopoietic stem cells and developing T lymphocytes.* Ann N Y Acad Sci, 2016. **1370**(1): p. 36-44.

204. Klein, L. and E. Petrozziello, *Antigen presentation for central tolerance induction.* Nat Rev Immunol, 2025. **25**(1): p. 57-72.
205. Spits, H., *Development of alphabeta T cells in the human thymus*. Nat Rev Immunol, 2002. **2**(10): p. 760-72.
206. Cose, S., *T-cell migration: a naive paradigm?* Immunology, 2007. **120**(1): p. 1-7.
207. Lee, M., et al., *The race for the prize: T-cell trafficking strategies for optimal surveillance*. Blood, 2012. **120**(7): p. 1432-8.
208. Harty, J.T., A.R. Tvinnereim, and D.W. White, *CD8+ T cell effector mechanisms in resistance to infection.* Annu Rev Immunol, 2000. **18**: p. 275-308.
209. Andreasen, S.O., et al., *Role of CD40 ligand and CD28 in induction and maintenance of antiviral CD8+ effector T cell responses.* J Immunol, 2000. **164**(7): p. 3689-97.
210. Weninger, W., N. Manjunath, and U.H. von Andrian, *Migration and differentiation of CD8+ T cells.* Immunol Rev, 2002. **186**: p. 221-33.
211. Gribonika, I., et al., *Peyer's patch T(H)17 cells are dispensable for gut IgA responses to oral immunization.* Sci Immunol, 2022. **7**(73): p. eabc5500.
212. Saravia, J., N.M. Chapman, and H. Chi, *Helper T cell differentiation.* Cell Mol Immunol, 2019. **16**(7): p. 634-643.
213. Ridgley, L.A., et al., *IL-6 Mediated Transcriptional Programming of Naive CD4+ T Cells in Early Rheumatoid Arthritis Drives Dysregulated Effector Function.* Front Immunol, 2019. **10**: p. 1535.
214. Zuniga, L.A., et al., *Th17 cell development: from the cradle to the grave.* Immunol Rev, 2013. **252**(1): p. 78-88.
215. Weaver, C.T., et al., *Th17: an effector CD4 T cell lineage with regulatory T cell ties.* Immunity, 2006. **24**(6): p. 677-688.
216. Berger, A., *Th1 and Th2 responses: what are they?* BMJ, 2000. **321**(7258): p. 424.
217. Munoz-Fernandez, R., et al., *Human decidual stromal cells secrete C-X-C motif chemokine 13, express B cell-activating factor and rescue B lymphocytes from apoptosis: distinctive characteristics of follicular dendritic cells.* Hum Reprod, 2012. **27**(9): p. 2775-84.
218. Denton, A.E., et al., *Type I interferon induces CXCL13 to support ectopic germinal center formation.* J Exp Med, 2019. **216**(3): p. 621-637.
219. Weinstein, J.S., et al., *TFH cells progressively differentiate to regulate the germinal center response.* Nat Immunol, 2016. **17**(10): p. 1197-1205.
220. Alam, S., et al., *CD4 T cell help is limiting and selective during the primary B cell response to influenza virus infection.* J Virol, 2014. **88**(1): p. 314-24.
221. Gage, E., et al., *Memory CD4(+) T cells enhance B-cell responses to drifting influenza immunization.* Eur J Immunol, 2019. **49**(2): p. 266-276.
222. Yin, L., et al., *CD4+ T cells provide intermolecular help to generate robust antibody responses in vaccinia virus-vaccinated humans.* J Immunol, 2013. **190**(12): p. 6023-33.
223. Abbas, A.K., H.J. Burstein, and S.A. Bogen, *Determinants of helper T cell-dependent antibody production.* Semin Immunol, 1993. **5**(6): p. 441-7.
224. Lanzavecchia, A., *Receptor-mediated antigen uptake and its effect on antigen presentation to class II-restricted T lymphocytes.* Annu Rev Immunol, 1990. **8**: p. 773-93.
225. Sette, A., et al., *Selective CD4+ T cell help for antibody responses to a large viral pathogen: deterministic linkage of specificities.* Immunity, 2008. **28**(6): p. 847-58.
226. Bergstrom, J.J., H. Xu, and B. Heyman, *Epitope-Specific Suppression of IgG Responses by Passively Administered Specific IgG: Evidence of Epitope Masking.* Front Immunol, 2017. **8**: p. 238.
227. Dangi, T., et al., *Pre-existing immunity modulates responses to mRNA boosters.* Cell Rep, 2023. **42**(3): p. 112167.
228. Dvorscek, A.R., et al., *Conversion of vaccines from low to high immunogenicity by antibodies with epitope complementarity.* Immunity, 2024. **57**(10): p. 2433-2452 e7.
229. Forsell, M.N.E., et al., *Regulation of Subunit-Specific Germinal Center B Cell Responses to the HIV-1 Envelope Glycoproteins by Antibody-Mediated Feedback.* Front Immunol, 2017. **8**: p. 738.
230. Goodwin, E., et al., *Influenza virus antibodies inhibit antigen-specific de novo B cell responses in mice.* J Virol, 2024. **98**(9): p. e0076624.
231. Inoue, T., et al., *Antibody feedback contributes to facilitating the development of Omicron-reactive memory B cells in SARS-CoV-2 mRNA vaccinees.* J Exp Med, 2023. **220**(2).

232. Jajosky, R.P., et al., *Antibody-mediated antigen loss switches augmented immunity to antibody-mediated immunosuppression.* Blood, 2023. **142**(12): p. 1082-1098.
233. McNamara, H.A., et al., *Antibody Feedback Limits the Expansion of B Cell Responses to Malaria Vaccination but Drives Diversification of the Humoral Response.* Cell Host Microbe, 2020. **28**(4): p. 572-585 e7.
234. Petro, C.D., et al., *Monoclonal antibodies against the spike protein alter the endogenous humoral response to SARS-CoV-2 vaccination and infection.* Sci Transl Med, 2024. **16**(772): p. eadn0396.
235. Schaefer-Babajew, D., et al., *Antibody feedback regulates immune memory after SARS-CoV-2 mRNA vaccination.* Nature, 2023. **613**(7945): p. 735-742.
236. Tas, J.M.J., et al., *Antibodies from primary humoral responses modulate the recruitment of naive B cells during secondary responses.* Immunity, 2022. **55**(10): p. 1856-1871 e6.
237. Xu, H., L. Zhang, and B. Heyman, *IgG-mediated immune suppression in mice is epitope specific except during high epitope density conditions.* Sci Rep, 2018. **8**(1): p. 15292.
238. Zhang, Y., et al., *Germinal center B cells govern their own fate via antibody feedback.* J Exp Med, 2013. **210**(3): p. 457-64.
239. Eckl-Dorna, J., et al., *Allergen-Specific Antibodies Regulate Secondary Allergen-Specific Immune Responses.* Front Immunol, 2018. **9**: p. 3131.
240. Andrews, S.F., et al., *High preexisting serological antibody levels correlate with diversification of the influenza vaccine response.* J Virol, 2015. **89**(6): p. 3308-17.
241. Sasaki, S., et al., *Influence of prior influenza vaccination on antibody and B-cell responses.* PLoS One, 2008. **3**(8): p. e2975.
242. Lu, X., et al., *Antibody-Mediated Suppression Regulates the Humoral Immune Response to Influenza Vaccination in Humans.* J Infect Dis, 2024. **229**(2): p. 310-321.
243. Liang, C.Y., et al., *Imprinting of serum neutralizing antibodies by Wuhan-1 mRNA vaccines.* Nature, 2024. **630**(8018): p. 950-960.
244. Garg, A.K., R. Desikan, and N.M. Dixit, *Preferential Presentation of High-Affinity Immune Complexes in Germinal Centers Can Explain How Passive Immunization Improves the Humoral Response.* Cell Rep, 2019. **29**(12): p. 3946-3957 e5.
245. Coelho, C.H., et al., *SARS-CoV-2 monoclonal antibody treatment followed by vaccination shifts human memory B cell epitope recognition suggesting antibody feedback.* bioRxiv, 2023.
246. Getahun, A. and B. Heyman, *Studies on the mechanism by which antigen-specific IgG suppresses primary antibody responses: evidence for epitope masking and decreased localization of antigen in the spleen.* Scand J Immunol, 2009. **70**(3): p. 277-87.
247. Watts, C. and A. Lanzavecchia, *Suppressive effect of antibody on processing of T cell epitopes.* J Exp Med, 1993. **178**(4): p. 1459-63.
248. Meyer-Hermann, M., *Injection of Antibodies against Immunodominant Epitopes Tunes Germinal Centers to Generate Broadly Neutralizing Antibodies.* Cell Rep, 2019. **29**(5): p. 1066-1073 e5.
249. Palm, A.E., et al., *Endogenous complement-activating IgM is not required for primary antibody responses but promotes plasma cell differentiation and secondary antibody responses to a large particulate antigen in mice.* Front Immunol, 2023. **14**: p. 1323969.
250. Karlsson, M.C., et al., *Efficient IgG-mediated suppression of primary antibody responses in Fcgamma receptor-deficient mice.* Proc Natl Acad Sci U S A, 1999. **96**(5): p. 2244-9.
251. Wernersson, S., et al., *IgG-mediated enhancement of antibody responses is low in Fc receptor gamma chain-deficient mice and increased in Fc gamma RII-deficient mice.* J Immunol, 1999. **163**(2): p. 618-22.
252. Nguyen, T.T.T., et al., *sIgM-FcmuR Interactions Regulate Early B Cell Activation and Plasma Cell Development after Influenza Virus Infection.* J Immunol, 2017. **199**(5): p. 1635-1646.
253. Vono, M., et al., *Maternal Antibodies Inhibit Neonatal and Infant Responses to Vaccination by Shaping the Early-Life B Cell Repertoire within Germinal Centers.* Cell Rep, 2019. **28**(7): p. 1773-1784 e5.
254. Fazekas de St, G. and R.G. Webster, *Disquisitions on Original Antigenic Sin. II. Proof in lower creatures.* J Exp Med, 1966. **124**(3): p. 347-61.
255. Fazekas de St, G. and R.G. Webster, *Disquisitions of Original Antigenic Sin. I. Evidence in man.* J Exp Med, 1966. **124**(3): p. 331-45.

256. Kaku, C.I., et al., *Broad anti-SARS-CoV-2 antibody immunity induced by heterologous ChAdOx1/mRNA-1273 vaccination.* Science, 2022. **375**(6584): p. 1041-1047.
257. Gerard, A., et al., *High-throughput single-cell activity-based screening and sequencing of antibodies using droplet microfluidics.* Nat Biotechnol, 2020. **38**(6): p. 715-721.
258. Hibi, T. and H.M. Dosch, *Limiting dilution analysis of the B cell compartment in human bone marrow.* Eur J Immunol, 1986. **16**(2): p. 139-45.
259. Ise, W., et al., *Memory B cells contribute to rapid Bcl6 expression by memory follicular helper T cells.* Proc Natl Acad Sci U S A, 2014. **111**(32): p. 11792-7.
260. Lee, J.H., et al., *Modulating the quantity of HIV Env-specific CD4 T cell help promotes rare B cell responses in germinal centers.* J Exp Med, 2021. **218**(2).
261. Scherle, P.A. and W. Gerhard, *Differential ability of B cells specific for external vs. internal influenza virus proteins to respond to help from influenza virus-specific T-cell clones in vivo.* Proc Natl Acad Sci U S A, 1988. **85**(12): p. 4446-50.
262. Johansson, B.E., T.M. Moran, and E.D. Kilbourne, *Antigen-presenting B cells and helper T cells cooperatively mediate intravirionic antigenic competition between influenza A virus surface glycoproteins.* Proc Natl Acad Sci U S A, 1987. **84**(19): p. 6869-73.
263. Ng, T., et al., *Intermediate Levels of Pre-Existing Protective Antibody Allow Priming of Protective T Cell Immunity against Influenza.* J Immunol, 2023. **210**(5): p. 628-639.
264. Eliasson, D.G., et al., *M2e-tetramer-specific memory CD4 T cells are broadly protective against influenza infection.* Mucosal Immunol, 2018. **11**(1): p. 273-289.
265. Angeletti, D., G.M. Frank, and J.W. Yewdell, *Flow-Cytometric Method Measuring B Cell Surface Immunoglobulin Avidity.* Methods Mol Biol, 2017. **1623**: p. 181-189.
266. Smith, A.P., et al., *Influenza Virus Infection Model With Density Dependence Supports Biphasic Viral Decay.* Front Microbiol, 2018. **9**: p. 1554.
267. Ferdous, S., et al., *B-cell epitopes: Discontinuity and conformational analysis.* Mol Immunol, 2019. **114**: p. 643-650.
268. Bromley, S.K., et al., *The immunological synapse.* Annu Rev Immunol, 2001. **19**: p. 375-96.
269. Besavilla, D.F., et al., *Pre-existing CD4 T cell help boosts antibody responses but has limited impact on germinal center, antigen-specific B cell frequencies after influenza infection.* Front Immunol, 2023. **14**: p. 1243164.
270. Ott, G., et al., *MF59. Design and evaluation of a safe and potent adjuvant for human vaccines.* Pharm Biotechnol, 1995. **6**: p. 277-96.
271. Einav, T., L.E. Gentles, and J.D. Bloom, *SnapShot: Influenza by the Numbers.* Cell, 2020. **182**(2): p. 532-532 e1.
272. Sabbaghi, A., et al., *A formulated poly (I:C)/CCL21 as an effective mucosal adjuvant for gamma-irradiated influenza vaccine.* Virol J, 2021. **18**(1): p. 201.
273. Wilkins, A.L., et al., *AS03- and MF59-Adjuvanted Influenza Vaccines in Children.* Front Immunol, 2017. **8**: p. 1760.
274. Morel, S., et al., *Adjuvant System AS03 containing alpha-tocopherol modulates innate immune response and leads to improved adaptive immunity.* Vaccine, 2011. **29**(13): p. 2461-73.
275. Green, J.A. and J.G. Cyster, *S1PR2 links germinal center confinement and growth regulation.* Immunol Rev, 2012. **247**(1): p. 36-51.
276. Green, J.A., et al., *The sphingosine 1-phosphate receptor S1P(2) maintains the homeostasis of germinal center B cells and promotes niche confinement.* Nat Immunol, 2011. **12**(7): p. 672-80.
277. Shinnakasu, R., et al., *Regulated selection of germinal-center cells into the memory B cell compartment.* Nat Immunol, 2016. **17**(7): p. 861-9.
278. Jafri, S., et al., *A sex-specific reconstitution bias in the competitive CD45.1/CD45.2 congenic bone marrow transplant model.* Sci Rep, 2017. **7**(1): p. 3495.
279. Busse, C.E., et al., *A Proposed New Nomenclature for the Immunoglobulin Genes of Mus musculus.* Front Immunol, 2019. **10**: p. 2961.
280. Palese, P., *The genes of influenza virus.* Cell, 1977. **10**(1): p. 1-10.
281. Gray, C. and L.K. Tamm, *pH-induced conformational changes of membrane-bound influenza hemagglutinin and its effect on target lipid bilayers.* Protein Sci, 1998. **7**(11): p. 2359-73.

282. Angeletti, D., et al., *Outflanking immunodominance to target subdominant broadly neutralizing epitopes.* Proc Natl Acad Sci U S A, 2019. **116**(27): p. 13474-13479.
283. Kosik, I., et al., *Neuraminidase inhibition contributes to influenza A virus neutralization by anti-hemagglutinin stem antibodies.* J Exp Med, 2019. **216**(2): p. 304-316.
284. Miller, M.A., et al., *Endogenous antigen processing drives the primary CD4+ T cell response to influenza.* Nat Med, 2015. **21**(10): p. 1216-22.
285. Johansson, B.E. and E.D. Kilbourne, *Dissociation of influenza virus hemagglutinin and neuraminidase eliminates their intravirionic antigenic competition.* J Virol, 1993. **67**(10): p. 5721-3.
286. Tarke, A., et al., *Impact of SARS-CoV-2 variants on the total CD4(+) and CD8(+) T cell reactivity in infected or vaccinated individuals.* Cell Rep Med, 2021. **2**(7): p. 100355.
287. Elsner, R.A. and M.J. Shlomchik, *Germinal Center and Extrafollicular B Cell Responses in Vaccination, Immunity, and Autoimmunity.* Immunity, 2020. **53**(6): p. 1136-1150.
288. Chen, L., et al., *Immunodominant CD4+ T-cell responses to influenza A virus in healthy individuals focus on matrix 1 and nucleoprotein.* J Virol, 2014. **88**(20): p. 11760-73.
289. Coffman, R.L., A. Sher, and R.A. Seder, *Vaccine adjuvants: putting innate immunity to work.* Immunity, 2010. **33**(4): p. 492-503.
290. Rossmann, L., et al., *Distinct single-component adjuvants steer human DC-mediated T-cell polarization via Toll-like receptor signaling toward a potent antiviral immune response.* Proc Natl Acad Sci U S A, 2021. **118**(39).
291. Linderman, S.L., et al., *Potential antigenic explanation for atypical H1N1 infections among middle-aged adults during the 2013-2014 influenza season.* Proc Natl Acad Sci U S A, 2014. **111**(44): p. 15798-803.
292. Liu, S.T., et al., *Antigenic sites in influenza H1 hemagglutinin display species-specific immunodominance.* J Clin Invest, 2018.
293. Andrews, S.F., et al., *Preferential induction of cross-group influenza A hemagglutinin stem-specific memory B cells after H7N9 immunization in humans.* Sci Immunol, 2017. **2**(13).
294. Loyal, L., et al., *Cross-reactive CD4(+) T cells enhance SARS-CoV-2 immune responses upon infection and vaccination.* Science, 2021. **374**(6564): p. eabh1823.
295. Murray, S.M., et al., *The impact of pre-existing cross-reactive immunity on SARS-CoV-2 infection and vaccine responses.* Nat Rev Immunol, 2023. **23**(5): p. 304-316.
296. Zhao, F., et al., *Challenges and developments in universal vaccine design against SARS-CoV-2 variants.* NPJ Vaccines, 2022. **7**(1): p. 167.
297. Andrabi, R., J.N. Bhiman, and D.R. Burton, *Strategies for a multi-stage neutralizing antibody-based HIV vaccine.* Curr Opin Immunol, 2018. **53**: p. 143-151.
298. Letourneau, S., et al., *Design and pre-clinical evaluation of a universal HIV-1 vaccine.* PLoS One, 2007. **2**(10): p. e984.
299. Stephenson, K.E. and D.H. Barouch, *A global approach to HIV-1 vaccine development.* Immunol Rev, 2013. **254**(1): p. 295-304.
300. Lozano, J.M., et al., *The Search of a Malaria Vaccine: The Time for Modified Immuno-Potentiating Probes.* Vaccines (Basel), 2021. **9**(2).
301. Mitran, C.J. and S.K. Yanow, *The Case for Exploiting Cross-Species Epitopes in Malaria Vaccine Design.* Front Immunol, 2020. **11**: p. 335.
302. Tajudeen, Y.A., et al., *A landscape review of malaria vaccine candidates in the pipeline.* Trop Dis Travel Med Vaccines, 2024. **10**(1): p. 19.
303. Graham, B.S., *Biological challenges and technological opportunities for respiratory syncytial virus vaccine development.* Immunol Rev, 2011. **239**(1): p. 149-66.
304. Sesterhenn, F., et al., *Boosting subdominant neutralizing antibody responses with a computationally designed epitope-focused immunogen.* PLoS Biol, 2019. **17**(2): p. e3000164.
305. Stephens, L.M. and S.M. Varga, *Considerations for a Respiratory Syncytial Virus Vaccine Targeting an Elderly Population.* Vaccines (Basel), 2021. **9**(6).