

Development of an RSV vaccine for infants derived from anti-idiotypic antibodies

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Abstract

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Respiratory syncytial virus (RSV) is a common pathogen that causes lower respiratory tract infections leading to significant morbidity and mortality at the extremes of age. Although vaccines have recently been approved for use in adults over 60, a vaccine that can prevent infection in the infant population is lacking. Current strategies to protect infants from RSV rely on passive immunity, either by the transfer of neutralizing monoclonal antibodies (mAbs) or maternal vaccination and transplacental antibody transfer. A vaccine that can rapidly elicit protective neutralizing antibodies and establish immunological memory in the infant population would fulfill a critical unmet need. A subset of antibodies that arise from the chromosomally encoded VH3-21/VL1-40 gene pairing are unique in that they are structurally pre-configured to bind to and neutralize RSV without the need to undergo affinity maturation, making them ideal candidates to be elicited through vaccination. Here, I describe a vaccine immunogen derived from anti-idiotypic monoclonal antibodies (ai-mAbs) that can recognize RSV-neutralizing VH3-21/VL1-40 B cell receptors from diverse pools of PBMCs. Additionally, I describe the characterization of a novel knock-in mouse model that expresses a bona fide germline VH3-21/VL1-40 RSV-neutralizing B cell receptor, ADI-19425, and demonstrate the utility of this model to evaluate the immunogenicity of the ai-mAb in pre-clinical vaccine/challenge studies. I found

that immunization with the ai-mAb vaccine elicited robust on-target B cell activation and a durable neutralizing serum response that functioned to reduce RSV lung titers upon viral challenge. Collectively, this data illustrates the efficacy of the ai-mAb as a novel infant RSV vaccine candidate and supports its further evaluation in preclinical studies.

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Chapter 1: Introduction to Respiratory Syncytial Virus – Epidemiology, Pathology, Process of Infection, and Preventative Measures

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RSV Burden of Disease and Epidemiology

Respiratory syncytial virus (RSV) was first identified and isolated from chimpanzees presenting with respiratory illness at Walter Reed Army Institute of Research in the United States in 1955¹. It was isolated from throat materials of an infected animal, and subsequently was identified in human patients who presented with respiratory disease and demonstrated a specific antibody response to the infectious agent^{1,2}. This infectious agent has since been deemed respiratory syncytial virus (RSV) and is an enveloped negative sense single-stranded RNA virus that is within the *Orthopneumovirus* genus of the Pneumoviridae family, closely related to human metapneumovirus (hMPV)³. This virus is transmitted through close contact as well as aerosolized droplets^{4,5}. Though primarily infecting the epithelium of the upper respiratory tract, during severe infection RSV can spread to the small bronchioles of the lower respiratory tract⁶. The host immune response to RSV is characterized by mucus production and inflammation, leading to narrowing of the airways which can be a serious complication for infants, older adults or those with chronic conditions⁷. RSV is the most common cause of acute lower respiratory infection in young children.

RSV is ubiquitous and almost all infants will become infected by the age of 2, contributing to a considerable global disease burden⁸. RSV infections are more prevalent during the winter seasons in temperate climates, similar to the periodicity of influenza, but are present year round in equatorial regions⁹. In the United States, it was estimated that RSV was responsible for 190,000-350,000 hospitalizations from October 1st 2024 through April 26th 2025¹⁰. Global approximations are likely to underrepresent the burden of RSV-related disease

due to lack of reporting; however, it was recently estimated that RSV is responsible for 3.6 million hospitalizations and >100,000 deaths in children under 5 each year¹¹. The RSV disease burden is highest for infants in the first 6 months of life, accounting for a fifth of all infections and almost 40% of all hospitalizations¹¹. While all children will become infected with RSV, those with underlying health conditions including congenital heart disease, lung disease and Down syndrome are predisposed to severe infection and hospitalization¹². Despite this predisposition, children in this population make up a minority of the total group affected by severe disease. The majority of infected children are healthy, full-term infants. A remaining gap in knowledge for RSV disease treatment is identifying which patients are likely to advance to severe lower respiratory tract disease (LRTD), and other than the risk factors mentioned above, there are no known predictors or biomarkers other than age.

Most children over 5 and adults infected with RSV will present with mild cold-like symptoms, yet those with underlying chronic health conditions or those under immunosuppression are at risk for severe infection¹³. While the pathogenesis of disease is widely studied in the pediatric population, less is known about the pathogenesis in older adults, and reinfections are common throughout life. The limitations of immune protection afforded by prior infection represent another current knowledge gap in the RSV research field^{14,15}.

Pathology and Clinical Assessment

RSV transmission is mediated through direct contact via inoculation of large droplets onto exposed mucosal surfaces such as the nose, mouth and eyes^{4,5}. Once within the host mucosal site, RSV will infect differentiated bronchiolar epithelium and type 1 alveolar pneumocytes¹⁶. Lower respiratory tract infection (LRTI) with reduced access to oxygen is common during primary infection due to inflammation and mucus deposition in the small airways (bronchiolitis), and can be particularly problematic for young infants due to their small air passages¹⁶. These aspects are thought to underlie the susceptibility of premature infants or

those with bronchopulmonary dysplasia for severe disease due to airway obstruction. Disease presents as a cough in infants with upper respiratory infection, and may proceed to bronchiolitis, hypoxia, lethargy, apnea, and wheezing following LRTI¹⁷. There are no methods to determine which infants will proceed to severe disease, hampering the establishment of efficacy measures in infant vaccine trials. There are, however, severity scores that have been used to define outcomes of RSV intervention trials¹⁸. Many of these symptoms such as pneumonia and bronchiolitis are diagnosed based on clinical evaluation, but there are both molecular and PCR-based methods for confirming RSV infection as the cause for symptoms¹⁹. Depending on infant disease presentation, certain hospital interventions may be taken to treat RSV-bronchiolitis and pneumonia; however, supportive care, including fluid hydration and respiratory support, is the only therapy for severe disease¹⁷.

Process of Infection and Proteins Involved

RSV is an enveloped negative sense RNA virus that has a genome of ~15 kilobases that transcribes 11 proteins of which three are displayed on its envelope surface: fusion (F), attachment (G) and the small hydrophobic ion channel protein (SH). F and G are the major glycoproteins expressed on the surface of the virion and are much more abundant than the SH protein. F and G have important roles in the process of infection, while SH is largely not incorporated in budding filaments from host cells and is dispensable for entry²⁰. RSV has two distinct subtypes or serotypes, defined as RSV-A and RSV-B²¹. These subtypes are characterized by sequence differences in the G protein which is the most varied structural protein of RSV. The two subtypes will commonly co-circulate, but RSV-A is often predominant²².

The G protein is important for attachment to the host cell surface through interaction with glycosaminoglycans (GAGs), which are unbranched polymers linked to many transmembrane proteins on mammalian cells, mediated largely by the heparin binding domain of G²³. Primary host receptors that interact with the G protein are CX3CR1, heparin sulfate proteoglycans and

CD14-Toll-like receptor 4 (TLR4)²⁴⁻²⁶. Laboratory variants lacking the G and SH proteins have been shown to grow well in culture, providing evidence that the F protein is also involved in attachment²⁷. The G protein can be secreted (sG) when transcription initiates at an alternative start codon. This is followed by a proteolytic event removing the transmembrane portion of the protein, allowing the ectodomain to be secreted from the host cell²⁸. sG has been implicated as an immune evasion mechanism, sequestering G-specific antibodies away from infected host cells or virions themselves and preventing Fc-mediated antiviral activity²⁹. While neutralizing antibodies targeting the G protein have been identified³⁰, the primary immune response targets the F protein, which is the basis for most preventative strategies.

The protein F is a class 1 fusion protein that exists as a trimer and is the main target of neutralizing antibody responses against RSV^{31,32}. Interestingly, while the G protein substantially varies between the two subtypes, the ectodomain sequences of F only differ by ~5% and does not exhibit substantial antigenic drift. The F ectodomain elicits neutralizing antibody responses in infected individuals, supporting the single RSV strain delineation with two predominant subtypes. The fusion protein F exists in two structurally distinct forms, pre-fusion (preF) and post-fusion (postF), and the conversion between these two conformations drives viral-host membrane fusion and facilitates entry into the host cell. To become fusion competent, F must be cleaved by host furin-like proteases to remove a 27 amino acid sequence, liberating the fusion peptide and separating two distinct subunits (F₁ and F₂) that are then linked through two disulfide bonds to generate a protomer^{33,34}. Three protomers then associate into the mature preF trimer where the fusion peptides (at the N-terminus of F₁) are buried within the central cavity. The preF conformation is inherently unstable with a low energy barrier to refold into the postF conformation, evidenced by an increase in postF displayed on virions as a function of time^{35,36}. When preF refolds into postF, the fusion peptides are projected away from the viral membrane as the secondary structure of the F₁ subunit reforms into a long α -helix. If a host

membrane is in close proximity to the virion during this process, the fusion peptides will be inserted into this membrane, followed by continued refolding of F into heptad repeats in F₁ that form a trimer of hairpins bringing the two membranes together, promoting fusion³⁷. The postF conformation is extremely stable and irreversible, and the folding energy liberated by the conversion of pre- to postF overcomes the Gibbs free energy required for cell fusion³⁸. PreF and postF share secondary and tertiary structural elements, however, are antigenically distinct despite the presence of some shared epitopes³⁹⁻⁴¹. The prefusion conformation of F has been found to be the main target of neutralizing antibodies against RSV, and those targeting epitopes specific to the preF conformation are much more potent than those targeting postF or shared epitopes^{32,40,42}.

There are two prevailing theories on the routes of RSV entry into a host cell: cell surface-based entry and endosomal entry following macropinocytosis⁴³. For cell surface based entry, the virion must first bind to the host cell through attachment with the G protein and potentially the F protein as well. The F protein has been found to interact with several host surface receptors such as insulin-like growth factor 1 (IGF1R), nucleolin (NCL), epidermal growth factor receptor (EGFR) and intracellular adhesion molecule 1 (ICAM-1)^{24,44,45}. Following attachment at the cell surface or macropinocytosis, the fusion protein undergoes the process of refolding in proximity with host cell membrane or endosomal membrane, leading to fusion of the virion and release of viral cargo into the cytoplasm⁴⁶. For class 1 fusion proteins, there are multiple mechanisms that trigger refolding: low pH, direct receptor binding and interaction with a second viral glycoprotein. However, there is conflicting evidence for all mechanisms surrounding RSV F refolding^{47,48}. In yet another possible process, preF spontaneously refolds with disregard to specific control mechanisms⁴⁹. This is evidenced by the basal rate of preF refolding into postF over time without any added triggers³⁶. Following fusion, RSV viral RNA is released, and transcription and replication take place in the cytoplasm of infected cells in inclusion bodies^{50,51}. Additionally, an

important mechanisms of cell-to-cell spread of RSV is through the generation of syncytia whereby F on the surface of host cells is sufficient to drive fusion between infected and non-infected cells, and led to the naming of RSV².

Prevention

There has been significant progress over the last two years towards preventing RSV infection, culminating in the approval of three RSV vaccines for individuals 60 years old and over. One of the approved vaccines has been granted implementation in the maternal population with the goal of passing on immunity to neonates through trans-placental transfer of protective IgG antibodies. Additionally, there have been newly approved monoclonal antibodies for the prevention of RSV infection in infants during their first RSV season. However, the history of RSV vaccine development has been fraught with failures, most notably a disastrous infant RSV vaccine clinical trial in the 1960s which led to vaccine-associated enhanced respiratory disease (VAERD). Additionally, as recently as last year an infant RSV vaccine trial carried out by Moderna was paused and subsequently terminated due to safety concerns following multiple infants in the vaccine arm presenting with severe LRTD. Though advances have been made recently, there are still many unanswered questions regarding the safety and implementation of a protective infant vaccine, as well as ways to stimulate lasting protection for adults.

Shortly following the isolation and identification of RSV, the first RSV vaccine clinical trial was held to test a formalin-inactivated version of RSV (FI-RSV) as a candidate in the infant population. During this trial, infants and children were administered one to three doses of FI-RSV vaccine which was immunogenic, resulting in increased binding titers to RSV antigens; however, these antibodies were largely non-neutralizing^{52,53}. During the subsequent RSV season following immunization of these participants, those who were seronegative prior to the start of the trial presented with enhanced respiratory disease. 80% of the infants who received the FI-RSV vaccine required hospitalization and two toddlers died following RSV infection⁵².

This failure set back the RSV infant vaccine field and led to in-depth analysis of the disease pathology and vaccine formulation. One of the factors that is associated with the enhanced respiratory disease (ERD or vaccine associated enhanced respiratory disease, VAERD) was that the FI-RSV displayed largely postF on its surface, and thus mostly elicited a non-neutralizing antibody response^{35,53,54}. These non-neutralizing antibodies are believed to have played a role in immune complex deposition and rampant complement activation in the lungs of infected participants⁵⁵. There was also an exaggerated infiltration of neutrophils and eosinophils in the airways of infected individuals, which has been linked to a type 2 (Th-2) biased response seen in animal infection models using FI-RSV, including heightened levels of IL-4, IL-5, IL-13 and IgE⁵⁶. This failure and the subsequent analysis of this trial has caused the RSV vaccine field to proceed cautiously, and with the marked goals of eliciting neutralizing antibodies and a Th-1 biased response.

While the development of an RSV vaccine has moved slowly, there have been prophylactic agents available for 30 years. The most efficacious interventions to prevent RSV infection for the infant population has been the development of RSV-neutralizing antibody treatments. The first prophylaxis available before the identification of monoclonal antibodies (mAbs) for the prevention of RSV-related LRTI was RSV-IVIG (RespiGam), or the intravenous delivery of RSV-specific polyclonal immunoglobulins⁵⁷. This immunoglobulin infusion, released in 1996, was available to high risk infants as monthly infusions and was efficacious in reducing severe-LRTI as well as hospitalizations. Palivizumab was the first approved monoclonal antibody for the prevention of RSV-related LRTI. It is a humanized (IgG1 isotype) mAb isolated from a murine hybridoma⁵⁸⁻⁶⁰. Palivizumab targets an epitope on site II of the RSV fusion protein, that is present on both preF / postF, and was approved for use in 1998. However, this prophylactic was again only available to high risk infants, such as those born prematurely or with chronic lung and heart diseases and required monthly infusions to maintain efficacy⁵⁸.

Despite this limited scope and intensive use regimen, palivizumab has been the gold standard for infant prophylaxis until very recently, with RSV-IVIG being phased out completely by 2004^{61,62}. The efficacy of palivizumab as well as the previous RSV-IVIG demonstrated the clear necessity and correlate of protection for vaccine strategies to elicit a neutralizing antibody response.

A landmark development underlying recent advances in RSV preventative measures was the engineering of a stabilized preF ectodomain led by Jason McLellan and the Kwong Lab at the Vaccine Research Center in 2013⁴¹. This advancement in protein engineering has given rise to a new class of potentially neutralizing monoclonal antibodies as prophylactics and provides the basis for the newly approved RSV vaccines as well as those in on-going clinical trials. Following structural studies of the preF protein in complex with a neutralizing antibody specific to this conformation³⁹, McLellan and colleagues were able to rationally introduce cystine pairs between protomers as well as cavity filling mutations and a trimerization domain to generate a stable construct that maintained the epitope of the preF-specific antibody from their original study⁴¹. This new preF construct, named DS-Cav1, was able to induce significantly higher neutralizing antibody titers in both mice and non-human primates following immunization when compared to those animals immunized with postF, and subsequently showed similar results in early human clinical trials^{41,63}. This advancement paved the way for the newly available vaccines for older adults and has allowed for the discovery of new antibody treatments.

The structure of RSV preF enabled subsequent structural studies to map neutralizing epitopes on both preF and postF, revealing shared and exclusively displayed epitopes relevant to mAb discovery and vaccine design. This has led to the field designating 6 antigenic sites on the RSV F protein, deemed sites Ø-V⁴⁰. Sites Ø, V and III are exclusively displayed on the preF configuration, with a limited maintenance of the site III epitope present on postF, while sites IV and II are displayed on both configurations of F, and site I is almost exclusively displayed on

postF^{39,40,64}. Additionally, sequencing studies have allowed for the interrogation of conservation of these different sites over the two RSV subtypes as well as contemporarily circulating strains. Work in this field has identified sites III and IV as the most conserved across all sequenced RSV strains^{65,66}. This comprehensive epitope mapping helped to identify sites exclusive to preF such as sites Ø, III and V as the most neutralization sensitive and has helped to inform antibody characterization efforts.

The culmination of over 80 years of RSV vaccine research has seen three vaccines (AREXVY, ABRYSV0 and mRESVIA) for adults 60 years and older gain FDA approval over the last two years, marking a significant milestone in addressing the RSV disease burden in this population⁶⁷⁻⁶⁹. All these vaccines deliver the RSV preF protein, with AREXVY and ABRYSV0 being subunit protein-based vaccines and mRESVIA being an mRNA delivery platform of preF. AREXVY and mRESVIA deliver a stabilized version of preF derived from an RSV-A subtype virus, while ABRYSV0 is a bivalent vaccine delivering preF derived from both RSV-A and -B. Additionally, ABRYSV0 has been approved for use in individuals aged 18-60 at heightened risk for severe disease, such as those who are immune compromised or have chronic health conditions. Despite these advances in RSV vaccine development, a vaccine for the infant population is still lacking.

Currently, there are multiple infant RSV vaccines in clinical development⁷⁰, though they have very recently faced yet another setback. Moderna's RSV trial of mRESVIA led to a marked increase in severe respiratory disease in 5 to 8 month old RSV-naïve participants: ~26% of the vaccinated infants with symptomatic RSV progressed to more severe disease versus 8% in the placebo group⁷¹; reminiscent of VAERD observed in the previous formalin inactivated vaccine. As a result of this finding, Moderna discontinued the Phase I trial in September 2024. Prompted by this finding and subsequent fact gathering, the FDA halted similar studies: "*Currently, enrollment of children <2 years of age and RSV-naïve children 2 through 5 years of age is on*

*hold for all clinical studies of RSV vaccine candidates under U.S. IND*⁷², highlighting the significant obstacles remaining to infant F-based RSV vaccines. In attempting to dissect the cause of the VAERD seen in the RSV-naïve infants who received this vaccine, researchers are still searching for an answer. The mRNA vaccine did stimulate neutralizing antibody responses in immunized infants, as well as a Th-1 biased cellular response, avoiding some of the primary pitfalls of the FI-RSV vaccine trial⁷¹. These results support the idea that there was a mechanism different than in the FI-RSV trial that led to this VAERD manifestation or potentially flaws in the underlying assumptions of the previous cause, and additional research is needed.

While the FDA has paused infant RSV vaccine trials delivering preF, development of live-attenuated and chimeric vaccine candidates has continued. The most advanced vaccine candidate for infants following the Moderna trial failure is a live attenuated RSV vaccine (LAV)^{73,74}. There is an on-going phase 3 trial sponsored by Sanofi of their LAV candidate in infants aged 6 to <22 months old (NCT06252285). Part of the rationale supporting the use of this vaccine platform in infants is that limited viral replication in the respiratory tract and intracellular processing/presentation of RSV antigens would induce a B and T cell response that may avoid the problems previously seen in VAERD¹⁶. Interestingly, the intracellular translation and presentation of preF encoded by the mRNA in the Moderna trial still led to VAERD in RSV-naïve infants, warranting further research into this area. LAV are additionally beneficial because they can be delivered intranasally which could promote local immune responses at the primary point of infection, with the potential to establish tissue resident immunity, and they present of multiple viral antigens leading to more a more diverse B/T cell response. Due to the historical and recent failures of infant RSV vaccines, there is a need for diverse and innovative approaches to elicit protective immunity in this challenging population.

While infant RSV vaccines are still in development, there have been multiple recently approved extended half-life monoclonal antibodies for use as prophylaxis in infants entering

their first RSV season. The first of these was nirsevimab, which is delivered as a single injection for infants under 8 months old and has demonstrated significant efficacy in preventing RSV-related LRTI^{75,76}. This antibody targets site Ø on preF and has displayed enhanced neutralization compared to the site II targeting monoclonal antibody palivizumab. Nirsevimab was approved for use by the FDA in 2023³⁹, followed more recently by clesrovimab, approved for use in 2025⁷⁷. Clesrovimab targets site IV, presented on both pre- and postF and has been harbors mutations in the Fc region that extend serum half-life, like nirsevimab⁷⁸. The added benefit of clesrovimab is that it is approved for use as a single dose regardless of infant weight, simplifying administration, whereas nirsevimab is delivered as a function of infant weight. These newly approved prophylactic antibody treatments come on the heels of a failed monoclonal antibody that was recently in clinical development, suptavumab, which targeted site V on preF but was discontinued after circulating RSV-B subtypes gained a mutation in the antibody binding domain rendering it ineffective at binding and neutralization⁷⁹.

As an additional route of prophylaxis, ABRYSVO has been approved for use in mothers at 32 to 36 weeks of gestational age to prevent RSV-related LRTD in infants from birth through 6 months⁸⁰. Maternal vaccination has the goal of stimulating a neutralizing polyclonal antibody response in immunized mothers who then pass on these protective antibodies to the fetus trans-placentally. In clinical trials, this vaccine demonstrated highest efficacy in preventing severe LRTD in infants at 90 days after birth (81.8%), which declined to 69.4% by 180 days⁸⁰. This data indicates that this treatment may only be efficacious for infants born close to the seasonal window of RSV transmission. The vaccine was less effective at preventing non-severe medically attended RSV-related LRTD, failing the statistical success criterion for this outcome. Moreover, the study excluded mothers that were at risk for preterm birth. While these prophylactic strategies have demonstrated efficacy, especially those which target the pre-fusion protein, an RSV vaccine for infants is still a global health priority.

Challenges to successful infant vaccine design

Development of an effective vaccine in infants is challenging for multiple additional reasons related to immunological immaturity and complications of maternally derived antibodies. The ability of infant B cells to undergo class switching and somatic hypermutation have been shown to correlate directly with age^{81,82}, and maternal antibodies have been shown to have a dramatic impact on infant immune responses to vaccination through epitope masking and antigen clearance via Fc interactions⁸²⁻⁸⁵. While maternal vaccination to protect newborns through transplacental antibody transfer has demonstrated short term protection⁸⁰, it may complicate direct vaccination of infants.

Recent studies have reported conflicting data on the effects of mAb transfer, specifically nirsevimab, on the infant immune response to natural infection or vaccination. An analysis of the phase 2b and phase 3 clinical trials testing the safety and efficacy of nirsevimab found that placebo recipients generated a statistically significant higher binding antibody response to the post-F antigen following diagnostically confirmed RSV infection at day 361 (the end of the study)⁸⁶. Nirsevimab binds an epitope specific to preF, thus analyzing binding responses to postF reveals serum response to natural infection or from maternally transferred antibodies. Accounting for the decay of maternal antibodies, higher responses to post-F in the placebo cohort indicated a larger or more long-lived response to natural infection than in the nirsevimab treatment group⁸⁶. The authors also compared the neutralizing antibody levels in placebo vs. nirsevimab groups from participants that were deemed seropositive to RSV (natural infection – based on a postF binding cutoff) and found them to be largely similar at this timepoint⁸⁶. They claimed that as participants analyzed had undetectable levels of nirsevimab at this timepoint, the comparable neutralizing antibody response in treated vs. placebo groups demonstrated equivalent humoral responses to natural infection⁸⁶. However, there was no data or methodology described to determine if these infants had undetectable nirsevimab levels. Thus, it remains possible that nirsevimab was still present at some low levels in the treatment group

which inflated the neutralizing antibody response measured for these participants. The authors claim that treatment with nirsevimab did not lead to sterilizing immunity and allowed the infants who received this treatment to still respond to the infection and generate a *de novo* neutralizing antibody response. However, considering the deficiencies in their study, more research is warranted to confirm this interpretation.

This phenomenon of mAb interference for *de novo* responses was also investigated as part of the halted phase 1 trial carried out by Moderna testing mRESVIA in RSV naïve infants. A small cohort (C) in this trial was comprised of RSV-naïve infants who had previously received treatment with nirsevimab prior to vaccination with the mRNA vaccine and the vaccine response in this group was compared to those who had not received this treatment⁷². This analysis revealed a striking discrepancy in the neutralizing antibody titers, with the nirsevimab-receiving cohort demonstrating a 0.7- and 1.0-fold rise in geometric mean titers of neutralizing antibodies against RSV-A and B, respectively following one dose of the RSV mRNA vaccine. The same the neutralizing titers rose 60.3- and 19.1-fold for the non-treatment group⁷². This lack of response to mRNA vaccination following treatment with nirsevimab highlights a large obstacle that must be overcome to design a vaccine that can effectively stimulate the infants' immune system to generate a *de novo* protective response in the presence of prophylactic antibodies either transferred maternally or through administration as a monoclonal.

A unique B cell population and germline targeting strategies for vaccine development

Studies characterizing the B cell and antibody responses to RSV in previously infected individuals have repeatedly identified B cell receptors (BCRs) derived from VH3-21/VL1-40 gene pairs that target the preF-specific site III and give rise to potently neutralizing antibodies^{40,81,87,88}. Most interestingly, VH3-21/VL1-40 antibodies lacking somatic hypermutation and capable of neutralizing RSV have been isolated from RSV infected infants, cord blood samples and naïve adult B cells⁸¹. These findings demonstrate the presence of a reproducible B

cell class that can be specifically targeted to generate neutralizing antibodies against RSV without the need for affinity maturation. Targeting this B cell class in an infant vaccine strategy would be particularly advantageous as it would allow for the generation of neutralizing antibodies while circumventing their unique immune environment. Unbiased high-throughput sequencing analysis of the naïve B cell repertoire found this gene pairing at frequencies of ~0.14%, indicating it to be an abundant cell population suitable for germline targeting immunogens^{89,90}.

Germline targeting vaccine strategies involve designing immunogens to stimulate a particular class of B cells that share common lineage features encoded by germline or somatic genes. These strategies rely on the fact that these specific B cell classes can give rise to an advantageous antibody response relevant to a particular pathogen, which may be difficult to target with wild-type or native antigens. This concept has been pioneered and advanced to the clinic in the realm of HIV vaccine development, where a class of broadly neutralizing antibodies (bnAbs) has been identified as VRC01-like, and these share common genetic features⁹¹⁻⁹⁴. Upon discovering this lineage found in multiple HIV+ donors, it was postulated that this class constitutes a public clonotype, that could be stimulated to produce bnAbs in a vaccination scheme with specifically designed immunogens. The problem with targeting this class is that although the highly somatically mutated VRC01 class antibodies readily bind diverse HIV-1 envelope proteins, the unmutated inferred germline precursor B cells do not^{93,94}. This challenge brought forth the idea of designing targeting immunogens to precisely react with unmutated precursors to VRC01 class antibodies, and sequential immunization schemes with more native-like HIV antigens would shepherd these B cells to produce bnAbs^{95,96}. Indeed, preliminary clinical trials using a VRC01 germline targeting immunogen in humans induced priming of on-target precursor bnAbs in 97% of participants (35/36), demonstrating the efficacy of these vaccine strategies⁹⁷. With the advent of this vaccine strategy from the HIV field, germline targeting has been employed for immunogen design in various disease contexts, such as

influenza^{98,99}. The relative success of these germline targeting approach forms a sound rationale for the development of an immunogen specific for the unmutated VH3-21/VL1-40 B cell subset described above to generate a protective RSV vaccine that may be specifically tailored for implementation in the infant population.

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Chapter 2: Targeting RSV-neutralizing B cell receptors with anti-idiotypic antibodies[°]

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Summary

Respiratory Syncytial Virus (RSV) causes lower respiratory tract infections with significant morbidity and mortality at the extremes of age. Vaccines based on the viral fusion protein are approved for adults over 60, but infant protection relies on passive immunity via antibody transfer or maternal vaccination. An infant vaccine that rapidly elicits protective antibodies would fulfill a critical unmet need. Antibodies arising from the VH3-21/VL1-40 gene pairing can neutralize RSV without the need for affinity maturation, making them attractive to target through vaccination. Here, we develop an anti-idiotypic monoclonal antibody (ai-mAb) immunogen that is specific for unmutated VH3-21/VL1-40 B cell receptors (BCRs). The ai-mAb efficiently engages B cells with bona fide target BCRs and does not activate off-target non-neutralizing B cells, unlike recombinant preF protein used in current RSV vaccines. These results establish proof of concept for using an ai-mAb-derived vaccine to target B cells hardwired to produce RSV neutralizing antibodies.

Keywords: respiratory syncytial virus, anti-idiotypic, neutralizing antibodies, vaccines, B cell sorting, germline-targeting

Introduction

Respiratory syncytial virus (RSV) is a common seasonal pathogen which generally causes mild respiratory symptoms in adults but can cause serious lower respiratory infection in infants and the elderly. RSV is responsible for ~60,000 recorded in-hospital deaths annually in children under 5^{1,2} and accounts for a substantial hospitalization burden in infants as well as aged adults^{1,3,4}. In 2023, two RSV vaccines, AREXVY™ (GSK) and ABRYSVO® (Pfizer), were approved for use in adults over 60 years old. Both are protein subunit vaccines based on the viral fusion protein (F). F can adopt a metastable pre-fusion (preF) state and a highly stable post-fusion state (postF). Most neutralizing antibodies in the sera of infected individuals map to preF, highlighting its importance for vaccine design^{5,6}. Epitope mapping of anti-F mAbs has identified distinct antigenic sites termed Ø-V. Some sites such as Ø, III, and V, are only present on preF, while the others are present on both conformations^{5,7-14}. Advances in antigen engineering have led to the development of stabilized preF immunogens¹⁵, which form the basis for AREXVY™ and ABRYSVO®¹⁶.

Despite these important advances in RSV vaccine development, a vaccine for use in infants remains a critical unmet need. Prophylaxis for infants currently relies on passive immunity. Since 1998, the site II-directed monoclonal antibody (mAb) palivizumab, under the commercial name of SYNAGIS® (Sobi), has been available for high-risk infants^{17,18}. More recently, the extended half-life site Ø-specific mAb, nirsevimab, under the commercial name Beyfortus™ (Sanofi), has been approved for all infants under 8 months of age entering their first RSV season^{10,19}.

As an alternative prophylactic strategy, ABRYSVO® is approved for use in pregnant individuals in the final month of gestation with the goal of protecting infants from birth through the first 6 months of age through transplacental transfer of protective antibodies^{16,20}. Prophylactic strategies afford transient protection against lower respiratory tract disease in

infants but are imperfect solutions because protection wanes as these antibodies are cleared. ABRYSSVO® is only administered after 32 weeks of gestation, providing a short window for implementation, and this timing may not be congruent with the seasonal pattern of RSV transmission. High costs prohibit the widespread use of passively transferred mAbs, particularly in low- and middle-income countries. While these prophylactic strategies are welcome tools for the prevention of RSV in the infant population, the development of an active vaccine that can rapidly establish durable protection in infants and toddlers could overcome the limitations of passive immunity.

Prevention of severe RSV infection in infants with palivizumab and nirsevimab suggest that neutralizing antibodies targeting F are important for protection against RSV-mediated disease in infants and indicate that a vaccine should seek to elicit high neutralizing antibody titers. However, raising protective antibodies in infants has historically been fraught with challenges. The earliest RSV vaccine was based on formalin-inactivated whole virus in which F preferentially adopted the post-fusion conformation²¹. Unfortunately, this vaccine formulation led to enhanced respiratory disease in infants which required hospitalization and in some cases led to death²². This manifestation has been attributed in part due to non-neutralizing anti-F antibodies²¹⁻²³. Due to this failure, infant RSV vaccine research has proceeded cautiously.

In addition to issues with formulation, infant vaccination faces unique immunological challenges. For example, the infant immune system is inefficient at generating somatically mutated antibodies^{24,25} which are often required for high affinity binding and/or neutralizing potency²⁶. Moreover, maternal antibodies can interfere with *de novo* immune responses in infants. Maternal antibodies can bind to vaccine antigens and suppress the infant response to immunization which can further complicate vaccination strategies^{27,28}. Indeed, infants tend to elicit higher neutralizing titers following RSV infection when maternal antibody levels are low^{29,30}.

Characterization of monoclonal antibodies elicited by RSV infection in infants has provided critical insight into their humoral responses. The isolation of F-reactive mAbs from infected infants⁹ identified a class of potentially neutralizing mAbs derived from the same antibody variable heavy (VH) and variable light (VL) chain genes: VH3-21 and VL1-40⁹. These VH3-21/VL1-40 mAbs mapped to an epitope on antigenic site III present only on preF^{9,11} and remarkably, were able to potentially neutralize RSV without undergoing affinity maturation.

The crystal structure of an unmutated neutralizing VH3-21/VL1-40 mAb in complex with preF revealed that the heavy chain (HC) contacts are primarily made with the complementarity determining heavy chain region 1 (CDRH1) and CDRH2, which are chromosomally encoded by VH3-21⁹. Similarly, the light chain (LC) contacts are made by the complementarity determining light chain region 1 (CDRL1) and CDRL2 regions encoded by the VL1-40 gene. In addition to infected infants, B cells expressing F-reactive VH3-21/VL1-40 B cell receptors (BCRs) were also found in cord blood and in naïve B cells from healthy adults⁹. Collectively these data indicate that unmutated VH3-21/VL1-40-derived antibodies are a public clonotype that is pre-configured to bind to and neutralize RSV, establishing them as an attractive, ubiquitous vaccine target.

We previously demonstrated that vaccine antigens derived from anti-idiotypic monoclonal antibodies (ai-mAbs) raised against unmutated precursor versions of neutralizing antibodies against HIV-1 and influenza could activate B cells *in vitro* and generate robust on-target B cell and serum responses using knock-in mice *in vivo*³¹⁻³⁵. Given that germline-encoded VH3-21/VL1-40 antibodies are preconfigured to neutralize RSV without the need for somatic mutation, we reasoned that they may be readily elicited by an anti-idiotypic vaccine. To this end, we raised ai-mAbs by immunizing mice with preF-reactive, unmutated VH3-21/VL1-40 RSV-neutralizing mAbs. By integrating binding, B cell sorting, and structural analysis, we identified two ai-mAb candidates. One bound to VH3-21 heavy chains and the other bound to VL1-40 light chains, both with high affinity and specificity. From these, we developed a bispecific ai-mAb that

combined the binding properties of both. The bispecific ai-mAb was able to select for B cells capable of producing RSV-neutralizing antibodies from a diverse pool of naïve B cells *ex vivo* and activate B cells engineered to express relevant target BCRs *in vitro*. These results provide proof of concept that an anti-idiotypic vaccine has the potential to elicit RSV neutralizing antibodies by specifically targeting and activating B cells expressing VH3-21/VL1-40 BCRs that are hard-wired to neutralize RSV.

Results

Isolation of monoclonal anti-idiotypic antibodies against mAbs encoded by VH3-21 and VL1-40 genes.

Hybridomas were generated from mice immunized with a cocktail of three RSV F site III-directed neutralizing mAbs derived from unmutated VH3-21 and VL1-40 genes: ADI-19425, ADI-25532, and ADI-14337⁹ (Fig. S1). Four hybridoma supernatants (2C1, 2C2, 2F1, and 1D3) showed binding to the mAbs used to immunize, and weaker or no binding to negative control mAbs. ai-mAbs were purified and evaluated for their ability to bind to a panel of recombinant mAbs by ELISA (Fig. 1A). The panel included ADI-19425, ADI-25532, and ADI-14337 as well as chimeric antibodies with a VH3-21-encoded heavy chain paired with a non-VL1-40 light chain or a VL1-40-encoded light chain but a non-VH3-21-derived heavy chain, and 37 other anti-EBV and anti-HIV-1 mAbs with diverse VH and VL gene usage³³⁻³⁶. All four ai-mAbs bound to ADI-19425, ADI-25532, and ADI-14337. 2C2, 2C1, and 2F1 bound to chimeric mAbs with a VL1-40 light chain, while 1D3 bound to chimeric mAbs with a VH3-21 heavy chain (Fig.1A). None of the ai-mAbs bound to any of the control antibodies (Fig.1A).

Fluorescently labeled ai-mAbs engage B cells expressing VH3-21 or VL1-40-derived BCRs in pools of naïve B cells

We next sought to determine whether the ai-mAbs secreted by the hybridomas- could recognize naïve B cells expressing bona fide VH3-21/VL1-40 BCRs. 2C1, 2C2, 2F1, and 1D3 ai-mAbs were biotinylated and individually complexed with a streptavidin fluorophore (SA-APC) conjugated to a unique oligonucleotide barcode. To counter-screen against B cells that bind outside of the anti-idiotypic paratope, we included a biotinylated isotype control, iv2³⁵, conjugated to SA-APC with a unique oligonucleotide barcode. 2-3% of naïve B cells stained with each labeled ai-mAb while <0.5% of B cells stained with the isotype control (Fig. 1B). APC⁺ naïve B cells were sorted and single cell VDJ antigen-barcode libraries were generated using paired heavy/light chain next generation sequencing. The paired BCR sequences of cells sorted by each ai-mAb were identified by bioinformatic demultiplexing of the libraries (Fig. S2B & C)³⁷. The percentage of ai-mAb sorted B cells that expressed VH3-21/VL1-40 pairs ranged from ~0.9 to ~5% (Fig. 1C), which is marginally higher than would be expected by chance (0.1-0.35%) based on high-throughput unbiased sequencing of paired BCRs from antigen-inexperienced B cells³⁸.

~95% of B cells sorted with 1D3 as bait expressed VH3-21 heavy chains (Fig. 1D), and ~85-95% of B cells sorted with 2C1, 2C2 and 2F1 expressed VL1-40 encoded light chains (Fig. 1E). Sequencing of the hybridomas revealed that 2C1, 2C2, and 2F1 were all clonal variants. Collectively these results indicate that the epitope of 1D3 is primarily encoded by the VH3-21 gene, and that of 2C1, 2C2 and 2F1 is primarily encoded by the VL1-40 gene.

Crystal structures of ADI-19425 complexed with 1D3 and 2C1

To understand the molecular basis behind the observed interactions between the ai-mAbs and BCRs derived from VH3-21 and VL1-40, we sought to obtain their co-crystal

structures with ADI-19425, a VH3-21/VL1-40-derived RSV neutralizing mAb for which the co-crystal structure with preF has been previously solved⁹. 1D3 and 2C1 were produced as recombinant IgGs and their binding to ADI-19425 antigen binding fragment (Fab) was confirmed by biolayer interferometry (BLI) (K_D = 0.4nM for 1D3 and 1.5 nM for 2C1, Fig. S3A & B). The ai-mAbs were then digested into Fabs and complexed with ADI-19425 Fab to enable structural analysis. We solved the crystal structures of the ADI-19425 Fab in complex with 1D3 Fab at 2.67 Å and ADI-19425 Fab in complex with 2C1 Fab at 2.41 Å (Fig. 2A & C, Fig. S3C-D, Table S1). ADI-19425 binds 1D3 with a total buried surface area (BSA) of ~ 1019 Å², primarily through its heavy chain with ~ 579 Å² (57% of total BSA) from the VH3-21-encoded region and ~ 190 Å² (19% of total BSA) from the CDRH3. The remainder, ~ 208 Å² (20% of total BSA), is contributed by the ADI-19425 VL1-40-encoded germline gene and ~ 42 Å² (4% of total BSA) from the lambda J gene (Fig. 2A & B). ADI-19425 binds 2C1 with a total BSA of ~ 994 Å², ~ 471 Å² (47% of the total BSA) of which is contributed from its VL1-40-encoded germline gene, while ~ 248 Å² (25% of the total BSA) comes from its VH3-21. The CDRH3 contributes ~ 275 Å² (28%) of the total BSA (Fig. 2C & D).

Superposition of the crystal structures showed that both ai-Fabs bound the ADI-19425 head-on with some overlap (Fig. S3C-E). Five ADI-19425 heavy chain residues (Tyr97 – Gly 100A, kabat numbering) and one light chain residue (Tyr91) interact with both ai-Fabs. These structural analyses support the observation that 1D3 and 2C1 preferentially engage BCRs derived from the VH3-21 and VL1-40 genes, respectively.

Generation of a VH3-21/VL1-40 directed bispecific anti-idiotypic mAb

Based on the ai-mAb binding and B cell sorting results (Fig. 1), none of the ai-mAbs show the desired specificity for paired VH3-21/VL1-40 BCRs. To enhance the specificity of these ai-mAbs to target VH3-21/VL1-40 BCR pairs, we sought to engineer a bispecific ai-mAb

that combines the binding properties of 2C1 and 1D3. We cloned the VH and VL sequences of 1D3 and 2C1 into an engineered heterodimeric Fc platform that permits the efficient purification of bispecific mAbs³⁹. The bispecific format contains mutations in the Fc region that lower the isoelectric point (Fig. 3A, yellow stars), promote heterologous Fc pairing (Fig. 3A, red stars), and disrupt binding to Fc receptors (Fig. 3A, green stars). The expression plasmids were co-transfected into 293E cells and purified by protein A-affinity followed by anion exchange chromatography, the latter of which yielded two peaks eluting at 13.1mS (~40mL elution volume) and 24.8 mS (~49.5mL elution volume) (Fig. 3B). We evaluated the binding of each peak, as well as the parental recombinant 2C1 and 1D3 ai-mAbs to the VH3-21/VL1-40 encoded anti-RSV Fab ADI-19425 as well as chimeric Fabs by biolayer interferometry (BLI) (Fig. 3C-F). 2C1 bound ADI-19425 and the ADI-19425LC chimera (Fig. 3C) while 1D3 bound ADI-19425 and the ADI-19425HC chimera (Fig. 3D). The ai-mAb that eluted in the first peak of the anion exchange column bound ADI-19425 and both chimeras, while the ai-mAb from the second peak only bound ADI-19425 and the ADI-19425HC chimera. We therefore conclude that the desired bispecific ai-mAb eluted in the first peak. We carried out subsequent experiments with the desired 2C1-1D3 bispecific ai-mAb from the ~13 mS/m peak.

Fluorescently labeled bispecific ai-mAb efficiently engages bona fide VH3-21/VL1-40 BCRs.

We used the fluorescently labeled bispecific ai-mAb as bait in single B cell sorting experiments (Fig. 4A). Naïve B cells that were bound to the labeled bispecific ai-mAb (Fig. 4A right) were single cell sorted, their VH and VL transcripts were recovered by RT-PCR⁴⁰ and sequenced to determine their gene usage using IMGT/V-QUEST^{41,42}. Sequencing results from three independent sorting experiments, each using PBMC samples from a unique donor were combined for analysis. In total 270 B cells were sorted, yielding 211 productive HCs, 216 productive LCs and 159 productive paired BCR sequences (Fig. 4B). 86% and 44% of the B cells sorted with the bispecific expressed VH3-21 HCs and VL1-40 LCs respectively,

representing 26.2- and 7.6-fold enrichments above the estimated gene frequencies determined by unbiased high-throughput paired sequencing of paired BCRs from naïve B cells from three unique donors³⁸ (Fig. 4B).

Importantly, the bispecific ai-mAb was able to efficiently engage B cells expressing the desired VH3-21/VL1-40 BCR gene pairing. Among ai-mAb-sorted B cells for which paired sequences of the heavy and light chain were recovered, 40% expressed VH3-21/VL1-40 pairs, representing a 207-fold enhancement over the estimated gene frequencies of 0.19% (Fig. 4B)³⁸. This enrichment demonstrates that the bispecific antibody has a high-degree of specificity for natively paired VH3-21/VL1-40 BCRs.

mAbs from Bispecific ai-mAb-sorted VH3-21/VL1-40 B cells cross-react with pre-fusion RSV F

In all, 64 discrete VH3-21/VL1-40 BCR⁺ B cells were recovered over three independent sorting experiments. To determine the functional properties of the BCRs, they were produced as recombinant mAbs. 60 of the 64 paired VH3-21/VL1-40 BCR sequences were successfully expressed as recombinant mAbs and screened for binding to RSV preF (DS-Cav1)¹⁵ and postF⁴³ by BLI. 18/60 or 30% of the sorted germline VH3-21/VL1-40 mAbs bound preF but not postF (Fig. 4C). In agreement with Goodwin et al., we were unable to identify a unique CDHR3 or CDRL3 signature among preF-binding VH3-21/VL1-40 mAbs, except for a slight preference toward usage of glycine, serine, and tyrosine residues at CDRH3 positions 96–100c⁹ (Fig. S5B). There was an enrichment for IGHJ4 gene usage in preF-binding mAbs (83.33%) vs. non-binding mAbs (42.86%, Fig. S5E & F) and a slight enrichment for IGLJ2 gene usage in preF-binding mAbs (61.11%) vs. non-binding mAbs (52.38%, Fig. S5K & L).

The majority of VH3-21/VL1-40 derived RSV-binding mAbs target antigenic site III on RSV preF^{5,9,11,44}. To evaluate whether the VH3-21/VL1-40 mAbs sorted with the bispecific ai-mAb also target this site, competitive binding assays to RSV preF were performed by BLI. 16 of the 18 VH3-21/VL1-40 mAbs that bound RSV preF showed near-complete inhibition by ADI-

14337 binding to preF, consistent with site III binding (Fig. 4D). Two mAbs showed relatively lower inhibition by ADI-14337 for binding to preF, potentially targeting an epitope partially overlapped by that of ADI-14337 (MLR_7 & MLR_19, Fig. 4D). Negative stain electron microscopy analysis of one representative Fab in complex with DS-Cav1, MLR_24, which showed the highest binding signal to preF (Fig. 4C), revealed that it binds preF comparably to ADI-19425 Fab⁹ (Fig. 4E & F). However, we were only able to resolve 1 Fab bound per trimer (Fig. 4E & F, Fig. S6), which differs from the AM22/ADI-19425/PR-DM crystal structure which showed binding of 3 site III Fabs per trimer. Collectively, these data demonstrate that the bispecific ai-mAb is capable of selectively engaging B cells expressing VH3-21/VL1-40 BCRs that recognize a site III epitope present on preF.

Unmutated VH3-21/VL1-40 mAbs sorted with the bispecific ai-mAb neutralize RSV subtypes A and B

The sorted VH3-21/VL1-40 mAbs were evaluated for neutralizing activity against RSV subtypes A (A2) and B (B1) in plaque reduction assays⁴⁴. All preF-binding mAbs were initially tested at 500 µg/mL (Fig. S7A & B). Three, MLR_15, MLR_24 and MLR_55, showed neutralizing activity and were titrated to determine their half-maximal inhibitory concentration (IC₅₀, Fig. 5A & B). MLR_15 exhibited weak (130.6 µg/mL) activity against RSV-A, while MLR_24 was more potent (24.2 µg/mL) (Fig. 5A & B). These mAbs showed a similar hierarchy but were more potent against RSV-B (MLR_15: 21.75 µg/mL; MLR_24: 0.22 µg/mL). MLR_55 weakly neutralized RSV-B (98.2 µg/mL) (Fig. 5A & B). MLR_24 was ~100-fold more potent against RSV-B than RSV-A and it showed comparable potency to other unmutated VH3-21/VL1-40 mAbs ADI-14337 and ADI-19425, and to palivizumab against RSV-B^{9,17} (Fig. 5A & B). In our assay, the state-of-the-art prophylactic D25 (nirsevimab) showed the most potent neutralization, ~500-fold more potent than MLR_24 against RSV-A and ~4.5-fold more potent than MLR_24 against RSV-B (Fig. 5B). Among the population of naïve VH3-21/VL1-40

antibodies sorted with the bispecific ai-mAb, 3 out of 60 (5%) had neutralizing activity against RSV, demonstrating that an anti-idiotypic immunogen can target RSV-neutralizing B cells and further illustrating the feasibility of using this vaccine platform to target defined, neutralizing, B cell lineages.

Binding kinetics of VH3-21/VL1-40 mAbs dictate neutralization potential

We next assessed whether differences in binding strength of the VH3-21/VL1-40 mAbs could discriminate between those that neutralize and those that do not. To this end, their relative steady-state binding to RSV-A preF was measured by BLI. mAbs MLR_15 and MLR_24 demonstrated the highest binding responses over a range of mAb dilutions (Fig. 5C, orange and green), while the MLR_55 response was lower (Fig. 5C, purple). An examination of the dissociation rates (K_D) from this data set revealed that MLR_55 had the slowest dissociation rate of all expressed mAbs (Fig. S7C). From this analysis, the neutralizing mAbs bifurcate from the non-neutralizers based on overall binding avidity and/or slow dissociation rates, indicating that binding kinetics are a key determinant of neutralization potency among VH3-21/VL1-40 mAbs. Interestingly, MLR_15 and MLR_55 utilized the same light chain sequence, suggesting that heavy chain differences accounted for the observed variation in neutralization potency and apparent affinity to RSV preF (Fig. S5M & N).

To further refine the avidity analysis of the neutralizing mAbs (MLR_15, MLR_24, MLR_55), the steady state binding to RSV-B⁴⁵ preF and RSV-A were compared (Fig. 5D, dotted vs. solid line). This analysis revealed that the neutralizing VH3-21/VL1-40 mAbs MLR_24 and MLR_55 bound more strongly to RSV-B preF than to RSV-A preF at every concentration tested (Fig. 5D). At the higher end of the dilution series, MLR_15 showed higher binding to RSV-A preF, but this reversed at the lower end of the dilution series, while the half maximal binding was nearly equivalent for both preF variants (Fig. 5D left), consistent with the smaller discrepancy between the neutralization potency against RSV-A and RSV-B (Fig. 5A & B). We also

determined the apparent affinity (K_D) for each neutralizing mAb against RSV-A / RSV-B preF (Fig. 5E). All mAbs demonstrated higher apparent affinity for RSV-B vs. RSV-A, with MLR_24 showing the greatest difference in apparent affinity (2.8-fold). The stronger binding to RSV-B preF by these mAbs supports their higher potency to neutralize this subtype.

Bispecific ai-mAb selectively activates B cells with desired BCR gene pairing

Previous studies have demonstrated that naïve B cells sorted with an antigen are representative of those that will respond to a matched immunization in humans⁴⁶⁻⁴⁸. Because the bispecific ai-mAb can engage natively paired BCRs with the VH3-21/VL1-40 gene pairing (Fig. 4), we hypothesized that this could be used as an immunogen to activate these target B cells. However, the binding and sorting data (Figs. 3 & 4) also indicate that the bispecific ai-mAb can bind off-target BCRs which have either the target HC or LC, but without the desired pairing. Since B cell activation requires cross-linking of adjacent cell-surface BCRs, we hypothesized that the bispecific ai-mAb would preferentially activate paired target B cells. To test this directly, we generated human B cell lines stably expressing the ADI-19425 BCR, the ADI-19425 HC paired with an irrelevant light chain, or an irrelevant heavy chain paired with the ADI-19425 light chain. The B cell lines were stained with fluorescently labeled 1D3 and 2C1 ai-mAbs, as well as the 1D3/2C1 bispecific ai-mAb (Fig. 6A-C). 1D3 bound to B cells expressing the ADI-19425 BCR (black) and the B cells expressing the ADI-19425HC chimera BCR (blue), while 2C1 bound to the ADI-19425 BCR and the ADI-19425LC BCR chimera (green) (Fig. 6A-C). The bispecific ai-mAb showed bright staining of the ADI-19425 cells (Fig. 6C, black), but only modest staining of the ADI-19425HC chimera cell line (Fig. 6C, blue). The bispecific ai-mAb did not bind the ADI-19425LC chimeric cells, likely because the chimeric BCR lacks contact residues encoded by the VH3-21 heavy chain (Fig. 2C & D), resulting in an increased dissociation rate and weaker binding (Fig. 3C & E).

We next measured the ability of the mono- and bispecific ai-mAbs to activate the BCR-expressing cell lines from Fig. 6A-C using a flow cytometry-based calcium flux assay (Fig. 6D-F)^{49,50}. 1D3 and 2C1 activated cells expressing the ADI-19425 BCR, as well as the VH3-21 and VL1-40 chimeric BCRs, respectively (Fig. 6D & E). In contrast, the bispecific ai-mAb only activated the ADI-19425 cell line (Fig. 6F). None of the ai-mAbs activated DG-75 cells expressing a non-VH3-21/non-VL1-40 control BCR (Fig. 6D-F, grey)³⁵.

To further evaluate the specificity of the bispecific ai-mAb to activate B cells harboring the VH3-21/VL1-40 gene pair, we engineered three additional B cell lines expressing unmutated, non-neutralizing, non-site III, preF-specific BCRs, none of which are derived from VH3-21 or VL1-40 (ADI-19470, ADI-24811, and ADI-25542)⁹. All cell lines, including ADI-19425, were activated by the addition of RSV-A preF or a positive control, however, only ADI-19425 was activated by the addition of the bispecific ai-mAb (Fig. 6G-I). Collectively these data indicate that the bispecific ai-mAb preferentially activates B cells harboring paired VH3-21/VL1-40 BCRs, but not off-target preF-specific non-neutralizing B cells that are activated by currently approved protein subunit vaccines based on preF.

Unmutated VH3-21/VL1-40 mAbs maintain neutralization potency in the presence of preF-binding, non-neutralizing mAbs

The calcium flux experiments demonstrate that the ai-mAb can engage and activate target neutralizing VH3-21/VL1-40 B cells, suggesting that these B cells could respond to immunization with the ai-mAb and secrete neutralizing antibodies. Assuming this is true, the B cell sorting experiments demonstrate that the ai-mAb also readily recognizes non-neutralizing VH3-21/VL1-40 B cells which would similarly respond to immunization. We therefore sought to determine whether the presence of an excess of non-neutralizing VH3-21/VL1-40 mAbs affects the potency of neutralizing VH3-21/VL1-40 mAbs. To test this experimentally, plaque reduction assays were set up against RSV-A and RSV-B where MLR_24 was present at a concentration

equivalent to its IC₅₀ or IC₈₀ along with a combination of 5 non-neutralizing, preF-binding mAbs at equal concentrations. The potency of MLR_24 against RSV-A and RSV-B was unaffected by a 5-fold excess of non-neutralizing preF-binding mAbs (Fig. 7A & B), indicating that the neutralizing activity of VH3-21/VL1-40 mAbs is maintained in the presence of an excess of non-neutralizing, site III-directed VH3-21/VL1-40 mAbs.

Discussion

Here we describe the isolation and characterization of a bispecific molecule derived from anti-idiotypic antibodies designed to target a conserved class of B cells capable of producing antibodies that potently neutralize RSV without undergoing affinity maturation. The bispecific ai-mAb readily engages B cells expressing target BCRs among diverse pools of naïve B cells *ex vivo* and selectively activates target B cells *in vitro*, providing proof of concept that the bispecific ai-mAb could be used to elicit protective antibodies if used as an immunogen.

Our binding and sorting experiments determined that the bispecific ai-mAb can also bind off-target cells, such as those with either a VH3-21 HC or a VL1-40 LC. However, the bispecific design is intended to promote specific BCR cross-linking and activation of target B cells through engagement of a VH3-21 HC with the 1D3 arm and a VL1-40 LC on an adjacent BCR with the 2C1 arm, while enhancing the strength of the binding interaction through avidity. In support of this, the calcium flux assays demonstrate specific activation of B cells expressing on-target BCRs. Moreover, the bispecific ai-mAb failed to activate B cells expressing non-neutralizing F-reactive BCRs that are otherwise activated by a clinically-tested preF vaccine immunogen, highlighting the ability of the bispecific ai-mAb to selectively activate B cells specific for a known neutralizing epitope on F.

Through naïve B cell sorting experiments, we demonstrated that the bispecific ai-mAb readily engages B cells expressing the desired germline-encoded VH3-21/VL1-40 BCRs, and they were enriched >200-fold above the estimated gene pair frequencies present in the naïve B

cell repertoire³⁸. When produced as recombinant mAbs, 30% showed measurable binding to preF while none bound postF. Nearly all of these mAbs mapped to site III, consistent with the binding profile of the VH3-21/VL1-40 antibody class. Among the F-binding VH3-21/VL1-40 mAbs, three (5%) neutralized RSV subtypes A and B, demonstrating that a non-F immunogen can readily target RSV-neutralizing BCRs in relevant samples. A recent study similarly used ai-mAbs to isolate primary B cells capable of producing neutralizing antibodies against influenza, providing additional proof concept that ai-mAbs can be used to target neutralization-competent B cell subsets with defined genetic features⁵¹.

Although only 5% of B cells sorted by the bispecific ai-mAb were neutralizing, based on the frequency of the VH3-21/VL1-40 gene pair in the naïve B cell repertoire, we estimate that the bispecific ai-mAb is capable of engaging 1 neutralizing B cell in ~5,500 to 22,000 B cells³⁸. Given that previous studies have shown that B cells sorted with germline targeting immunogens are representative of those that respond to immunization in humans^{46-48,52}, these data suggest that vaccination with the bispecific ai-mAb will elicit a VH3-21/VL1-40-dominated response which will include RSV-binding and neutralizing antibodies.

Our results demonstrate that not all germline VH3-21/VL1-40 mAbs bind to preF, and of these, only a fraction is capable of neutralizing RSV. This is in agreement with a study by Goodwin et al.⁹ which found that 40% of naïve, preF reactive, VH3-21/VL1-40 (or the related VH3-11/VL1-40) B cells from cord blood or naïve B cells from adults were able to neutralize RSV. The lower percentage observed here may be due to differences in the method used to select cells for analysis: using a preF probe for sorting, vs. targeting the gene pairing itself. Although the bispecific ai-mAb engaged B cells expressing VH3-21/VL1-40 BCRs that were both neutralizing and non-neutralizing, mAbs derived from the neutralizing B cells bound more tightly to preF, suggesting that they would have a competitive advantage in binding F over the non-neutralizing mAbs. This notion is supported by the observation that MLR_24 maintained

neutralization potency even in the presence of an excess of non-neutralizing, VH3-21/VL1-40 preF-binding mAbs.

Previous studies found that VH3-21/VL1-40-derived mAbs neutralize both subtypes of RSV with comparable potency, likely due to the relative conservation of site III on preF^{5,9,11,53,54}. However, the VH3-21/VL1-40 mAbs isolated here, and the ADI-19425 control mAb, exhibited overall higher potency against RSV-B than RSV-A (~6-fold for MLR_15, ~100-fold for MLR_24, and ~3-fold for ADI-19425). This trend (average IC₅₀ of 3.73 µg/mL against RSV-A, vs. 2.98 µg/mL for RSV-B) was observed among the 25 unmutated VH3-21/VL1-40 mAbs that showed neutralization potency isolated by Goodwin et al.⁹. The larger discrepancy seen in this study can in part be attributed to the difference in neutralization assays (microneutralization vs. plaque reduction) across studies and may not be the case among all F-reactive VH3-21/VL1-40 BCR⁺ B cells that could be targeted by the bispecific ai-mAb. Nevertheless, the ai-mAb efficiently targeted the naïve B cells of the VH3-21/VL1-40 BCR class, including those that are neutralizing.

In our plaque-reduction assays MLR_24, the most potent mAb isolated here, was ~4.5- and ~500-fold less potent than the current state of the art prophylactic mAb D25 (nirsevimab) against RSV-B and RSV-A, respectively. Passive transfer studies in susceptible animals with the mAbs isolated herein could define whether and at what levels these mAbs would need to be present for protection^{11,12,44}, providing a benchmark for future vaccine studies. Despite only isolating mAbs with moderate potency against RSV-A, it is plausible that the ai-mAb could engage BCRs encoding more potent mAbs. This notion is supported by the observation that the ai-mAb binds with high affinity to ADI-19425 and activates B cells expressing this BCR. The fact that we did not identify more potent mAbs here could be attributed to inefficiencies in B cell sorting and VH and VL amplicon recovery as well as the inherent under sampling of the B cell

repertoire by analyzing peripheral blood samples. Nevertheless, our data demonstrate that the ai-mAb is capable of engaging BCRs that correspond to RSV-neutralizing mAbs.

Based on the naïve B cell sorting analyses by Goodwin et al.⁹ and this study, both the ai-mAb and preF can engage and activate unmutated VH3-21/VL1-40 RSV neutralizing B cells. The bispecific ai-mAb differs from F in that it specifically engages site-III directed B cells and avoids activating non-neutralizing B cells directed at other epitopes on F. This approach has potential advantages over F-based vaccine strategies to protect infants from RSV infection. An ai-mAb with high affinity and specificity for VH3-21/VL1-40 class antibodies may be particularly well suited to elicit neutralizing antibodies and establish immunological memory in the first 6 months of life while the infant immune system has diminished ability to generate somatically mutated antibodies^{25,55}.

A theoretical drawback of using ai-mAbs to target B cells is that the activated B cells may enter germinal centers, undergo somatic mutation and gain affinity for the ai-mAb at the cost of losing affinity for preF. Although some B cells could plausibly suffer this fate, it is also likely that a substantial portion of activated B cells, including those that encode neutralizing antibodies will rapidly become antibody-secreting plasmablasts⁵⁶⁻⁵⁹. In support of this, we previously showed that a single immunization with a high-affinity ai-mAb rapidly induced a robust on-target plasmablast response and corresponding high-titer serum antibody responses that persisted for 6 months in a murine adoptive transfer model³³. Further, elevated levels of early memory B cells were also identified in this ai-mAb immunization group³³. Another theoretical consequence of using ai-mAbs as immunogens is that they may bind and mediate killing through Fc mediated functions. To ameliorate this possibility, we included mutations in our bispecific design to ablate Fc binding³⁹.

Because the bispecific ai-mAb is antigenically distinct from RSV, it may circumvent interference of the infant immune response caused by epitope masking and antigen clearance

mediated by maternal antibodies^{27,28,30}. The strategy to elicit high titers of anti-F antibodies in pregnant people may prove to be a double-edged sword in this regard; high titers will provide transient protection to infants, while at the same time hampering their humoral response to vaccination. Vaccination of infants with an antigenically disparate ai-mAb could augment protection afforded by maternal vaccination or prophylactic monoclonal antibody transfer by eliciting a *de novo* serum neutralizing response that persists beyond the point at which transferred antibodies no longer provide protection.

Collectively, these highlight potential advantages of an ai-mAb-based vaccine strategy as it can selectively target a B cell class pre-programmed to bind a conserved antigenic site on preF and neutralize RSV without the need for affinity maturation and it is antigenically distinct from RSV, circumventing epitope masking by maternal antibodies. The bispecific ai-mAb described herein represents a step towards a safe and effective infant vaccine to prevent severe RSV infection.

Limitations

Our analysis was restricted to *in vitro* and *ex vivo* investigation. Although the calcium flux assays here indicate that the ai-mAb can activate target cells of interest, these experiments cannot predict the fate of VH3-21/VL1-40 B cells following immunization *in vivo*. The development of a small animal model that expresses target VH3-21/VL1-40 BCRs at relevant target frequencies would enable *in vivo* analysis in the future. This would further enable us to determine the fate of activated B cells, their BCRs, the antibodies they secrete; and whether protective levels of serum antibodies can be achieved by ai-mAb immunization.

Another limitation of this study was the limited number of donors screened with the bispecific ai-mAb in sorting studies (N=3), and the corresponding number of unmutated VH3-21/VL1-40 mAbs assayed for binding and neutralization potential.

Resource Availability

Lead Contact

Requests for resources and reagents should be directed to and will be fulfilled by Andrew T. McGuire (amcguire@fredhutch.org).

Material Availability

All materials generated herein are available upon request under an MTA from the corresponding author (amcguire@fredhutch.org). The pTT3 vectors are used under license from the National Research Council of Canada.

Data and Code Availability

- Data presented in this study are deposited in the following databases: Coordinates and structure factors for both ADI-19425 Fab – 1D3 Fab and ADI-19425 Fab – 2C1 Fab structures have been deposited to the Protein Data Bank (PDB) under accession codes 8VS8 (ADI-19425 Fab – 1D3 Fab) and 8VS7 (ADI-19425 Fab– 2C1 Fab). In addition, the following publicly available datasets are mentioned: PDB ID: 1D5I⁶⁰, PDB ID: 5I76⁶¹, PDB ID: 6P67⁶², PDB ID: 6APC¹¹, PDB ID: 6APD⁹, and PDB ID: 5TDG⁶⁰. Accession numbers for produced VH3-21/VL1-40 mAbs can be found in Table S2. All other data is available upon request from the lead contact.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this work is available from the lead contact upon request.

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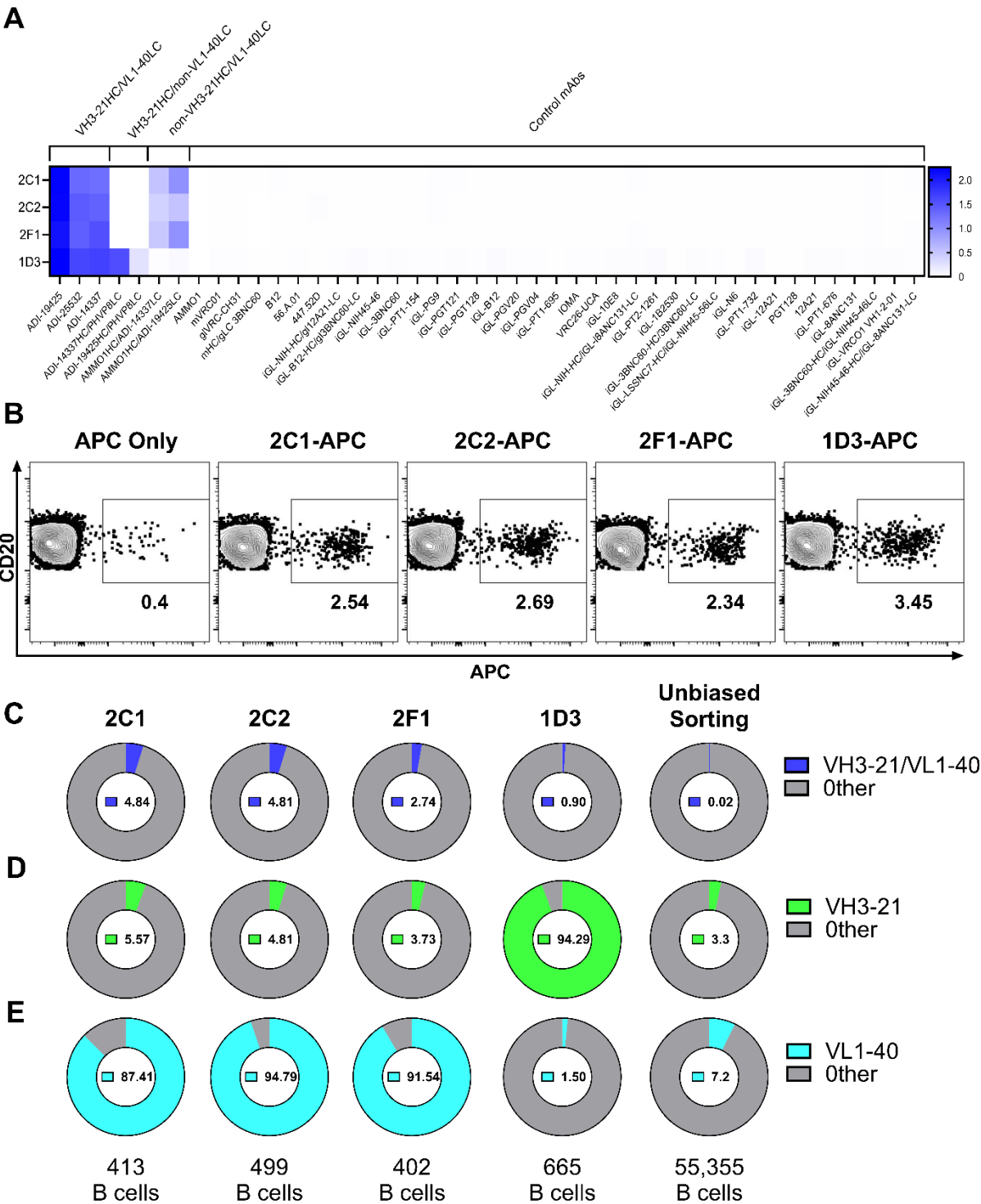
Author Contributions

Conceptualization, A.T.M and S.C.S.; investigation, S.C.S., Y-H.W., L.J.H., G.K., A.H., B.P., I.R.S., N.A., A.P., M.B., C.C., and M.B.P.; formal analysis, S.C.S., Y-H.W., L.J.H., G.K., A.H., B.P., I.S., Z.M., and A.T.M; writing – original draft, S.C.S, L.J.H., G.K., A.H., M.P., and A.T.M.; writing – review and editing, all authors; visualization, S.C.S., G.K., A.H., and A.T.M.; supervision, A.T.M., J.B., M.P., A.E., and M.J.M.; funding acquisition, A.T.M., M.P., and A.E.

Declaration of Interests

A.T.M, and S.C.S. are co-inventors on a patent application 63/635,785 pertaining to the ai-mAbs described herein. All other authors declare no competing interests.

Figure Titles and Legends



1D3 were evaluated for their ability to bind to a panel of recombinant mAbs by ELISA. The scale indicates the A_{450} signal as measured by ELISA. Data shown is average of duplicate wells from one biological replicate. **(B)** ai-mAbs were fluorescently labeled with APC conjugated to a unique oligonucleotide barcode and used to stain naïve B cells from human PBMCs. The frequency of APC-positive B cells is shown. Gating strategy is shown in Fig. S2. **(C-E)** APC⁺ naïve B cells were sorted, and the paired B cell receptor transcripts obtained through next-generation sequencing. **(C)** The percentage of VH3-21/VL1-40 pairs among all BCR sequences sorted by each ai-mAb bait (blue). **(D)** The percentage of BCRs expressing a heavy chain derived from the VH3-21 gene sorted by each ai-mAb (green). **(E)** The percentage of BCRs expressing a light chain derived from the VL1-40 gene sorted by each ai-mAb bait (teal). The number of B cells analyzed is shown at the bottom of **C-E**. The frequency of naïve B cells expressing these genetic features identified by high-throughput unbiased sorting is included for comparison in³⁸ **C-E**. Data are representative of one biological replicate for each ai-mAb in **B-E**.

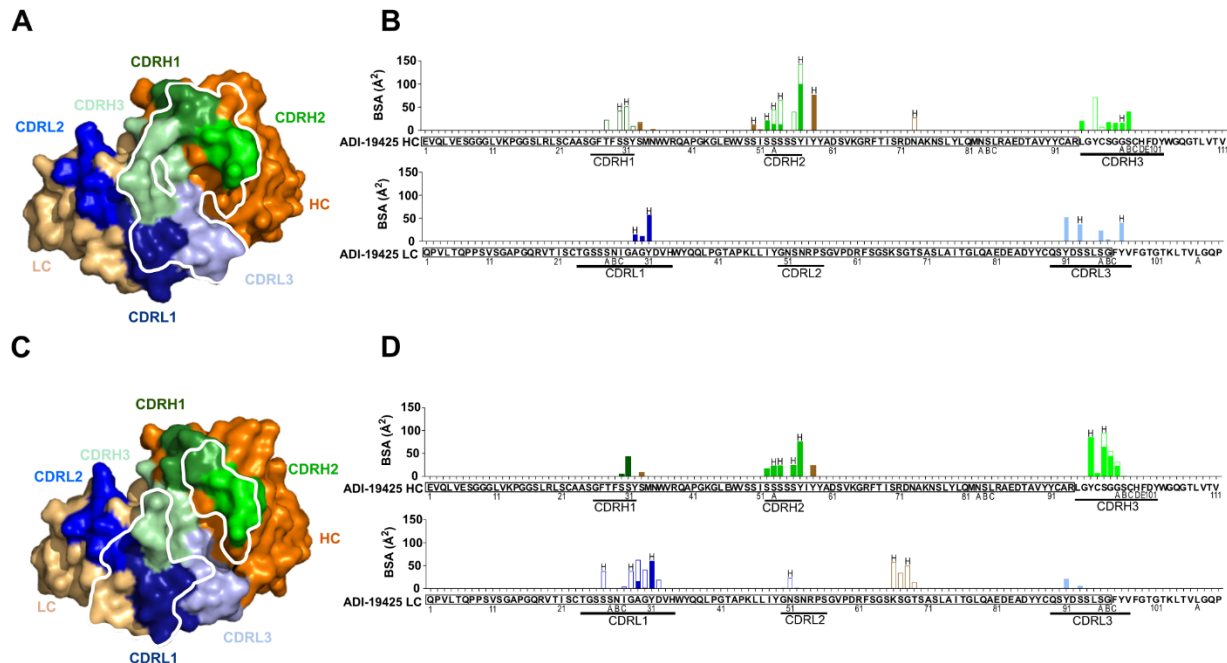


Figure 2: Crystal structures of ai-mAbs in complex with ADI-19425. (A, C) Surface representation of ADI-19425 Fab bound to (A) 1D3 Fab and (C) 2C1 Fab. Views are looking down on the ADI-19425 paratope. Residues within 5Å of (A) 1D3 or (C) 2C1 are outlined in white. (B, D) Buried surface area (BSA) plots shown as stacked bar graphs of ADI-19425 HC (top) and ADI-19425 LC (bottom) bound by (B) 1D3 Fab or (D) 2C1 Fab. Interactions with the HC of either 1D3 or 2C1 are shown as solid-colored bars while interactions with the LC of either 1D3 or 2C1 are shown as empty bars. Residues participating in hydrogen-bond interactions are labeled with an “H”. ADI-19425 HC and LC sequences are shown underneath the graph. VH3-21 and VL1-40 sequences are boxed. See also Fig. S3 and Table S1.

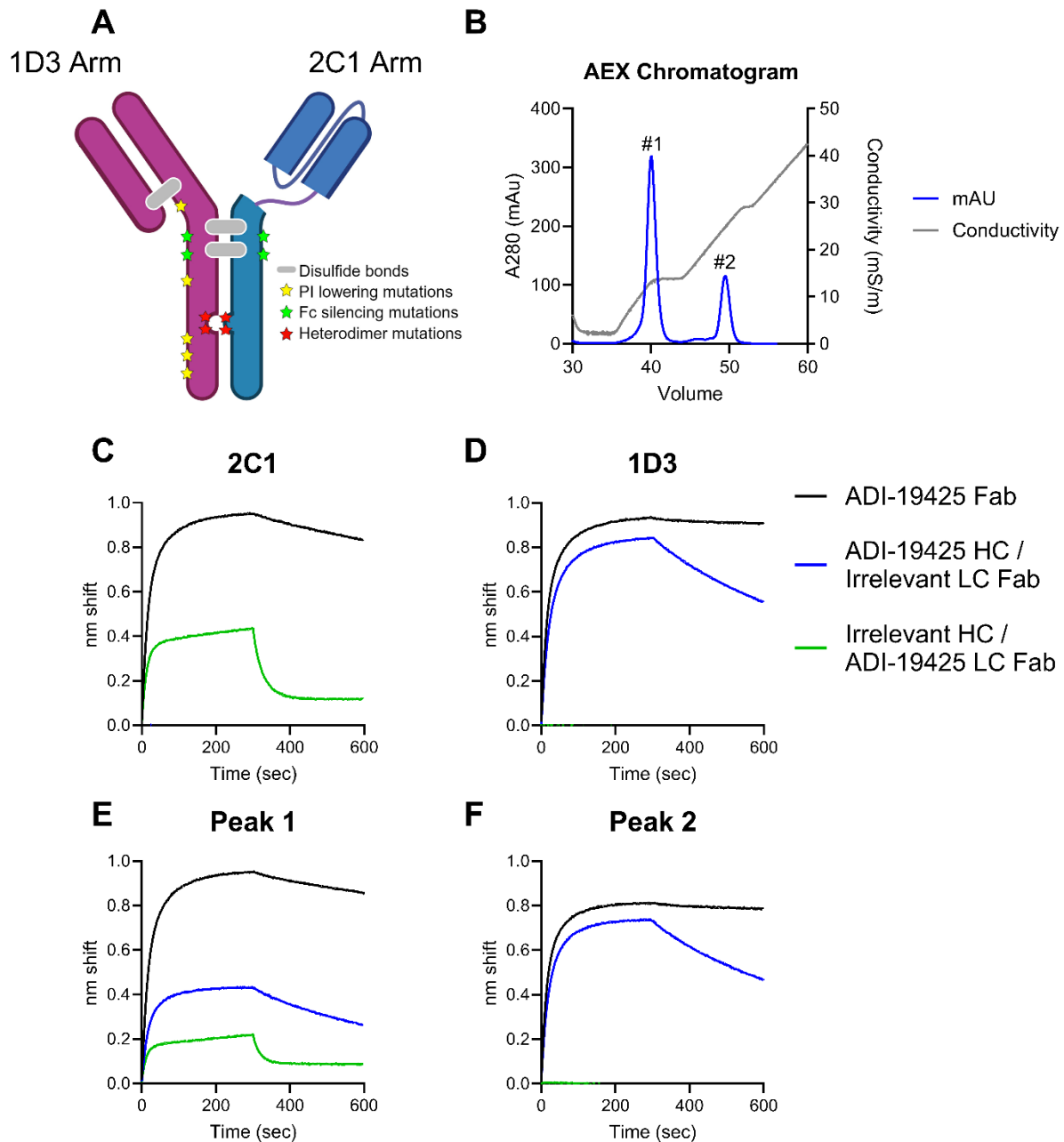


Figure 3: Engineering a VH3-21/VL1-40 BCR-targeting bispecific ai-mAb. (A) Schematic of the bispecific platform used³⁹ created with BioRender.com. (B) Anion exchange (AEX) chromatogram of bispecific ai-mAb production. Representative data from one biological replicate is shown. (C-F) Binding of ADI-19425 Fab and the indicated chimeric Fabs to

recombinant 2C1-IgG (**C**), 1D3-IgG (**D**), IgG from peak 1 (**E**), or IgG from peak 2 (**F**) was measured by BLI. Representative data from one biological replicate.

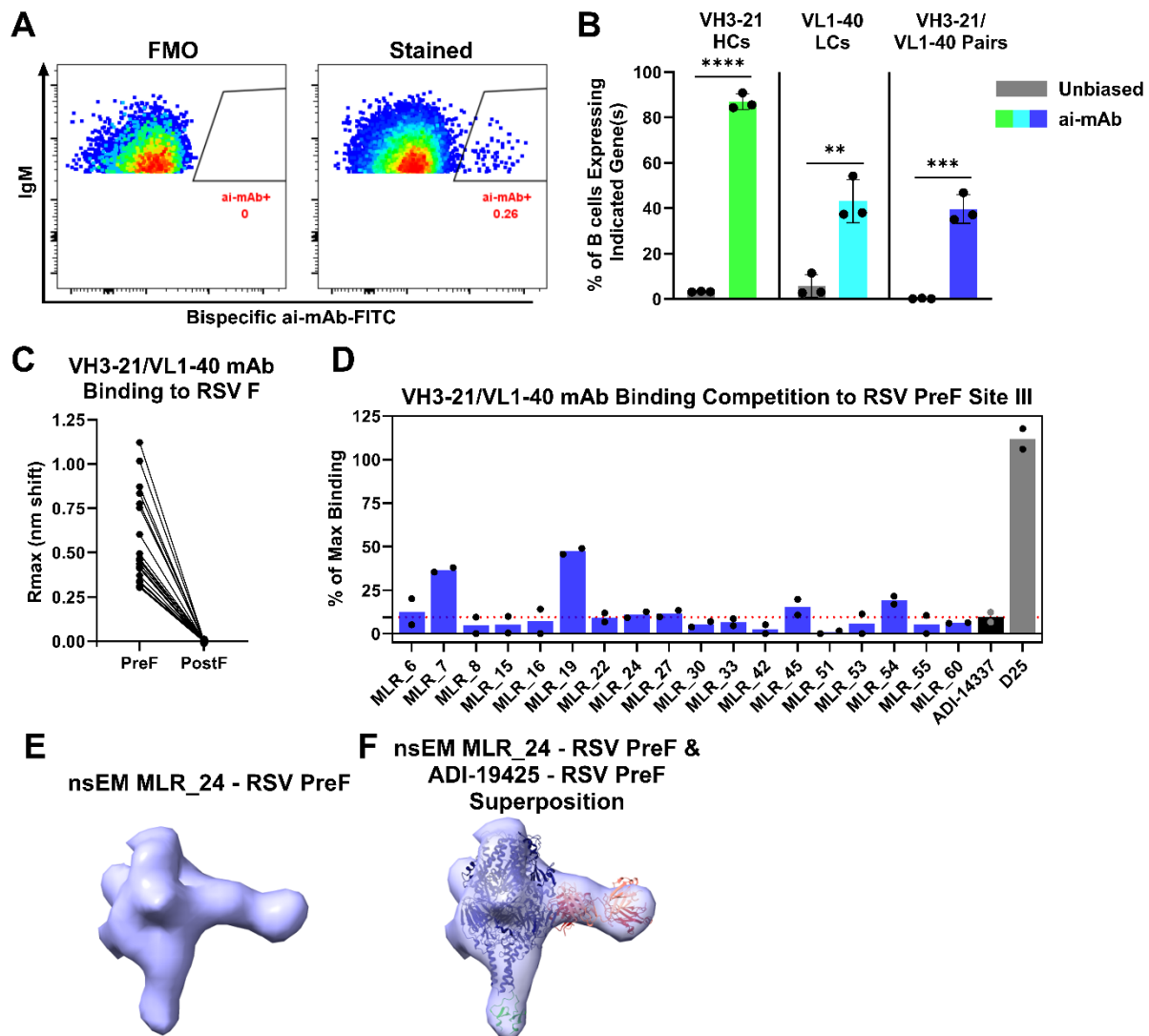


Figure 4: Bispecific ai-mAb-specific B cell sorting and characterization. (A) Fluorescently-labeled 1D3/2C1 bispecific ai-mAb was used to stain and sort naïve human B cells, representative sorting plot shown. See Fig. S4 for gating strategy. (B) From the sorted cells, the percentage of productive VH3-21 HCs (green) and VL1-40 LCs (teal) are shown. From successfully recovered pairs, the percentage of VH3-21/VL1-40 (blue) are shown. Each data point represents the frequency of gene usage from n=3 independent sorting experiments. Bars represent the mean $\pm$ standard deviation. Naïve BCRs obtained from unbiased high-throughput sequencing of n=3 unique donors³⁸ are shown for comparison (grey). Significant differences

were determined using Student two-tailed t tests. $**p = 0.001-0.01$, $***p = 0.0001-0.001$, $****p < 0.0001$. **(C)** VH3-21/VL1-40 BCRs from **B** were expressed as recombinant mAbs and tested for binding to RSV preF and postF by BLI. Data shown is average from 2 biological replicates. **(D)** mAbs from **C** were tested for their ability to compete for binding with ADI-14337⁹ to preF by BLI. Each data point represents a biological replicate and the bars represent mean. The red dotted line represents the average ADI-14337 competition against itself. D25 is an RSV preF site $\emptyset$ mAb¹², and included as a negative control. **(E)** Side view of a representative nsEM 3D class of the DS-Cav1 – MLR_24 Fab complex. **(F)** Superposition of the crystal structure of ADI-19425 Fab bound to preF⁹ (PDB ID: 6APD) into the representative 3D volume. The crystal structure is colored as follows: RSV preF (midnight blue), ADI-19425 heavy chain (dark red), ADI-19425 light chain (salmon). Density was also seen for the T4 fibrin trimerization domain, which is modeled by the foldon region of the GPP-Foldon crystal structure (PDB ID: 1NAY) in green. See also Figs. S4-6 and Table S2.

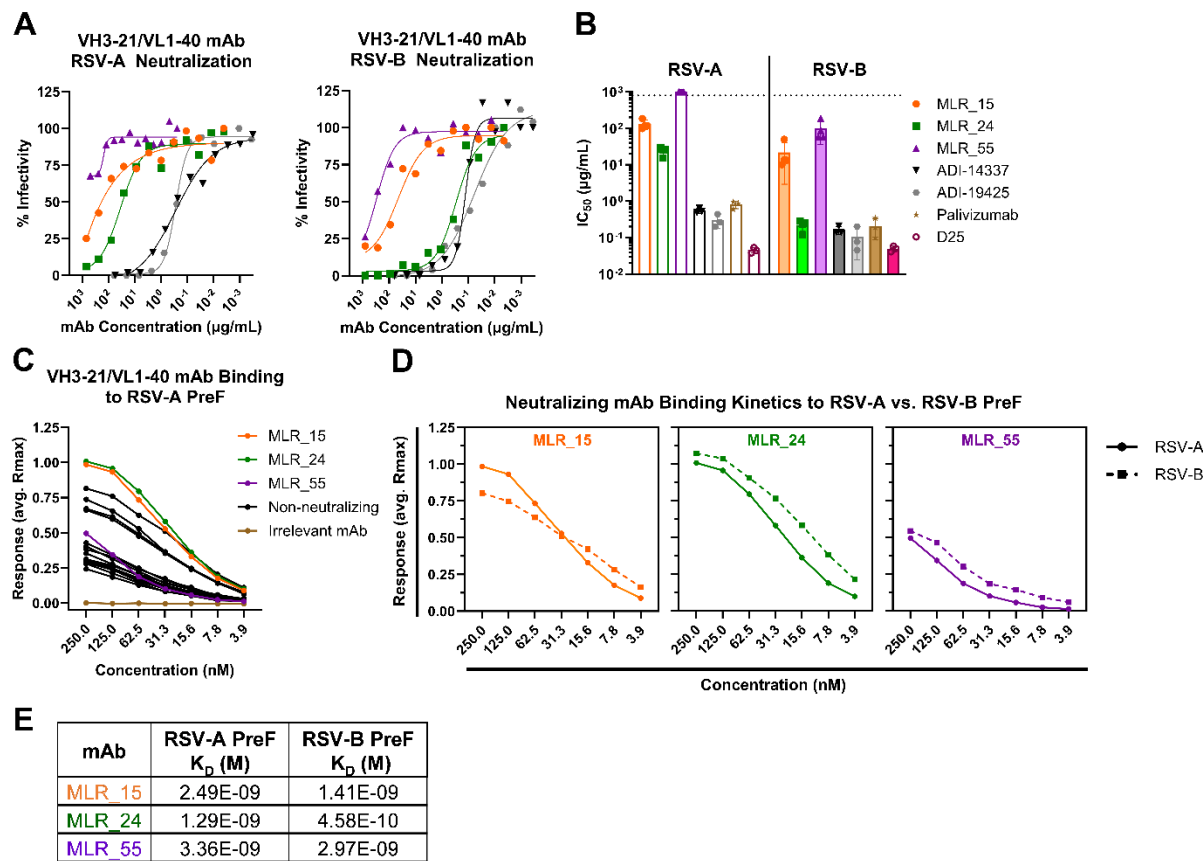


Figure 5: VH3-21/VL1-40 mAb RSV neutralization and affinity (A) Neutralization curves of selected VH3-21/VL1-40 mAbs against RSV-A (left) and RSV-B (right). Points shown are mean of 2 technical replicates from a single biological replicate. (B) Mean IC₅₀ values of selected mAbs against RSV-A and RSV-B. Each dot represents a biological replicate (n=3-4), and bars represent mean ± standard deviation. Dotted line represents maximum tested concentration. (C) Steady-state analysis of VH3-21/VL1-40 mAb binding response to RSV-A preF measured by BLI. Each data point represents the mean response from two biological replicates. (D) Steady-state kinetic analysis of neutralizing VH3-21/VL1-40 mAbs binding to RSV-A (solid lines) and RSV-B (dashed lines) preF measured by BLI. Each data point represents the mean response from two biological replicates. (E) Apparent affinity of neutralizing VH3-21/VL1-40 mAbs to RSV-A / RSV-B preF. See also Fig. S7.

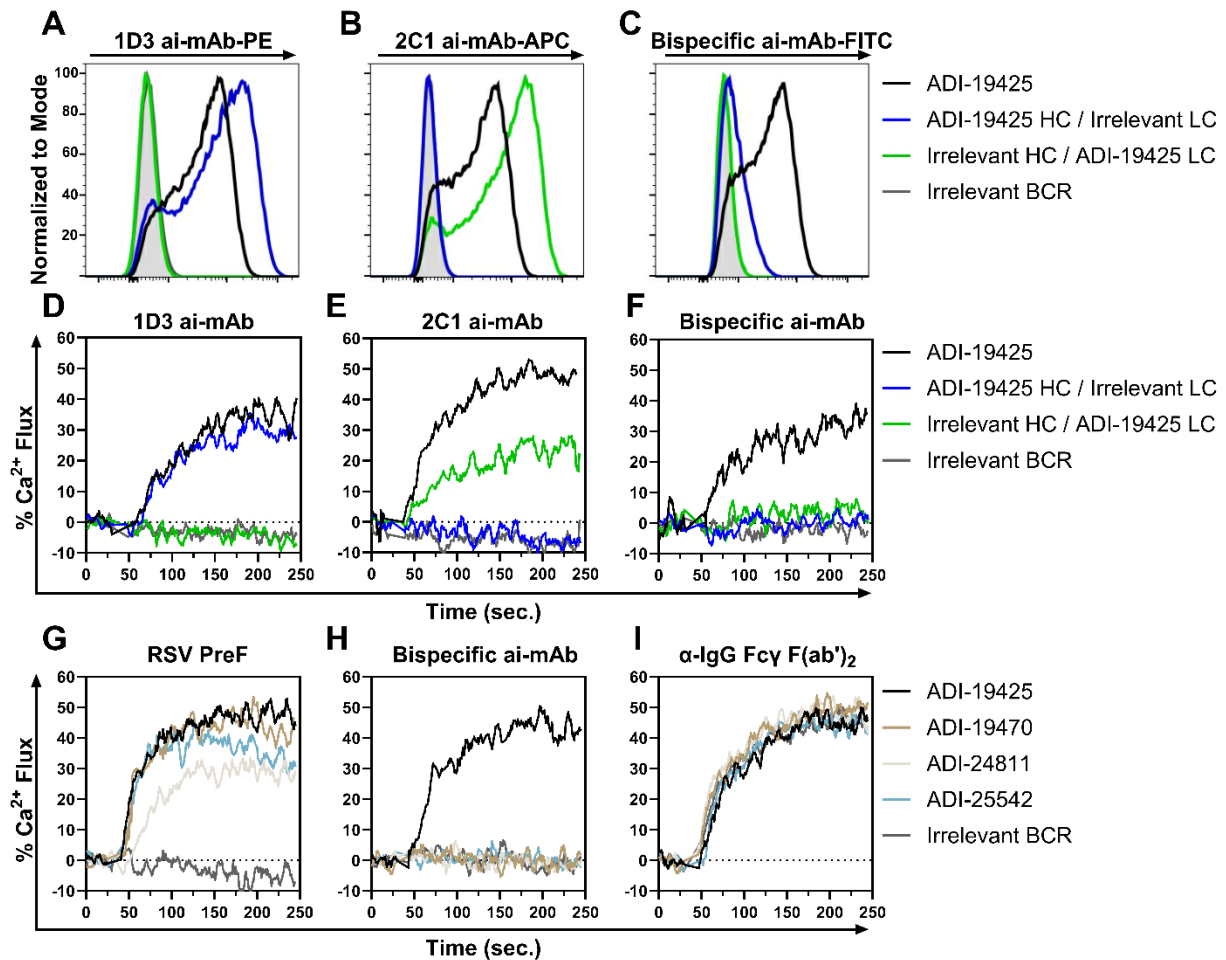


Figure 6: Activation of on and off-target BCR-expressing cell lines by calcium flux assays

(A-C) Staining of DG75 B cell lines transduced to express the indicated BCRs shown as histograms, with signals normalized to mode. Representative of a single biological replicate. (A) Staining with 1D3 ai-mAb. (B) Staining with 2C1 ai-mAb. (C) Staining with bispecific ai-mAb. (D-F) Calcium flux in response to the addition of the 1D3 ai-mAb D, 2C1 ai-mAb E, or the 1D3/2C1 bispecific ai-mAb F as immunogen. Signals shown are representative of 2 biological replicates. (G-I) Calcium flux in non-neutralizing RSV preF specific BCR-expressing cell lines as indicated. Cell lines were stimulated with RSV-A preF G, the bispecific ai-mAb H, and as a positive control an α -IgG Fc γ F(ab')₂ I. Signals shown are representative of 2 biological replicates.

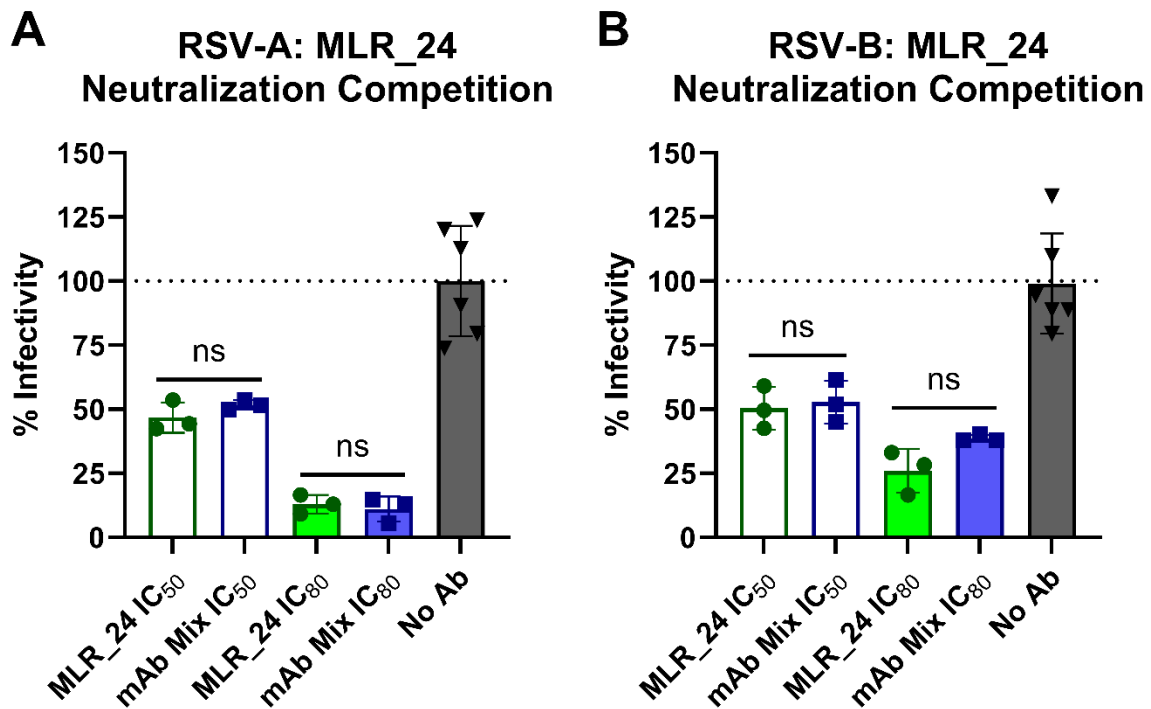


Figure 7: Neutralization competition assays. (A & B) MLR₂₄ was formulated at its IC₅₀ (green, open) or IC₈₀ (green, shaded) concentration alone or in combination with 5 non-neutralizing, pre-F-binding mAbs at matched concentrations “mAb Mix” (blue open, blue shaded) (See Fig. S7A & B) and tested inhibition of RSV-A **A** and RSV-B **B** in a plaque reduction assay. Each dot represents a single biological replicate carried out in triplicate, bars represent mean $\pm$ standard deviation. Significance determined by Mann-Whitney tests comparing MLR₂₄ to mAb Mix at each concentration.

Star Methods

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Human Subjects

PBMC were collected from adults without HIV who were recruited at the Seattle HIV Vaccine Trials Unit (Seattle, Washington, USA) as part of the study “Establishing Immunologic Assays for Determining HIV-1 Prevention and Control”, also referred to as Seattle Assay Control (SAC) Cohort. All participants signed informed consent, and the Fred Hutchinson Cancer Center (Seattle, Washington, USA) Institutional Review Board approved the SAC protocol (FHIRB0005567) prior to study initiation. Donors were selected randomly and no considerations were made for age or sex.

Cell lines

All cell lines were incubated at 37°C in the presence of 5% CO₂ and were not tested for mycoplasma contamination. 293-6E cells (human female) and suspension adapted 293T cells (human female) were maintained in Freestyle 293 media (ThermoFisher Cat# 12338018) with gentle shaking. DG-75 cells (human male) were maintained in RPMI (Corning Cat# 15-040-CV) + 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin (cRPMI). HEp-2 (human male) and Vero cells (African green monkey) were maintained in DMEM (Gibco Cat#11965-092) + 10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin (cDMEM).

Method Details

Recombinant Antibodies

The heavy and light chain variable regions of ADI-19425 (VH GenBank: MG524063, VL GenBank: MG524528), ADI-25532 (VH GenBank: MG524182, VL GenBank: MG524647) and ADI-14437 (VH GenBank: MG524251, VL GenBank: MG524716) were codon optimized, cloned

into pTT3-based IgG1 expression vectors with human constant regions³⁶ using 5x In-Fusion HD Enzyme Premix (Takara Bio) according to the manufacturer's instructions.

BCRs from VH3-21/VL1-40⁺ B cells were expressed as recombinant antibodies as described above. Accession numbers for produced VH3-21/VL1-40 mAbs can be found in Table S2.

Generation of anti-idiotypic monoclonal antibodies

Mice were injected 3 or 5 times with a cocktail of ADI-19425, ADI-25532 and ADI-14337. Three days after the final injection, spleens were harvested and used to generate hybridomas at the Fred Hutchinson Antibody Technology Center. Hybridoma supernatants were initially screened for binding to a panel of mAbs including ADI-19425, ADI-25532, ADI-14337, the anti-EBV mAb AMMO1, a chimeric mAb with a VL1-40 light chain and a non-VH3-21 heavy chain (AMMO1-HC/ADI-19425LC) and a chimeric mAb with a VH3-21 heavy chain and a non-VL1-40 light chain (ADI-19425HC/CL40 light chain). Hybridomas from wells containing supernatant that showed a strong binding signal to ADI-19425, ADI-25532, ADI-14337 and weaker or no binding to the control mAbs were plated onto semi-solid media and single colonies were used to establish monoclonal cell lines using a Clonepix instrument. To purify mAbs, hybridomas were cultured in serum free media cells and cellular debris were removed by centrifugation at 4,000 × *g* followed by filtration through a 0.22 µm filter. The clarified supernatant was diluted with an equal volume of Pierce™ Protein G IgG Binding Buffer

and then passed over a Protein G Agarose column, washed with 5 column volumes of Protein G IgG Binding Buffer and then eluted in 1 mL fractions of Pierce IgG Elution Buffer, pH 2.0 into 0.1 mL of Tris HCl, pH 8.0. Purified mAbs were then concentrated and buffer exchanged into PBS using an Amicon filter unit. Aliquots of purified mAbs were biotinylated using the EZ-Link™

NHS-PEG4-Biotin kit at a theoretical 1:1 ratio of mAb to biotin. Purified mAbs were flash frozen and stored at -20°C until use.

To produce recombinant anti-idiotypic mAbs, RNA was extracted from 1×10^6 cells using the Monarch® Total RNA Miniprep Kit and cDNA encoding the heavy and light chain variable regions of the murine hybridomas were by reverse transcribed and amplified using the procedures outlined in Meyer, *et al.*⁶¹. Amplicons were sanger sequenced directly or TOPO cloned and then sequenced. The sequences were codon optimized and cloned into pTT3-based IgG expression vectors with human constant regions³⁶ using 5x In-Fusion HD Enzyme Premix, expressed in 293-6E cells and purified using Protein A chromatography.

ELISA Screening of Anti-idiotypic Antibodies

50 µL/well of target mAbs at 2µg/mL (ADI-19425, ADI-14337 etc.) in ELISA coating buffer (0.1 M NaHCO₃ pH 9.4-9.6) were added to 96-well half volume plates (Thermo Scientific Cat# 3455) and incubated overnight at 4°C. The plates were then washed 3 times with ELISA wash buffer (PBS with 0.02% Tween 20). Plates were then blocked with 100 µL/well with ELISA blocking buffer (10% nonfat milk with 0.03% Tween 20) for 2 hours at 37°C. After blocking, plates were washed 3 times with ELISA wash buffer. Followed washing, purified IgG from ai-mAb hybridoma supernatants were added to each well at 2µg/mL in 50ul in ELISA blocking buffer and plates were incubated for 1 hour at 37°C. Following primary hybridoma ai-mAb staining, plates were washed 3 times with ELISA wash buffer and then stained with goat anti-mouse IgG human ads-HRP (Southern Biotech) at a 1:30,000 dilution in 50 µL/well of ELISA blocking buffer and incubated for 1 hour at 37°C. Plates were then washed 3 times in ELISA wash buffer and developed with 50 µL/well of SureBlue Reserve TMB Microwell Peroxidase substrate (SeraCare) for 5 minutes, followed by neutralization with 50 µL/well of 1 N sulfuric acid. A₄₅₀ of each well was read on a Molecular Devices SpectraMax M2 plate reader.

Recombinant Protein Expression

Plasmids antibody heavy and light chains were transfected into 293-6E cells at a density of 10^6 cells/mL in Freestyle 293 media (ThermoFisher) using the 293Free transfection reagent (EMD Millipore) according to the manufacturer's instructions. Expression was carried out in Freestyle 293 media for 6 days after which cells and cellular debris were removed by centrifugation at $4,000 \times g$ followed by filtration through a $0.22 \mu\text{m}$ filter. Clarified cell supernatant containing recombinant mAbs were passed over Protein A resin (GoldBio), pre-equilibrated with phosphate buffered saline (PBS; 1 mM KH_2PO_4 , 155 mM NaCl, 3mM Na_2HPO_4) washed with 10 column volumes of PBS and then eluted in 1 mL aliquots of Pierce IgG Elution Buffer, pH 2.0 (ThermoFisher) into 0.1 mL of Tris HCl, pH 8.0. Purified antibodies were concentrated, and buffer exchanged into PBS using an Amicon filter unit.

Plasmids encoding RSV-A preF (DS-Cav1) and postF were kind gifts of Jason McLellan, and the plasmid encoding RSV strain B1 preF was a kind gift of Peter Kwong^{15,43,45}. All RSV viral protein-encoding plasmids were transfected into 293-6E cells as described above. Imidazole and sodium chloride were added to the cellular supernatant prior to resin capture at final concentrations of 0.01M and 0.5M respectively. Clarified cell supernatants were then passed over Ni-NTA resin (Thermo Scientific), pre-equilibrated with NTA-A buffer (500mM NaCl, 10mM Tris, 0.02% azide, 10mM Imidazole, pH 7.1), washed with 10 column volumes of NTA-A buffer and then eluted in 1mL aliquots of NTA-B buffer (500mM NaCl, 10mM Tris, 0.02% azide, 500mM Imidazole, pH 8). Purified viral proteins were then concentrated and purified by size exclusion chromatography using a HiLoad 16/600 Superdex S200 (Miliopore Sigma) column fitted to a BioRad NGC system. Final fractions containing the RSV proteins were concentrated, analyzed by SDS-Page and BLI, and flash frozen for future use.

ADI-19425, 1D3 and 2C1 IgGs were digested into Fabs using Endoproteinase Lys-C (New England BioLabs) by adding 1µg of Lys-C per 10mg of IgG and the mixture incubated overnight at 37°C. Following digestion, mixtures were incubated with Protein A resin for 1-2 hours to remove any remaining Fc or undigested IgG. Resulting Fabs were further purified using an Enrich SEC 650 (Bio-Rad) column and concentrated.

Generation of a VH3-21/VL1-40 targeting bispecific ai-mAb

A recombinant VH3-21 and VL1-40 targeting bispecific ai-mAb with a “silent Fc” was developed using the platform developed by Moore et al ³⁹. Codon optimized cDNA encoding a signal peptide, the 1D3 variable heavy chain region and the human IgG1 constant domain harboring N208D, Q295E, N384D, Q418E, N421D, L368D, K370S, E233P, L234V, L235A, G236del, and S267K mutations (Chain A) was synthesized by Twist Biosciences and cloned into pTT3. A leader peptide followed by a 2C1 scFv (VH- GKPGS₄-VL) fused to Human IgG1 residues 216 through 447 containing a C220S, E357Q, and S364K mutations (Chain B) was synthesized by Twist Biosciences and cloned into pTT3. pTT3 plasmids encoding Chain A, Chain B and the 1D3 light chain were co-transfected into 293-6E cells and purified using Protein A chromatography as described in “recombinant protein expression”. Purified antibodies were then concentrated, buffer exchanged into 50 mM Tris pH 8.5, and then purified on a Hi-Trap Q HP (Cytiva) anion exchange column pre-equilibrated with 50 mM Tris, pH 8.5 and eluted over a gradient to 50% elution buffer (50 mM Tris, 1 M NaCl, pH 8.5) over 10 column volumes. Fractions containing the bispecific antibody were concentrated and then further purified by size exclusion chromatography on a HiLoad 16/600 Superdex S200 (Milipore Sigma) column. Final fractions containing the bispecific antibody were analyzed by SDS-PAGE, tested for binding specificity, concentrated, buffer exchanged into PBS, snap frozen and stored at -80°C until use.

B cell sorting

Bulk Human B cell sorting

Cryopreserved PBMCs were thawed and resuspended in 200µL of EasySep buffer (1X PBS, 2% heat inactivated fetal bovine serum, 1µM EDTA). B cells were isolated using the Human B Cell Enrichment Kit (Stem Cell) according to the manufacturer's instructions. Enriched B cells were resuspended in 200µL EasySep buffer and incubated with 10µL rat serum, 10µL Mouse Serum, 10µL mouse-anti-human CD32 (BD), 10µL mouse-anti-human CD23 (BD) and 10µL mouse-anti-human CD16 (BD). Cells were then washed with EasySep buffer and resuspended in 200µL EasySep buffer containing CD19-BV711 (BioLegend) at a 1:200 dilution, CD27-PE-Cy7 (eBioscience) at a 1:600 dilution, CD14-FITC (BD Pharmingen) at a 1:60 dilution, CD3-FITC (BD Pharmingen) at a 1:60 dilution, CD20-AF700 (BioLegend) at a 1:300 dilution, IgD-PerCP-Cy5.5 (eBioscience) at a 1:120 dilution, IgM-BV605 (BioLegend) at a 1:120 dilution, Fixable Viability Dye, V500 (eBioscience) at a 1:300 dilution, and i) biotinylated murine anti-idiotypic mAbs conjugated to Total SeqC-0960 APC streptavidin (BioLegend), Total SeqC-0959 APC streptavidin (BioLegend), Total SeqC-0958 APC streptavidin (BioLegend), Total SeqC-0957 APC streptavidin (BioLegend), or Total SeqC-0956 APC streptavidin (BioLegend), ii) recombinant anti-idiotypic mAbs labeled with APC and PE (separately labeled pools) using Zenon™ Human IgG Labeling Kits (ThermoFisher Scientific) murine anti-idiotypic antibodies labeled with Zenon-PE. Cells were then washed with 5mL of EasySep buffer and suspended in 0.5 mL of EasySep buffer and subjected to analysis on a FACSymphony S6 (BD Biosciences). Naive B cells were defined as live (V500-), CD14⁻, CD3⁻, CD19⁺, CD20⁺, IgM⁺, IgD⁺, CD27⁻. Naive ai-mAb⁺ (APC⁺, PE⁺) B cells were bulk sorted and BCR libraries were prepared using the Chromium Single Cell Human BCR Amplification Kit (10X Genomics).

Libraries from naïve B cells that were positive for murine anti-idiotypic mAbs conjugated to TotalseqC streptavidin APC were generated using the Chromium Single Cell Human BCR Amplification Kit (10X Genomics) and the 5' Feature Barcode Kit (10X Genomics). All single cell

libraries were separately indexed using the Dual Index Kit TT Set A (10X Genomics) and sequenced on an Illumina NovaSeq or NextSeq instrument at the Fred Hutch Genomics Core. Analysis of flow data was processed using FlowJo 10.8.1 software (Tree Star).

Analysis of barcoded NGS sequences

Sequence files were obtained from the Fred Hutchison Genomics Core. FASTQ data was analyzed using Cell Ranger (Version 3.0.0) on an independent computing cluster. Subsequently, enclone⁶² was used for clustering and visualization of B cell clonotypes in association with specific barcodes. Both enclone and Cell Ranger are programs developed and published by 10x Genomics used for downstream analysis of 10x compatible sequencing datasets (Pleasanton, CA). Briefly, Cell Ranger was run using sequencing data mentioned above in order to demultiplex using unique molecular identifier (UMI), index, and unique 10x compatible barcodes (totalSeqC Barcodes) present in the sequencing data. The output of Cell Ranger is demultiplexed data compatible with downstream use in enclone software. Enclone was then used to enumerate and visualize the grouping of VDJ sequences in association with respective 10x feature barcode. By extension, BCRs were assigned to the ai-mAb they were tagged with during sorting. Datasets produced in enclone were exported as CSV files and R studio was used to visualize data further.

Analysis was limited to B cells with a single heavy-light chain pair. The feature barcode counts for each antigen were analyzed using the *chnopt* package for R statistical software (R Foundation for Statistical Computing, Vienna, Austria) for a segmented generalized linear model³⁷ to determine a threshold for positivity (Fig. S2). B cells above the threshold were considered positive for a given ai-mAb. We included the iv2 mAb labeled with a barcoded APC-barcode conjugate as a control for non-specific binding to murine IgG constant regions, or the SA-APC

reagents. B cells that were above the threshold for the iv2 barcode were excluded from subsequent analysis.

Single B cell Sorting and Sequencing

In single B cell sorting experiments, B cells were thawed and enriched using magnetic beads from cryopreserved PBMCs as described above. Enriched B cells were resuspended in 200µL EasySep buffer and incubated with 10µL rat serum, 10µL mouse serum, 10µL mouse-anti-human CD32 (BD), 10µL mouse-anti-human CD23 (BD) and 10µL mouse-anti-human CD16 (BD). Cells were then washed with EasySep buffer and resuspended in 200µL EasySep buffer containing CD19-BV711 (BioLegend) at a 1:200 dilution, CD27-PE-Cy7 (eBioscience) at a 1:200 dilution, CD14- PE (BD Pharmingen) at a 1:100 dilution, CD3-PE (BD Pharmingen) at a 1:100 dilution, CD20-AF700 (BioLegend) at a 1:250 dilution, IgD-PerCP-Cy5.5 (eBioscience) at a 1:100 dilution, IgM-BV605 (BioLegend) at a 1:100 dilution, and Fixable Viability Dye-V500 (eBioscience) at a 1:200 dilution. The bispecific antibody was conjugated to DyLight 488 NHS Ester (Thermo Scientific) according to the manufacturer's instructions, and used at a 1:5,000 dilution. After a 30-minute incubation at 4°C, cells were washed with 5mLs of EasySep buffer, resuspended in 400µL PBS + 2% BSA, and subjected to analysis on a FACS Aria II (BD Biosciences). Naïve B cells that stained positive for the bispecific antibody (AF488⁺) were single-cell sorted into individual wells of 96 well plates and then were snap frozen on dry ice. Analysis of flow data was processed using FlowJo 10.8.1 software (Tree Star).

To sequence the VH/VL pairs from single sorted B cells, cDNA was generated using Superscript IV (Invitrogen) and the VH and VL sequences were recovered using gene-specific primers and cycling conditions previously described⁴⁰. VH and VL amplicons were Sanger sequenced (Genewiz) and assigned antibody gene usage using IMGT V-quest⁴¹.

Biolayer interferometry:

BLI assays were performed on the Octet Red 96E Instrument (Sartorius) at 30°C with shaking at 1,000 RPM.

Bispecific ai-Ab Binding screens:

The purified bispecific antibody was captured using Anti-Human IgG capture (AHC, Sartorius) biosensors by immersing sensors into 250µL KB buffer (1X PBS, 0.01% BSA, 0.02% Tween 20, and 0.005% NaN₃) with the bispecific ai-mAb at a concentration of 80nM for 200s. 1D3 IgG and 2C1 IgG were captured in the same fashion in parallel. After loading, the baseline signals were recorded for 60s in KB. The sensors were then immersed into wells containing ADI-19425 Fab, ADI-19425HC / AMMO1 LC chimeric Fab, iv8 HC / ADI-19425 LC chimeric Fab, or a negative control Fab at concentrations of 200nM for 300s (association phase). Following this, sensors were immersed in KB for an additional 300s (dissociation phase). The background signal obtained from sensors immersed in the negative control Fab was subtracted from each individual ai-mAb or the bispecific antibody.

Small Scale mAb screens:

Total antibody recovered from the small-scale production pipeline was assayed for binding to RSV-A preF (DS-Cav1) and postF by BLI^{15,43}. Briefly, antibodies were captured by immersing AHC biosensors into 250µL of supernatant harvested from mAb-transfected 293E cultures on day 5 for 200s. After loading, baseline signals were recorded for 60s in KB. The sensors were then immersed in wells containing RSV-A preF or postF at concentrations of

140nM in 250ul KB for 300s. Following this, sensors were immersed in KB for an additional 300s. The binding screens included ADI-19425 and palivizumab as positive controls and VRC01^{9,17 63} as a negative control.

Kinetic Analysis of ai-mAbs and Neutralizing VH3-21/VL1-40 mAbs:

AHC sensors were immersed in KB containing 67nM of 1D3 IgG or 2C1 IgG for 300s. After loading, the baseline signal was recorded for 60s in KB. Next, sensors were immersed in wells containing ADI-19425 Fab at serial dilutions ranging from 250nM to 0nM for 300s (association), followed by dissociation in KB for an additional 300s. As a negative control, wells containing no mAb were used, and the background signal from these wells were subtracted from the mAb-containing wells. Curve fitting was performed using a 1:1 binding model and the ForteBio data analysis software. Mean K_a and K_D values were determined by averaging all binding curves that matched the theoretical fit with an R^2 value of > 0.99 . The average K_D for each ai-mAb was determined by dividing the average K_D by the average K_a .

Steady State Kinetic Analysis of Sorted VH3-21/VL1-40 mAbs:

Purified VH3-21/VL1-40 mAbs recovered from sorting experiments that showed measurable binding to RSV-A preF, were further analyzed for steady state binding kinetics by BLI. VH3-21/VL1-40 mAbs were immobilized on AHC sensors at a concentration of 67nM in KB for 300s. Following loading, baseline signals were recorded for 60s in KB. As the association step, sensors were immersed in wells containing serial dilutions of RSV-A preF or postF from 250 nM to 0nM in KB for 300s, followed by immersion into wells containing KB to measure dissociation for 300s. Double subtractions of parallel wells containing an irrelevant EBV mAb (E1D1)⁶⁴ as well as non-mAb loaded sensors associated in RSV-A preF were applied to each

sample. Curve fitting was performed using a 1:1 binding model in the ForteBio data analysis software. Steady state Response values (average $R_{\max}$ values for final 5s of association step) were plotted for each mAb concentration in the dilution series. All experiments were performed twice, and the mean Response was plotted. Additionally, the average dissociation rates (K_D) measured over the whole concentration series under these conditions were plotted for each mAb.

For RSV-neutralizing VH3-21/VL1-40 mAbs (MLR_15, MLR_24, MLR_55), steady state binding kinetics were also calculated for RSV-B preF. The assay described above was repeated using RSV-B preF, after which curve fitting was performed using a 1:1 binding model and the ForteBio data analysis software. Steady state Response values (average $R_{\max}$ values for final 5s of association step) were plotted for each mAb concentration in the dilution series. In addition, average apparent affinity (bivalent K_D) was calculated for these mAbs as described above for antibodies to ADI-19425.

Competition Assays:

VH3-21/VL1-40 mAbs were biotinylated using the EZ-Link™ NHS-PEG4-Biotin kit (ThermoFisher Scientific) at a theoretical 1:1 ratio of mAb to biotin. Biotinylated mAbs were immobilized on streptavidin biosensors (SA, Sartorius) at 100nM in KB buffer for 300s, after which a baseline signal was read for 60s in KB. Sensors were then immersed for 300s in wells containing 25nM of RSV-A preF that had been pre-incubated with 250nM of the previously characterized RSV mAb ADI-14337(site III) or VRC01 (control mAb) in KB for 1 hour at 37°C^{9,63}. Following this, the dissociation rate was measured in KB for 300s. Assays were repeated twice, and mean values shown. The following equation was used to calculate the % of Max Binding in the presence of competing mAbs:

$$\% \text{ of Max Binding} = (R_{\max} \text{ preF} + \text{ADI-14337} / R_{\max} \text{ preF} + \text{VRC01}) * 100$$

Generation of B cell lines

BCR expression constructs were designed as previously described^{49,65}. cDNA consisting of a signal peptide-rearranged VDJ-IgG constant regions (membrane anchored splice variant)-furin cleavage site-T2A self-cleaving peptide-signal peptide-rearranged VK or VL and kappa or lambda constant regions were codon-optimized and synthesized, and cloned into pTwist Lenti SFFV Puro WPRE to create pTwist Lenti SFFV Puro WPRE-ADI-19425-BCR, pTwist Lenti SFFV Puro WPRE-ADI-19425HC/AMMO1LC-BCR, pTwist Lenti SFFV Puro WPRE-iv8HC/ADI-19425LC-BCR, pTwist Lenti SFFV Puro WPRE-ADI-19470-BCR, pTwist Lenti SFFV Puro WPRE-ADI-24811-BCR and pTwist Lenti SFFV Puro WPRE-ADI-25542-BCR. pTwist Lenti-based BCR expression constructs were co-transfected with psPAX2 (a gift from Didier Trono, Addgene) and pMD2.G (a gift from Didier Trono, Addgene) at a 4:2:1 ratio into suspension adapted 293T cells in Freestyle 293 media (ThermoFisher Scientific) using 293-Free Transfection reagent (Millipore Sigma) according to the manufacturer's instructions. 4 days later the culture was centrifuged at $300 \times g$ to remove cells and debris and the 1mL of viral supernatant was added to 4mLs of DG-75 (human male) cells at 1×10^6 cells/mL in cRPMI plus 2 $\mu\text{g/mL}$ polybrene. 24-48 hours post-transduction and indefinitely thereafter, puromycin was added to the cell cultures at a concentration of 1 $\mu\text{g/mL}$. Generation of the DG-75 cell line transduced with the gl12A21 BCR was previously described³⁵.

Ai-mAb staining of BCR-transduced cell lines

Surface expression of BCR-transduced cell lines were confirmed by staining with fluorescently labeled ai-mAbs and subsequent analysis via flow cytometry. Briefly, parental ai-

mAbs 1D3 and 2C1 were labeled with PE and APC, respectively, using the Zenon™ Human IgG Labeling Kits (ThermoFisher Scientific). These labeled antibodies in addition to the DyLight-488 labeled bispecific ai-mAb were used to surface stain BCR-transduced cell lines. BCR expression was assessed on a FACSymphony A5 (BD Biosciences) flow cytometer and analyzed using FlowJo 10.8.1 software (Tree Star).

B cell activation in BCR-transduced cell lines

Calcium mobilization in B cells stably expressing BCRs upon bispecific antibody stimulation was monitored as previously described^{49,50}. Briefly, cells were loaded with Fluo-4 Direct calcium indicator (Invitrogen) and mixed 1:1 with complete RPMI at 37°C for 1 hour. Cells were pelleted and stained with PE-conjugated anti-human Fcγ Fab (Jackson ImmunoResearch) (1:100 dilution in 100 μL complete RPMI plus Fluo-4 Direct) for 30 min. The cells were washed with 5 mL of complete RPMI and resuspended at $\sim 2 \times 10^6$ cells/mL in complete RPMI and subjected to Ca^{2+} flux analysis at a medium flow rate on a FACSymphony A5 (BD Biosciences) flow cytometer. In all cases BCR transduced cells were mixed with untransduced cells as an internal control at a 5:2 ratio.

Levels of background fluorescence (Min_{FL}) were determined by averaging the background Fluo-4 absorbance in cells for 30s. After that, activation of B cells expressing exogenous BCRs by the various immunogens was determined by monitoring changes in Fluo-4 fluorescence associated with cells expressing the exogenous BCRs (PE^+ cells) for 210s. The bispecific ai-mAb, parental ai-mAbs (1D3, 2C1), and control mAb (VRC01), were added at a final concentration of 100 nM. RSV-A preF was added to final concentration of 1 μM. An α -IgG Fcγ F(ab')₂ (Jackson ImmunoResearch) was added to a final concentration of 100nM as a positive control. Ionomycin was added to a final concentration of 150 nM for 60s following the

immunogen addition, and maximum Fluo-4 fluorescence (Max_{FL}) was established by averaging the Fluo-4 fluorescence signal recorded during the last 10s. The percent of maximum Fluo-4 fluorescence at each time point t was determined using the formula: $(\text{Fluorescence at } t - \text{Min}_{\text{FL}}) / (\text{Max}_{\text{FL}} - \text{Min}_{\text{FL}}) \times 100$. This analysis was performed on both the BCR positive (anti-Human Fc γ -PE⁺) and BCR negative cells (anti-Human Fc γ -PE⁻) simultaneously. The background Fluo-4 fluorescence signal from the BCR negative cells was subtracted from that of the BCR positive population at each time point. All flow analysis was done with FlowJo 10.8.1 software (Tree Star).

Fab-Fab complex formation

ADI-19425 and 1D3 Fabs were mixed at a 1.5:1 (ADI-19425:1D3) molar ratio and kept at 4°C rotating overnight. The resulting complex was purified using HiLoad 16/600 Superdex S200 (Milipore Sigma) column, resulting in a major peak consisting of the Fab-Fab complex and a minor peak consisting of the excess ADI-19425 Fab. Fractions from the major peak were pooled and concentrated to 10mg/mL. ADI-19425 and 2C1 Fabs were mixed at a 1.2:1 (ADI-19425:2C1) molar ratio and complexed and purified the same way as the ADI-19425 – 1D3 complex. The ADI-19425 – 2C1 complex was concentrated to 9.8mg/mL.

Crystallization and data collection

For both complexes, crystallization conditions were screened at RT using the sitting drop vapor diffusion method with a Formulatrix NT8 drop setting and Rock Imager.

ADI-19425 Fab – 1D3 Fab complex screening was done with Clear Strategy I and II, MCSG1-3, ProPlex (Molecular Dimensions), WPS1 and 2 (Rigaku), Xtal HT, and an Additive

screen (Hampton Research). Crystals from the MCSG2 B1 condition with Additive Screen H5 (0.1M MES pH 6.5, 12% w/v PEG 20K, 4% formamide) were further optimized using the hanging drop method at RT. Final crystals were grown in a solution of 0.1M MES pH 6.5, 12% PEG 20K, and 4% formamide. A cryoprotectant of 0.1M MES pH 6.5, 12% PEG 20K, 4% formamide and 40% ethylene glycol was used. Crystals diffracted to 2.67 Å. Data was collected at ALS beamline 5.0.2 and processed using XDS⁶⁶. ADI-19425 Fab – 2C1 Fab complex screening was done with MCSG1-3, Morpheus, ProPlex (Molecular Dimensions), WPS1 (Rigaku) and Xtal HT (Hampton Research). Crystals from the WPS1 H1 condition (0.1M Imidazole pH 6.5, 10% PEG 3350, 30% MPD and 0.2M (NH₄)₂SO₄) were further optimized using the hanging drop method at RT. Final crystals were grown in a solution of 0.1M Imidazole pH 6.5, 12% PEG 3350, 30% MPD, and 0.2M (NH₄)₂SO₄. A cryoprotectant of 0.1M Imidazole pH 6.5, 12% PEG 3350, 30% MPD, and 30% ethylene glycol was used. Crystals diffracted to 2.41 Å. Data was collected at Advanced Photon Source beamline 19-ID and processed using HKL3000⁶⁷. The data collection statistics are summarized in Table S1.

Structure solution, refinement, and analysis

The structures of both ADI-19425 Fab – 1D3 Fab and ADI-19425 Fab – 2C1 were solved by molecular replacement with Phaser in Phenix⁶⁸. Chain H (heavy chain) of PDB ID: 1D5I⁶⁹ and Chain A (light chain) of PDB ID: 5I76⁷⁰ were used as the search model for 1D3 Fab. The anti-IL-7Ralpha 2B8 Fab of PDB ID: 6P67⁷¹ was used as the search model for 2C1 Fab. An existing ADI-19425 structure (PDB ID: 6APC⁹) was used as the search model for ADI-19425 Fab. All search models were divided into VL:VH and CL:CH1 components. Model building was done using Coot^{72,73} and refinement was performed in Phenix⁶⁸. The ADI-19425 Fab – 1D3 Fab structure had eight Fab-Fab complexes in the unit cell, but electron density was missing for four ADI-19425 CL:CH1 domains, which were not placed in the structure. The ADI-19425 Fab – 2C1

Fab structure had one Fab-Fab complex in the unit cell. The data collection and refinement statistics are summarized in Table S1. Structural figures were made in PyMOL⁷⁴ and Inkscape⁷⁵. BSA analysis was done using PISA^{76,77}. For the ADI-19425 Fab – 1D3 Fab complex structure, chains E, F, G and H were used for BSA analysis.

Negative stain electron microscopy and analysis

Purified DS-Cav1 was diluted to 0.03mg/mL in Tris buffer saline (10mM Tris-HCl, 150mM NaCl, pH 7.5). 4 μ L of this sample was applied to formvar/carbon Ted Pella 400 mesh copper grids and stained with 2% (w/v) uranyl formate. Grids were imaged at 120kV on a Talos L120C Transmission Electron Microscope. Data was collected using the Leginon software⁷⁸ at 1.58 Å pixel size and 2mm spherical aberration with a 40 e/Å² total exposure dose. Micrographs were loaded into Cryosparc⁷⁹ and processed. Particles were picked using Blob Picker, and unbound particles and noise were filtered out until 10,059 particles remained.

Purified DS-Cav1 was spiked with 3.2 molar excess Fab per DS-Cav1 trimer and grids were immediately stained and later imaged as described for the unbound DS-Cav1 protein. Micrographs were loaded into Cryosparc and processed. Particles were picked using Blob Picker, and unbound particles and noise were filtered out. 14,158 total particles were run through Ab-Initio and Homogeneous Refinement before a 3D class was generated. The 3D model was analyzed in ChimeraX⁸⁰.

Virus production

Recombinant RSV subtype strain A2-GFP and subtype strain B1-GFP were obtained from ViraTree. Viruses were cultured and viral titers determined as previously described⁴⁴.

Briefly, RSV viruses were cultured on HEp-2 cells (human male) in cDMEM and harvested by physical disruption. Viral supernatant was purified from cell pellets by centrifugation at $4,000 \times g$, followed by decanting. Viral supernatant was aliquoted in 1mL vials, flash frozen, and stored at -80°C . Viral titers were determined through infection of Vero cells (African green monkey) in 24-well plates over serial dilutions of the virus, overlaying with DMEM including 0.8% methylcellulose (Sigma-Aldrich). Fluorescent scans were completed using a Typhoon imager (GE Life Sciences) at 5 days post-infection, and plaques were quantified using ImageJ (LOCI, University of Wisconsin).

Plaque Reduction Neutralization Assays

As a primary screen of neutralization activity of VH3-21/VL1-40 mAbs, plaque reduction assays were run on Vero cells at a set mAb concentration of 500 $\mu\text{g/mL}$. Vero cells were seeded at 100,000 cells/mL in 24 well plates and cultured for 48-72 hours. VH3-21/VL1-40 mAbs were diluted to 500 $\mu\text{g/mL}$ in DMEM and then mixed with an equal volume of RSV-A-GFP or RSV-B-GFP diluted to 2,000 pfu/mL and incubated for 1 hour at 37°C . Vero cells were then incubated with 100 μL of the antibody/virus mixture for 1 hour at 37°C , followed by addition of 500 μL DMEM containing 0.8% methylcellulose. Fluorescent images were taken at 5 days post-infection using a Typhoon imager, and plaques were counted using ImageJ. Plaque counts were transformed into % infectivity by dividing the well counts by those from no antibody control wells and multiplying by 100%. Antibodies that had visible neutralization capacity, reduced % infectivity vs. no antibody containing wells, were followed up for IC_{50} titer calculation. All antibodies were run in triplicate over a single experiment for these initial screens.

mAb IC_{50} and IC_{80} values were further determined by plaque reduction assays. Vero cells were prepared as above, and monoclonal antibodies were serially diluted in 80 μL of DMEM

mixed with 80 μ L of RSV-A-GFP or RSV-B-GFP diluted to 2,000 pfu/mL and incubated for 1 hour at 37°C. Vero cells were then incubated with 100 μ L of the antibody/virus mixture for 1 hour at 37°C, followed by addition of 500 μ L DMEM containing 0.8% methylcellulose. Fluorescent images were taken at 5 days post-infection using a Typhoon imager, and plaques were counted using ImageJ. Plaque counts were transformed into % infectivity by dividing the well counts by those from the highest antibody dilution for each respective antibody multiplied by 100%. % infectivity was plotted as a function of antibody dilution. The neutralization curves were fit using the log(inhibitor) versus response - variable slope (four parameters) analysis in Prism 9.4.0. The half maximal inhibitory antibody dilution IC₅₀ and IC₈₀ were interpolated from the curve in Prism 9.5.0. Undetermined values were reported as 2x the highest concentration tested. Within each assay antibodies were run in duplicate, and assays were repeated 3-4 times.

Plaque Reduction Neutralization Competition Assays

IC₅₀ and IC₈₀ concentrations for MLR_24 against RSV-A and RSV-B were determined as above. MLR_24 at its IC₅₀ and IC₈₀ concentrations against RSV-A and RSV-B were prepared in 80 μ L of DMEM mixed with 80 μ L of RSV-A-GFP or RSV-B-GFP diluted to 2,000 pfu/mL in triplicate wells and incubated for 1 hour at 37°C. In parallel, MLR_24 was mixed at its IC₅₀ and IC₈₀ concentrations against RSV-A and RSV-B with matched amounts of 5 preF-binding, non-neutralizing mAbs (MLR_27, MLR_30, MLR_33, MLR_42, MLR_45) in 80 μ L of DMEM mixed with 80 μ L of RSV-A-GFP or RSV-B-GFP diluted to 2,000 pfu/mL in triplicate wells and incubated for 1 hour at 37°C. Vero cells were then incubated with 100 μ L of the antibody/virus mixture for 1 hour at 37°C, followed by addition of 500 μ L DMEM containing 0.8% methylcellulose. Fluorescent images were taken at 5 days post-infection using a Typhoon imager, and plaques were counted using ImageJ. Plaque counts were transformed into % infectivity by dividing the well counts by the average of those from infection of cells without antibody multiplied by 100%.

QUANTIFICATION AND STATISTICAL ANALYSIS

Data were analyzed, and graphs created using GraphPad Prism 9.5.0 software. Statistical tests used are included in figure legends where applicable as well as sample sizes and replicates.

Supplemental Figures and Tables

A

	10	20	30	40	50	60	70	80	90	100	110	120	Accession Number
IGHV3-21*01	EVQLVESGGGLVKPGGSLRLS	CAASGFTFSSYSMNWVRQAPGK	GLEWVSSISSSSSYYIYADSVKGRFTISRDNAKNSLYLQMNSLRAEDTAVYYCAR										
ADI-19425 HC										LGYCSGGSCH	FDYWGGTLVTVSS		MG524063
ADI-14337 HC										EYYDSSGYTNWFDPWGQGT	TLVTVSS		MG524251
ADI-25532 HC	Q		Q										MG524182

B

	10	20	30	40	50	60	70	80	90	100	110	Accession Number
IGLV1-40*01	QSVLTQPPSVSGAPGQRVTISCTGSSSNIGAGYDVHWYQQLPGTAPKLLIYGNSNRPSGVDPDRFSGSKSGTSASLAITGLQAEDEADYYCQSYDSSLG											
ADI-19425 LC	P										FYVFGTGTKLTVL	MG524528
ADI-14337 LC	P										A--WVFGGGTQLTVL	MG524647
ADI-25532 LC	V										--YVFGTGTKVTVL	MG524716

Figure S1: Alignment of unmutated VH3-21/VL1-40 mAbs used to generate anti-idiotypic hybridomas. Related to Figure 1. **(A)** Alignment of ADI-19425, ADI-14337, and ADI-25532⁹ heavy chains to germline VH3-21*01. **(B)** Alignment of ADI-19425, ADI-14337, and ADI-25532 light chains to germline VL1-40*01.

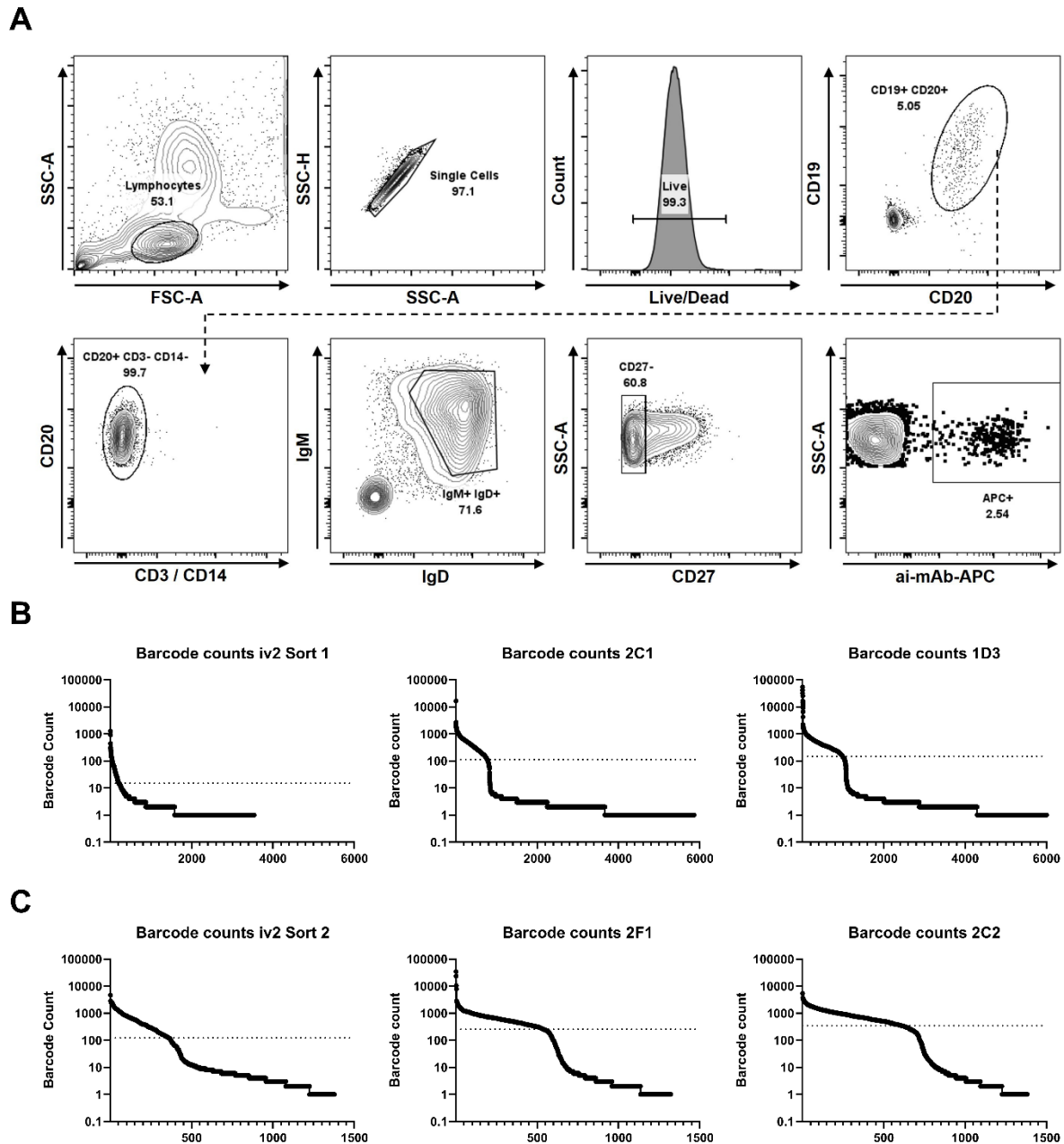


Figure S2: Gating strategy to sort ai-mAb specific naïve B cells and barcode threshold setting. Related to Figure 1. **(A)** Gating as follows: Single cells → Lymphocytes → Live cells → CD3⁻ CD14⁻ → CD19⁺ CD20⁺ → IgD⁺ IgM⁺ → ai-mAb-APC⁺ B cells. Cell percentages of parent gate are shown, representative staining plots from 1 group of sorted cells. **(B & C)** ai-mAbs were labeled with APC conjugated to a unique oligonucleotide feature barcode and used to stain

naïve PBMC in two cocktails containing iv2, 2C1, 1D3 (**B**) and iv2, 2F1, and 2C2 (**C**). Individual B cell libraries were generated using the chromium platform from 10X Genomics. Each panel shows the number of times the indicated feature barcode was observed for a given cell plotted from highest to lowest where each dot represents a single B cell. The thresholds, represented by the horizontal dotted line in each panel, were estimated by segmented generalized linear models using the *chngpt* R package³⁷. BCR sequences from B cells above the threshold were considered positive for a given ai-mAb. B cells above the threshold from the iv2 isotype control in each sort were discarded from subsequent analysis.

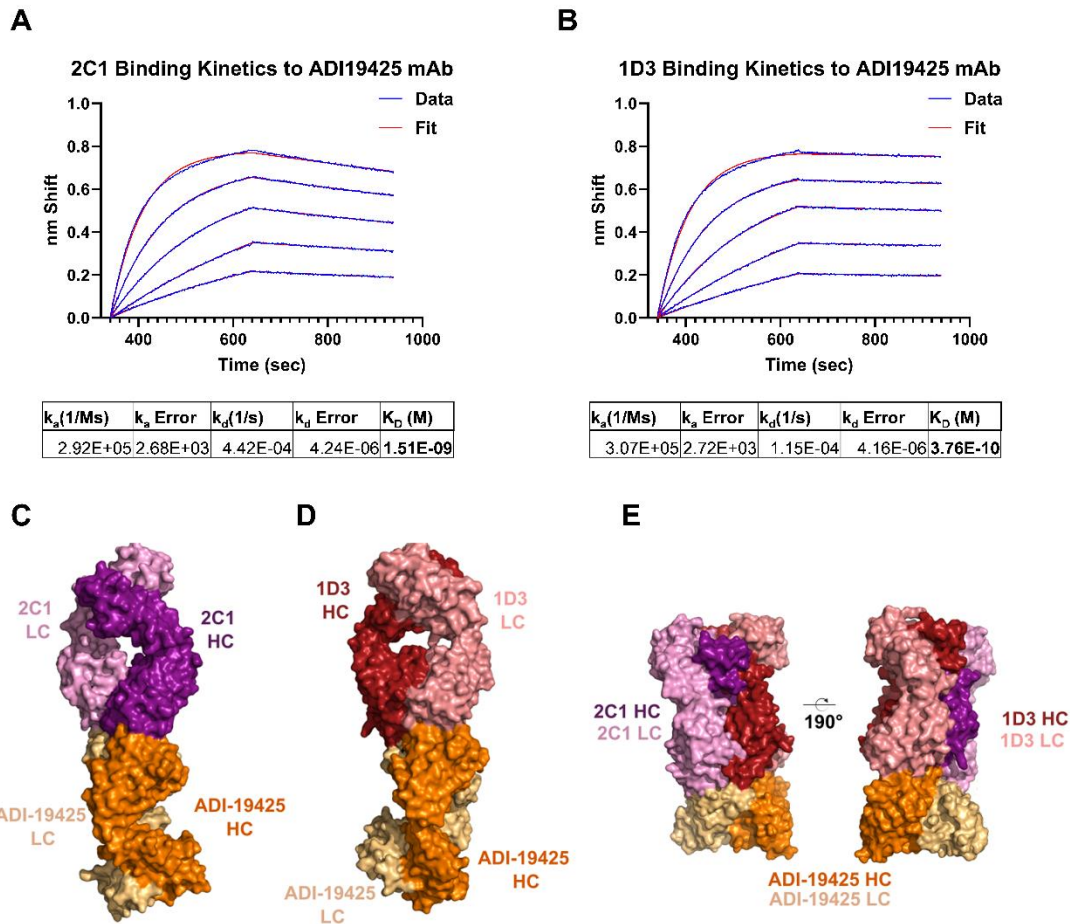


Figure S3: Binding kinetics of ai-mAbs to ADI-19425 Fab and Fab-Fab structures. Related to Figure 2. **(A & B)** Binding kinetics of recombinant 2C1 **(A)** or 1D3 **(B)** IgG to ADI-19425 Fab at the indicated concentrations were measured by biolayer interferometry. Blue lines represent observed data, and red lines are theoretical fit. The calculated kinetic parameters are shown below each plot. Data shown from a single biological replicate. **(C-E)** Full view of complexes between **(C)** 2C1 Fab (dark purple – heavy chain, light purple – light chain) and **(D)** 1D3 Fab (red – heavy chain, pink – light chain) with ADI-19425 Fab (orange – heavy chain, wheat – light chain). **(E)** Overlays of both complexes are shown aligned by the VH/VL region of ADI-19425 Fab and rotated on the y-axis by 190°.

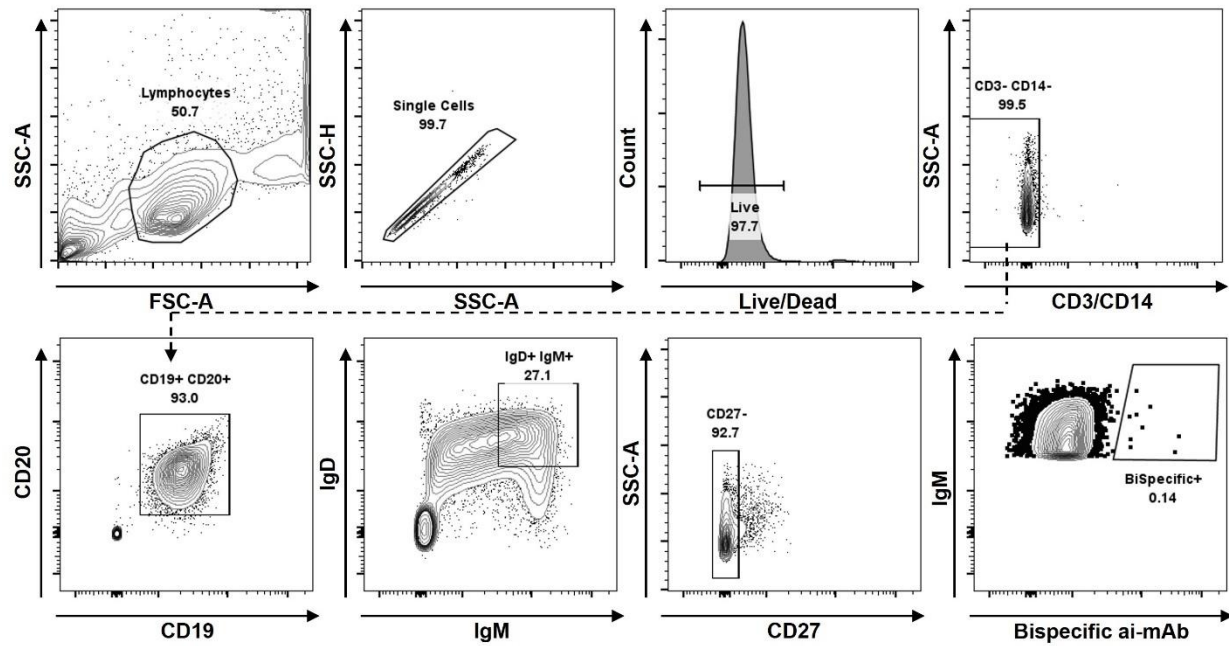


Figure S4: Gating strategy to identify and single cell sort ai-mAb specific naïve B cells.

Related to Figure 4. Gating as follows: Lymphocytes → Single cells → Live cells → CD3⁻, CD14⁻ → CD19⁺ CD20⁺ → IgD⁺ IgM⁺ → CD27⁻ → IgM⁺ Bispecific ai-mAb⁺. Percentages of parent gate indicated, representative gating from one sorting experiment.

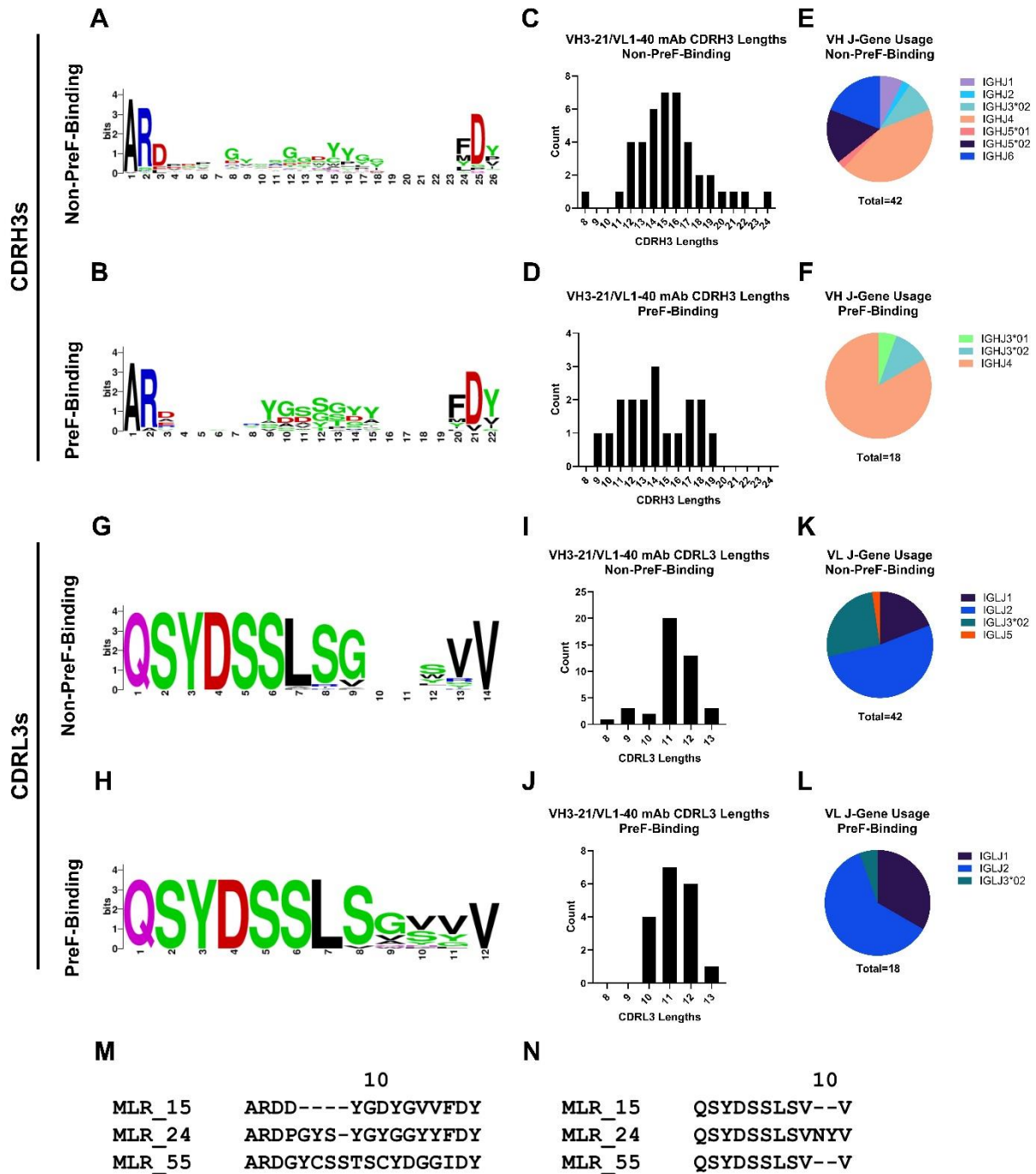


Figure S5: CDR3 and J-gene analysis of ai-mAb-sorted VH3-21/VL1-40 mAbs. Related to Figure 4. **(A & B)** Sequence logo plots of non-preF-binding **A** and preF-binding **B** CDRH3s of sorted VH3-21/VL1-40 mAbs created with <https://weblogo.berkeley.edu/logo.cgi>. **(C & D)** Histograms of CDRH3 lengths of non-preF-binding **C** and preF-binding **D** VH3-21/VL1-40 mAbs.

(**E & F**) Heavy chain J-gene usage of non-preF-binding **E** and preF-binding **F** VH3-21/VL1-40 mAbs. J-gene alleles with the same amino acid usage combined for analysis. (**G & H**) Sequence logo plots of non-preF-binding **G** and preF-binding **H** CDRL3s of sorted VH3-21/VL1-40 mAbs. (**I & J**) Histograms of CDRL3 lengths of non-preF-binding **I** and preF-binding **J** VH3-21/VL1-40 mAbs. J-gene alleles with the same amino acid usage combined for analysis (**K & L**) Light chain J-gene usage of non-preF-binding **K** and preF-binding **L** VH3-21/VL1-40 B cells. (**M & N**) Alignments of CDRH3s **M** and CDRL3s **N** of RSV-neutralizing VH3-21/VL1-40 B cells.

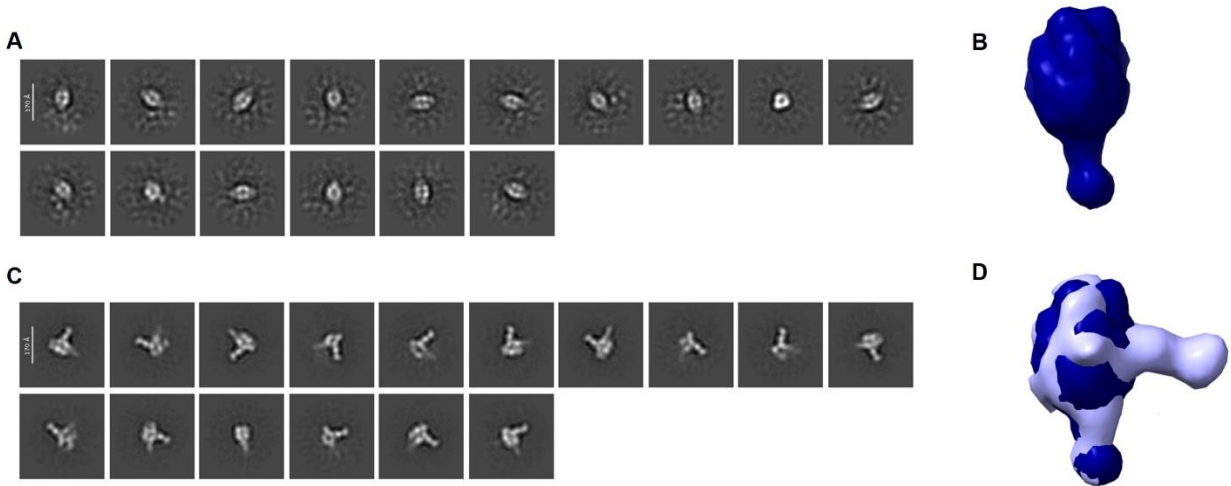


Figure S6: nsEM Images of DS-Cav1 and MLR_24 Fab – DS-Cav1 Complex. Related to Figure 4. **(A)** Representative 2D classes of unbound DS-Cav1¹⁵. **(B)** A 20Å map of a crystal structure of RSV preF with a trimerization domain (PDB ID: 5TDG). **(C)** Representative 2D classes of the DS-Cav1 – MLR_24 Fab complex. **(D)** The 20Å map of the RSV preF crystal structure (dark blue) superimposed onto a representative 3D class of the DS-Cav1 – MLR_24 Fab complex (light purple).

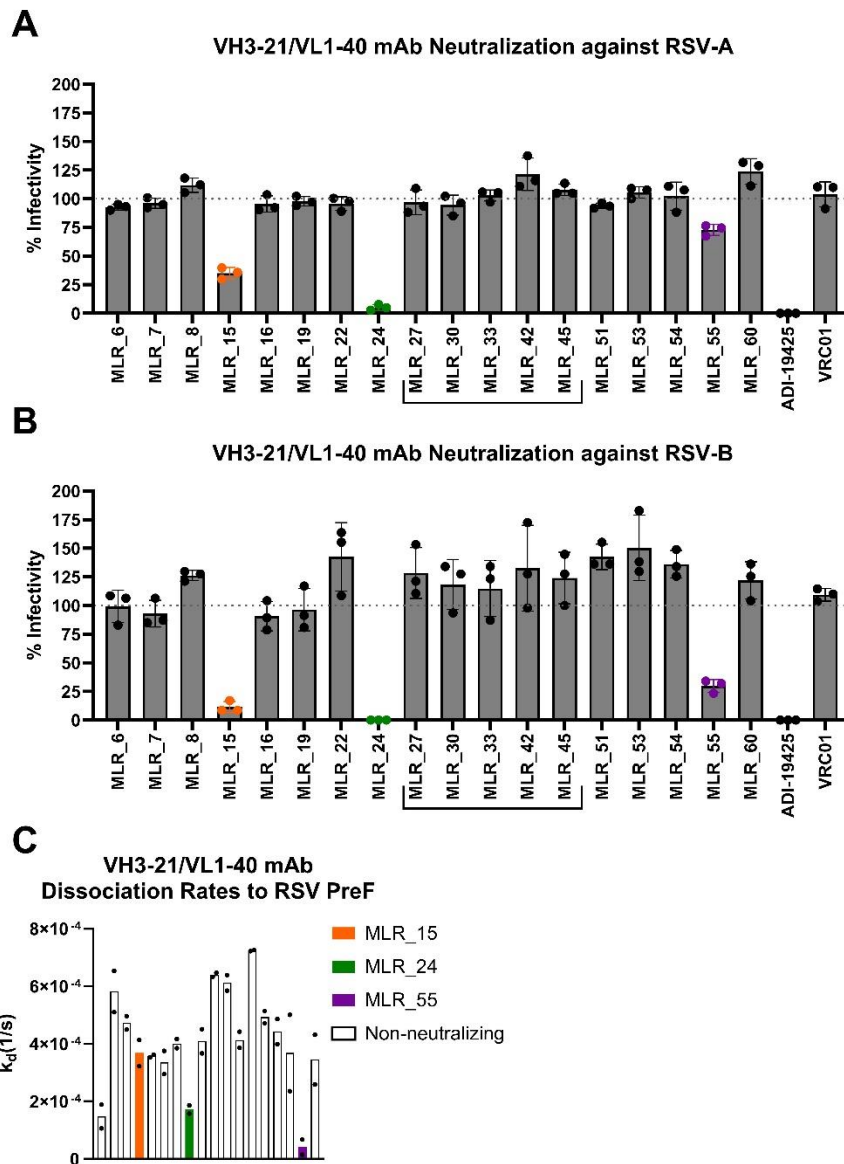


Figure S7: Neutralization screen of VH3-21/VL1-40 sorted mAbs against RSV-A / B and Steady State Affinity Measurements. Related to Figures 5 and 7. All recombinant VH3-21/VL1-40 preF-binding mAbs sorted with the bispecific ai-mAb were screened for neutralization activity against RSV-A (**A**) or RSV-B (**B**) at 500 $\mu\text{g/mL}$ final dilution using a plaque reduction assay in triplicate. % Infectivity is the infectivity in the presence of mAb $\div$ infectivity in the absence of mAb $\times 100\%$. Colored mAbs indicate those followed up for IC_{50} determination. VRC01⁶³ is an irrelevant HIV mAb and included as a negative control. Bracketed mAbs were

used in neutralization competition assays in **Fig. 7A & B**. Each point represents a technical replicate from a single experiment. Bars represent mean $\pm$ standard deviation. **(C)** Dissociation rates for VH3-21/VL1-40 mAbs averaged over the dilution series in **Fig. 5C**, coloring consistent as above. Each data point is is a biological replicate, bars indicate mean.

	ADI-19425 Fab - 1D3 Fab	ADI-19425 Fab - 2C1 Fab
Data collection		
Space group	P1	P12 ₁
Cell dimensions		
<i>a</i> , <i>b</i> , <i>c</i> (Å)	95.07, 149.98, 164.43	66.80, 66.93, 130.91
α , β , γ (°)	83.98, 89.90, 74.61	90, 103.74, 90
Resolution (Å)	49.45-2.72 (2.72 – 2.67)	50.00–2.44 (2.44-2.40)
R_{merge}^a	0.116 (0.566)	0.242 (0.891)
$\langle I/\sigma(I) \rangle$	4.0 (0.7)	15.4 (2.1)
$CC_{1/2}$	0.961 (0.319)	0.953 (0.844)
Completeness	92.5 (92.4)	98.4 (92.5)
Redundancy	1.9 (1.9)	6.6 (6.4)
Refinement		
Resolution (Å)	49.33 – 2.67 (2.77 – 2.67)	42.16 – 2.41 (2.50 – 2.41)
No. unique reflections	228772 (22653)	42798 (3991)
$R_{\text{work}}^b/R_{\text{free}}^c$	23.1/27.3 (32.6/37.2)	20.5/25.3 (26.9/33.1)
No. atoms	45020	6660
Protein	44852	6464
Water	178	166
Ligand	0	60
B-factors (Å ²)	56.24	34.79
Protein	56.31	34.80
Water	39.65	33.19
Ligand	0	41.86
RMS bond length (Å)	0.003	0.005
Ramachadran Plot Statistics^d		
Residues	5948	861
Most Favored region	94.16	95.28
Allowed Region	5.48	4.37
Disallowed Region	0.36	0.35
Clashscore	6.10	6.66
PDB ID	8VS8	8VS7
^a $R_{\text{merge}} = [\sum_h \sum_i I_h - I_{hi} / \sum_h \sum_i I_{hi}]$ where I_h is the mean of I_{hi} observations of reflection h . Numbers in parenthesis represent highest resolution shell. ^b R_{factor} and ^c $R_{\text{free}} = \sum F_{\text{obs}} - F_{\text{calc}} / \sum F_{\text{obs}} \times 100$ for 95% of recorded data (R_{factor}) or 5% data (R_{free}). ^d Determined using MolProbity (10.1002/pro.3330)		

Table S1: Data collection and refinement statistics for crystal structures

Name	Donor	RSV Pref Rmax (DS-Cav1)*	RSV-A Neut. IC ₅₀ (µg/mL)**	RSV-B Neut. IC ₅₀ (µg/mL)**	GenBank Accession Number (VH)	GenBank Accession Number (VL)
MLR_1	A	N.B.	N.N.	N.N.	PP429379	PP429439
MLR_2	A	N.B.	N.N.	N.N.	PP429380	PP429440
MLR_3	A	N.B.	N.N.	N.N.	PP429381	PP429441
MLR_4	A	N.B.	N.N.	N.N.	PP429382	PP429442
MLR_5	A	N.B.	N.N.	N.N.	PP429383	PP429443
MLR_6	A	0.311	N.N.	N.N.	PP429384	PP429444
MLR_7	A	0.834	N.N.	N.N.	PP429385	PP429445
MLR_8	A	0.437	N.N.	N.N.	PP429386	PP429446
MLR_9	A	N.B.	N.N.	N.N.	PP429387	PP429447
MLR_10	A	N.B.	N.N.	N.N.	PP429388	PP429448
MLR_11	A	N.B.	N.N.	N.N.	PP429389	PP429449
MLR_12	A	N.B.	N.N.	N.N.	PP429390	PP429450
MLR_13	A	N.B.	N.N.	N.N.	PP429391	PP429451
MLR_14	B	N.B.	N.N.	N.N.	PP429392	PP429452
MLR_15	B	1.017	130.57	21.75	PP429393	PP429453
MLR_16	B	0.333	N.N.	N.N.	PP429394	PP429454
MLR_17	B	N.B.	N.N.	N.N.	PP429395	PP429455
MLR_18	B	N.B.	N.N.	N.N.	PP429396	PP429456
MLR_19	B	0.465	N.N.	N.N.	PP429397	PP429457
MLR_20	B	N.B.	N.N.	N.N.	PP429398	PP429458
MLR_21	B	N.B.	N.N.	N.N.	PP429399	PP429459
MLR_22	B	0.303	N.N.	N.N.	PP429400	PP429460
MLR_23	B	N.B.	N.N.	N.N.	PP429401	PP429461
MLR_24	B	1.122	24.2	0.22	PP429402	PP429462
MLR_25	B	N.B.	N.N.	N.N.	PP429403	PP429463
MLR_26	B	N.B.	N.N.	N.N.	PP429404	PP429464
MLR_27	B	0.494	N.N.	N.N.	PP429405	PP429465
MLR_28	B	N.B.	N.N.	N.N.	PP429406	PP429466
MLR_29	B	N.B.	N.N.	N.N.	PP429407	PP429467
MLR_30	B	0.776	N.N.	N.N.	PP429408	PP429468
MLR_31	B	N.B.	N.N.	N.N.	PP429409	PP429469
MLR_32	B	N.B.	N.N.	N.N.	PP429410	PP429470
MLR_33	B	0.753	N.N.	N.N.	PP429411	PP429471
MLR_34	C	N.B.	N.N.	N.N.	PP429412	PP429472
MLR_35	C	N.B.	N.N.	N.N.	PP429413	PP429473
MLR_36	C	N.B.	N.N.	N.N.	PP429414	PP429474
MLR_37	C	N.B.	N.N.	N.N.	PP429415	PP429475
MLR_38	C	N.B.	N.N.	N.N.	PP429416	PP429476
MLR_39	C	N.B.	N.N.	N.N.	PP429417	PP429477
MLR_40	C	N.B.	N.N.	N.N.	PP429418	PP429478
MLR_41	C	N.B.	N.N.	N.N.	PP429419	PP429479
MLR_42	C	0.362	N.N.	N.N.	PP429420	PP429480
MLR_43	C	N.B.	N.N.	N.N.	PP429421	PP429481
MLR_44	C	N.B.	N.N.	N.N.	PP429422	PP429482
MLR_45	C	0.882	N.N.	N.N.	PP429423	PP429483
MLR_46	C	N.B.	N.N.	N.N.	PP429424	PP429484
MLR_47	C	N.B.	N.N.	N.N.	PP429425	PP429485
MLR_48	C	N.B.	N.N.	N.N.	PP429426	PP429486
MLR_49	C	N.B.	N.N.	N.N.	PP429427	PP429487
MLR_50	C	N.B.	N.N.	N.N.	PP429428	PP429488
MLR_51	C	0.500	N.N.	N.N.	PP429429	PP429489

MLR 52	C	N.B.	N.N.	N.N.	PP429430	PP429490
MLR 53	C	0.381	N.N.	N.N.	PP429431	PP429491
MLR 54	C	0.430	N.N.	N.N.	PP429432	PP429492
MLR 55	C	0.606	N.N.	98.21	PP429433	PP429493
MLR 56	C	N.B.	N.N.	N.N.	PP429434	PP429494
MLR 57	C	N.B.	N.N.	N.N.	PP429435	PP429495
MLR 58	C	N.B.	N.N.	N.N.	PP429436	PP429496
MLR 59	C	N.B.	N.N.	N.N.	PP429437	PP429497
MLR 60	C	0.426	N.N.	N.N.	PP429438	PP429498
*N.B., non-binder; **N.N. non-neutralizing.						

Table S2: Properties and accession numbers for sorted and produced VH3-21/VL1-40 mAbs

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Chapter 3: Characterization of an anti-idiotypic RSV vaccine in an engineered mouse model

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Introduction

Development of an infant RSV vaccine has faced numerous challenges over the years, including failed clinical trials as recently as 2024¹. These obstacles have made it clear that a single vaccine strategy may not be amenable to all infants, especially those who are RSV-naïve, necessitating the development of novel and diverse immunization approaches. To address this, we have developed and completed *in vitro* characterization of an RSV-vaccine tailored for the infant population based on anti-idiotypic antibodies² (and Chapter 2). This vaccine relies on targeting a reproducible B cell class expressing VH3-21/VL1-40 immunoglobulin genes that can give rise to antibodies that neutralize RSV without the need for affinity maturation^{2,3}. Additionally, this class of B cells produce antibodies that recognize a conserved epitope present only RSV preF, the main target of neutralizing antibody responses in humans^{4,5}. Using B cell sorting, antibody characterization and *in vitro* assays, we demonstrated that the anti-idiotypic immunogen (ai-mAb) could target and activate B cells expressing B cell receptors (BCRs) from this specific class and engage primary B cells expressing bona fide RSV neutralizing BCRs². This initial work provides proof of concept of the efficacy of the ai-mAb to target RSV-protective B cells and warrants further development as a vaccine candidate.

Evaluation of the bispecific anti-idiotypic vaccine in a relevant small animal model would provide compelling evidence to advance the immunogen further into pre-clinical and early-stage clinical investigation. Multiple animal models have been used for evaluation of RSV vaccine candidates and monoclonal antibodies, but the most common are cotton rats and BALB/c mice⁶⁻

¹³. These animals exhibit defined pathology and measurable viral loads in the nasal and lung tissues as well as lose weight in response to RSV challenge. Common C57BL/6 mice have also been leveraged as an animal model for RSV infection and therapeutic development¹⁴⁻¹⁷. Evaluation of our anti-idiotypic vaccine in a mouse model is hampered by the fact that mice do not express the human-derived VH3-21/VL1-40 BCR genes. To address this problem, we engineered a knock-in (KI) C57BL/6 mouse that constitutively expresses the RSV-neutralizing, germline-encoded VH3-21/VL1-40 BCR, ADI-19425³. After determining successful KI of the target BCR, and normal development of B cells in this mouse, we employed it in an congenic adoptive transfer model where the transgenic VH3-21/VL1-40 B cells are present at physiological frequencies observed in humans (~0.14%)^{18,19}. Using this adoptive transfer model, we demonstrate that immunization with the bispecific anti-idiotypic vaccine generates a sustained RSV preF specific serum response that can neutralize RSV *in vitro*. The ADI-19425 BCR is reactive only with RSV preF, and thus this is the expected outcome. We further demonstrate that immunization with the ai-mAb activates KI-B cells and that some of these B cells differentiate into plasma cells that traffic to the long-lived bone marrow niche. Finally, we demonstrate that immunization with the ai-mAb prior to nasal challenge with RSV leads to reduced viral loads in the lung tissue of mice compared to mock immunized animals. Collectively these results provide compelling evidence that the ai-mAb can engage target B cells *in vivo* and induce a protective neutralizing antibody response, supporting the further development of the bispecific anti-idiotypic as an alternative vaccine immunogen for protection against RSV specifically tailored for the infant population.

Results

Generation of a knock-in mouse to express the VH3-21/VL1-40 BCR ADI-19425

Mice lack VH3-21 and VL1-40 antibody genes, and thus we generated a KI-mouse constitutively expressing the unmutated, RSV-neutralizing VH3-21/VL1-40 BCR, ADI-19425³.

Specifically, we used CRISPR-Cas9 to introduce the rearranged Ab heavy chain (HC) and light chain (LC) to replace the variable-joining and kappa light chain-joining gene clusters, respectively, in C57BL/6 CD45.2⁺ mice (Fig. 1A-B)²⁰. Binding of mouse B cells to DS-Cav1 fluorescent tetramers (RSV preF⁸) demonstrated a successful KI for both chains, as ~65% of KI B cells stained with the tetramer while tetramer binding was not observed in B cells from wild-type (WT) mice (Fig. 1C). These data confirmed that the ADI-19425 BCR was successfully knocked in, but that allelic exclusion is incomplete since not all B cells bind preF.

Establishment of adoptive transfer conditions to recapitulate human VH3-21/VL1-40 B cell frequency

Adoptive transfer mouse models have been used widely by us and others to better recapitulate the human B cell repertoire for vaccination studies²¹⁻²³. These systems rely on the differential marking of donor or recipient cells to be able to differentiate them. Here, C57BL/6 mice expressing the CD45.1 allele were used as recipients so that the transgenic B cells, expressing CD45.2, could be identified by differential CD45.1 vs. CD45.2 cell surface staining. To generate a mouse model of VH3-21/VL1-40 B cell frequency reflective of that seen in humans, we transferred 5,000, 50,000 and 500,000 KI B cells into groups of 4 recipient CD45.1 mice intravenously and enumerated the frequency of transferred cells in spleens 24 hours later by CD45 staining (Fig. 1D-E). Based on the average of 4 mice in each experimental group, transfer of 5,000 B cells resulted in a frequency of 0.0004% CD45.2 B cells, transfer of 50,000 B cells resulted in a frequency of 0.00275% CD45.2 B cells and transfer of 500,000 B cells resulted in a frequency of 0.0371% CD45.2 B cells in the spleen 24 hours after infusion (Fig. 1E-F). Based on our previous research, 5% of VH3-21/VL1-40 B cells that are targeted by the ai-mAb are expected to be able to neutralize RSV². Extrapolating from the 0.14% frequency of naïve VH3-21/VL1-40 B cells present in the human population^{18,19}, we expect the frequency of

naïve VH3-21/VL1-40 B cells capable of neutralizing RSV and recognized by the ai-mAb to be ~0.007%. As such, we opted to transfer 500,000 B cells for subsequent studies.

Immunization with the ai-mAb in the adoptive transfer model generates sustained serum RSV neutralizing titers

Following the establishment of adoptive transfer conditions to recapitulate the human frequency of germline VH3-21/VL1-40 RSV-neutralizing naïve B cells, we performed immunization experiments to assess the immunogenicity and serum responses in animals receiving the ai-mAb compared to those receiving an isotype control immunization. Specifically, we transferred 500,000 ADI-19425⁺ KI B cells into CD45.1 recipient mice and immunized them via intramuscular (IM) injection 24 hours later with 10µg of the bispecific ai-mAb or an isotype control mAb (AMMO1²⁴) formulated in 5µg of SMNP adjuvant²⁵ (Fig. 2A). We used this approach to mimic the common route of human vaccine administration, while using a recently characterized highly immunogenic adjuvant that is being tested in on-going clinical trials (NCT06033209). Analysis of serum antibody responses 5 weeks post-immunization revealed binding responses to RSV preF⁸ in mice given the ai-mAb, while those immunized with the isotype control showed little reactivity to preF (Fig. 2B). Additionally, ai-mAb immunized mice demonstrated serum neutralizing antibodies to RSV-A that were maintained up to five weeks (Fig. 2C-D). Together these data demonstrates that a single immunization with the ai-mAb engages target B cells in the adoptive transfer model stimulating a sustained humoral response that can bind and neutralize RSV.

Immunization with the ai-mAb leads to plasma cell entry into the bone marrow niche

As immunization with the ai-mAb could activate transferred on-target VH3-21/VL1-40 B cells (ADI-19425⁺ KI) and elicit a sustained neutralization response in the serum (Fig. 2), we next assessed the differentiation and tissue localization of these cells. Activated B cells become

memory or plasma cells, and some of these plasma cells accumulate in the bone marrow (BM) niche to provide long-term antibody secretion and immunological memory. These cells provide the basis for long-term immunity and generation of this differentiated B cell phenotype is a prime objective of most vaccination schemes²⁶. To determine if our immunization approach was capable of stimulating plasma cell differentiation and BM entry of VH3-21/VL1-40 cells, we modified our conditions by transferring a larger amount (2×10^6) of B cells to facilitate identification of rare cells in this restricted niche (Fig. 3A). We initially attempted these experiments with our standard transfer conditions but found this population challenging to visualize (Data not shown). As in previous experiments, ai-mAb immunization elicited a robust and sustained serum RSV preF binding response (Fig. 3B) that corresponded with *in vitro* RSV-A neutralization (Fig. 3C-D). Examination of the plasma cell population in the BM niche of mice five weeks post-immunization revealed the presence of a small, but detectable population of KI-CD45.2⁺ cells in multiple mice (Fig. 3E, red quadrant). These data demonstrates that immunization with the ai-mAb can generate on-target plasma cell entry into the BM niche and supports the possibility of lasting protection afforded by antibody secretion from these long-lived cells.

Establishment of RSV challenge and protection

To determine whether immunization with the bispecific ai-mAb in our adoptive transfer model could protect against subsequent RSV infection, we leveraged a model of RSV challenge in C57BL/6 mice (Fig. 4). To first determine if antibodies generated from ADI-19425-KI B cells would, in theory, protect against RSV challenge as well as to establish infection conditions in our model, groups of 4 mice were transferred with either 500 μ g of ADI-19425 IgG or the isotype control antibody AMMO1²⁴ by intraperitoneal injection (Fig. 4A). 24 hours post-transfer, mice were bled to determine levels of human IgG present in their sera using antigen-specific ELISA, which revealed successful transfer of the antibodies at comparable levels (Fig. 4B). Additionally,

neutralization assays demonstrated that the sera of mice transferred with ADI-19425 were able to neutralize RSV-A *in vitro*, while the sera of mice transferred with AMMO1 could not. At this same day 0 timepoint, both groups of mice were challenged with 1×10^6 plaque forming units (pfus) of RSV-A GFP intranasally (Fig. 4A). All mice were sacrificed 5 days post infection, and their lungs were harvested and assayed for RSV-A viral titers. All mice that were transferred with ADI-19425 IgG had absent viral lung titers, while the mice that received the isotype control had substantial lung titers (Fig. 4D). These results demonstrate that the mice were successfully infected with RSV, exhibiting detectable lung titers, and mice that received transfer of ADI-19425 IgG were completely protected from lung infection. These data helped to establish in our model a productive mouse RSV infection, and to confirm the premise that antibodies derived from the ADI-19425⁺ KI B cells would protect against lung infection *in vivo* if present at adequate frequencies.

Immunization with the ai-mAb reduces RSV lung infection

To determine whether immunization with the ai-mAb would stimulate a response that protects against subsequent RSV infection, a similar vaccination experiment was carried out as described in Figure 2. Groups of 8 mice received adoptive transfer of 500,000 ADI-19425⁺ KI B cells followed 24 hours later by immunization with either 10 μ g of ai-mAb or an isotype control formulated in 5 μ g SMNP adjuvant via IM injection (Fig. 5A). Mice were then bled at 7, 14 and 19 days post immunization, and ELISA demonstrated sustained preF binding titers in the sera of mice that received immunization with the ai-mAb as previously seen (Fig. 5B). Additionally, the sera of these mice were capable of RSV-neutralization *in vitro*, while the sera of mice that received immunization with AMMO1 were not (Fig. 5C-D). Finally, mice were challenged with 1×10^6 pfus of RSV-A GFP intranasally at day 14, and then sacrificed 5 days later and lungs were harvested to measure viral titers. Mice that received immunization with the ai-mAb demonstrated significantly reduced RSV lung titers compared to the mice that received

immunization with AMMO1, demonstrating the efficacy of this vaccine to protect against RSV infection *in vivo* (Fig. 5E).

Conclusions

We have engineered a mouse that constitutively expresses the unmutated, RSV-neutralizing VH3-21/VL1-40 BCR, ADI-19425. Using this KI mouse, we established an adoptive transfer model that recapitulates the estimated frequency of unmutated VH3-21/VL1-40 RSV-neutralizing B cells found in the human repertoire, and used this model to evaluate the immunogenicity and efficacy of a bispecific ai-mAb as an RSV vaccine candidate. In this adoptive transfer model, we demonstrate activation of on-target ADI-19425⁺ B cells that leads to a durable RSV-neutralizing serum response in mice immunized with the ai-mAb. Additionally, we show immunization with the ai-mAb leads to a population of plasma cells in the long-lived BM niche that are comprised of on-target ADI-19425⁺ KI cells, demonstrating efficacy in eliciting this critical memory cell population. This long-lived population of cells is often a primary goal of vaccination efforts and has been difficult to elicit in some contemporary immunization schemes^{26,27}. Studies to confirm this population and to determine the duration of protection conferred by this memory population are future goals of this project.

Finally, immunization with our bispecific ai-mAb leads to a significant reduction in RSV lung titers following intranasal challenge compared to a control immunization, demonstrating the protective efficacy of our immunogen. While the model generated here focuses on activating a B cell subset that has a fixed BCR capable of generating RSV-neutralizing antibodies, our data presents critical proof of concept that our immunogen would generate a protective response within a polyclonal VH3-21/VL1-40 B cell environment if neutralizers are present in a similar frequency to what we have estimated. While our immunization data demonstrates that we can elicit sustained serum neutralizing titers and partial protection from challenge, the passive transfer study illustrates that sterilizing immunity could be accomplished through more robust

on-target cell activation and higher neutralizing antibody titers. Future studies will be directed at increasing the immunogenicity of the ai-mAb to reach the goal of sterilizing immunity as well as immunization experiments in more relevant conditions such as in animals with RSV-specific maternal serum antibodies present (Chapter 4).

Limitations of the current study include the use of the C57BL/6 mouse as the animal model. While these mice have been used previously for the development of RSV treatments, they do not present with the same pathology seen in other animal models or humans¹⁴⁻¹⁷. While this was necessitated due to the reliance and methodology of generating an engineered mouse model, future studies could focus on engineering a BALB/c strain, using other humanized systems or C57BL/6 mice harboring an immune deficiency to lead to enhance pathology. Another limitation was relying on a supraphysiological frequency of transferred cells to detect ADI-19425⁺ plasma cells in the bone marrow following immunization with the ai-mAb. While this larger transfer population was used to enable imaging of this rare plasma cell population by flow cytometry, we note that the sustained serum responses seen in immunized mice under the standard transfer conditions hints that this population may be generated under all transfer and immunization conditions but may be difficult to visualize due to sampling inefficiencies. Future experiments optimizing the adjuvant or immunogen formulation will help determine if this population of cells can be enhanced or detected using the standard transfer conditions. A further limitation of this study is that it relied upon activating a single VH3-21/VL1-40 B cell population with a fixed BCR that is known to generate RSV-neutralizing antibodies. Future studies in more complex adoptive transfer schemes, or with humanized animals with multiple VH3-21/VL1-40 BCRs present, some of which may be non-RSV reactive, would provide important efficacy data in a more relevant-polyclonal B cell environment. Despite these limitations, these data support the continued evaluation of the ai-mAb immunogen as a promising alternative RSV vaccine candidate specifically tailored to the infant population into more advanced preclinical testing.

Figure Titles and Legends

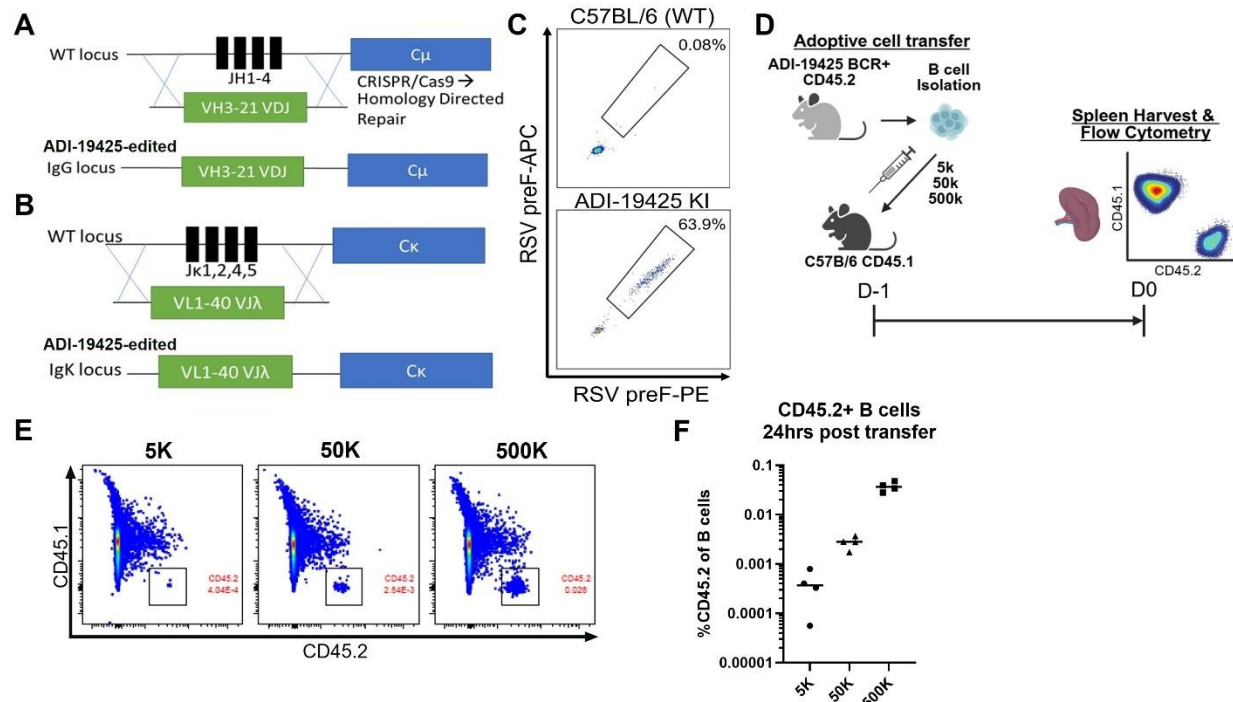


Figure 1: Generation of knock-in mice and establishment of adoptive transfer model. (A-B) CRISPR-Cas9 was used to edit rearranged ADI-19425 VDJ into the murine Ig locus **A** and the rearranged ADI-19425 VJλ into the murine Igk locus **B**. **(C)** Peripheral B cells from a wildtype (WT) mouse or a KI ADI-19425 BCR+ mouse were stained with RSV preF conjugated to APC or PE. **(D)** Schematic of adoptive transfer test conditions. Created with BioRender.com. **(E)** Representative CD45.1/CD45.2 staining plots of splenic B cells 24h post transfer from a mouse in each group. **(F)** Percentage of CD45.2+ B cells as a function of total B cells from each mouse transfer group.

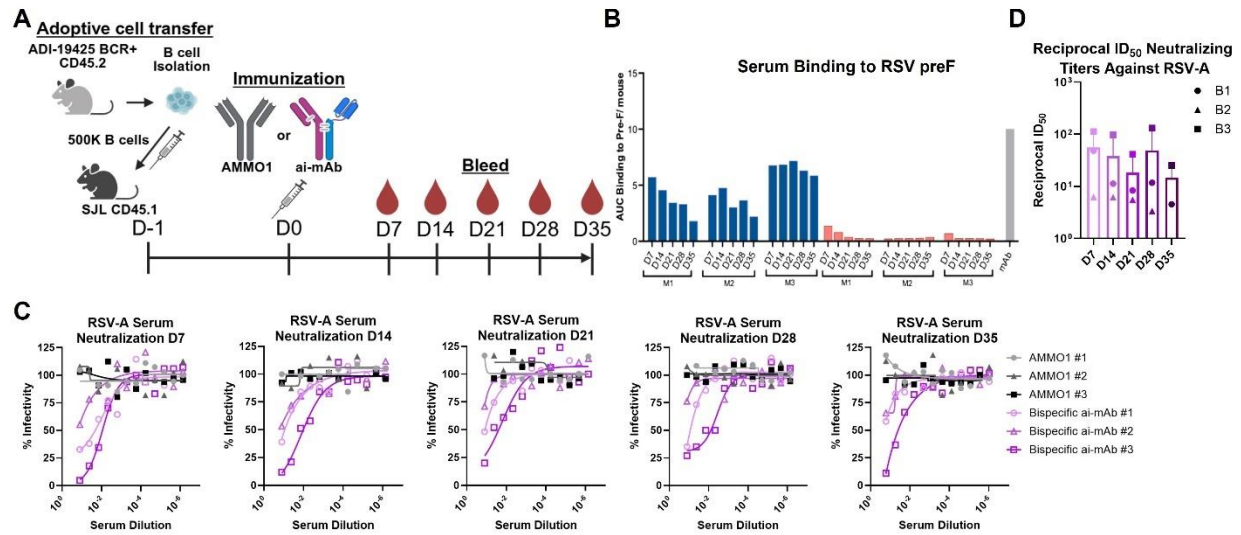


Figure 2: Immunization and serum responses in adoptive transfer model. (A) Schematic of experimental timeline. Created with BioRender.com. (B) Serum binding to RSV preF measured by ELISA of individual mice at indicated timepoints. Mice immunized with the bispecific ai-mAb shown in blue and mice immunized with the isotype control shown in salmon. (C) RSV-A *in vitro* neutralization curves of sera collected from mice immunized with the bispecific ai-mAb (purple shades) or the isotype control (AMMO1, grey shades) over time. (D) Reciprocal ID₅₀ RSV-A neutralizing titers of sera from the bispecific ai-mAb immunized mice over time. Individual mice identified by separate shapes and labeled B1-3 to denote immunization with the bispecific ai-mAb.

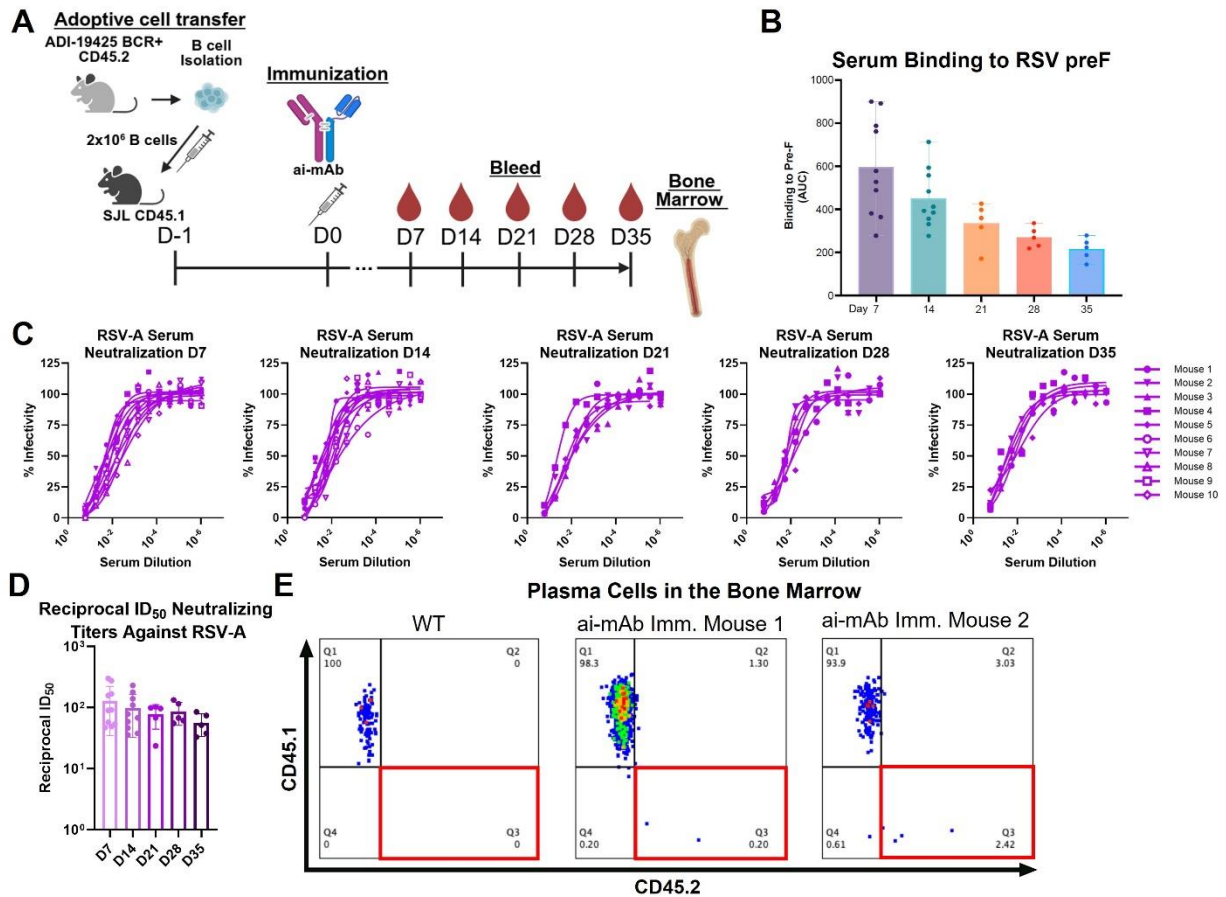


Figure 3: Immunization leads to on-target plasma cell entry into the bone marrow. (A) Schematic of experimental timeline. Created with BioRender.com. **(B)** Serum binding to RSV preF measured by ELISA of individual mice at indicated timepoints. **(C)** RSV-A *in vitro* neutralization curves of sera collected from immunized mice over time. **(D)** Reciprocal ID₅₀ RSV-A neutralizing titers of sera from immunized mice over time. **(E)** Representative CD45.1/CD45.2 staining plots of a wildtype CD45.1 mouse (WT) or ai-mAb immunized mice following adoptive transfer (ai-mAb Imm.) in the plasma cell compartment within the bone marrow at D35. Bone marrow gating strategy as follows: Lymphocytes → Live cells → CD138⁺ Sca-1⁺ → TACI⁺.

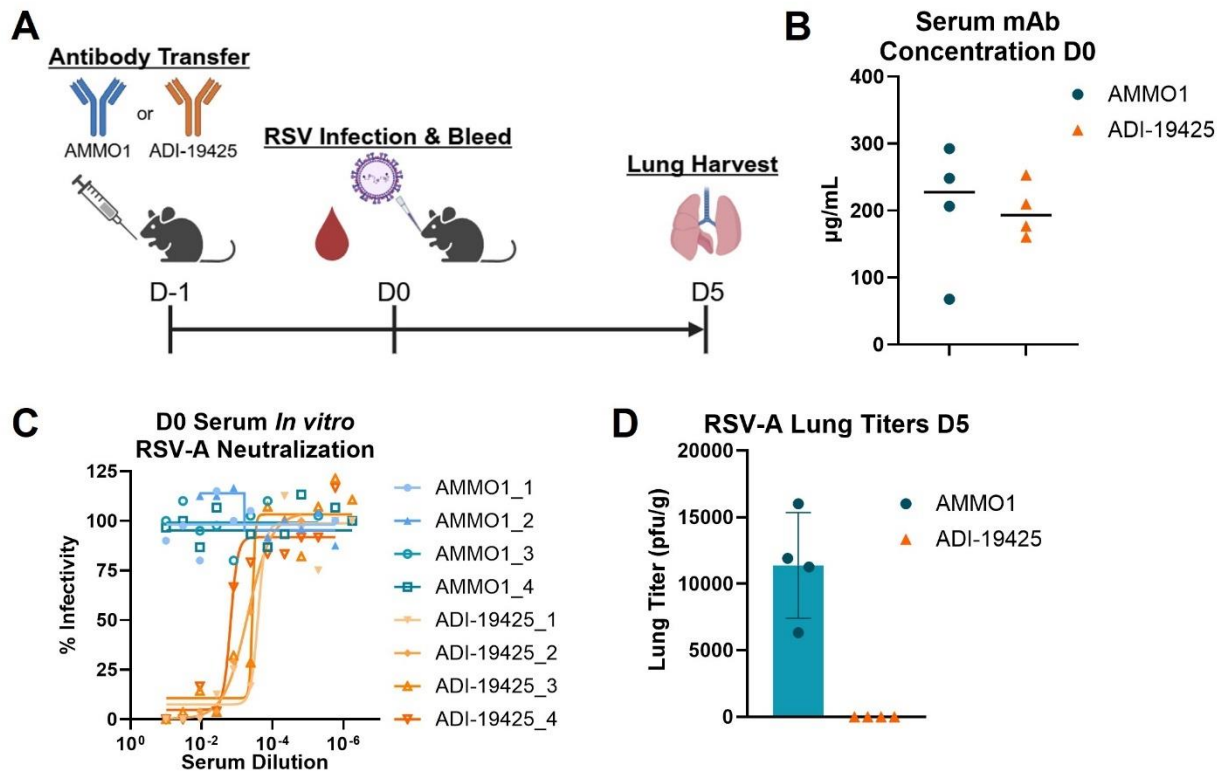


Figure 4: Prophylactic antibody transfer and RSV challenge. (A) Schematic of experimental timeline. Created with BioRender.com. (B) Serum concentration of human IgGs measured by ELISA of individual mice 24h post transfer. (C) RSV-A *in vitro* neutralization curves of sera collected from transferred mice at D0. (D) RSV-A plaque forming units per gram of lung tissue at 5 days post infection.

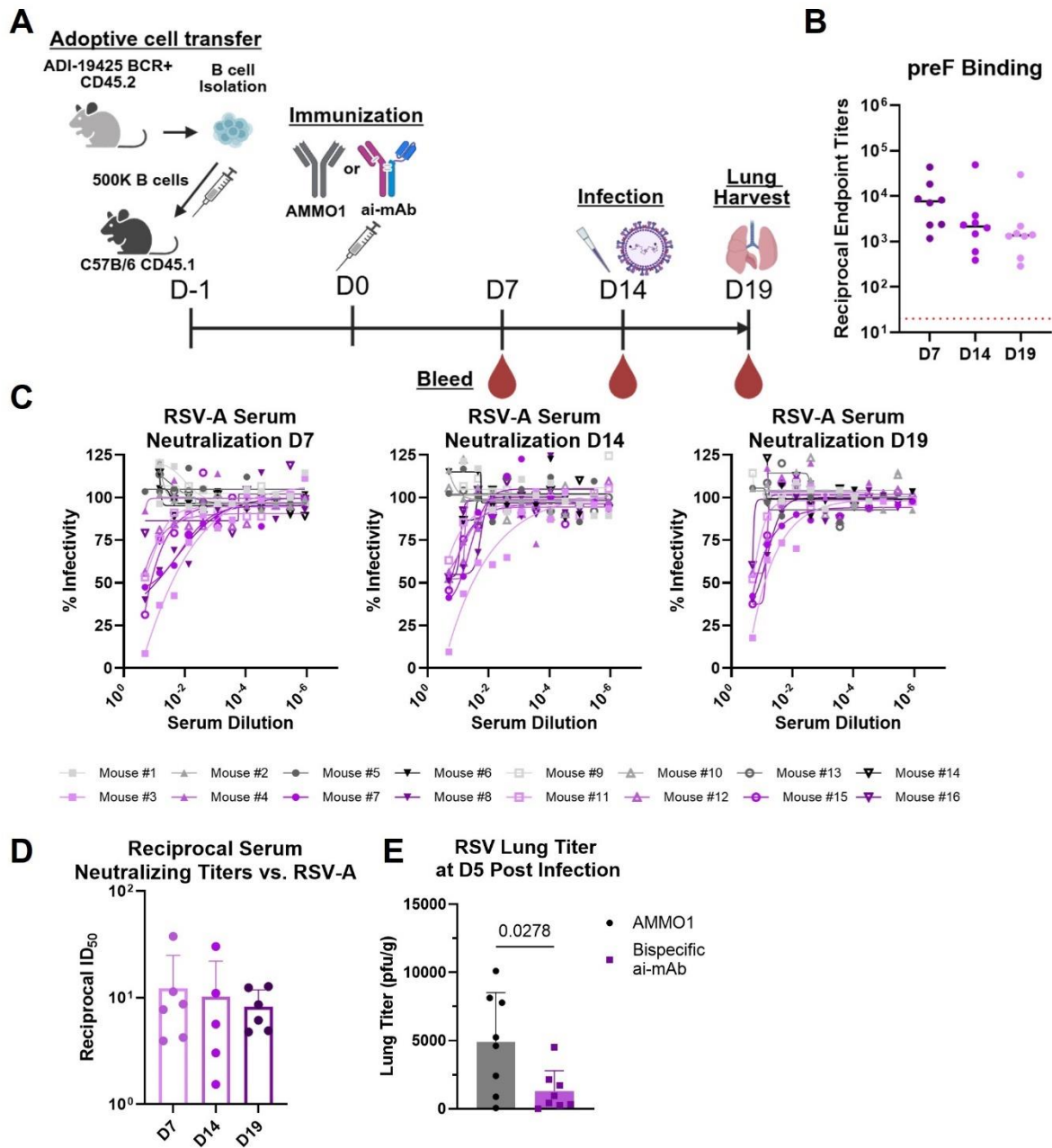


Figure 5: Immunization and RSV challenge in adoptive transfer model. (A) Schematic of experimental timeline. Created with BioRender.com. **(B)** Serum binding to RSV preF measured by ELISA of ai-mAb immunized mice at indicated timepoints. **(C)** RSV-A *in vitro* neutralization curves of sera collected from ai-mAb immunized mice (purple shades) and AMMO1 immunized mice (grey shades) over time. **(D)** Reciprocal ID₅₀ RSV-A neutralizing titers of sera from ai-mAb

immunized mice over time. (**E**) RSV-A plaque forming units per gram of lung tissue at 5 days post infection. Statistical analysis calculated with Welch's t test, n = 8 animals per group.

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Chapter 4: Summary and Future Directions

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RSV continues to pose a significant health burden in the infant population despite decades of research and the advent of newly approved vaccines for the adult population. While advances in protein engineering and antibody discovery have led to greater understanding of protective immune responses to RSV and shepherded a wave of next generation therapeutics, an effective vaccine for the infant population that faces the greatest burden of disease is still lacking. Due to the recent infant RSV vaccine failure¹ and limitations of monoclonal antibody treatment^{2,3}, there is a clear need for diverse and alternative strategies to protect this vulnerable population. Previous work identified that B cells arising from the VH3-21/VL1-40 BCR gene pairing have the ability to produce RSV-neutralizing antibodies without the need for somatic hypermutation⁴, and B cells of this class have repeatedly been identified in antibody screening campaigns of RSV infected individuals⁵⁻⁷. B cells of this germline class capable of neutralizing RSV have also been identified in cord blood samples and from naïve populations of adult PBMCs⁴, demonstrating this to be a reproducible repertoire that could be a promising vaccine target. Unbiased high-throughput sequencing analysis of the naïve B cell repertoire found the VH3-21/VL1-40 BCR pairing at an average frequency of 0.14% suggesting it to be an abundant target suitable for germline targeting immunogens^{8,9}.

Development of an effective vaccine in infants is challenging for multiple reasons related to immune environment and complications of maternally-derived antibodies. The ability of infant B cells to undergo class switching and somatic hypermutation have been shown to correlate directly with age^{10,11}, and maternal antibodies can have a dramatic impact on infant immune responses to vaccination through epitope masking and antigen clearance via Fc interactions¹¹⁻¹³. While maternal vaccination to protect newborns through transplacental antibody transfer has

demonstrated short term protection¹⁴, it may complicate direct vaccination of infants. This complication is highlighted by the recent mRNA vaccine trial in infants who previously received nirsevimab, who showed significantly reduced responses to the vaccine compared to participants who had not received the monoclonal antibody³. Collectively, these challenges call for novel vaccine strategies to circumvent the immunological obstacles faced by infants.

To address these challenges, we designed a bispecific anti-idiotypic antibody (ai-mAb) immunogen capable of targeting germline VH3-21/VL1-40 B cells that give rise to RSV-neutralizing antibodies¹⁵. The ai-mAb immunogen itself is novel and has distinct advantages over conventional RSV F displaying vaccines: i) this immunogen does not present non-neutralizing epitopes to the immune system, which has been implicated in driving VAERD in previous clinical trials in infants using virally-derived immunogens. ii) the ai-mAb is structurally unrelated to RSV F and will likely evade epitope masking by maternal/monoclonal Abs that interfere with infant immune responses. iii) VH3-21/VL1-40 Abs do not require affinity maturation to neutralize RSV and thus can provide rapid protection, even in infants.

Through initial characterization studies, we have shown this immunogen is capable of targeting unmutated VH3-21/VL1-40 B cells capable of giving rise to RSV-neutralizing antibodies from diverse pools of PBMCs¹⁵ (Chapter 2). Additionally, we demonstrated that this immunogen can activate cell lines engineered to express on-target VH3-21/VL1-40 BCRs, and not non-neutralizing preF-reactive BCRs¹⁵ (Chapter 2). To evaluate the efficacy of this immunogen in an animal model of vaccination, we engineered a mouse to constitutively express an unmutated VH3-21/VL1-40 BCR capable producing RSV-neutralizing antibodies (Chapter 3). This was necessary as mice do not express VH3-21 or VL1-40 BCR genes, and this engineered mouse allowed us to set up adoptive transfer experiments to create a model where target VH3-21/VL1-40 B cells were present a frequency that is representative of the human repertoire. Using this adoptive transfer model, we were able to show that immunization with the ai-mAb formulated in adjuvant was able to activate target B cells and elicit a sustained RSV-neutralizing

serum response. Further, we showed that immunization with the ai-mAb led target B cells to differentiate into plasma cells and enter the long-lived niche of the bone marrow, a critical goal of vaccination strategies¹⁶. Finally, we demonstrated that immunization with the ai-mAb generated a protective response against RSV challenge that significantly reduced viral lung titers compared to a control immunization. These results provide critical proof-of-concept that our ai-mAb immunogen is a promising RSV vaccine candidate specifically tailored to the infant population and support its further development in pre-clinical testing.

While the initial *in vivo* characterization of this vaccine demonstrated efficacy in reducing viral infection in the lungs of immunized animals, its immunogenicity and induced serum neutralizing titers could be improved. Relying on a single dosing scheme is preferable for implementation, however it puts this vaccine at a deficit to other contemporary strategies that use prime - boost, or multiple boosting regimens. A single dose vaccination in this case is preferable because multiple immunizations with a non-native antigen have shown to drive affinity maturation away from the target of interest and potentially towards the non-related vaccine antigens¹⁷. One way to improve immunogenicity and response to vaccination without increasing doses is to directly conjugate adjuvants to our immunogen to allow for cell-specific immune activation. One potential strategy within this is directly linking the well-studied molecular adjuvant C3d, a component of the complement pathway¹⁸, directly to the ai-mAb. Recently, linking of this adjuvant to an HIV antigen demonstrated a more robust GC response and enhanced antibody secretion¹⁹. Another potential molecular adjuvant that could be conjugated to the ai-mAb is a CpG ODN, an agonist of TLR-9, which has been shown to lead to cell-directed activation and improved humoral responses to vaccination²⁰. In addition to conjugating adjuvants directly to the ai-mAb, another strategy to improve immunogenicity is to generate multimer constructs or mRNA delivery of the ai-mAb. However, with the current bispecific platform and the required purification technique this is not feasible, but different bispecific platforms such as tandem scFvs could enable nucleic acid delivery. Another way to address this

would be to mutate the parental anti-idiotypic antibodies via yeast display or machine learning techniques to engineer a single binding domain immunogen that can target the paired VH3-21/VL1-40 unmutated BCRs. If a single binding domain construct was engineered, then multimerization or delivery via mRNA would be appealing next steps to see how these improve immunogenicity and durability of protection. While we have demonstrated that immunization with the ai-mAb in a supraphysiological environment of target cells leads to plasma cell differentiation and entry into the bone marrow, optimizing this pathway and stimulating a productive memory response will be key to life-long protection. Future studies will test the ability of new immunogen-adjuvant formulations to generate plasma and memory B cell responses and how these can be best tuned to establish lasting protection.

Another avenue for directed research with the ai-mAb will be to investigate its efficacy in the presence of maternal or prophylactic monoclonal antibodies. The benefit of the ai-mAb is that it is antigenically distinct from RSV, and thus we hypothesize that it will not be blocked from the infant's immune system by these transferred antibodies. Experiments in which monoclonal or polyclonal RSV specific antibodies are transferred into mice prior to immunization will provide critical evidence to support this hypothesis. Furthermore, it may be feasible to vaccinate pregnant dams with a preF-subunit vaccine, like the Abrysvo maternal vaccination, and then immunize neonates following adoptive transfer of ADI-19425-KI cells to probe the efficacy of this vaccine in a more relevant infant immune environment. These next steps will provide additional evidence to support the progression of the ai-mAb immunogen into early clinical development, with the goal of developing an efficacious vaccine to protect infants from RSV infection while overcoming the critical challenges to immunizing this population.

Given the successes of current prophylactic treatments such as monoclonal antibodies and maternal vaccination for protecting infants during their first 6 months of life, I believe it will be critical to develop a complementary vaccine that can be delivered concurrently. All infants that have access and the means to receive one of these protective treatments should seek them

out to greatly reduce the risk of serious infections in the earliest stages of life. However, designing vaccines that can begin to stimulate the infant's own immune system while they are being protected by prophylactic treatments will be critical provide protection spanning the newborn's complete childhood, their most vulnerable period. While we have demonstrated the early efficacy of our vaccine, it is still far from being implemented in the public. The most advanced live-attenuated vaccines will be posting efficacy data in the coming months to years and hopefully will provide a first marketable immunization to protect infants from RSV. It is currently unknown how these vaccines will work in infants who have received the prophylactic treatments, and this will be critical to their success. Given the failures of infant RSV vaccines, more options and diverse strategies will be needed to address this health challenge, as well as an appetite and understanding in the public realm for successful adoption of these treatments in the future.

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