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CELLULAR AND MOLECULAR MECHANISMS FOR INDUCTION OF BROAD ANTI-VIRAL B CELL RESPONSES THROUGH VACCINATION

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Cover illustration: Created in Adobe Illustrator and Adobe Photoshop by Sebastian Ols. Depicts from left to right, the prefusion conformation of the HRSV fusion protein, the icosahedral nature of a designed multivalent nanoparticle, and the molecular structure of an antibody.

CELLULAR AND MOLECULAR MECHANISMS FOR INDUCTION OF BROAD ANTI-VIRAL B CELL RESPONSES THROUGH VACCINATION

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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Grit: the power of passion and perseverance

– Angela Lee Duckworth

Dedicado a mis abuelas, Laila y Ligia,
a las que extraño mucho

POPULAR SCIENCE SUMMARY OF THE THESIS

What makes a vaccine a good vaccine? It depends on who you ask.

The public would like to have a vaccine that protects against disease completely, one must be immune in the literal sense. That protection must also be long-lasting, preferably longer than a year or two. Administration of a vaccine should preferably not be felt either, which means no side effects. These demands set the bar high, but it is not impossible that future vaccines will be such “dream vaccines”.

For us researchers, the focus must therefore be on improving how we design vaccines today. It requires a better understanding of how the components and methods used in the preparation of vaccines affect the end result. As in good cooking, a well-thought-out and proven recipe is required. This thesis has focused on precisely this. To study different vaccine ingredients and how we can bring them together to elicit better vaccine responses.

A good vaccine response gives an increased quantity and quality of antibodies. Antibodies are target-seeking molecules that can block infection and are produced by different types of B cells in our immune system. Antibodies and B cells protect us against future infection by remembering the invader in case it returns. Antibodies of high quality are needed in large quantities because it guarantees a longer lifespan of the protection. It is also important that the antibodies have broad specificity which means that they can also recognize close relatives of previous invaders. The fact that viruses mutate and change is one of the reasons why, for example, vaccine protection against COVID-19 today is not as long-lasting as we had hoped.

In my thesis, I first studied whether where we give the vaccine injection is critical. We studied vaccination in the muscle and under the skin, but there were no large differences in the immune responses stimulated. However, we discovered that there were differences in where the vaccine goes, i.e., which lymph nodes, to trigger the immune response. This is important information for future studies where other immunization routes, such as the nose are to be tested.

Further, I also studied how small spiky spheres, nanoparticles, can be used as vaccines. We found that immunizing with nanoparticles broadened the antibody response to RSV compared to a traditional vaccine with soluble surface proteins from the virus. The response was broadened because the nanoparticles better stimulated B cells using repetitive patterns of viral surface proteins. The antibodies could even become so good that they could also bind to a related virus. This could mean that the vaccine might protect against two infections at the same time.

The thesis has thus increased our understanding of how the immune system's reaction to vaccination can be controlled and corrected by various vaccine components. This is important for how we design and think about future vaccine recipes to provide long-lasting protection that is resilient and able to withstand new virus variants.

POPULÄRVETENSKAPLIG SAMMANFATTNING AV AVHANDLINGEN

Vad gör ett vaccin till ett bra vaccin? Det beror på vem du frågar.

Allmänheten vill gärna ha ett vaccin som skyddar mot sjukdom helt och hållet; man ska vara immun i bokstavlig mening. Det skyddet ska samtidigt också vara långvarigt, helst längre än ett år eller två. Ett vaccin ska man helst inte heller känna av, det vill säga det ska inte ge biverkningar. Dessa önskemål sätter ribban högt men det är inte omöjligt att framtidens vacciner är just sådana drömvaccin. För oss forskare måste fokus därför ligga på att förbättra hur vi komponerar vaccin idag. Det kräver en detaljerad förståelse över hur komponenterna och metoderna som används i tillverkningen påverkar slutresultatet. I liknelse med god matlagning så krävs ett väl genomtänkt och beprövat recept. Den här avhandlingen har haft fokus på just detta. Att studera olika vacciningredienser och hur vi kan sammanföra dessa för att stimulera bättre vaccinsvar.

Ett bra vaccinsvar ger en ökad mängd antikroppar av hög kvalitet. Antikroppar är målsökande molekyler som kan blockera infektion. De produceras av olika typer av B-celler i vårt immunförsvar. Antikroppar och B-celler skyddar oss mot framtida infektioner genom att minnas inkräktaren ifall den återkommer. Det behövs antikroppar i stora mängder för att säkerställa en längre livslängd på skyddet. Det är även viktigt att våra antikroppar har bred specificitet, det vill säga att de även kan känna igen nära släktingar till tidigare inkräktare. Att virus muterar och förändrar sig är en av anledningarna att till exempel vaccinskyddet mot COVID-19 idag inte är så långvarigt som vi hade hoppats.

I min avhandling studerade jag först om vävnaden i vilken vi ger vaccinsprutan har betydelse. Vi undersökte vaccininjektion i muskeln och under huden, men fann inga stora skillnader i de immunsvar som stimulerades. Vi upptäckte dock att det fanns skillnader i vart vaccin tar vägen i kroppen, dvs till vilka lymfkörtlar, för att starta immunsvaret. Detta är viktig information för framtida studier som vill utforska andra immuniseringsvägar, som till exempel via näsan.

Vidare studerade jag hur små taggiga bollar, nanopartiklar, kan användas som vaccin. Vi upptäckte att då vi immuniserade med nanopartiklar breddades antikroppssvaret mot RS-virus jämfört mot ett traditionellt vaccin baserat på lösliga ytprotein från viruset. Anledning till detta var att nanopartiklarna bättre stimulerade B-celler med hjälp av sitt repetitiva mönster av virusytprotein. Antikropparna kunde till och med bli så bra att de även kunde binda till ett besläktat virus. Detta betyder att vaccinet möjligen skulle kunna skydda mot två infektioner samtidigt.

Avhandlingen har därmed ökat förståelsen kring hur immunförsvarets reaktion mot vaccinering kan styras och ändras av olika vaccinkomponenter. Detta är viktigt för hur vi utformar och tänker kring framtida vaccinrecept för att ge ett långvarigt skydd som är motståndskraftigt och kan mota bort nya virusvarianter.

RESUMEN GENERAL DE LA TESIS

¿Qué hace que una vacuna sea una buena vacuna? Depende de a quién le preguntes.

Al público le gustaría tener una vacuna que proteja completamente contra la enfermedad, uno debe ser inmune en el sentido literal. Esa protección también debe ser duradera, preferiblemente más de un año o dos. Preferiblemente, la administración de la vacuna no tiene que provocar efectos secundarios. Estas exigencias ponen el listón muy alto, pero no es imposible que las futuras vacunas sean esas "vacunas de ensueño".

Para nosotros, los investigadores, el enfoque debe estar, por lo tanto, en mejorar la forma en que diseñamos las vacunas en la actualidad. Requiere una mejor comprensión de cómo los componentes y métodos utilizados en la preparación de la vacuna afectan el resultado final. Como en la buena cocina, se requiere una receta bien pensada y contrastada. Esta tesis se ha centrado precisamente en esto. Estudiar diferentes ingredientes de vacunas y cómo podemos unirlos para obtener mejores respuestas de vacunas.

Una buena respuesta a la vacuna genera una mayor cantidad y calidad de anticuerpos. Los anticuerpos son moléculas que buscan dianas que pueden bloquear la infección y son producidos por diferentes tipos de células B en nuestro sistema inmunológico. Los anticuerpos y las células B nos protegen contra futuras infecciones al recordar al invasor en caso de que regrese. Se necesitan anticuerpos de alta calidad en grandes cantidades porque esto garantiza una mayor vida útil de la protección. También es importante que los anticuerpos tengan una amplia especificidad, lo que significa que también pueden reconocer parientes cercanos de invasores anteriores. El hecho de que los virus muten y cambien es una de las razones por las que, por ejemplo, la protección vacunal frente a la COVID-19 hoy en día no es tan duradera como esperábamos.

En mi tesis, primero estudié si el lugar donde aplicamos la inyección de la vacuna es crítico. Estudiamos la vacunación en el músculo y debajo de la piel, pero no hubo grandes diferencias en las respuestas inmunes estimuladas. Sin embargo, descubrimos que había diferencias en el lugar al que se dirige la vacuna, es decir, a qué ganglios linfáticos, para desencadenar la respuesta inmunitaria. Esta es información importante para futuros estudios donde se probarán otras vías de inmunización, como la nariz.

Además, también estudié cómo pequeñas esferas puntiagudas, nanopartículas, pueden usarse como vacunas. Descubrimos que la inmunización con nanopartículas amplió la respuesta de anticuerpos contra el RSV en comparación con una vacuna tradicional con proteínas de superficie solubles del virus. La respuesta se amplió porque las nanopartículas estimularon mejor a las células B utilizando los patrones repetitivos de las proteínas de la superficie viral. Los anticuerpos podrían incluso volverse tan buenos que también podrían unirse a un virus relacionado. Esto podría significar que la vacuna podría proteger contra dos infecciones al mismo tiempo.

La tesis ha aumentado así nuestra comprensión de cómo la reacción del sistema inmunitario a la vacunación puede ser controlada y corregida por varios componentes de la vacuna. Esto es importante para la forma en que diseñamos y pensamos en futuras recetas de vacunas para brindar una protección duradera que sea resistente y capaz de resistir nuevas variantes de virus.

ABSTRACT

Viral infections contribute to significant morbidity and mortality worldwide. The immense diversity across and within viral families poses a substantial challenge for the development of prophylaxis and therapeutics. Vaccines are one of the most effective medical interventions to prevent infectious diseases. This thesis focused on characterizing anti-viral B cell responses elicited by vaccination and the mechanisms for inducing diversified responses that encompass broad reactivity. All studies were performed in the non-human primate (NHP) model to mimic the human immune system and increase the translational value of the results.

In **paper I**, we characterized how two common routes of parenteral immunization, intramuscular (IM) and subcutaneous (SC) injections, differentially affect the early innate immune responses and the development of adaptive immune responses. The main difference observed between the SC and IM routes was the specific lymph node (LN) clusters to which the vaccine was transported. SC immunization targeted the more superficial LNs in the SC fat while the IM route targeted LNs deeper in the tissue located near major veins. The induction of vaccine-specific adaptive immune responses did not differ.

In **papers II and III**, we performed a detailed analysis of the responses to a novel self-assembling protein nanoparticle that displayed multiple copies of the surface fusion (F) glycoprotein of human respiratory syncytial virus (HRSV). In mice, the multivalent display by the nanoparticle enhanced antibody responses compared to single copies of the HRSV-F protein. This occurred in a valency-dependent manner and relied on the assembly of the multivalent nanoparticle. Importantly, the improved responses were also observed in NHPs. In NHPs, the increased antigen display valency skewed antibody specificities, epitope-focused B cells, and led to an increase in the genetic diversity of responding B cell clonotypes. This resulted in the elicitation of pneumovirus cross-reactive antibodies. We could partly attribute this effect to increased avidity and/or B cell receptor cross-linking from repetitive arraying of antigen on the nanoparticle surface. To follow up on this phenomenon and understand the development of antibody breadth, in **paper IV** we characterized a pneumovirus cross-neutralizing antibody lineage elicited by nanoparticle immunization. Through molecular and structural analyses of antibody variants and evolutionary intermediates, we found that this antibody had acquired cross-reactivity through affinity maturation, with critical residues located in the second heavy chain complementarity determining region (HCDR2), and that similar antibody lineages with the potential to also acquire breadth may have been elicited in multiple other animals.

In conclusion, this thesis improves our understanding of the mechanisms by which vaccine formulation and delivery can modulate the quality and breadth of anti-viral B cell responses. This type of information is important for development and refinement of vaccines that are broadly protective, “universal”, within viral families.

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II. Marcandalli J*, Fiala B*, **Ols S**, Perotti M, de van der Schueren W, Snijder J, Hodge E, Benhaim M, Ravichandran R, Carter L, Sheffler W, Brunner L, Lawrenz M, Dubois P, Lanzavecchia A, Sallusto F, Lee KK, Veesler D, Correnti CE, Stewart LJ, Baker D, Loré K, Perez L*, King NP*.

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III. **Ols S**, Lenart K*, Arcoverde Cerveira R*, Skotheim R, Philomin A, Kochmann J, Corcoran M, Cagigi A, Brunette N, Fiala B, Wrenn S, Marcandalli J, Hellgren F, Thompson EA, Lin A, Kumar A, Chen M, Phad GE, Graham BS, Perez L, Borst AJ, Karlsson Hedestam GB, Ruckwardt TJ, King NP*, Loré K*.

Multivalent Antigen Display on Nanoparticles Elicits Diverse and Broad B cell Responses

Manuscript. *Equal Contribution.

IV. **Ols S***, Borst AJ*, Arcoverde Cerveira R*, Brunette N, Kochmann J, Skotheim R, Philomin A, Lenart K, Corcoran M, Ruckwardt TJ, Karlsson Hedestam GB, Perez L, King NP*, Loré K*

Lineage Evolution of Vaccine-Induced Pneumovirus Cross-Neutralizing Antibodies

Manuscript. *Equal Contribution.

SCIENTIFIC PAPERS NOT INCLUDED IN THE THESIS

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SII. Cagigi A, Yu M, Österberg B, Svensson J, Falck-Jones S, Vangeti S, Åhlberg E, Azizmohammadi L, Warnqvist A, Falck-Jones R, Gubisch PC, Ödemis M, Ghafoor F, Eisele M, Lenart K, Bell M, Johansson N, Albert J, Sälde J, Pettie DD, Murphy MP, Carter L, King NP, **Ols S**, Normark J, Ahlm C, Forsell MN, Färnert A, Loré K, Smed-Sörensen A. Airway antibodies emerge according to COVID-19 severity and wane rapidly but reappear after SARS-CoV-2 vaccination. *JCI Insight.* 2021 Nov 22;6(22).

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CONTENTS

LIST OF ABBREVIATIONS

Ab(s)	Antibody or antibodies
AID	Activation-induced (cytidine) deaminase
AIDS	Acquired immunodeficiency syndrome
APC	Antigen-presenting cell
ASC	Antibody-secreting cell
BLI	Biolayer interferometry
CCR	C-C motif chemokine receptor
CDR	Complementarity determining region
CSR	Class-switch recombination
DAMP	Damage-associated molecular pattern
DC	Dendritic cell
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
ELISpot	Enzyme-linked immunospot
EM	Electron microscopy
Env	Envelope glycoprotein of HIV-1
F	Fusion glycoprotein of pneumoviruses
Fab	Fragment antigen binding
Fc	Fragment crystallizable
FcR	Fc-receptor
FDC	Follicular dendritic cell
FR	Framework region
G	Attachment glycoprotein of pneumoviruses
GC	Germinal center
HCDR	Heavy chain complementarity determining region
HIV	Human immunodeficiency virus
HMPV	Human metapneumovirus
HRSV	Human respiratory syncytial virus
ID	Intradermal
Ig	Immunoglobulin

IGHV	Immunoglobulin heavy chain variable region
IGKV	Immunoglobulin kappa chain variable region
IGLV	Immunoglobulin lambda chain variable region
IL	Interleukin
IM	Intramuscular
LN	Lymph node
MHC	Major histocompatibility complex
NFL	Native-flexibly linked
NHP	Non-human primate
PAMP	Pathogen-associated molecular pattern
PBMCs	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PostF	Postfusion conformation of pneumovirus F protein
PRR	Pattern recognition receptor
PreF	Prefusion conformation of pneumovirus F protein
RNA	Ribonucleic acid
SC	Subcutaneous
SEB	Staphylococcus enterotoxin B
SHM	Somatic hypermutation
SLO	Secondary lymphoid organ
SWE	Squalene oil-in-water emulsion
Tfh	T follicular helper
Th	T helper
TLR	Toll-like receptor
UCA	Unmutated common ancestor
VLP	Virus-like particle

1 INTRODUCTION

Vaccines are regarded as the most successful and cost-effective medical interventions to-date. They have the capacity to instruct the immune system on how to clear or prevent infections. This is accomplished by harnessing the same immune mechanisms that fight natural infections and generate immunological memory to more rapidly clear pathogens upon re-exposure. Historically, vaccines have largely been developed through empirical methods, yet numerous pathogens have evaded the standard vaccine strategies and pose significant challenges that require new approaches to vaccine development. Today, the majority of licensed vaccines provide protection through the elicitation of antibody (Ab) responses that can neutralize or help clear specific pathogens [1]. The quality and durability of elicited Ab responses vary widely between vaccines, a dilemma that is still not fully understood [2]. Non-live vaccines are generally safe but are less immunogenic and may not generate broad humoral responses, which poses challenges in providing protection against highly variable pathogens, such as HIV and the respiratory viruses Influenza A and SARS-CoV-2. Therefore, a better understanding of the induction and maintenance of broad and potent Ab responses is needed to inform next-generation vaccine strategies.

To study the mechanisms of vaccination, we must first understand the immune system that provides the protection offered by vaccines. Broadly, the immune system is divided into two arms, the innate and the adaptive. The innate immune system is crucial for rapid clearance of pathogens as well as to alert and activate cells. Phagocytic cells of the innate immune system are particularly important for their capacity to activate or educate the cells of the adaptive immune system. In contrast, the adaptive arm consists of a diverse and specific repertoire of T and B cells that make up the cellular and humoral immune response, respectively. These cells can recognize specific components of pathogens and can remount robust secondary responses through the maintenance of immunological memory.

In this thesis, I aimed to study the generation of high-quality, durable, and broad B cell responses. This means that both the early immune events after vaccination and the induction and evolution of adaptive responses have been the focus of my work. Hence, I will introduce the two arms of the immune system and their interplay, with a particular focus on the generation of B cell responses. I will also describe the main problems faced in vaccinology today and the proposed solutions in the form of vaccine antigen design, antigen formulation, adjuvant choice, and route of delivery. Detailed knowledge of the immune responses to these vaccine modalities can help inform rational vaccine design in the future.

2 LITERATURE REVIEW

2.1 IMMUNOLOGY

2.1.1 Innate immune responses

The innate immune system is the first line of defense against foreign agents that invade the body. It is recognized as a rapid system that lacks an educatable memory yet still has the specificity to discriminate self from non-self [3]. This segregation is accomplished by pattern recognition receptors (PRRs) that can detect pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs). PAMPs are conserved patterns on microbial pathogens that do not readily exist at steady state, while DAMPs are components of host cells that are released upon cell death or damage. PRRs are differentially expressed in the cells of the innate immune system and their signaling dictate their effector functions and how they educate the adaptive immune system [4]. The cells of the innate immune system include phagocytic cells, such as monocytes, macrophages, dendritic cells (DCs), and neutrophils, as well as eosinophils, basophils, mast cells, and natural killer cells.

The phagocytic cells of the innate immune system are the most prominent at the initial site of infection or vaccination (Figure 1) [5, 6]. Certain subsets of monocytes, macrophages, and DCs reside at the interfaces of the body and the environment, ready to respond to danger. Upon alerting the rest of the immune system to a present danger, an influx of cells from the blood is recruited that consists mainly of monocytes and neutrophils. These cells excel at phagocytosing foreign antigens and degrading them. Antigen-presenting cells (APCs), such as DCs, have the capacity to efficiently load peptide antigens on major histocompatibility complexes (MHC) to prime or activate cognate T cells [7]. DCs are seen as the bridge between the innate and adaptive immune systems because of their unique potency at initiating robust adaptive responses (reviewed in [8, 9]). The role of multiple other phagocytic cells in the initiation of adaptive responses have in recent years also begun to be appreciated (reviewed in [10]), such as the highly abundant neutrophils [11].

How distinct vaccine formulations and routes of delivery affect the innate immune response to vaccination and influence the subsequent interactions with the adaptive immune system is an understudied area. More knowledge in this area would guide how new vaccines can be designed and administered to be more effective.

2.1.2 The lymphatic system

The lymphatic system plays an important role in immune defense and the function of the immune system. It consists of lymphatic vessels, lymphoid organs, lymph nodes (LNs), and lymphoid tissues. Lymphatic vessels carry a clear fluid termed lymph, with a similar composition to plasma in blood and additionally contains waste products, cellular debris, and foreign particles such as viruses and bacteria. Activated innate immune cells can travel from peripheral tissues of infection or inflammation through the lymph to the nearest LNs to initiate an immune response. Lymph also transports lymphocytes between LNs and other lymphoid

organs and tissues. The lymphatic vessels can thereby transport both intact pathogen/vaccine to LNs and APCs that process the pathogen/vaccine for antigen presentation in LNs (reviewed in [12]). Optimization of vaccine formulations to harness the lymphatic system for efficient vaccine trafficking and initiation of adaptive immune responses is currently an intense research area (see chapter 2.3 on Vaccinology) [13].

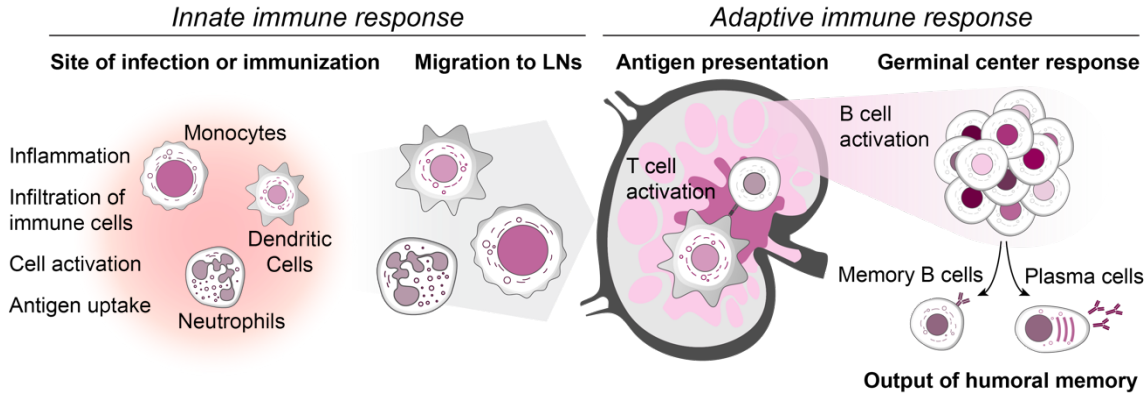

Figure 1. ***The early events after infection or vaccination.*** *Inflammation is induced at the site of infection or immunization by sensing of foreign materials by resident innate immune cells, epithelial cells, and stromal cells. The release of cytokines and chemokines by these cells leads to the recruitment of additional immune cells to infiltrate the tissue and to the activation of responding cells. Neutrophils and monocytes dominate the response to inflammation in numbers although additional DCs are also recruited. Activated cells will phagocytose the foreign material (i.e.., pathogen or vaccine antigens) in an effort to clear the cause of inflammation but also to initiate an adaptive immune response that will remember the encounter. Phagocytic cells carrying foreign antigen will migrate to the LNs through the lymphatic system. In LNs, APCs will activate cognate T cells through the antigen presentation process. Antigens that have mainly floated freely through the lymphatic system to the LNs will activate cognate B cells to proliferate and instigate the germinal center (GC) response through cooperation with activated cognate T cells. The output of the GC reaction is affinity matured B cells that constitute humoral memory, quiescent memory B cells that are ready to respond upon future encounters and Ab-secreting plasma cells that sustain Ab levels. Abs can block future infections from taking hold and thereby inhibit the initiation of inflammation or potentiate and speed up the innate immune systems reaction time to future encounters.*

2.1.3 Adaptive immune responses

The hallmarks of the adaptive immune system include a diverse and antigen-specific receptor repertoire, a capacity to be educated, and a capability to retain a memory of encountered threats [5]. Adaptive immune responses utilize two main constituent cell types, T cells and B cells, which mediate cellular and humoral immunity, respectively. Both T cells and B cells undergo early development in the bone marrow, but mature and go through central tolerance in separate primary lymphoid organs, T cells in the thymus (reviewed in [14]) and B cells in the bone marrow (reviewed in [15]). Central tolerance mediates negative selection of lymphocytes that recognize self (i.e., autoreactive). Upon exit from the thymus or the bone marrow, naïve T cells and B cells will circulate through the body and secondary lymphoid organs (SLOs) in search of their cognate antigen. Peripheral tolerance will eliminate autoreactive clones that manage to escape central tolerance by clonal deletion, conversion to regulatory cells, or induction of anergy.

Naïve T cells are licensed in LNs by APCs that present their cognate antigen (Signal 1) and simultaneously provide the necessary co-stimulatory molecules (Signal 2) and cytokines (Signal 3) [7]. The combination of these signals dictates how the T cell will be polarized (i.e., their functional properties). Cellular immunity is mediated by two main subtypes of T cells, the CD8 T cells that can kill infected cells via antigen-specific cytolytic activity and the CD4 T cells that have a supporting role and provide necessary survival and activation signals to B cells and CD8 T cells. CD8 T cells are crucial for the clearance of ongoing infections, but their role in preventing infection is less clear. They are usually induced in small, but detectable, numbers to most vaccines, especially to live vaccines [16]. CD4 T cells are divided into T helper (Th) subsets based on their production of specific cytokines or the transcription factor that controls their fate (e.g. Th1: IFNγ/T-bet; Th2: IL-4/GATA-3; Th17: IL-17A/RORγt; Tfh: IL-21/Bcl6) [17]. Each CD4 T cell subset excels at a certain task when combatting infection (Th1: intracellular pathogens; Th2: extracellular parasites; Th17: extracellular bacteria; Tfh: B cell selection), but can also play a part in autoimmunity and allergy.

Humoral responses play a fundamental role in the protective effect by vaccination [1]. However, much remains to be elucidated on how high-quality, durable, and broad Ab responses are induced after vaccination. The study of B cells, the cells that mediate the Ab response, are therefore central in my thesis work and will be discussed in more detail below.

2.1.4 Antibody structure, genetics, and diversity

Antibodies (Abs) are disulfide-linked heterodimeric proteins of pairs of immunoglobulin heavy and light chains (Figure 2A) [18, 19]. The heavy and light chains have N-terminal variable regions and C-terminal constant domains that are functionally distinct. The variable domains are responsible for the specificity of the Ab (i.e., the antigen binding fragment (Fab)) and can be divided into the complementarity determining regions (CDR1-3) and the framework regions (FR1-4). The CDR loops are where the majority of the variability is concentrated and in particular the heavy chain CDR3 is widely regarded as the principle determinant of Ab specificity [20]. However, the CDR1 and CDR2 loops can also contribute significantly to Ab specificity and in some cases the light chain is the dominant determinant. The FRs are generally more conserved and help to stabilize the CDR loops at the Ab binding interface (a.k.a. the paratope) through the formation of β-sheets. The heavy chain constant domains encode for the fragment crystallizable (Fc) region that determines the Ab class and effector functions.

The naïve B cell repertoire achieves its breadth through somatic gene recombination during B cell development in the bone marrow. The variable (V), diversity (D), and joining (J) segments make up the variable domain of the heavy chain, while the light chain variable domain contains only the V and J segments (Figure 2B). A selection of each germline segment is made from multiple available segments, and they are recombined through the process of V(D)J recombination. The addition of random nucleotides in the junctions between gene segments allows for further diversity to be encoded early in development. Following these recombination events, the Ab can be expressed as a membrane-bound B cell receptor (BCR) or secreted in the form of a soluble Ab. It is estimated that combinatorial diversity can generate a primary naive

B cell repertoire of >10^{11} BCRs/Abs [21, 22]. Population-level variation in germline genes may have effects on the elicitation of Ab responses but our understanding is incomplete and its study remains a challenge [23, 24]. High degrees of genetic variation in IGHVs have been observed in studies of both humans and NHPs [25, 26]. A recent study has also suggested that population-level gene usage variance in the heavy chain locus may be explained by heritable factors such as polymorphisms [27].

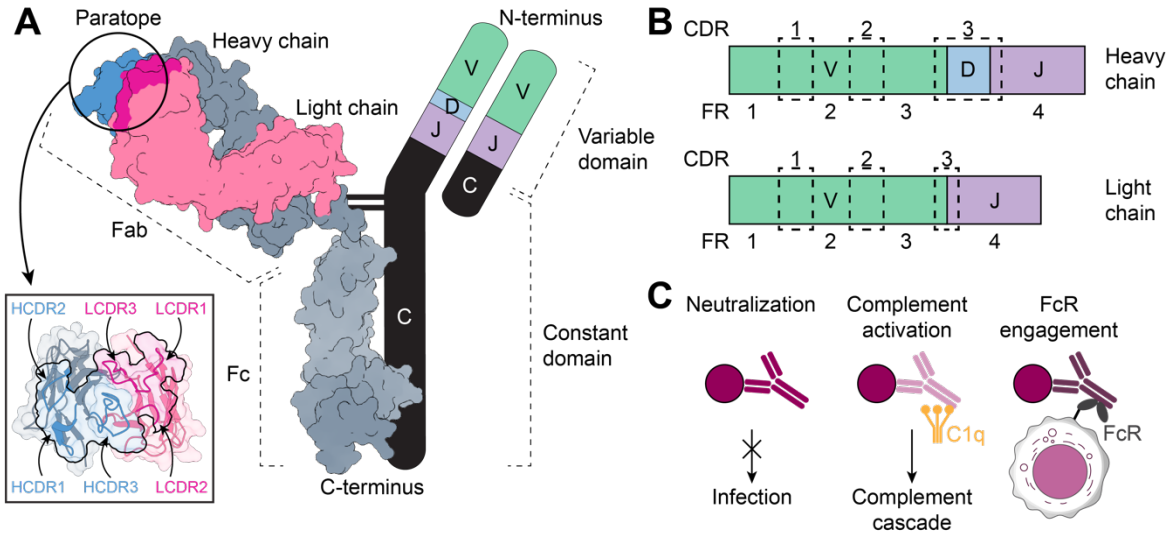

Figure 2. ***Ab structure, genetics, and function.*** *(A) Abs are disulfide-linked heterodimers consisting of a bivalent Fab for binding and an Fc region for mediating effector functions. The Fab domain is made up of a heavy and light Ig chain and encompasses the variable domains of the Ab which through the combinatorial diversity of gene recombination and the process of affinity maturation can be made to bind virtually any molecule. The paratope or antigen-binding region of the Ab is formed by the CDR loops of the variable domains (boxed insert) (PDB: 1HZH). The constant domain of the Ab encodes for much of the structure of the molecule as well as its effector functions. (B) The variable domains of the heavy and light chain are generated through the process of V(D)J recombination which determines the specificity of a given Ab by encoding for the CDR1-3 loops. (C) The function of Abs is to bind to foreign threats and to eliminate them either through direct blocking of infection by neutralization, Fc-mediated activation of the complement system, or FcR-dependent activation of other immune cells.*

Upon recognition of cognate antigen, the BCR may undergo further diversification through the processes of somatic hypermutation (SHM) and class-switch recombination (CSR). Both processes are controlled by the activation-induced (cytidine) deaminase (AID) and substantially contribute to a more effective humoral immune response [28]. During SHM, AID deaminates cytosine to uracil, which is mutagenic when paired with guanosine, leading to error-prone DNA repair of the mismatch. This leads to mutations at the site of deamination (reviewed in [29, 30]) and these are mainly introduced in AID hotspot motifs [31, 32]. The introduction of random point-mutations in the V(D)J coding regions is a process to enhance binding affinity (i.e., binding strength/quality) for the target antigen. This is regulated and tested in a process termed affinity maturation (see chapter 2.1.5 on Antigen-specific B cell responses). On the other hand, CSR or isotype switching is a process of deletional DNA recombination that replaces the constant region genes with their downstream exons [33]. This results in B cells in humans switching from IgM and IgD expression to IgG3, IgG1, IgA1, IgG2, IgG4, IgE, or

IgA2. The Ab isotypes are specialized against different types of pathogens, control different effector functions through their Fc domain, have divergent half-lives in circulation, and excel in distinct anatomical locations.

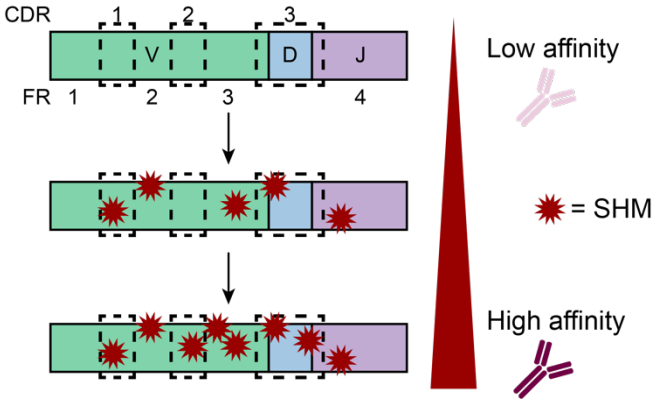

Figure 3. ***SHM and affinity maturation.*** *The acquisition of multiple rounds of somatic mutations in the variable domains of Abs is critical in the affinity maturation process which increases the affinity to the cognate antigen.*

The role of Abs is to bind to their cognate antigen. This could be a molecule, molecular structure, or any foreign particulate matter that are present in the body. Pathogen-specific Abs can mediate multiple effector functions once they have bound their target (Figure 2C). Neutralization of a pathogen by inhibiting its ability to infect target cells can be achieved through binding of specific epitopes of surface proteins on the pathogen. This is one of the most established effector functions for anti-viral Abs and this is mainly determined by the specificity of the Fab. The Fc region of the Ab can mediate additional effector functions once bound to its target including activation of the complement system and activation of innate immune cells through Fc receptor engagement (reviewed in [34]). Innate immune cells can be triggered to perform opsonophagocytosis, to release cytokines or chemokines, to degranulate cytotoxic molecules, or to mature into professional APCs. The Fc-dependent effector functions are less common to assess but may exhibit critical functions for the effect of non-neutralizing Abs.

2.1.5 Antigen-specific B cell responses

Antigen-specific B cell responses are triggered by BCR recognition of cognate antigen and lead to B cell activation that may or may not require T cell help. The type of antigen determines if the response is T-dependent or T-independent, with "conventional" protein antigens generating the former and polysaccharide antigens or other highly repetitive structures are associated with the latter. T-independent responses mainly lead to rapid generation of Ab-secreting plasma cells [35]. Vaccines tend to generate T-dependent responses. The initial affinity of responding B cells to complex protein antigens is low and needs to be improved. This immunological phenomenon is one focus of my thesis and will be discussed below.

In SLOs, such as LNs, B cells that have recognized their cognate antigen via their BCR will capture the antigen in its native form and proceed to internalize, process, and present antigen peptides on MHC class II molecules [36, 37]. The activated B cell will upregulate CCR7 to migrate to the B cell-T cell border in search of a cognate CD4 T helper cell that can recognize their presented peptide:MHC complexes. The T helper cell will instruct the B cell through co-

stimulatory signals and cytokines to undergo a burst of proliferation and to differentiate through one of three distinct pathways (Figure 4). An activated B cell may either enter the germinal center (GC) reaction to undergo affinity maturation, rapidly differentiate into a short-lived non-GC plasma cell that can provide an immediate Ab response of low affinity, or regain a quiescent state as an early non-GC memory B cell [38-40]. The strength of BCR signaling and the availability of T cell help shape the choice between the three lineages. Recently, also the rapidly diminishing access to cognate antigen has been implicated in the choice of differentiation path of activated B cells [41].

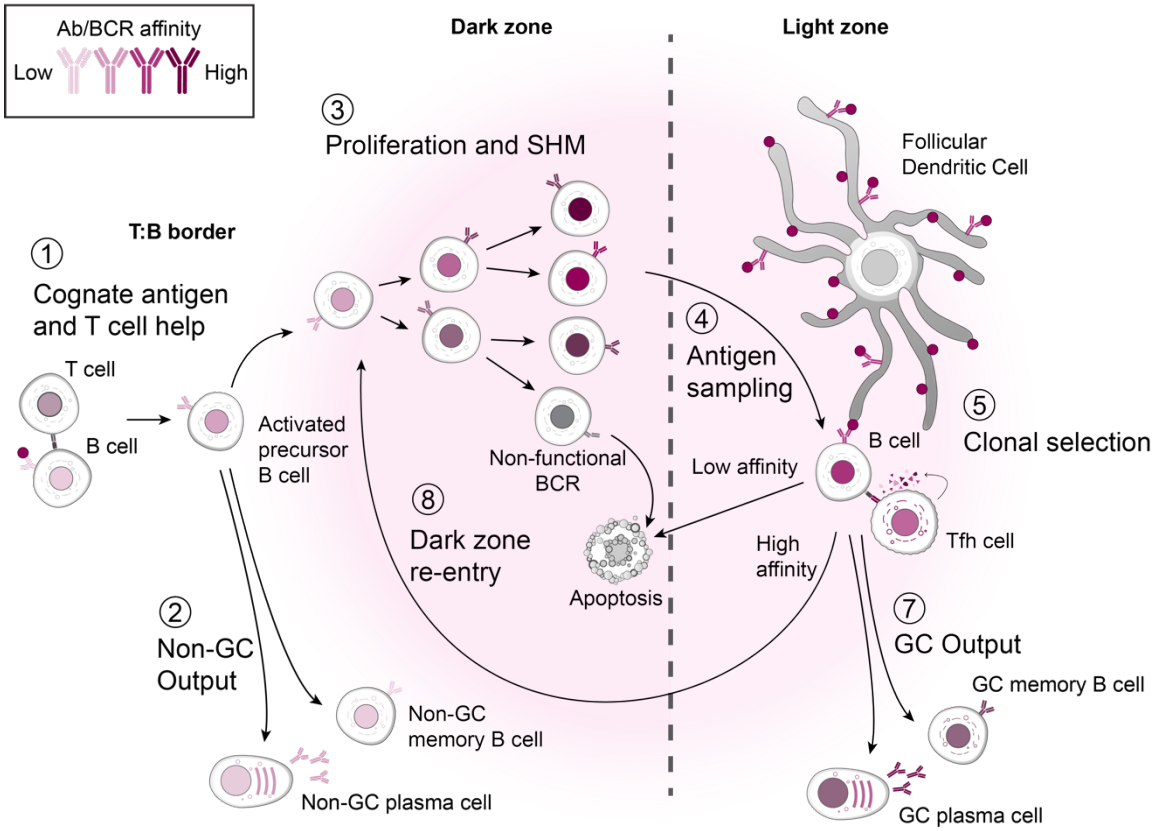

Figure 4. ***B cell activation and the GC reaction.*** *Upon recognition of cognate antigen, B cells will find CD4 T cell help at the T:B border in LNs (1). T cell help will activate the B cell and instruct it to either differentiate into non-GC memory B cells or plasma cells (2), or to enter the GC reaction to undergo proliferation and SHM (3). After multiple rounds of SHM in the dark zone, GC B cells will test their affinity in the light zone by sampling antigen captured on FDCs (4) and presenting it to GC Tfh cells. Tfh cells are responsible for clonal selection (5) by providing the necessary signals for B cells to either exit the GC and differentiate into memory B cells or plasma cells, or to re-enter the dark zone (8) for continued affinity maturation. SHM that leads to BCRs that are non-functional or of reduced affinity will lead to apoptosis by neglect from Tfh cells. This iterative process leads to the generation of high-affinity Abs and the output of humoral memory.*

Affinity maturation involves the interrelated processes of SHM and clonal selection. In the GC reaction (Figure 4), B cells proliferate and undergo SHM in the dark zone with the intention to improve their affinity for the target antigen. GC B cells then cycle through the light zone to compete for antigen displayed by follicular dendritic cells (FDCs) and thereby test their affinity to the antigen. FDCs display immune complexed antigen on FcRgIIb and on complement

receptors 1 (CD21) and 2 (CD35) [42]. FDCs can also protect antigen from degradation for extended periods through a mechanism of cycling immune complexes in non-degrative endosomal compartments [43]. Clonal selection of the best suited B cells is assisted by a limited number of T follicular helper (Tfh) cells. The competition for survival signals is mainly affinity-based and dependent on B cells acquiring enough antigen from FDCs to efficiently process and present on MHC class II to Tfh cells [38, 44-46]. Tfh cells can provide secreted and membrane-bound signals including IL4, IL-21, and CD40L. B cells that survive competition in the light zone will either re-enter the dark zone for additional rounds of proliferation and SHM or exit the GC by differentiating into memory B cells or long-lived Ab-secreting plasma cells [36, 47]. The current notion is that B cells of lower affinity differentiate into memory B cells while those of higher affinity differentiate into long-lived plasma cells as a result of a temporal switch in the output from the GC reaction [48]. Additionally, targeting vaccine antigen to FDCs and increasing their retention time within GCs are thought to enhance the magnitude and quality of B cell responses [49].

In a primary immune response, the output of adaptive immune responses has a gradual rise in magnitude (Figure 5A) [18]. The education of both T cells and B cells can take between days and weeks to reach significant quantities. As described earlier, Abs are produced continuously in multiple waves of successively increasing affinity. This ensures that the cause of inflammation can be combatted progressively and that Abs start to act before the response of highest quality. Upon antigen re-encounter (i.e., recall responses) (Figure 5B), the memory responses generated as a result of the first encounter are quicker to respond and initiate the secondary response. This response is not only faster but also starts at a higher quality as it takes advantage of already affinity-matured B cells. The presence of circulating Abs also aids in potentiating the response through increased activation of innate immune cells and enhanced deposition of immune complexed antigen on FDCs. New GC responses are initiated in secondary responses, both from the recruitment of new naive B cells and the activation and differentiation of memory B cells [50].

Memory B cells are long-lived quiescent cells that patrol the body through the blood, spleen, and SLOs in search of their cognate antigen [51, 52]. Following antigen re-encounter, memory B cells are activated and proliferate as activated B cells or differentiate into Ab-secreting plasmablasts to rapidly increase the Ab titers in circulation (Figure 5B) [53], which aids in more rapid clearance of the antigen [54-56]. Memory B cells are also believed to seed the formation of new GC reactions upon antigen re-encounter, although it is still unclear how this happens and if all memory B cells have this capacity [57-59]. Memory B cells may therefore play an important role in the adaptation of Ab responses to evolving threats such as virus variants.

Long-lived plasma cells are terminally differentiated Ab-secreting cells (ASCs) that secrete large amounts of high-affinity Abs and are the basis of sustained humoral immunity [60, 61]. These cells maintain a steady level of Ab titers in the circulation to provide a first line of defense upon re-challenge. Long-lived plasma cells mainly reside in the bone marrow where they are

dependent on a special survival niche of factors such as APRIL and BAFF [62]. The durability of Ab titers secreted from the bone marrow varies greatly and it is not known how longevity is imprinted during infection or vaccination [2]. The disparity in Ab properties induced by licensed vaccines, especially in terms of longevity, is fundamental to understand to inform effective future vaccine development (reviewed recently in [63]).

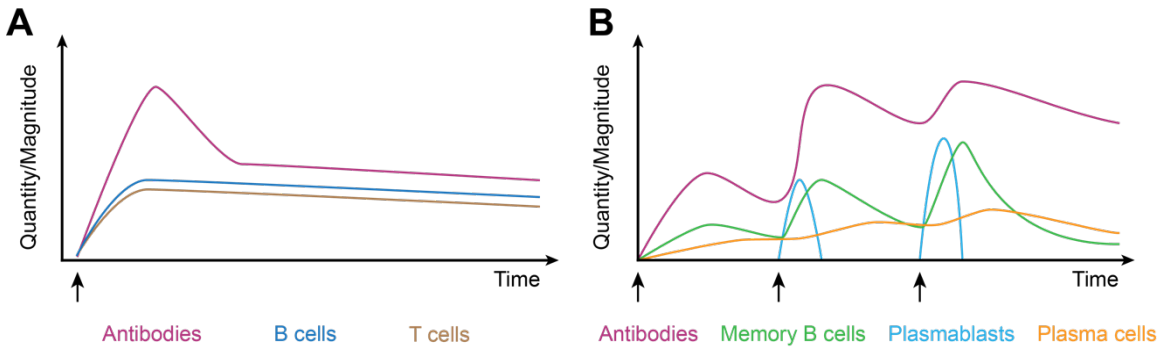

Figure 5. ***Dynamics of the adaptive immune response.*** *A) In a primary immune response, Abs, B cells and T cells increase to reach a plateau that may remain relatively stable. Abs wane quite rapidly from their peak magnitude but stabilize at a level that is sustained by Ab-secreting plasma cells. B) During subsequent encounters with a pathogen, the memory response reacts quickly, and Ab titers rapidly increase from the differentiation of memory B cells to Ab-secreting plasmablasts. The magnitude of humoral responses reaches transient peaks after each exposure and then declines to lower but stable levels.*

In fact, there are multiple models of how durable humoral immunity is maintained and they remain a subject of debate [60]. The models can be divided into two categories, memory B cell dependent or independent. The dependent models suggest that memory B cells are regularly re-activated by either cross-reactivity, repeated infections/vaccinations, persistent antigen on FDCs, or polyclonal/bystander activation [64]. However, increasing evidence suggests that the memory B cell independent models are more plausible since it has been demonstrated that plasma cells and Ab titers can be sustained either when memory B cells are depleted [65-68], in the absence of antigen [69], and that no increases are observed despite bystander activation [70, 71]. Because of the varying Ab half-lives observed, two models for plasma cell survival have also been proposed. The first model is based on competition for limited space in the survival niche in the bone marrow [72, 73] and the second is based on that imprinting of lifespan occurs in the GC reaction [60]. The competition model predicts that Ab titers would wane more quickly with increasing age as niche space would become more scarce and "irrelevant" specificities would be outcompeted, but this has not yet been a solid observation [2]. Instead, the imprinting model may account for differences in T cell help and the strength of BCR signaling. These are factors that can be influenced by vaccine formulations and thereby underscores that a better understanding of the GC reaction and the fate of antigen-specific B cells is needed to improve future vaccines.

2.2 VIROLOGY

2.2.1 Pneumoviruses

A major part of my thesis (**paper II-IV)** has been studying the immune responses to a vaccine candidate for human respiratory syncytial virus (HRSV). This virus belongs to the *Pneumoviridae* family which are enveloped negative-stranded RNA viruses representing a recently reclassified virus family of the order *Mononegavirales* previously considered a subfamily of the *Paramyxoviridae* family [74]. Respiratory tract infections are associated with these viruses and natural hosts include humans, cattle, rodents, and birds. The family of five species is divided into two genera: *Metapneumovirus* and *Orthopneumovirus*. The *Orthopneumovirus* genus is the most famous as it contains *Human orthopneumovirus*, *Bovine orthopneumovirus*, and *Murine orthopneumovirus* which are more commonly known under the names human respiratory syncytial virus (HRSV), bovine respiratory syncytial virus, and murine pneumonia virus, respectively. The *Metapneumovirus* genus contains *Human metapneumovirus* (HMPV) and *Avian metapneumovirus*.

The viral genomes of the two pneumovirus genera are conserved in terms of content but differ slightly in their architecture (Figure 6A) [74]. A major difference is also the lack of the nonstructural proteins NS1 and NS2 among *Metapneumoviruses*. NS1 and NS2 together inhibit apoptosis and interferon responses in infected host cells. The lipid bilayer of the pneumovirus virion displays the fusion (F), attachment (G), and small hydrophobic (SH) proteins (Figure 6B) [75]. The role of SH as an ion channel is believed to delay apoptosis in infected cells. The F and G glycoproteins are the most abundant on the surface of the virus and play important roles in entry.

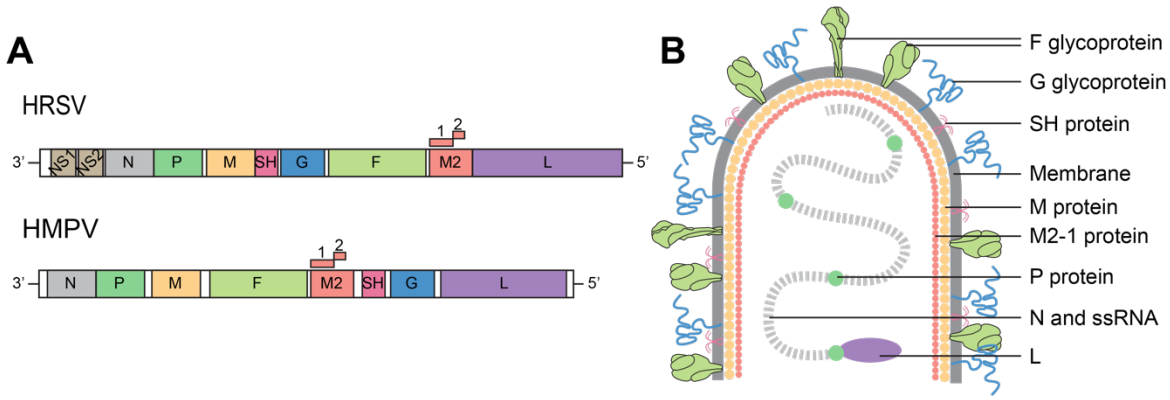

Figure 6. ***Structure of the pneumovirus genome and virion.*** *A) The viral genomes of pneumoviruses are single-stranded RNA (ssRNA) and encode for multiple proteins, including the non-structural proteins (NS1 and NS2; limited to Orthopneumoviruses such as HRSV), the nucleoprotein (N), the phosphoprotein polymerase cofactor (P), the membrane protein (M), the small hydrophobic protein (SH), the attachment protein (G), the fusion protein (F), the transcription processivity factor (M2), and the large polymerase subunit (L). B) The virion buds with a filamentous morphology that progressively becomes more spherical. The localization of the different viral proteins is shown.*

The G protein's role, as its name suggests, is to interact with host cell attachment factors on cell surfaces, such as glycosaminoglycans [76]. Although the G protein facilitates virion

attachment, it is not necessary for HRSV propagation *in vitro* [77, 78] which implies a role in attachment for the F protein. Both the G and F glycoproteins have been shown to interact with heparin or heparan sulfate [79]. Additionally, several proteins have been proposed to facilitate HRSV entry through interaction with the F protein, including intercellular adhesion molecule 1 (ICAM1) [80], epidermal growth factor receptor (EGFR) [81], nucleolin [82], and insulin-like growth factor-1 receptor (IGF1R) [83]. Of note, none of these putative attachment factors has yet been mapped to bind directly to specific domains on the F protein.

Pneumoviruses cause significant disease burden in humans and cause reinfections throughout life. The viruses are ubiquitous pathogens that infect nearly all infants by the age of two for HRSV [84, 85] and the age of five for HMPV [86, 87]. The symptoms of pneumovirus infections are indistinguishable and transmission is generally from close contact. In adults, pneumoviruses cause an upper respiratory tract infection and are one of the virus families implicated in causing the common cold. In infants, pneumoviruses are the primary cause of hospitalization from respiratory tract infections [84-86, 88]. The disease burden in children under the age of five of HRSV- and HMPV-associated lower respiratory tract infections are estimated to 33.1 million and 14.2 million cases with 3.2 million and 643,000 hospitalizations, respectively [84, 85, 88]. Ninety-nine percent of deaths occur in the developing world. In the elderly, the disease burden is only starting to be appreciated for pneumoviruses with considerable morbidity and mortality estimated [89].

2.2.1.1 Pneumovirus vaccine development

There are no approved vaccines or therapeutics for pneumoviruses. HRSV vaccine development commenced in the 1960s, shortly after the discovery of the virus in 1956, but has progressed cautiously for many decades because of aberrant immune responses elicited to natural infection after early vaccine clinical trials. Multiple clinical trials in the 1960s of formalin-inactivated whole-virus HRSV vaccines caused vaccine-enhanced disease in infants [90-93]. Neutrophil infiltration of the lungs [94] and immune complex deposition in the small airways [95] are believed to enhance the disease course. In fact, a recent study in a human challenge model has shown that neutrophils in the respiratory mucosa predispose to HRSV infection and disease [96]. Human challenge models have also shown local IgA responses in the respiratory mucosa to correlate with protection from infection and not systemic Ab responses [97]. Although, systemic Ab responses are still important at preventing lower respiratory tract infections and severe disease, as will be discussed later.

Today, vaccine development for HRSV focuses on three target populations: infants, pregnant women, and the elderly (reviewed in [98-100]). Each target population will likely require tailored vaccines because of unique challenges and considerations needed. Maternal immunization is the main target profile of protein subunit vaccines and will rely on boosting pre-existing neutralizing Abs. Vaccination in the late second or early third trimester could enhance placental transfer of anti-HRSV Abs to protect the infant from severe disease in the crucial first six months of life [101]. Placental transfer of maternal Abs was recently shown to

be regulated by Fc glycosylation patterns [102, 103], an aspect which some research suggests can be modulated by vaccine adjuvants [104, 105].

Maternal immunization is a feasible strategy as the role of Abs as a correlate of protection is well-established for HRSV. Neutralizing Ab responses are highly correlated with protection from HRSV disease in multiple age groups [106-108]. Also, passive immunizations with polyclonal anti-HRSV Ig preparations (RespiGam) [109] or the recombinant monoclonal Ab palivizumab (Synagis) [110] can prevent severe disease in high-risk infants. Palivizumab, which binds the surface F glycoprotein, has limited efficacy [111] but it has been highlighted that the F protein is an important target of prophylactic and therapeutic interventions. Today, multiple vaccines and monoclonal Abs targeting the F protein are in advanced clinical trials for HRSV while for HMPV only early-stage clinical trials are underway (reviewed in [98-100]).

2.2.1.2 The pneumovirus fusion (F) glycoprotein

The indispensable role of the F protein in pneumovirus infectivity means it is the primary target of neutralizing Abs and the focus of vaccine development efforts. The pneumovirus F protein mediates fusion of the viral and host-cell membranes [75, 112]. Through a significant structural rearrangement, from the metastable prefusion conformation to the highly stable postfusion conformation, the F protein brings the viral and host-cell membranes together (Figure 7A). Where fusion occurs is not fully understood (reviewed in [75]), but it is possible that fusion happens at the plasma membrane and in endocytic vesicles depending on the target cell. Unfortunately, even less is known about the trigger for fusion. The F protein is a class I viral fusion protein, but it does not exhibit any of the three well-defined triggers for other such fusion proteins, including low pH, direct receptor binding, and provocation by a second viral glycoprotein. A lack of spatiotemporal control of triggering is an alternative hypothesis that argues that the reduced efficiency of productive fusion events may be outweighed by the benefits for conformational immune evasion. The presence of both prefusion and postfusion conformations of the F protein on the surface of virions is well-documented [113]. The postfusion conformation is taller than the functional prefusion conformation and could thereby shield the prefusion protein from neutralizing Abs.

Structural analyses of the HRSV-F protein in the prefusion and postfusion conformations [114-116], and later the HMPV-F protein in the postfusion conformation [114-116], have been important milestones in the antigenic characterization of the F protein. They have enabled structure-based vaccine design of prefusion-stabilized F proteins for HRSV [117-119] and for HMPV [120, 121]. The F proteins of HRSV and HMPV are structurally very similar in their prefusion conformation despite sharing only ~33% amino acid identity [122]. This has aided in the structural and protein engineering work needed to stabilize the HMPV-F protein in the prefusion conformation [120, 121].

Ab neutralization of HRSV predominantly targets the prefusion conformation of the F protein [123, 124](refs). Vaccine development has therefore focused on prefusion-stabilized F antigens during the past decade and multiple phase III clinical trials of stabilized immunogens are now

ongoing (reviewed in [98-100]). Postfusion F vaccine candidates, or that resemble the postfusion morphology, have failed in several late-stage clinical trials but are still under development.

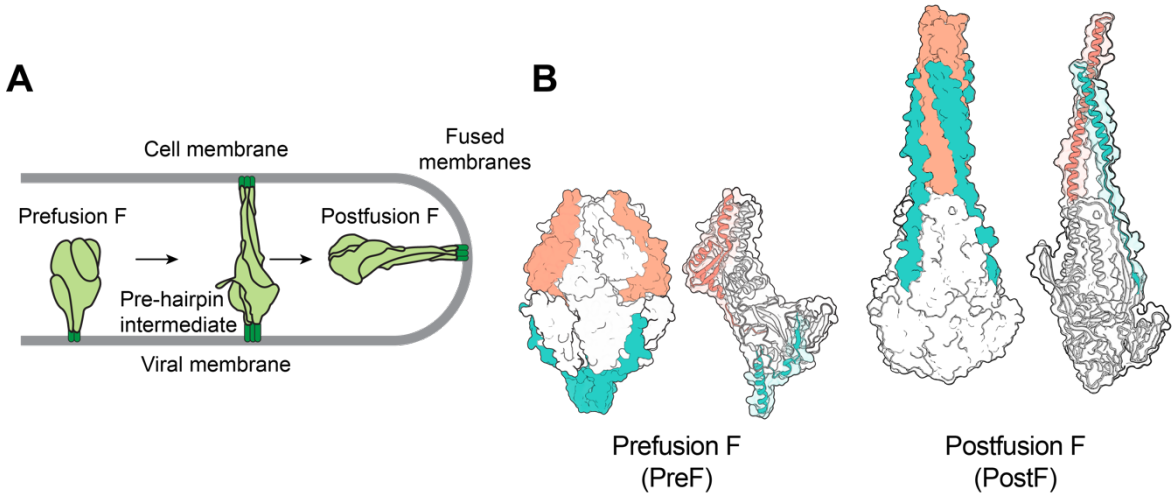

Figure 7. ***Pneumovirus F protein function and structure.*** *A) The F protein fuses the viral and cell membranes by transitioning from the metastable prefusion conformation and into the pre-hairpin intermediate where the fusion peptide has been buried in the host cell membrane. Refolding of the F protein onto itself and into the postfusion conformation brings the two membranes together. B) Structure of trimeric and monomeric prefusion (PDB: 4jhw) and postfusion (PDB: 3rrr) HRSV-F. Colored surfaces depict residues that change their conformation in the transition from the prefusion to postfusion conformation.*

The antigenic landscape of HRSV-F consists of six major antigenic sites (Ø, I, II, III, IV, V) enumerated on the prefusion conformation of which some are also retained in the postfusion conformation (I, II, III, IV) (Figure 8A) [75, 125]. In fact, the prefusion and postfusion conformations share almost two thirds of their surface area (i.e., the three dimensional structure remains intact) [117]. The antigenic sites vary in their degrees of neutralization sensitivity as deduced by functional characterization of a large quantity of human monoclonal Abs [126]. Some HRSV-F-specific Abs are up to 50-fold more potent than the clinically used Ab palivizumab and recognize epitopes exclusive to the prefusion conformation. Abs targeting the apex-proximal sites (Ø and V) are generally the most potent at neutralizing as they are prefusion-specific and are believed to "lock" the F protein in the prefusion conformation, inhibiting the fusion machinery and thereby infection of host cells [115, 127]. Similarly, Abs targeting quaternary epitopes can also inhibit transition to the pre-hairpin intermediate conformation and are potent neutralizers [128]. The shared surface area of the prefusion and postfusion conformation contains antigenic sites I-IV and Abs targeting these sites are generally less potent at neutralizing HRSV [126]. Binding to antigenic sites I-IV are believed to inhibit infection by blocking the transition from the pre-hairpin intermediate to the postfusion conformation through steric effects. Despite their lower potency, Ab reactivity to the shared surface area makes up a dominant proportion of the neutralizing Ab response in humans [124].

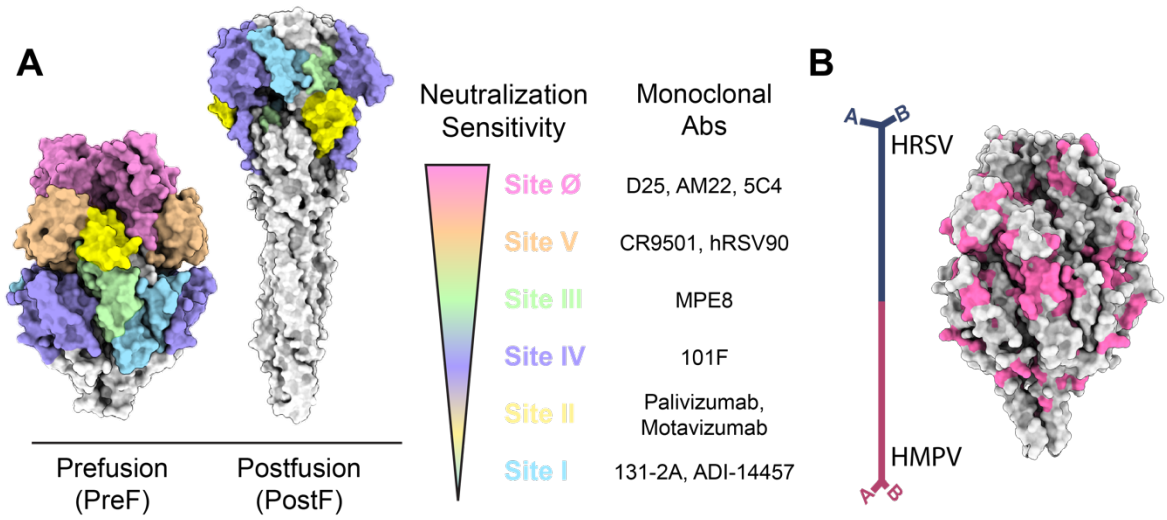

Figure 8. ***The antigenic landscape of the HRSV-F protein.*** *A) Antigenic sites denoted by color on the surface of the prefusion (PDB: 4mmu) and postfusion (PDB:3rrr) HRSV-F protein. The established neutralization sensitivity hierarchy is depicted and representative monoclonal Abs for each antigenic site are listed. B) Amino acid homology of HRSV-F and HMPV-F proteins depicted in a phylogenetic tree and in a model of the prefusion HRSV-F protein (PDB: 6ous). Pink color denotes conserved residues.*

Similarly, characterizations of the antigenic landscape on the prefusion HMPV-F protein have been performed in the past year and borrow the nomenclature of antigenic sites from HRSV-F because of their high structural similarity [129-131]. The neutralization sensitivity hierarchy of antigenic sites differs slightly between the two pneumoviruses, in part because of N-linked glycosylation sites differentially masking certain sites. For HMPV, prefusion/postfusion shared surface reactive Abs targeting sites II, III, and IV are the most potent at neutralizing [129-131]. Additional antigenic sites embedded in the trimer interface of the prefusion conformation have also been described for both HRSV and HMPV [129, 131, 132]. These internal epitopes are believed to be exposed during the natural "breathing" of the F trimer on the virion surface or through trimer destabilization induced by Ab binding [133].

2.2.1.3 Pneumovirus cross-reactive Abs

HRSV-F and HMPV-F cross-reactive antibodies have been identified in multiple studies of the human monoclonal Ab response [126, 131, 134-137]. Repeated sequential exposures with HRSV and HMPV are believed to affinity mature these Ab responses. A handful of Abs targeting antigenic sites III, IV, and V, which reside in surfaces of high conservation between the two pneumoviruses (Figure 8B), have been shown to possess this cross-reactivity [126, 131, 134-137]. The Ab MPE8 is the most well-characterized and was the first to be identified [121, 134, 136]. MPE8 defines an Ab class of site III-specific Abs that are restricted to IGHV3-21:IGLV1-40 or IGHV3-11:IGLV1-40 gene pairing and require little affinity maturation for neutralization of HRSV [138]. Cross-neutralization of HMPV requires affinity maturation of MPE8's light chain [134, 136]. Germline-encoded features in the HCDR1 and HCDR2 have been shown to contribute significantly to binding and distinct HCDR3s are tolerated to achieve

cross-neutralization [126, 136, 137]. These findings have made the elicitation of pneumovirus cross-neutralizing Abs an attractive target of more recent vaccine development efforts.

2.2.2 Human immunodeficiency virus type 1 (HIV-1)

In the first part of my thesis (**Paper I**), a vaccine candidate to the human immunodeficiency virus type 1 (HIV-1) was used. HIV-1 is an enveloped positive-stranded RNA virus of the *Lentivirus* genus of the *Retroviridae* family of the order *Ortervirales*. HIV-1 is one of the two causative agents, along with HIV-2, of acquired immunodeficiency syndrome (AIDS) [139]. The infection is primarily sexually transmitted but non-sexual transmission can also occur through pregnancy and childbirth or intravenous drug use. HIV/AIDS is a pandemic that has claimed an estimated 40 million lives since its discovery in the 1980's and where 38 million people currently live with the disease, 1.5 million people acquire HIV every year, and around 650,000 deaths are estimated annually [140]. Effective antiretroviral treatments exist today as well as pre- and post-exposure prophylaxis. Efforts to develop an HIV-1 vaccine have been numerous but unsuccessful to date, in part because of the viral diversity generated from the extremely error-prone replication mediated by the viral enzyme reverse transcriptase. The vaccine development efforts over the past nearly 40 years have been credited for pushing the forefront of vaccine technologies (see chapter 2.3.1 on Vaccine development) [141].

2.2.2.1 The HIV-1 envelope (Env) glycoprotein

The envelope (Env) glycoprotein is a metastable class I fusion protein and the sole viral protein on the surface of HIV-1 virions [142]. Hence, it is also the sole target of neutralizing Abs and of particular interest for vaccine development. The Env glycoprotein uses extensive glycan- and conformational-shielding to evade host immune responses. Additionally, the highly error-prone replication of HIV-1 has generated an immense diversity of circulating Env variants that pose a significant challenge for the development of neutralizing Ab responses. The metastable nature of Env is also a form of immune evasion and remains a challenge for vaccine design efforts. Multiple prefusion-stabilized, "native-like", Env trimers have been created using different structure-based antigen design approaches (reviewed in [143]). In **Paper I** of this thesis, a prefusion-stabilized version of the Env trimer was used as a model antigen to study the immune responses induced by different immunization routes.

2.3 VACCINOLOGY

2.3.1 Vaccine development

Traditionally, vaccines have been produced by methods of inactivation or attenuation of pathogens. This approach to develop a vaccine can be simplified into three broad steps, namely identification of the pathogen, inactivation or attenuation of the pathogen, and inoculation with the whole pathogen or parts of it. However, this strategy is no longer the most viable as it is either considered too dangerous for certain highly mutation-prone viruses (e.g. HIV and influenza) or it does not promote a focused enough immune response that is protective (e.g. HRSV). Instead, modern vaccine design combines expertise in genomics, immunology, bioinformatics, and structural biology in a process termed "reverse vaccinology" [144, 145]. This process of vaccine design involves identifying a protein antigen target through sequencing of the pathogen, characterizing a site of vulnerability on the target protein through studies of the human Ab repertoire after natural infection, and stabilization of the target epitope through structure-based protein engineering (reviewed in [146-149]). These processes need to be further combined with appropriate antigen formulation, adjuvant selection, and route of delivery to elicit the desired immune response (Figure 9), which will be discussed below.

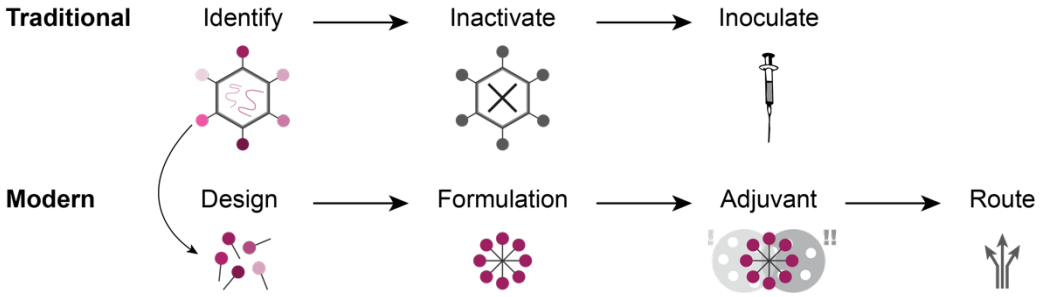

Figure 9. ***Traditional and modern approaches to vaccine development.*** *The traditional approach involves identifying the disease-causing pathogen, inactivating the pathogen, and then inoculating with whole or parts of the inactivated pathogen. The modern approach is more interdisciplinary and reduces the target to protein subunit antigens that contain neutralization sensitive epitopes, the antigens are designed to optimally present these epitopes by structure-guided methods and formulated to improve their recognition by the immune system. The choice of adjuvant and route of immunization can be used to further skew the type and localization of the elicited adaptive immune response.*

2.3.2 Nanoparticle formulation

Protein subunit vaccines are generally considered poorly immunogenic. An increasingly popular avenue of enhancing the adaptive immune response to these antigens is by means of formulation inside or on the surface of a nanoparticle vehicle [13, 150]. Nanoparticles are highly customizable and can be produced in different sizes, multiple synthetic or recombinant materials, and with an array of different antigen densities. They can enhance antigen targeting to and retention within LNs [151-156], APC uptake and presentation to T cells [157, 158], and B cell activation [159-163] (Figure 10). The increased size of particles is an important factor in their enhancement of immune responses as small molecules (<5-10nm) will rapidly diffuse into systemic circulation, while intermediate sized particles (10-100nm) may enter the

lymphatics through multiple mechanisms and thereby traffic more efficiently to LNs [13, 150, 164]. Large particles (>100nm) need APC-mediated uptake and trafficking to target LNs.

With the variety of vaccine platforms available today, multiple aspects can be considered in the selection of the most suitable one, such as the type and quality of the immune response required, the ease of production, and antigen stability during formulation. Synthetic particles include polymer- or lipid-based particles, such as poly(lactic-co-glycolic acid) (PLGA), multilamellar vesicles, micelles, ISCOMs, liposomes, and lipid nanoparticles [165]. Self-assembling protein nanoparticles include virus-like particles (VLPs), virosomes, ferritin, lumazine synthase, and recently also *in silico* designed nanocages [166, 167]. Many of these platforms have not yet been licensed for human use but are currently being tested in clinical trials. Licensed nanoparticle platforms include the VLPs used for the hepatitis B virus and human papillomavirus vaccines. The hepatitis B VLP platform has also successfully been used as a scaffold for display of heterologous antigens, with the licensed malaria vaccine RTS,S employing it. Additionally, virosome platforms have also been licensed, with influenza and hepatitis A being examples. Mounting evidence suggests that multivalent display with these platforms, and others, may also have considerable effects on the imprinting of Ab response durability (reviewed in [168]). Nanoparticle formulations therefore offer exciting opportunities to tune and improve vaccine responses. It is thus timely to perform detailed analyses of their mechanisms of action and how they influence the Ab response in terms of both quality and specificity.

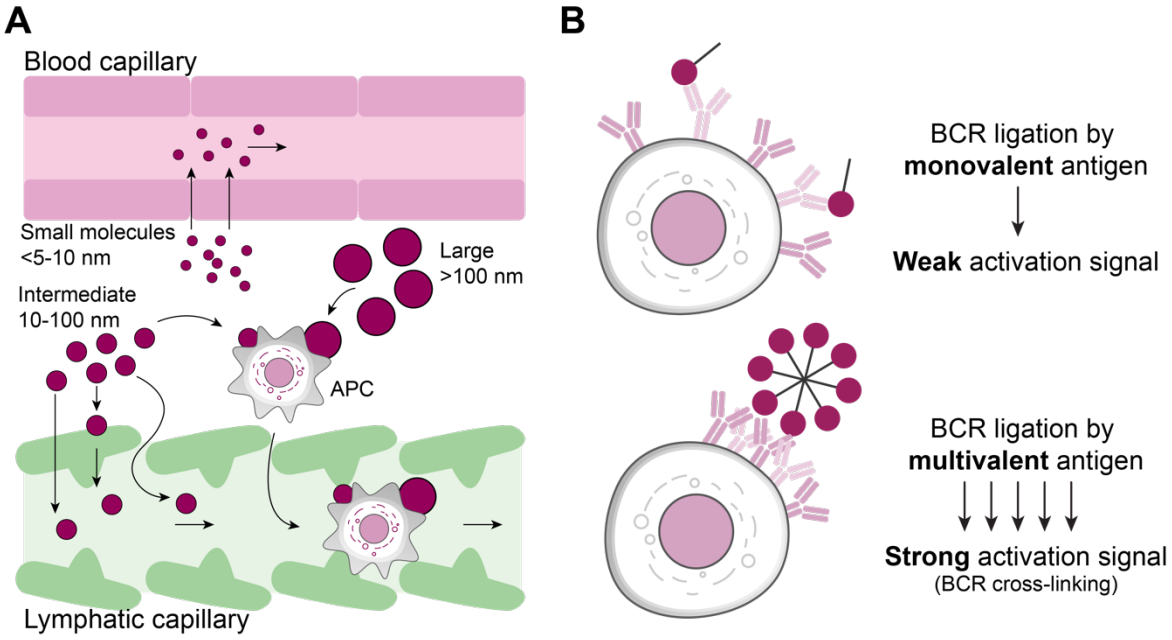

Figure 10. ***The role of nanoparticle formulation in biodistribution and antigen display.*** *A) The size of immunogens governs their transport through blood, the lymphatics, or by cell-mediated uptake and shuttling. B) Multimerization of antigens by display on nanoparticles better activates B cells by cross-linking their BCRs.*

2.3.3 Adjuvants

Adjuvants are components added to vaccines to improve immunogenicity and increase the magnitude of adaptive immune responses. Adjuvants can also influence the type of immune response elicited by their specific activation of innate immunity. The most common adjuvant in licensed vaccines is different variants of aluminum salts (Alum) and these have been used almost since the inception of vaccines [169]. Later, oil-in-water emulsion adjuvants, such as MF59, have also been approved for human use and have strong immune stimulatory activity [170]. However, the mechanism of action of Alum and MF59 are still not fully understood [171]. With the discovery of PRRs and their role in innate immunity, receptors such as the Toll-like receptor (TLR) family have become targets for vaccine adjuvants [4, 171]. By harnessing natural or synthetic ligands of TLRs, the immune response can be tailored due to the select expression on different cell types [172]. Recently, it has also been shown that the immune stimulatory effect of adjuvants, such as TLR agonists, can be modulated and systemic activation reduced by nanoparticle encapsulation [173, 174]. Although the number of available adjuvants has increased, a lack of understanding their mechanism of action and their role in durable adaptive responses, hinders us from making rational adjuvant choices in vaccine development today.

2.3.4 Route of delivery

Route of vaccine administration can have a substantial impact on the elicited immune responses and may therefore be considered during vaccine development. Routes are broadly classified as enteral, administered through the gastrointestinal tract, or parenteral, not administered by the enteral route. Several licensed vaccines are administered today by the oral (enteral) route, including vaccines against cholera, polio, rotavirus, and typhoid [175]. However, parenteral routes are more commonly used in clinical vaccination settings, such as intramuscular (IM), intradermal (ID), and subcutaneous (SC) delivery (Figure 11). Additionally, intravenous and intraperitoneal administration are popular in small animal models but are rare in clinical practice for vaccination in humans. IM administration is the standard route for most licensed vaccines, with a good safety and immunogenicity profile as well as being easy to perform. Although delivery into the skin has gathered a lot of interest due to its dense network of resident APCs and clinical trials of ID delivery have resulted in higher immune responses and antigen dose-sparing when compared to IM [176, 177]. Similarly, the use of SC delivery has increased with the development of new nanoparticle formulations, but their testing has mainly been conducted in mice. However, mechanistic insights into SC delivery are largely lacking and extensive clinical trial data have demonstrated that there is no significant difference in the adaptive immune responses induced compared to the IM route (reviewed in [177]). As a result, the choice of administration route is motivated by balancing immunogenicity and reactogenicity, which has primarily favored IM delivery to date.

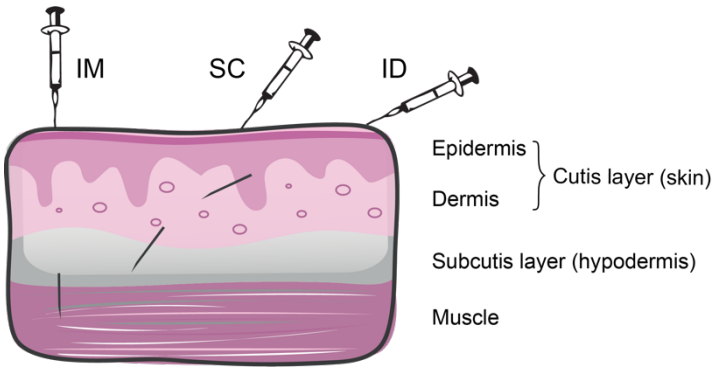

Figure 11. ***Common routes of vaccine administration.*** *IM immunization is an injection into the muscle, SC immunization is an injection into the subcutis layer or the fat under the skin, and ID immunization is an injection into the dermis layer of the skin.*

2.3.5 Models for vaccination

Preclinical evaluation and mechanistic studies of vaccines are predominantly carried out in mice. However, results from mice studies are not always translatable to humans. This may be a result of differences in immune cell subsets [178-180], receptor expression [179], microanatomy [181], Ab repertoire [182], or the route of administration used. In contrast, non-human primates (NHPs) share more similarities with humans in all these aspects and can function as a bridge from mice to human testing. NHPs, such as rhesus macaques, are therefore valuable models that allow for more invasive and regular sampling than humans, thus enabling more complete mechanistic studies of vaccine formulations.

3 RESEARCH AIMS

The main aim of this thesis was to characterize immunological parameters that influence the induction of high-quality and durable Ab responses through vaccination. This included studying how the responses were affected by utilizing nanoparticle vs single antigen vaccines as well as different routes of immunization. The work evolved to address how broad anti-viral Ab responses with cross-reactivity to related viruses are elicited. Tackling viral diversity is a major challenge in vaccine development and much remains to be elucidated on how B cells can be selected and matured to target highly conserved neutralizing epitopes.

The specific aims of this thesis were to:

- Characterize and compare the innate and adaptive immune responses induced by intramuscular and subcutaneous vaccine administration (**Paper I**).
- Assess the Ab responses induced by a novel nanoparticle vaccine candidate displaying multivalent HRSV-F antigen (**Paper II**).
- Gain mechanistic insight into how multivalent display of the HRSV-F antigen modulates B cell responses (**Paper III**).
- Determine the acquisition of breadth in the development of an Ab lineage induced by homologous prime-boost immunization with the nanoparticle HRSV-F vaccine candidate (**Paper IV**).
- Define Ab properties required for pneumovirus cross-neutralization (**Paper IV**).

4 MATERIALS AND METHODS

4.1 ETHICAL CONSIDERATIONS

As mentioned earlier, the work in this thesis is primarily based on experiments with the NHP model. NHPs are important models in biomedical research because of their similarities to humans. However, using animals that are so similar to us raises serious ethical concerns. Their use is therefore strictly regulated and monitored. In our studies we took careful consideration of the "3Rs principle": to Replace NHPs with viable alternatives when feasible (such as *in vitro* systems or smaller animal models), to Reduce the use of animals to the minimum number needed to obtain sufficient results, and to Refine the scientific procedures and study designs as well as the care and treatment of the animals. In practical terms, this meant that all our animals were recycled from other studies, human blood samples were used for *in vitro* assays wherever possible, and if animals were to be euthanized as much sample material as possible was harvested and stored for future analyses. Importantly, our immunizations with fluorescent vaccines (**Papers I and III**) were terminal experiments where we made use of the fact that parenteral administration routes lead to highly localized trafficking [6, 183-185] and therefore all limbs of the animal could be used to maximize data collection and minimize the number of animals needed.

Responses to vaccination are complex and involve multiple tissues and several parts of the immune system. Hence, these responses cannot be properly evaluated in solely reductionistic systems such as *in vitro* assays or cell lines. Animal models allow for an interrogation of the response of the whole immune system to immunization as well as the durability and effectiveness of the response that develops. The primate immune system is very similar to that of humans, which is different in several aspects to that of rodents. The ability to test relevant doses of vaccines and the anatomical similarities between monkey and human is a great advantage to draw conclusions on future clinical evaluation of vaccine candidates. NHPs, such as rhesus macaques, are therefore indispensable for the testing of important vaccines intended to be used in humans.

4.2 SAMPLE MATERIAL

Animal studies were approved by the Local Ethical Committee on Animal Experiments in Stockholm (**Papers I-IV**) or by the Animal Care and Use Committee of the Vaccine Research Center, National Institutes of Health (NIH) (**Paper III**). Indian and Chinese rhesus macaques (*Macaca mulatta*) were housed in the Astrid Fagraeus laboratory at Karolinska Institutet (**Papers I-IV**) or at Bioqual Inc., USA (**Paper III**), according to the guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care.

Human blood samples were obtained from an anonymous sample bank and were collected from healthy blood donors after informed consent in accordance with the declaration of Helsinki.

4.3 IMMUNIZATIONS

In **Paper I**, rhesus macaques were immunized with 50 μg HIV-1 Env native-flexibly linked (NFL) trimer covalently conjugated to liposomes and formulated with 37.5 μg Matrix-M adjuvant (Novavax). Immunizations were given either IM or SC. Fluorescently labeled vaccines were used for *in vivo* tracking of immune cell interactions in tissues. The Env trimers and liposomes were labeled with Alexa Fluor 680 (AF680; Invitrogen) or TopFluor Cholesterol (Avanti Polar Lipids), respectively, according to the manufacturer's protocols.

In **Papers II-IV**, rhesus macaques were immunized with 50 μg HRSV F (DS-Cav1) trimer and formulated in squalene oil-in-water emulsion (SWE) adjuvant (Vaccine Formulation Institute). DS-Cav1 was given either as a soluble trimer or displayed as 20 or 10 copies on the I53-50 nanoparticle by genetic fusion. Immunizations were given IM. For *in vivo* biodistribution experiments the vaccines were labeled with Alexa Fluor 647 (AF647; Invitrogen) according to the manufacturer's protocols.

4.4 RHESUS TISSUE AND BLOOD SAMPLING

Mononuclear cells from peripheral blood (PBMCs) and bone marrow were purified by density-gradient centrifugation using Ficoll-Paque (Cytiva) according to standard protocols (**Papers I-III**). For biodistribution studies (**Papers I and III**), tissues were collected after euthanasia and processed to a single-cell suspension by mechanical disruption and filtering through a 70 μm cell strainer. Injection site tissues (i.e., muscle and skin) were digested with Liberase and DNase treatment, mechanically disrupted, and filtered.

4.5 IMMUNOFLUORESCENCE STAINING OF TISSUE CRYOSECTIONS

In situ immunofluorescence experiments of optimal cutting temperature (OCT) media embedded and snap frozen LN biopsies were used to visualize the spatial localization of fluorescently labeled vaccine (**Paper I**). Glass mounted cryosections were blocked to prevent unspecific signals, permeabilized with a 0.1% saponin solution, and stained with a panel of primary Abs specific for immune cell-specific markers. This was followed by sequential addition of biotinylated secondary Abs specific for the species of the respective primary Abs used, streptavidin-conjugated fluorophores, and extra blocking steps. A tyramide signal amplification (TSA) kit (Invitrogen) was used to boost signal of *in situ* vaccine when detected with the anti-Env Ab VRC01. Cover slips were mounted with Prolong Diamond antifade mounting media and cured overnight. Images were captured using an automated confocal slide scanner (Pannoramic MIDI II FL, 3DHistech) with a Zeiss 20x objective. Accompanying CaseViewer software (3dHistech) was used to prepare images and pseudo-coloring.

4.6 MULTIPARAMETER FLOW CYTOMETRY

Multiparameter flow cytometry was used extensively for the characterization of immune cell subsets, phenotyping of cell activation status, and identification of the presence of fluorescently labeled vaccine or antigen-specificity with fluorescent antigen probes in single-cell suspensions of samples (**Papers I and III**). Cells were incubated with a viability dye, blocked with an FcR-

blocking reagent, and then stained with a panel of fluorescently labeled antibodies. Cells were fixed and permeabilized for intracellular staining before staining with a panel of fluorescently labeled antibodies. Samples were resuspended in 1% formalin solution prior to acquisition on a LSR Fortessa flow cytometer (BD Biosciences). All antibodies were titrated for their specific application and the various panels were optimized before use. Analysis was performed using FlowJo v10 (BD Biosciences).

4.7 T CELL STIMULATIONS

For the assessment of antigen-specific cytokine production by memory T cells, single-cell suspensions were restimulated *in vitro* (**Papers I and III**). Samples were stimulated with overlapping peptide pools (15-mers overlapping by 11 amino acids) of the immunizing antigen (HIV-1 Env or HRSV-F) or with recombinant protein (HIV-1 Env, HRSV-F, or I53-50 nanoparticle). The addition of brefeldin A to the overnight culture, which inhibits protein transport from the endoplasmic reticulum to the Golgi apparatus, ensured that cytokines could be detected by intracellular staining with a multiparameter flow cytometry panel. An unstimulated condition was used for background subtraction of non-specific cytokine production. Restimulation of cells with staphylococcal enterotoxin B (SEB) was used as a positive control.

The antigen-specific T cell response was also quantified through a multi-day proliferation assay (**Paper I**). Single-cell suspensions were labeled with CellTrace Violet (Invitrogen) and restimulated for five days with overlapping peptide pools, recombinant protein, or in media only. T cell proliferation was quantified by dye dilution using flow cytometry. An unstimulated condition was used for assessment of non-specific background proliferation. Restimulation of cells with SEB was used as a positive control.

4.8 B CELL ELISPOT ASSAYS

An enzyme-linked immunospot (ELISpot) assay was used to determine the frequency of two distinct antigen-specific B cell populations (**Papers I and III**). Briefly, ELISpot plates were coated with polyclonal anti-human IgG Abs, single-cell suspensions were added directly to wells for enumeration of ASCs or after a four-day polyclonal stimulation to recall quiescent memory B cells. Cells were cultured overnight in the ELISpot plates and spots were detected with biotinylated antigen probes (Env or DS-Cav1) followed by streptavidin-conjugated alkaline phosphatase and visualized with BCIP/NBT substrate. The plates were imaged, and spots counted, using an AID ELISpot reader. Background subtraction was performed using detection with an irrelevant biotinylated antigen, ovalbumin.

4.9 AB ELISAS

Multiple enzyme-linked immunosorbent assay (ELISA) formats were used and developed for the assessment of the quantity, quality, and specificity of both the polyclonal and monoclonal Ab responses (**Papers I-IV**).

Most Ab ELISAs followed a similar protocol. In brief, ELISA plates were coated with antigen, either through direct coating or HIS-tag capture, and serial dilutions of samples were incubated on the plates. Detection of Abs bound to the antigen-coated plates was carried out with polyclonal anti-human or anti-NHP reagents conjugated with horseradish peroxidase (HRP) followed by sequential incubation with a TMB substrate and then a sulfuric acid stop solution. Absorbance values (optical density; OD) were measured at 450 nm using a Varioskan LUX Multimode Microplate Reader (ThermoFisher). The EC50/ED50 titer, which corresponds to the titer leading to 50% of maximal binding, was calculated for each sample using a four-parameter non-linear regression curve fit.

The addition of a soluble protein antigen competitor in solution during sample incubation was used to quantify the proportions of different Ab specificities through quantification of the reduction in binding in the presence of the competitor. Also, the addition of a chaotropic wash was used to assess the strength of binding or avidity of Abs. A sodium thiocyanate (NaSCN) wash was added at different concentrations after sample incubation to dissociate weakly binding Abs and the reduction in binding was quantified [186, 187].

For epitope binning experiments (**Papers III and IV**), the ELISA protocol was modified further. Instead of directly measuring the amount of bound Abs from a sample, an indirect measurement was performed where the differential binding of a panel of reference monoclonal Abs was quantified in the presence of the sample. In brief, serial dilutions of sample were incubated on antigen-coated plates with the addition of a fixed concentration of biotinylated monoclonal reference Ab of known specificity. The binding of the biotinylated Ab was detected using neutravidin-HRP and its reduction in binding was used to calculate the degree of competition from Abs in the sample. The assay setup was individually optimized for each reference Ab to obtain a satisfactory dynamic range and lower limit of detection, as described previously [188].

4.10 VIRUS NEUTRALIZATION ASSAYS

Virus neutralization assays were used to assess the functionality of Abs in polyclonal serum/plasma (**Papers I-III**) or of recombinant monoclonal Abs (**Papers III and IV**). The IC50 titer, which corresponds to the titer leading to 50% inhibition of infection, was calculated for each sample using a four-parameter non-linear regression curve fit.

In **Paper I**, an HIV-1 Env pseudovirus with a luciferase readout was used in a single round infectious assay with TZM-bl target cells at the Vaccine Research Center, NIH, as previously described [189].

In **Paper II**, a recombinant HRSV A2 virus encoding for the fluorescent protein GFP as a reporter was used with Hep2 cells (ATCC: CCL-23) in a flow cytometry-based assay at the Institute for Research in Biomedicine, as previously described [190].

In **papers III and IV**, high-throughput fluorescence microplate reader-based HRSV and HMPV neutralization assays were optimized and established by me at the Karolinska Institutet

by modification of a previously established high-throughput HRSV assay [191]. For HRSV, a recombinant HRSV A2 virus encoding for the fluorescent protein mKate as a reporter was used with Hep2 cells (ATCC: CCL-23). For HMPV, a recombinant HMPV A2 virus encoding for the fluorescent protein GFP as a reporter was used with LLC-MK2 cells (ATCC: CCL-7). In brief, cells were seeded in 384-well plates the day prior to the experiment and the next day serial dilutions of samples were incubated with a fixed concentration of virus for one hour at 37 °C prior to addition to the cells. Fluorescence endpoints were read after virus incubation with cells at 26 hours with HRSV and at 48 hours with HMPV using a Varioskan LUX Multimode Microplate Reader (ThermoFisher).

4.11 SINGLE-B CELL SORTING, BCR CLONING, AND AB EXPRESSION

To perform a molecular characterization of the monoclonal Ab repertoire we single-cell sorted HRSV-F-specific memory B cells, polymerase chain reaction (PCR) amplified and sequenced their BCRs, and selected a subset of BCRs for recombinant expression as Abs to test their function and specificity (**Papers III and IV**). Frozen PBMCs were thawed, stained with a panel of fluorescent antibodies and HRSV-F antigen probes, and index-sorted at single-cell density into 96-well PCR plates using a BD Aria III Fusion cell sorter. Plates were immediately placed on dry ice and stored at -80 °C. Complementary DNA (cDNA) was retrieved in one step by single-cell reverse transcription (RT-PCR) and then stored at -80 °C for future amplification of BCR variable region transcripts. Variable regions from IgG heavy, lambda, and kappa chains were amplified using nested PCR protocols adapted from Sundling et al. [192, 193], where forward and reverse primers anneal to the leader sequence and constant region, respectively. This enables amplification of the full VDJ segment. The PCR products were purified and sequenced using Sanger sequencing by Genewiz.

Recombinant expression of monoclonal Abs from NHPs was performed by Genscript. In brief, variable regions encoding Abs were sequence optimized and genes were synthesized and cloned into human IgG1, lambda, or kappa expression vectors. CHO cells were transfected with the plasmids for expression and Abs were purified from supernatants using protein A affinity columns. Purified IgG Abs were used as is for characterization of binding, specificity, and function.

Fabs were used for affinity determinations and structural analyses. To digest IgG Abs to Fabs for downstream analyses, the IgG was incubated with endopeptidase Lys-C (Merck) and Fab fragments were purified using spin columns packed with CaptureSelect FcXP affinity matrix (ThermoFisher).

4.12 BULK BCR REPERTOIRE SEQUENCING

Bulk BCR repertoire sequencing was performed on IgM and IgG libraries prepared from frozen PBMC samples (**Papers II and III**). 5-prime multiplex PCR was used to generate the libraries with rhesus macaque specific primers as previously described [26, 194, 195] and the libraries were sequenced by high-throughput sequencing with Illumina MiSeq 2x300-bp. IgM libraries were prepared from pre-immunization PBMC samples of naive animals and IgG libraries were

prepared from PBMC samples from two weeks after the second and third immunizations (i.e., boost 1 and boost 2).

4.13 BCR REPERTOIRE ANALYSES

To gain biological insights from the large amounts of BCR repertoire sequencing data obtained in **Papers III and IV**, we further developed a previously described approach that integrates single-cell and bulk sequencing datasets to find related antigen-specific clonal sequences [195]. This analysis is a multistep process that includes pre-processing (e.g., quality filtering and error correction), V(D)J germline gene annotation, and clonotyping (i.e., grouping by clonal family). We built our analysis pipeline around IgDiscover [25, 195], which can conduct multiple steps of this process, with the addition of custom R scripts for pre-processing of antigen-specific Sanger sequences and for extraction of antigen-specific clonotypes after dataset integration.

Germline gene annotation is a critical part of BCR repertoire analysis and is highly dependent on the accuracy and comprehensiveness of available databases. We utilized IgDiscover and our IgM repertoire sequencing datasets to infer individualized IGHV allele databases for each of our animals, as previously described [25]. Additionally, we also performed gene annotation with previously published rhesus macaque databases for comparison, including the international immunogenetics information system (IMGT) [196], Cirelli et al. [197], and the KI macaque database (KIMDB) [26].

Clonotyping BCR sequences is not trivial as there is currently no consensus on a robust definition for grouping of related sequences. The most common approaches cluster sequences by IGHV allele and IGHJ allele assignment as well as HCDR3 length and a set threshold of HCDR3 homology. This is to reflect that these sequences are descendants of an unmutated common ancestor (UCA) with a specific V(D)J recombination, as the HCDR3 is formed during this process. The homology threshold used for the HCDR3 varies from 80-100% at nucleotide or amino acid level depending on the publications available. We used an 80% amino acid level homology threshold with the addition of the criteria that at least one HCDR3 junction should be identical, as previously described [195].

Lastly, we performed clonotype diversity estimations to draw conclusions on the total HRSV-F-specific BCR repertoire in our animals. Estimations are necessary as it is not possible to accurately sample the full BCR repertoire of an individual. Instead, the abundance of different clonotypes in a sample can be used to estimate how diverse the total repertoire is. We utilized two distinct species richness estimators, Chao1 [198] and Recon [199]. Chao1 uses a non-parametric estimator and Recon uses a maximum-likelihood estimator that assumes a large and well-mixed repertoire. Chao1 tends to underestimate richness while Recon tends to overestimate, hence it is common to use both to get a more accurate representation of species diversity [200, 201].

4.14 BCR TRANSGENIC RAMOS CELLS

A B cell line expressing the Ab D25 as a membrane-bound BCR was generated by lentiviral transduction of surface-IgM-negative RAMOS cells as previously described [202, 203]. In brief, the V(D)J regions of the heavy and light chain of D25 were synthesized and cloned by Genscript into separate expression vectors containing distinct fluorescent reporter genes and the respective consensus human IgM constant regions. These vectors were packaged into lentiviral particles by co-transfection in HEK293T cells from which clarified supernatants were used for co-transduction of RAMOS cells. Cells were enriched by multiple rounds of cell sorting for stable fluorescence from both reporter proteins as well as high surface-IgM expression. For Ca^{2+}-flux experiments, D25 IgM RAMOS cells were stained with FuraRed (ThermoFisher) in serum-free media. Acquisition was performed on a LSR Fortessa flow cytometer (BD Biosciences) and baselines were recorded for each sample before addition of antigens to measure BCR-specific activation. Anti-IgM Ab was used as a positive control and to measure the maximal signal. The FuraRed ratio of bound and unbound calcium over time was used for analysis.

4.15 AB KINETICS EXPERIMENTS

Affinity determinations were performed using biolayer interferometry on an Octet Red 96 system (Sartorius). Antigens were immobilized on anti-Penta-HIS biosensor tips which were dipped in wells containing serial dilutions of Fabs to measure association for 200 seconds followed by measurement of dissociation in wells containing kinetics buffer. Baseline subtraction and fitting of a 1:1 binding model was performed with the accompanying software (Sartorius). Additionally, BLI was used to assess indirect competition with Fab AM14 by saturating tips in competitor Fabs prior to measuring AM14 association. Degree of competition was calculated by comparison with response shift measurements of AM14 association without the presence of the competitor.

4.16 ELECTRON MICROSCOPY

Electron microscopy (EM) was performed at the Institute for Protein Design, University of Washington. Negative-stain EM (nsEM) was used to obtain low-resolution structures of vaccine components to verify structural integrity (**Papers II and III**) and of mAb:antigen complexes to corroborate epitope binning experiments (**Papers III and IV**). In brief, for nsEM, samples were applied to thin layer carbon grids with 2% uranyl formate as previously described [204]. Data were collected on an Talos L120C 120kV electron microscope. A total of ~350 images were collected per sample. For cryogenic EM (cryoEM), complexed SC-DM/LOR24 Fab was applied to glow-discharged holey carbon grids and vitrified. Data collection was performed on an FEI Titan Krios Electron Microscope. Approximately 4000 micrographs were collected. All EM data processing was carried out in CryoSPARC.

4.17 STATISTICAL ANALYSES

The sample sizes are generally small when working with NHPs, as discussed under Ethical Considerations, and therefore careful reflection of the limitations and assumptions of statical tests must be employed. Throughout the work presented in my thesis, two-tailed non-parametric statistical tests were primarily used because the distribution of the data with a small sample size will violate the assumptions of parametric tests. Corrections for multiple comparisons were also employed to minimize the risk for erroneous inferences. Parametric tests were only used when comparing larger datasets, such as BCR sequences, as the assumptions of normality and variance were met.

5 RESULTS & DISCUSSION

5.1 THE SUBCUTANEOUS AND INTRAMUSCULAR ROUTES OF VACCINATION HAVE MAINLY SIMILAR EFFECTS ON THE IMMUNE RESPONSES (PAPER I)

As discussed earlier, the route of immunization can be used to modulate the elicited immune response. Also, the skin, with its dense network of APCs, is a popular target for several novel vaccine candidates including nanoparticle formulations. ID vaccine administration is technically challenging and SC administration is therefore more commonly used in the preclinical development of novel vaccine candidates. The benefits of SC delivery are unclear from vaccine studies in humans [205-212] although recent NHP studies [213] have proposed a significant benefit in the elicitation of neutralizing Ab responses over IM delivery.

The lack of mechanistic insights into SC delivery, such as if the skin or muscle or both are the target of vaccine antigen and inflammation, motivated us to study SC administration and benchmark it against the standard IM route for comparison. Our group had previous experiences of mechanistic biodistribution vaccine studies in the NHP model [6, 183, 185]. Using the NHP model, we could study the early innate immune events at the site of vaccine administration and the biodistribution of vaccines *in vivo* in an animal model with many similarities in physiology and immunology to humans. Hence, the focus of my first study (**Paper I**) was to characterize the early immune events at the site of injection and the vaccine-draining LNs after SC and IM nanoparticle vaccine administration. We studied this using NFL HIV-1 Env trimers displayed on liposome nanoparticles [163, 214] as the model vaccine and fluorescently labeled it to enable visualization of biodistribution and immune cell targeting *in vivo*.

By sampling both the skin and muscle at each injection site, we were able to determine which tissue compartment that was targeted by vaccine-induced inflammation and immune cell recruitment for both the SC and IM routes. We found that vaccine uptake by CD45+ immune cells was strictly restricted to the muscle for IM administration and the skin for SC administration (Figure 12A). This is in line with the IM route having highly localized inflammation at 24 hours, which our group and others have shown for other vaccine modalities as well [6, 183, 215]. The dominant immune cells subsets were monocytes and neutrophils for both routes (Figure 12B). SC immunization targeted a slightly more diverse subset of cells since skin-resident APCs also internalized the vaccine. Importantly, we showed that SC administration resulted in inflammation highly localized to the skin and not visible in the muscle. It remains to be determined if SC administration targets the skin with the same efficiency as ID delivery. Most likely it does not, as vaccine dose sparing has only been reported for ID delivery [176, 177], which is possibly the result of targeting critical skin-resident APCs for antigen trafficking to LNs as suggested in mice [216]. More recently, a comparison of ID, SC, and IM delivery in NHPs with a vector-based vaccine mainly found differences in the early systemic response through transcriptomics analysis, but the site of injection was not extensively analyzed [217]. A head-to-head comparison of early innate

immune events in the skin by ID and SC delivery is warranted to understand the mechanism behind their differential effects on adaptive immune responses.

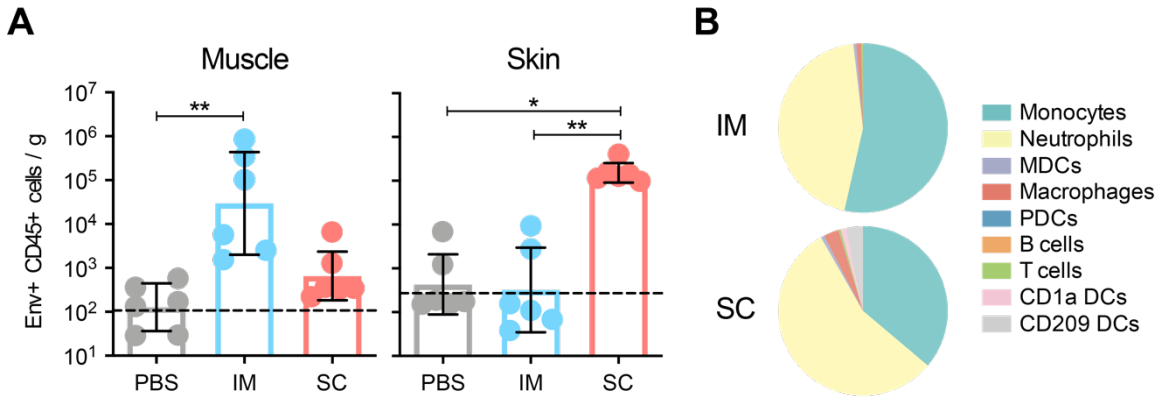

Figure 12. ***Tissue targeting and uptake of vaccine at the site of injection.*** *A) Uptake of fluorescently labeled Env immunogen by CD45+ immune cells in the muscle or skin by route of vaccination. B) Immune cell subset distribution of Env+ cells in muscle after IM delivery and in skin after SC delivery.*

Efficient delivery of vaccine antigen to the LNs is crucial for the induction of strong adaptive immune responses. Thus, important factors to evaluate include both the trafficking of internalized antigen by APCs for the priming of T cells and the lymph drainage of free-floating intact antigen for activation of cognate B cells in LN follicles. We sampled multiple LN clusters draining the immunized limbs (Figure 13A) as biodistribution experiments with Evans blue dye in NHPs had demonstrated that the route of immunization may differentially target LNs [213, 218]. Indeed, we found that detection of vaccine-carrying cells in LN clusters differed between IM and SC delivery (Figure 13B). SC immunization primarily targeted shallow LN clusters located in SC fat (i.e., axillary and inguinal LNs) which we termed primary (1°) LNs because of their relative proximity to the injection site. IM immunization, on the other hand, mainly targeted LN clusters located deeper in tissues and proximal to major veins (i.e., apical and iliac LNs) which we in turn termed secondary (2°) LNs as they were further from the injection site. We also found that the priming of vaccine-specific adaptive immune responses, both T cells and GC B cells as demonstrated by proliferation assays and Env-probing experiments, respectively, were mostly restricted to the respective LN clusters. Subsequent vaccine biodistribution studies in NHPs with IM administration by us (**Paper III**) and others [219] recapitulated the preferential targeting of 2° LNs by this route. It remains to be determined if the LN targeting dynamics observed in NHPs will translate to humans although such evaluation would be challenging. Studies of LN vaccine responses in humans have been limited to the superficial 1° LNs because of the practicality of identification and sampling when utilizing ultrasound-guided fine-needle aspiration (FNA), which is minimally invasive. Vaccine responses have indeed been measurable and sustained in the 1° LNs of IM vaccine recipients [220-224]. With our own data in mind, I can only speculate that potentially more robust responses would be detectable in the respective 2° LNs but this remains to be elucidated.

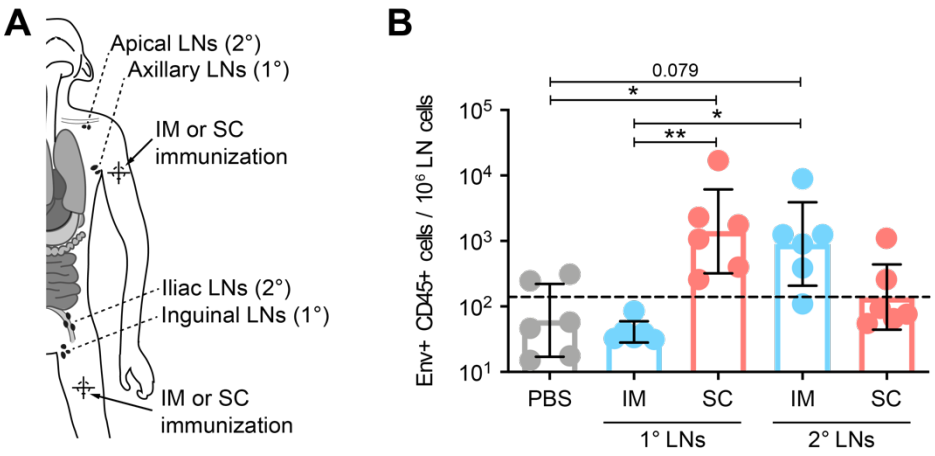

Figure 13. ***LN targeting of vaccine by route of immunization.*** *A) Schematic of sites of immunization and location of 1° and 2° draining LNs. B) Quantification of Env+ CD45+ immune cells in 1° and 2° LNs by route of immunization.*

With most vaccines given as multi-dose regimens or as booster doses to individuals with pre-existing immunity from infection, we wondered if this could influence the trafficking of a vaccine. We found that pre-existing immunity, with high levels of circulating Abs, increased uptake of vaccine and the activation of innate immune cells at the site of injection. We speculate that this is primarily through immune complex formation of antigen and Abs and consequent Fc-receptor engagement on infiltrating innate immune cells. Transport to LNs was not drastically altered at 24 hours, although a detectable reduction after IM delivery could be indicative of a change in draining kinetics. Our study was limited in its assessment to a singular time point after vaccine administration and thus it is difficult to conclude if these effects would be beneficial or unfavorable to the boosting of adaptive immune responses. In NHP experiment by others, boosting and passive Ab administration both resulted in increased deposition of fluorescently labeled antigen in LN follicles at two and seven days after immunization [225, 226]. Passive Ab administration even had a dose-dependent effect [225] from which one could infer that similar biodistribution may be recapitulated when assessing the time interval and waning immunity between booster doses. Thus, the implications of pre-existing immunity for vaccine boosting are far from uncovered and are especially timely with the observations during the SARS-CoV-2 mass-vaccination that extending the time intervals between doses can enhance adaptive immune responses [227, 228].

In conclusion, we primarily observed differences between SC and IM administration in tissue targeting both at the site of injection and for the vaccine-draining LNs. These differences did not translate into a quantifiable difference in adaptive immune responses (i.e., T cells, B cells, and Abs) induced by SC and IM administration, which is in line with what has been reported in many human clinical trials (reviewed in [177, 229, 230]). Ultimately, the clinical practicality, reactogenicity profile, and immune responses from clinical trials favor the IM route over the SC route for vaccine administration. The differential tissue targeting described by us is an important addition to be aware of for the design of vaccine studies that rely on injection site or LN tissue sampling.

5.2 MULTIVALENT DISPLAY OF THE HRSV-F PROTEIN ENHANCES THE MAGNITUDE AND MODULATES THE SPECIFICITY AND DIVERSITY OF THE B CELL RESPONSE (PAPERS II AND III)

The main aim of my thesis was to characterize how Ab and B cell responses can be improved through vaccination. In this regard, repetitive arrays of antigen in vaccine formulations are associated with improved induction of Ab responses. Multiple nanoparticle technologies attempt to harness this effect through multivalent antigen display on their surface, including synthetic nanoparticles and naturally occurring self-assembling protein nanoparticles (reviewed in [166]). The latter has the advantage of being highly ordered and monodisperse structures where protein antigens can be displayed through genetic fusion. Naturally occurring scaffolds have been used to present HRSV-F [231], influenza hemagglutinin [152, 232, 233], HIV-1 Env [234-238], Epstein-Barr virus glycoprotein [239], and coronavirus spike proteins [240-242]. In all cases, improved Ab responses were observed with multivalent display as compared to single 'soluble' antigen and even an effect of elicited Abs to be focused to certain epitopes (epitope-focusing) was observed in some cases [233, 239, 243]. Yet, a mechanistic understanding of multivalent display is largely lacking in the field. In **Papers II and III**, we characterized a novel self-assembling nanoparticle platform for multivalent display and sought to understand the immunological mechanisms for its enhancement of B cell responses.

Only a few naturally occurring scaffolds are available for multivalent display, all with fixed structural properties and spontaneous self-assembly within the cell expression system. This limits the structural and functional space for design and optimization of multivalent immunogens. The development of novel self-assembling nanoparticles through computational design methods [244-247] offers new opportunities to design multivalent immunogens. In **Paper II**, as a proof-of-concept, we showed that the prefusion-stabilized HRSV-F protein (DS-Cav1) can be displayed on the designed two-component self-assembling protein nanoparticle I53-50 through genetic fusion to the trimeric component I53-50A (Figure 14A). The two-component nature of the nanoparticle allowed for *in vitro* assembly and tunable valency of antigen display down from a maximum of 20 copies of HRSV-F trimer (Figure 14B). We found by experiments in mice that the increase in Ab responses was dependent on the display valency of the immunogen and on the assembly of the nanoparticle before immunization. This suggests that both stronger B cell activation through BCR cross-linking and potentially effects on immunogen trafficking and localization through an increase in immunogen size may play important roles [164, 165]. Recently, an elegant study in mice found that antigen valency also dictates the composition of the B cell response and the proportion of lower-affinity B cells recruited into the GC response [248]. To further our understanding of multivalent immunogen-induced B cell responses, we reasoned that these immunological effects warranted investigation in outbred higher-order mammals.

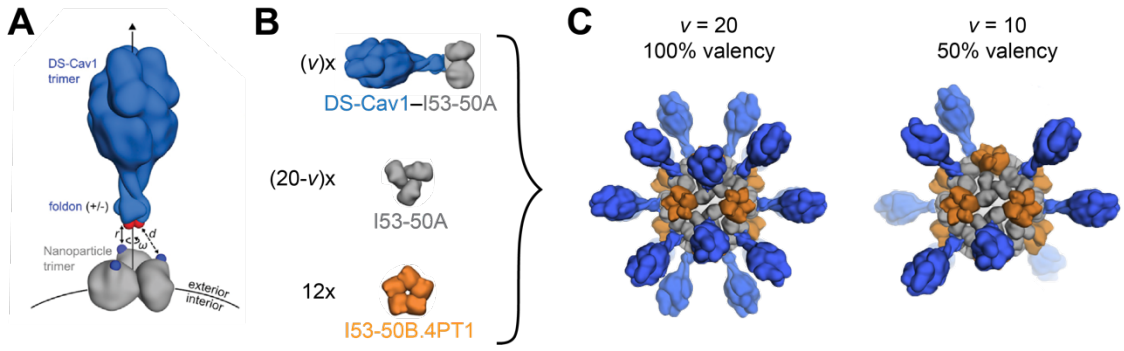

Figure 14. ***Valency of antigen display can be modified in vitro with designed nanoparticle scaffolds.*** *A) Schematic of strategy for fusion of DS-Cav1 antigen to the trimeric building block component of the I53-50 nanoparticle. B) Ratios of components necessary to in vitro assemble the two-component I53-50 nanoparticle. The addition of non-antigen bearing trimeric I53-50A component can be used to decrease the valency (v) of antigen display. C) Depiction of a full (100%) valency nanoparticle displaying 20 copies of DS-Cav1 and a half (50%) valency nanoparticle displaying 10 copies of DS-Cav1. Both these immunogens were used in subsequent NHP experiments.*

We therefore used our NHP model to study the nanoparticle's immunogenicity (**Papers II and III**) and its biodistribution kinetics (**Paper III**) as well as to perform detailed qualitative analyses of the B cell and Ab response (**Paper III**). Prime-boost immunization of NHPs recapitulated the improved Ab responses observed in mice although the differences in magnitude between nanoparticle and single antigen were more subtle and a third immunization diminished the difference further (Figure 15A and B). However, B cell responses both in blood and bone marrow were enhanced in magnitude by nanoparticle immunization as quantified by ELISpot (Figure 15C). This observation also held true across the B cell compartment in multiple tissues. Thus, improvements in magnitude were not solely limited to circulating Abs but also the output of B cell subsets from the GC, suggesting potentially direct effects of multivalent immunogens on B cells.

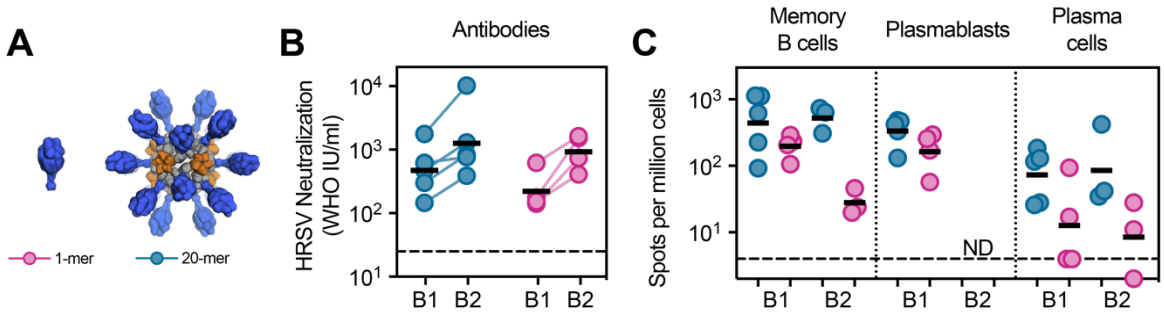

Figure 15. ***Ab and B cell responses induced by a multivalent HRSV-F nanoparticle vaccine.*** *A) NHPs were immunized with either the single 'soluble' DS-Cav1 trimer (1-mer) or with the full valency nanoparticle (20-mer). B) HRSV neutralizing Ab responses in serum of NHPs after boost 1 (B1) and boost 2 (B2). C) Quantification of DS-Cav1-specific memory B cells and plasmablasts in the blood, and plasma cells in the bone marrow by ELISpot. ND = not determined.*

As discussed previously, immunogen size can directly modulate the kinetics of lymphatic drainage and thereby vaccine-elicited Ab responses [249, 250]. We therefore investigated

differences in vaccine biodistribution using fluorescently labeled immunogens as we had similarly performed in **Paper I**. This time we performed longitudinal experiments to capture the kinetics of the early immune response and vaccine trafficking. Lymphatic drainage *in vivo* of labeled immunogens differed significantly at the earliest time points studied (Figure 16). LN targeting followed the dynamics observed in **Paper I**, with the IM delivered immunogens primarily draining to 2° LNs. The quantifiable peak of immunogen signal was at two hours for the single 'soluble' protein while the nanoparticle reached a peak at day three, suggesting that size indeed can delay the transport kinetics and retain antigens longer *in vivo*. This may contribute to the improved immunogenicity observed in NHPs and is reminiscent of slow-release immunization strategies [49, 197].

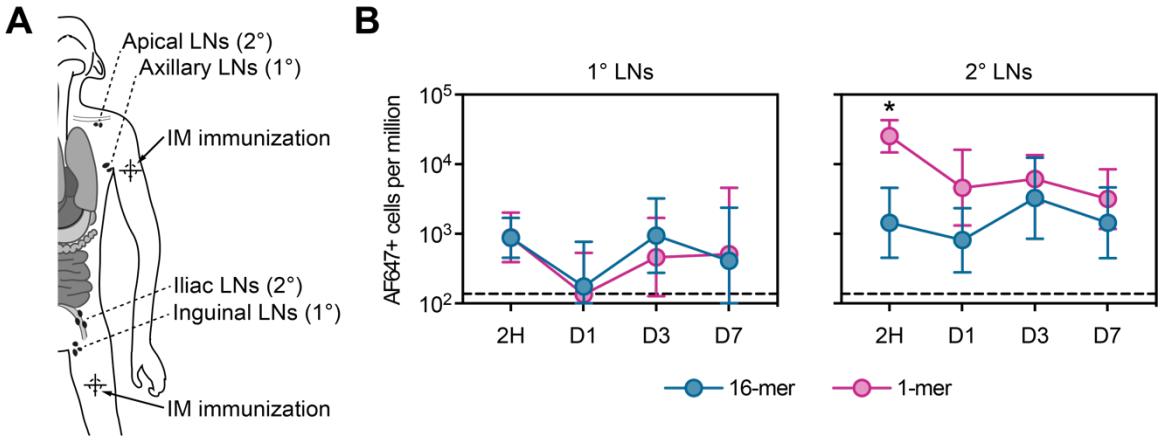

Figure 16. ***LN draining kinetics of multivalent nanoparticle vs single 'soluble' protein.*** *A) Schematic of sites of immunization and location of 1° and 2° draining LNs. B) Quantification of fluorescently labeled vaccine-positive CD45+ immune cells in the LNs at 2 hours, 1 day, 3 days, or 7 days after IM immunization.*

To evaluate the effects of valency, we expanded our NHP study with the addition of a group immunized with a partial valency nanoparticle (i.e., 50% valency or 10 copies of HRSV-F trimer). The magnitude of Ab responses was largely equivalent between the two nanoparticle valencies (Figure 17A) which may indicate that the equal immunogen size may have the largest effect on the magnitude of the B cell response. We reasoned that valency may instead influence Ab specificities by modulating epitope accessibility. Indeed, skewing of Ab epitope reactivity was clear (Figure 17B) and the prefusion/postfusion shared surface area was predominantly targeted in nanoparticle immunized animals (Figure 17C). Unfortunately, the interpretation of these results was complicated by our later discovery that the multivalent nanoparticles co-displayed both the prefusion and postfusion conformations of HRSV-F which could significantly contribute to the observed specificity skewing. This highlighted that utilizing improved prefusion-stabilized constructs of HRSV-F [118, 231] may be a necessity for advancement of a clinically relevant multivalent vaccine with a controlled antigenicity profile. However, we could demonstrate that despite the less than 15% presence of the prefusion HRSV-F conformation, multivalent nanoparticles could better activate transgenic prefusion-specific B cells *in vitro* (Figure 17D). Increased avidity or BCR cross-linking can thereby act directly on B cells to overcome suboptimal antigenicity.

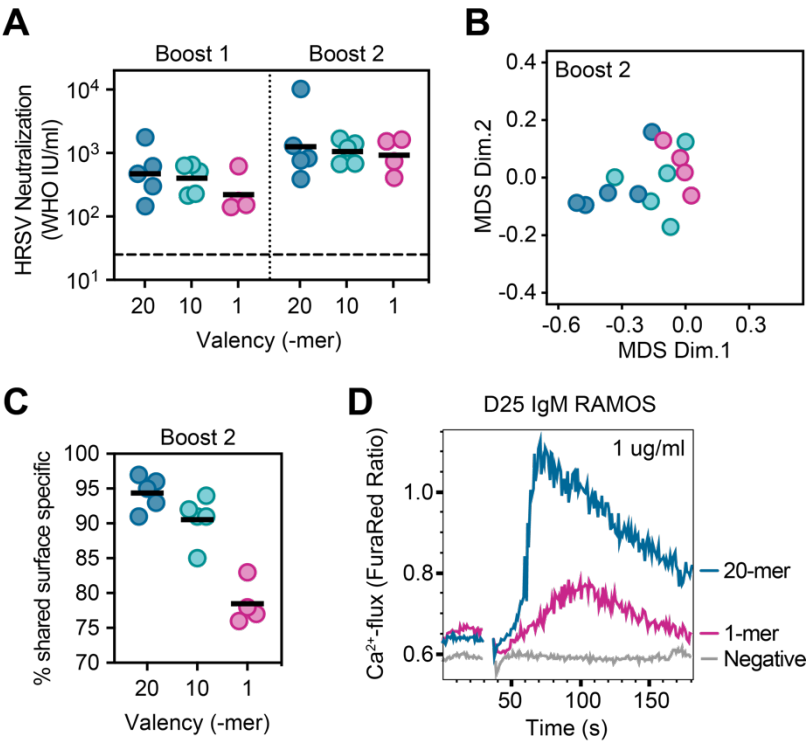

Figure 17. ***Valency skews Ab specificities and enhances B cell activation.*** *A) HRSV neutralization potency of serum samples from different immunogen groups. B) Multidimensional scaling projection of competition ELISA data with reference Abs and plasma samples. C) Proportion of prefusion-specific Abs in plasma that is outcompeted by postfusion competitor. D) Activation of D25 transduced B cells by immunogens in vitro as measured by Ca^{2+}-flux.*

We next analyzed the specificity of circulating memory B cells to decipher the molecular underpinnings of Ab specificity skewing. Staining with separate prefusion and postfusion HRSV-F probes allowed us to quantify the differential proportions of vaccine-specific B cells in the blood (Figure 18A). Not only was the specificity of memory B cells skewed (Figure 18B), similar to Ab responses in plasma, but the increased magnitude in total F-specific B cells was explained by a dominant expansion of prefusion/postfusion shared surface-specific B cells (Figure 18C). At the B cell level, this could be the result of either an oligoclonal response of narrow specificity or a polyclonal response of diverse specificities. To distinguish between these possibilities, we performed BCR sequencing on both F-specific single-cells and bulk

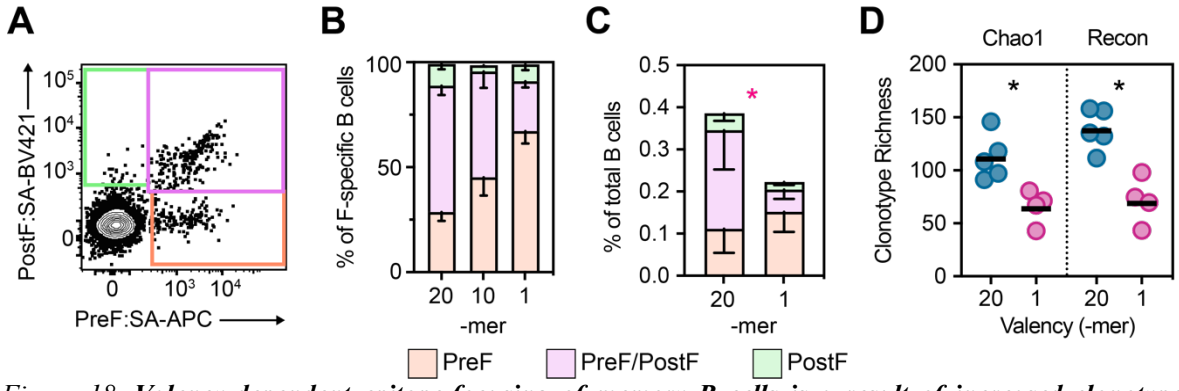

Figure 18. ***Valency-dependent epitope-focusing of memory B cells is a result of increased clonotype recruitment.*** *A) Representative flow cytometry plot of prefusion and postfusion HRSV-F probe binding by memory B cells in PBMCs. B) Proportion of HRSV-F-specific memory B cells reactive to different probe gates in (A). C) Quantification of probe binding as percentage of total B cells. D) Clonotype richness estimated for prefusion/postfusion-specific B cells using Chao1 and Recon. A-D) All analysis performed for boost 2 time point.*

PBMCs to generate an integrated dataset with improved depth and coverage of the receptor repertoire. This approach was necessary to accurately evaluate clonal expansions and clonotype diversity as the single-cell datasets were highly polyclonal. Assessment of clonotype diversity in the integrated datasets using the species richness estimators Chao1 and Recon revealed that B cell focusing was a result of highly polyclonal expansions (Figure 18D). Thus, through lowering of the affinity threshold needed for activation, multivalent display can in an avidity-dependent manner recruit a more diverse pool of B cells into an immune response. This may have implications for the type, potency, and breadth of resulting Ab responses.

We characterized the functional consequences of increased clonotype diversity and simultaneously also verified the specificity of our prefusion/postfusion antigen-specific B cell sorts. Consequently, we characterized 51 monoclonal Abs generated from recombinant expression of a selection of BCRs from our integrated datasets. The specificity of our Abs had high concordance with our sort-defined specificities as assessed by ELISA (Figure 19A) and were mapped to bind the whole antigenic surface of the F protein by epitope binning ELISAs and nsEM. A large fraction (24 of 51) of Abs could not be mapped to a known antigenic site and the nsEM was inconclusive or not attainable because of low Fab affinity to prefusion HRSV-F (SC-DM). We suspect that a considerable fraction of these Abs binds to the trimer interface as this surface may become accessible *in vivo* through trimer 'breathing', degradation, or Ab-induced destabilization. As expected, the HRSV neutralization potency of the Abs followed the established neutralization sensitivity hierarchy described earlier (Figure 19B). The exception was the site II-specific Ab LOR21 that exhibited exceptionally high potency of 21 ng/ml despite its reactivity to the prefusion/postfusion shared surface. Two other Abs, LOR24 and LOR47, were found to cross-neutralize the related pneumovirus HMPV with similar potency to HRSV (Figure 19C). These results suggested that increased clonotype diversity may help shape more potent and broad Ab responses through activation of unique precursor B cells.

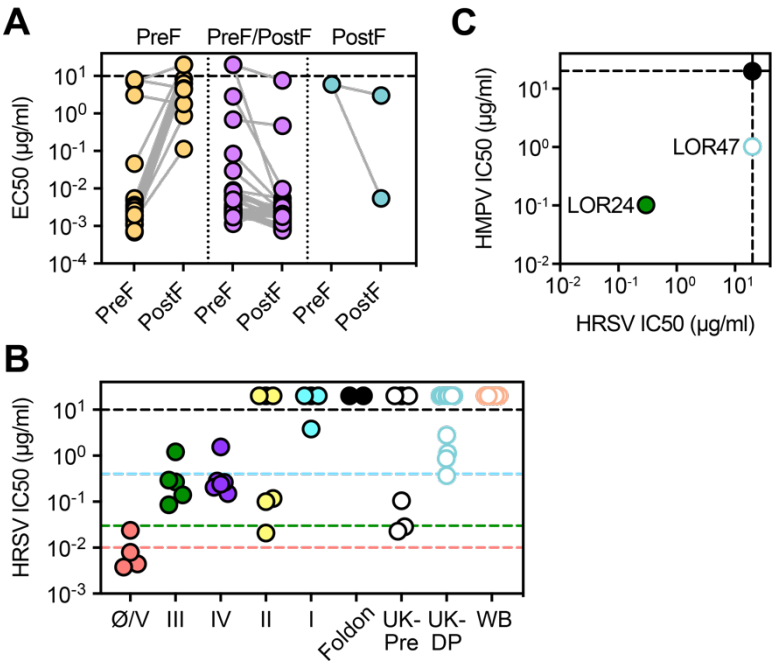

Figure 19. ***Induced monoclonal Abs target the whole antigenic surface of HRSV-F and two cross-neutralize HMPV.*** *A) Binding reactivity to prefusion (PreF) and postfusion (PostF) of 51 characterized LOR Abs grouped by sorting specificity. B) HRSV neutralization potency of LOR Abs by epitope bin. UK = unknown. WB = weak binder. Colored dotted lines denote reference Abs: palivizumab (blue), MPE8 (green), and D25 (red). C) HRSV and HMPV neutralization of the four HMPV-F cross-reactive LOR Abs. Colored by epitope bin as in (B).*

The two Abs with neutralization breadth (LOR24 and LOR47) were both isolated from a single nanoparticle-immunized animal and therefore we tested how frequent such a response was in other animals. Analysis of the polyclonal serum revealed that Ab binding breadth was solely elicited by multivalent nanoparticle immunization, with all nanoparticle-immunized animals having quantifiable anti-HMPV-F Abs (Figure 20A). We also found that the increased clonotype diversity specific to the prefusion/postfusion shared surface area correlated strongly with the acquisition of breadth (Figure 20B). Thus, we hypothesized that increased recruitment of genetically diverse B cell clones, potentially consisting of a wide range of initial affinities, increases the probability that individual clones evolve to develop breadth later. The role of valency in reducing the affinity threshold needed for activation is in line with previous observations of affinity-based clonotype competition being the primary constraint to GC entry [39, 234, 248, 251-253].

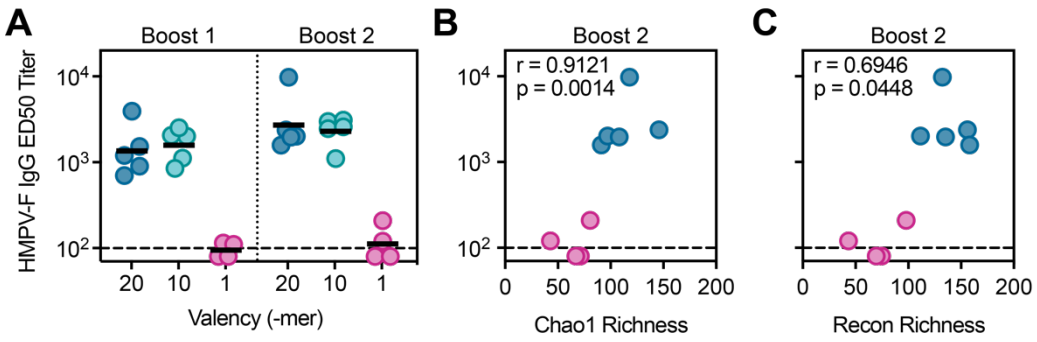

Figure 20. ***HMPV-F cross-binding is exclusively elicited by nanoparticle immunization and correlates with increased B cell clonotype diversity.*** *A) Prefusion HMPV-F binding of plasma IgG from immunized NHPs. B-C) Spearman correlation of HMPV-F binding from (A) with prefusion/postfusion shared surface-specific clonotype richness estimated by Chao1 (B) or Recon (C) at Boost 2.*

In brief, in **Paper II**, we demonstrated that computationally designed protein nanoparticles are functional vaccine candidates for multivalent antigen display and can offer increased control of design parameters over naturally occurring scaffolds. Demonstrating their utility, these scaffolds have now been widely applied for display of diverse viral surface antigens [254-259]. A SARS-CoV-2 vaccine based on this platform [256, 260] has recently reached approval for human use in South Korea [261], further validating the efficacy of the platform. Finally, from our in-depth studies in the NHP model in **Paper III**, we concluded that multivalent display has multifaceted effects on the B cell response, most likely through a combination of trafficking kinetics and broad B cell activation by modulation of BCR avidity. These effects may help improve the elicitation of broad and ultrapotent neutralizing Abs against complex viral antigens through vaccination. While every antigen may offer a unique scenario, I believe that our results offer broad approximations of translational results that can have substantial utility in vaccine design.

5.3 VACCINE-INDUCED MONOCLONAL AB LOR24 REPRESENTS A NOVEL CLASS OF PNEUMOVIRUS CROSS-NEUTRALIZING AB (PAPER IV)

Pneumovirus cross-neutralizing Abs have so far only been identified from adult human donors [126, 134-137] and it is therefore hypothesized that recurrent exposures to HRSV and HMPV throughout life select for these Abs. Thus, from our findings in **Paper III**, it was unclear how cross-neutralization of HMPV could be selected for through homologous prime-boost immunization with a multivalent nanoparticle immunogen expressing only HRSV-F and not any antigen from HMPV. The study in **Paper IV** was therefore born out of my interest to understand Ab evolution, specifically the acquisition of neutralization breadth, in the context of vaccination. We focused our efforts to study the nanoparticle-induced Ab LOR24 as we had mapped its epitope to antigenic site III and we knew it had similar neutralization potency against both HRSV and HMPV. Additionally, through serendipity, we had already expressed the clonally related Ab LOR19 which did not possess cross-neutralization. This offered us a starting point for interrogating the functional evolution of this Ab lineage. To undertake this project, I took inspiration from multiple studies of Ab lineage development in the context of infection [262-265] and vaccination [195, 266, 267] to select my methodology and approach. Importantly, we made sure to use our extensive BCR sequencing datasets from **Paper III** to characterize the development of the LOR24 Ab lineage as well as other potentially similar Abs. Published studies of the well-documented prototypic pneumovirus cross-neutralizing Ab MPE8 [134, 136] were also helpful guidance in our approach.

We constructed maximum-likelihood phylogenetic trees from LOR24-related sequences found in our BCR datasets (Figure 21A). This revealed that the Abs LOR24 and LOR19 had diverged early in their development, a possible explanation for their differential cross-neutralization capacity of HMPV. We also found that a separate nanoparticle-immunized animal had induced an Ab lineage, LOR74, which evolved into a replica of LOR24. This suggests that a conserved evolutionary path may exist to acquire breadth in this Ab lineage. By expressing the Ab of the UCA of the LOR24 lineage as well as single-chain germline reversions of LOR24, among other Ab variants, we could interrogate the relative contribution of the heavy and light chains in the acquisition of breadth. HMPV cross-neutralization was non-existent in the UCA (i.e., the naive B cell precursor) of LOR24 and the reversion of the heavy chain to germline was also sufficient to abolish cross-neutralization (Figure 21B). These results focused our analyses to the mutational landscape of the mature LOR24 heavy chain and its longitudinal development. Specifically, our focus fell on the HCDR loops as they make up the binding interface of the Fab. LOR24 and LOR19 differed only in their acquisition of substitutions in the HCDR2 and some surrounding residues. A switch of this region between the two Abs was sufficient to transfer cross-neutralization to LOR19 and abolish it in LOR24 (Figure 21B). Next, we inferred the Ab intermediates in the evolution of the LOR24 branch and expressed Ab variants with stepwise substitutions in the HCDR2 to dissect the time point of acquisition of breadth. HMPV cross-neutralization was achieved with the T56Y substitution in intermediate I4 (Figure 21C). This highlights the importance of the tyrosine at position 56, which is lacking in LOR19, but it remains to be elucidated if it is sufficient on its own or if the substitutions at positions 57 and

58 acquired earlier are required in concert. The importance of the shared mutational landscape between LOR24 and LOR19, including HCDR1 and FR3 substitutions, also remains to be investigated.

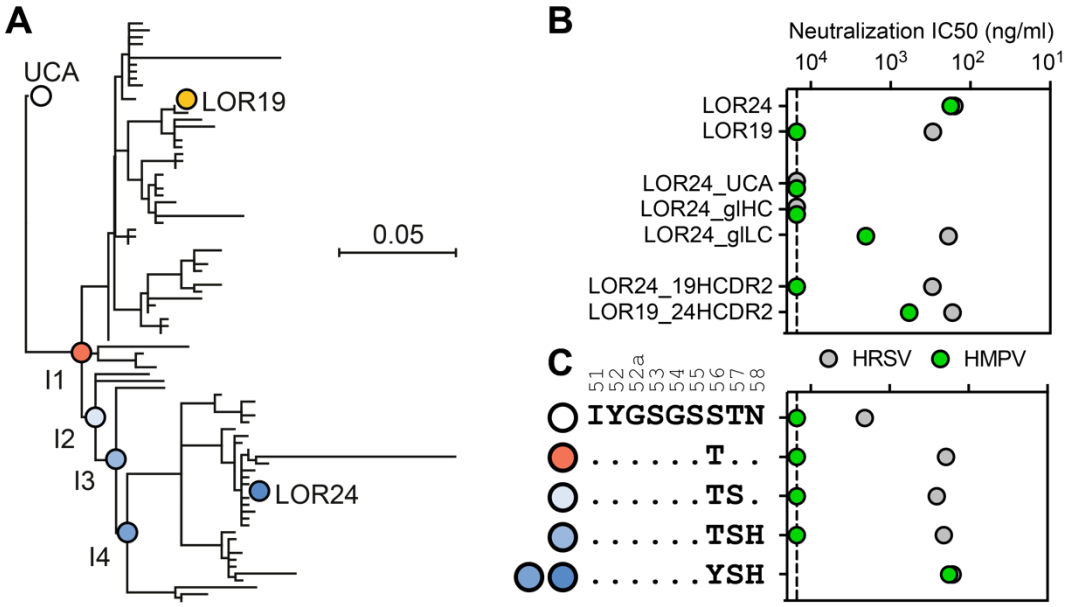

Figure 21. ***LOR24 Ab lineage acquired HMPV cross-neutralization through affinity maturation of the HCDR2.*** *A) Maximum-likelihood phylogenetic tree of heavy chain sequences of the LOR24/LOR19 clonotype. B) HRSV and HMPV neutralization of LOR24 and LOR19 as well as germline reverted variants and HCDR2 switch variants. C) HRSV and HMPV neutralization of Ab LOR24 with HCDR2 substitutions mirroring inferred evolutionary intermediates in (A).*

To gain further insight into the binding interface of LOR24 and its critical binding interactions we employed a structural approach. Using cryo-EM, we imaged the complex of LOR24 Fab with prefusion HRSV-F (SC-DM) to obtain a 3.1 Å map (Figure 22A). Unfortunately, insufficient resolution of the binding interface was obtained to confidently assess side-chain interactions. This was most likely the result of a preferred orientation of the complex on the grid. Future imaging using tilted grids will hopefully give us a better resolution of the interface. Utilizing a structure-guided approach we aim to further interrogate which residues of the LOR24 Ab lineage are critical for its neutralization breadth.

However, with the cryo-EM map we obtained, we could compare the binding angle and approach of LOR24 in comparison to other HRSV-F-specific Abs with published structural information, including MPE8 (Figure 22B). This analysis revealed that LOR24 and MPE8 bind an overlapping epitope as suspected, antigenic site III, but that LOR24 differs primarily in its binding approach (i.e., angle to the antigen) and thereby their binding footprint. It will be important to elucidate if this novel binding footprint explains the lower potency of LOR24 in comparison to MPE8.

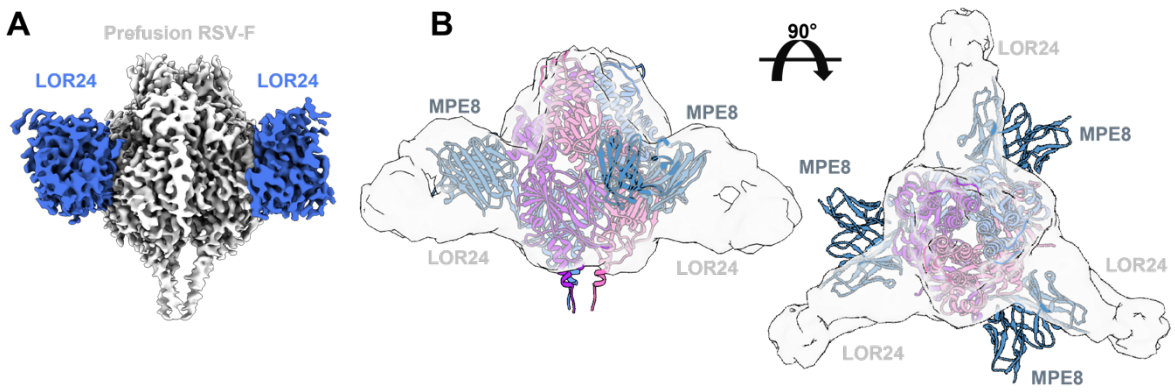

Figure 22. ***Structural basis for LOR24 binding to prefusion HRSV-F antigenic site III.*** *A) 3.1 Å cryo-EM map of LOR24 Fab complexed with prefusion HRSV-F (SC-DM). B) Superimposition of cryo-EM map from (A) with published structure of MPE8 bound to prefusion HRSV-F (PDB: 5U68).*

During our early attempts at structurally characterizing the LOR24 Fab complex with prefusion HRSV-F (DS-Cav1) by nsEM we noticed a heterogenous population of complexes. A considerable amount of the particles resembled monomeric F subunits that were tethered together by the foldon trimerization domain (Figure 23A). This was reminiscent of the trimer destabilization caused by the antigenic site V-specific antibody CR9501 [133]. We therefore designed a sequential binding experiment to assess LOR24-mediated trimer destabilization using the Ab AM14 which binds a quaternary epitope of trimeric prefusion HRSV-F [128] and does not directly compete with LOR24 for epitope binding. Saturation of prefusion HRSV-F (DS-Cav1) with LOR24 reduced binding of AM14 as measured by BLI (Figure 23B). This suggests that LOR24 does indeed disrupt trimerization of the F protein and thereby inhibits binding of AM14. Trimer destabilization could be a potential mechanism of pneumovirus neutralization. It is unclear if induced destabilization also triggers the conformational change to the postfusion state as has been described for certain Abs against the HIV-1 Env glycoprotein [268, 269] and the SARS-CoV Spike glycoprotein [270].

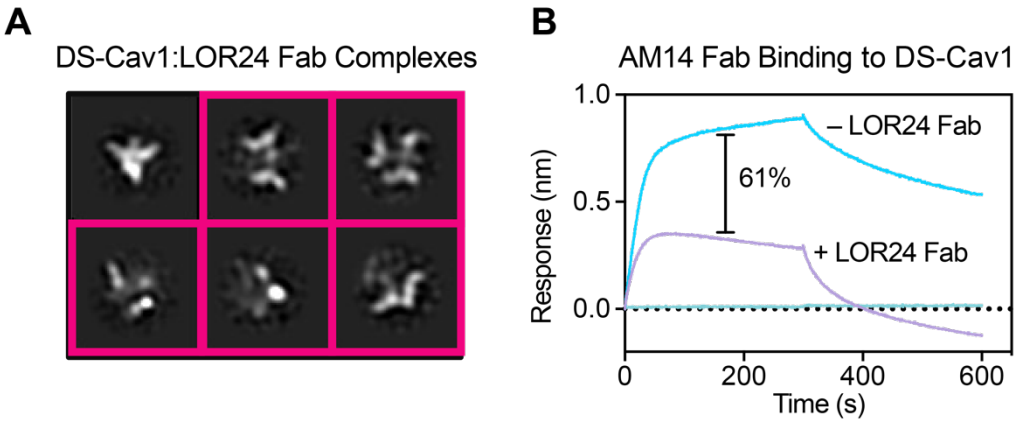

Figure 23. ***LOR24-mediated destabilization of the prefusion HRSV-F trimer.*** *A) Representative two-dimensional class averages of prefusion HRSV-F (DS-Cav1) complexed with LOR24 Fab showing prominent trimer destabilization (pink boxes). B) Saturation of prefusion HRSV-F (DS-Cav1) with LOR24 Fab shows 61% reduction in AM14 Fab binding (purple line) compared to AM14 alone (blue line) by BLI.*

Lastly, we hypothesized that we could make the clonotype definition of LOR24 wider to identify other Abs in our sequence datasets that may possess LOR24-like properties. Our strategy to define a potential LOR24 Ab class focused on IGHV allele homology, specifically HCDR1 and HCDR2 identity. Through this approach we identified 172 clonotypes with natively paired light chains from our immunized animals of which we expressed eight for characterization. Three of the eight Abs (LOR69, LOR72, and LOR73) had a similar competition profile as LOR24 (Figure 24A) and had a comparable binding approach as assessed by nsEM. These three Abs had diverse HCDR3s and light chain usage which validated our wider definition approach. None of the Abs could bind HMPV-F but we found that cross-binding could be transferred to Ab LOR69 by switching the HCDR2 to that of LOR24 (Figure 24B). HMPV cross-neutralization was not acquired by this change (Figure 24C), suggesting that additional residues critical for LOR24's breadth remain to identified.

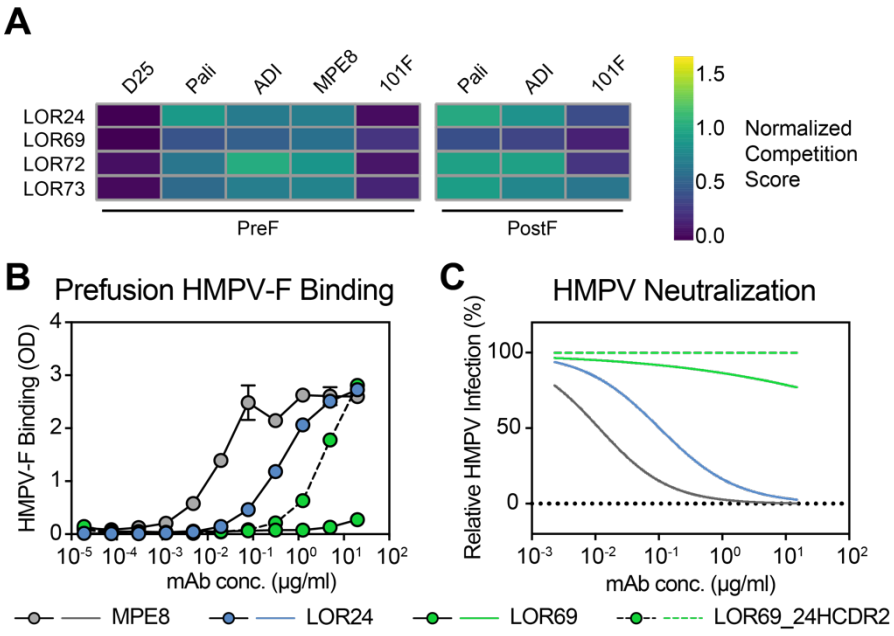

Figure 24. ***LOR24-like Abs can acquire binding breadth through additional affinity maturation.*** *A) Heatmap of epitope binning by ELISA using reference mAbs D25 (site Ø), Palivizumab (site II), ADI-14457 (site I), MPE8 (site III), and 101F (site IV). B-C) Addition of LOR24 HCDR2 to Ab LOR69 transfers partial HMPV-F binding (B) but not HMPV neutralization (C).*

In conclusion, I think the combination of taking a deep dive into the development of a specific Ab lineage and at the same time attempting to broaden its definition by identifying homologous Abs proved highly informative. Determining detailed evolutionary pathways for the acquisition of breadth are crucial for the design of epitope-specific germline-targeting immunogens [161, 236, 271-273]. Complementary approaches to study Ab lineages such as mutation probability modelling using mathematical models of AID targeting [272, 274, 275] and identification of improbable mutations can help elucidate the evolutionary bottlenecks in the elicitation of broadly neutralizing Abs. Also, the reductionist approach of additive substitutions to the UCA Ab for functional testing of the minimally viable Ab [262, 276] aligns well with our approach of performing reversions or substitutions on the mature Ab. The combination of both approaches is labor intensive but has been successfully used by others [267]. Ultimately, the

capacity to elicit a LOR24-like response in humans needs to be investigated and the precursor frequency of the necessary naive B cells quantified to assess the viability of a targeted vaccine approach [277, 278]. To-date, only the pneumovirus cross-neutralizing human Ab M2B6 has been characterized as using the human orthologue gene IGHV4-59 of LOR24 [137]. A more comprehensive analysis of the pneumovirus cross-reactive B cell repertoire in humans is needed to grasp its prevalence and diversity.

6 CONCLUSIONS

The aim of this thesis was to gain a better understanding of how vaccine modalities shape the quality, breadth, and durability of B cell responses. This involved studies of the early innate immune response at the site of immunization and in the vaccine-draining LNs as well as detailed studies of the development of B cell responses and polyclonal and monoclonal Ab responses.

The key findings from the studies included in this thesis are:

- Intramuscular (IM) and subcutaneous (SC) immunization routes elicit highly similar innate and adaptive immune responses (**Paper I**).
- Immunization route affects which lymph nodes that are primarily targeted by the vaccine (**Paper I**).
- Multivalent display of antigen on a nanoparticle can significantly improve the magnitude and quality of Ab responses in both mice and macaques (**Paper II**).
- Nanoparticle formulations can result in slow antigen trafficking to LNs, most likely through a size increase, which may contribute to retention of antigen and an improved immune response (**Paper III**).
- Epitope-focusing of Abs and B cells can be modulated through multivalent display (**Paper III**).
- Epitope-focusing of B cells is coupled to increased engagement of naive cells leading to increased clonotype diversity of memory cells. Avidity and/or BCR cross-linking due to multivalent display may play a role in this (**Paper III**).
- Abs with broad pneumovirus cross-reactivity can be elicited through homologous prime-boost immunization (**Paper III and IV**).
- The monoclonal Ab LOR24 defines a novel class of site III-specific and potentially pneumovirus cross-reactive Abs that share sequence features (**Paper IV**).
- Destabilization of the trimeric structure of the pneumovirus fusion protein may define the mode of virus neutralization by LOR24 (**Paper IV**).

The results and conclusions presented in this thesis add to our understanding of how B cell responses develop and how they can be modulated by vaccination. These findings offer insights that may improve future vaccine design for the induction of broad anti-viral B cell responses. The constantly changing antigenic landscape of multiple viruses is a challenge to in vaccine design which has been explicitly highlighted by the ongoing SARS-CoV-2 pandemic and its multiple virus variants that can evade humoral immunity. As such, a better vaccine design toolbox is needed with well-understood components that can be assembled through rational vaccine design.

7 FUTURE PERSPECTIVES

This thesis improves our understanding of how anti-viral B cell responses can be modulated by choice of vaccine platform and immunization route, but there are still several open questions related to our mechanistic understanding of the induction of vaccine responses.

With regards to our findings in **Paper I**, it will be important to study other routes than SC and IM immunization in terms of biodistribution, target cells and tissues, and elicited vaccine immunity. A priority should be a better understanding of the induction of mucosal immune responses through vaccination as they may be superior at inhibiting respiratory viral infections (reviewed in [279]). Optimization of intranasal delivery for different vaccine platforms and characterization of the differences between mucosal priming and "prime-and-pull" approaches (i.e., systemic priming and mucosal boosting) will also be needed.

As discussed earlier, the time intervals between vaccine doses remain to be thoroughly assessed. The observations of vaccine-induced GC responses potentially lasting months in humans and NHPs [222, 280] may require us to rethink how vaccine regimens are designed. But first, detailed studies are needed of how vaccination affects ongoing GC responses and the contributions of pre-existing memory and circulating Abs. The improvements in Ab responses elicited by extending dosing intervals [227, 228], as well as the lack of a boosting effect in infection-experienced individuals given two doses four weeks apart [281, 282], exemplify that potentially detrimental effects may exist by timing a vaccine dose incorrectly.

Our understanding of the immunological effects of multivalent antigen display are only just beginning to be elucidated. I strongly believe that designed nanoparticle scaffolds will be the necessary tools to systematically assess the individual contribution of different parameters such as valency, display geometry, and immunogen size. Designed nanoparticle immunogens are the only platform that offer the necessary versatility and modifiability (reviewed in [283]). Importantly, structure-based vaccine design is changing how we design vaccines, and multivalent display on nanoparticles is evidently an important part of structure-based design. Several features of nanoparticle immunogens can influence the magnitude and quality of vaccine-elicited antibodies. More well-controlled studies of these features will be important guides for future nanoparticle vaccine design efforts.

It is also important to consider that multivalent display may not be the best strategy to improve vaccine responses against all viral antigens. We need increased knowledge on whether valency consistently enhances the immune response compared to single 'soluble' antigen to guide future vaccine design. As observed in our study (**Paper III**), there may be diminishing returns in improvements of the magnitude of B cell responses with subsequent immunizations. Since every antigen is unique, a detailed understanding of the antigenic landscape is needed to design multivalent display around the geometric constraints for targeting epitopes of interest. Multimerizing an antigen through display on a scaffold without a regard for the location of sites of vulnerability may explain the mixed results that have been achieved by display of certain antigens, including the highly glycan masked HIV-1 Env trimer [254, 284, 285].

That multivalent display of the HRSV-F antigen could elicit pneumovirus cross-reactive B cell responses was unexpected (**Paper III**). I am tempted to speculate that a higher magnitude of cross-reactive responses could be achieved through the co-display of HRSV-F and HMPV-F on the same nanoparticle. Multivalent co-display of coronavirus and influenza surface antigens have in animal models shown promise in inducing broad Ab responses [241, 242, 258, 286]. Although, a recent study of 'soluble' prefusion HRSV-F (DS-Cav1) immunization in humans showed that HMPV-F cross-reactive memory B cells could be activated and expanded [287]. Multivalent co-display may enhance the activation of cross-reactive B cells relative to a single 'soluble' protein as suggested by Ab epitope-focusing to the influenza hemagglutinin stem in NHPs primed with inactivated virus vaccines [258]. Thus, the benefit of co-display in the setting of pre-existing immune memory remains to be extensively investigated, although epitope-focusing may very well be antigen-dependent as discussed earlier. Co-display of diverse antigen variants has been hypothesized to focus the B cell response on conserved epitopes shared between the displayed antigens. A detailed molecular characterization of the B cell response induced by co-display and the contribution of avidity-dependent activation of diverse B cell clones is still lacking. More studies are needed on how we can harness increasing B cell diversity to stimulate potent and broadly neutralizing Ab responses to also protect against other related viruses or cover variants of virus strains, as exemplified by the continuous emergence of SARS-CoV-2 variants.

One can also speculate that multivalent display may have a potential role as a tool for breaking epitope imprinting or immunodominance. The activation and selection of lower affinity naive B cells by increased avidity due to repetitive antigen display may be beneficial to circumvent the presence of high-affinity memory B cells. Although the concept of original antigenic sin is debated (reviewed in [288]), the generation of codominant Ab epitope-specificities may be beneficial for preventing viral escape from neutralization [289]. Further investigation of vaccine platforms, such as nanoparticles, that can be utilized to broaden Ab reactivities and the molecular diversity of responding B cells is needed.

Lastly, studying Ab lineage development is intriguing, and it will be important to understand its implications for future vaccine design. It remains to be shown if we can use this type of information to know when during an immunization regimen a neutralizing Ab response can be expected and whether certain epitopes should be modified or masked to avoid disadvantageous skewing of elicited Abs. Reductionist approaches such as singular epitope scaffolding [290, 291] to remove off-target responses warrant further investigation in this context. Ultimately, I believe coupling studies of Ab evolution with measurements of epitope:paratope adaptations could provide important insights into the selection processes taking place during affinity maturation in the GC response. This may be a natural next step in improving our understanding of how broad Ab reactivity can be selected for and potentially guide vaccine design to preferentially elicit these responses.

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