Toward an Effective HIV-1 Vaccine: Multivalent Nanoparticle Immunogens and Public Antibodies

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CHAPTER 1

INTRODUCTION

1.1 Development of the Antibody Repertoire and Shared Public Clonotypes

1.1.1 Antibody and B cell Development

Before residing in peripheral lymphoid organs as memory B cells waiting for secondary exposure to an infectious agent or fighting off infections as antibody-secreting plasma cells, B cells begin their journey in primary lymphoid organs. B cells begin as stem cells and progress as progenitor, or "pro"-B cells before being classified as pre-B cells. Successful gene rearrangement of the heavy-chain locus followed by rearrangement of one of the two light-chain loci (kappa and lambda), results in an immature B cell with an IgM immunoglobulin B cell receptor (BCR) (Murphy and Weaver, 2016). These B cells undergo selection to eliminate self-reactive B cells in a process known as central tolerance, before migrating to peripheral lymphoid organs. In the periphery, B cells differentiate into mature but antigen-naïve B cells expressing IgD as well as IgM. These B cells are activated when their BCR binds to an encountered antigen. Activated B cells may then undergo class switching and may alternatively express IgA, IgE or IgG on the surface.

While some B cells can provide an immune response immediately after antigen binding in a process known as a T-cell independent B cell response, other B cells require the help of T-helper cells. These B cells interact with T cells in the germinal center where BCRs are fine-tuned to recognize antigen in a process known as somatic hypermutation. Not all B cells survive this process, but the ones that do differentiate either into plasmablasts that then become antibody-

secreting plasma cells, or memory B cells, which are long-lived and are sensitive to the antigen they recognized, and are ready to rapidly respond to future exposures (Murphy and Weaver, 2016).

1.1.2 Diversity in the Antibody Repertoire

An individual's antibody repertoire is an integral tool of the adaptive compartment of their immune system that theoretically could respond to any antigen that it encounters. This is particularly important considering the number of pathogens capable of infecting humans is approximated to be about 1,400 and includes bacteria, parasites, viruses and the like (2011). Antigen recognition is imparted by the variable fragment (Fv) of the antigen binding fragment (Fab) of an antibody (Inbar et al., 1972) and is comprised of four framework regions (FW1, 2, 3, 4) that bound and separate three complementarity-determining regions (CDR1, 2, 3) (Tonegawa, 1983). These regions are defined by recombination events of the V, D and J genes in the heavy chain and the V and J genes in the light chain. The first three framework regions are encoded by the V gene whereas the fourth is encoded by the J gene. The first two CDRs are also encoded by the V gene, but the third (CDR3) is formed by the combinatorial rearrangement of the V gene, D gene (in the heavy chain only), and the J gene, includes non-templated residues, and is a source of significant sequence variation (Figure 1) (Murphy and Weaver, 2016). These regions altogether possess astronomical diversity initially imparted through the development of the B cell in primary lymphoid organs. This in turn allows the immune system the flexibility to respond to the myriad of antigens that it may face.

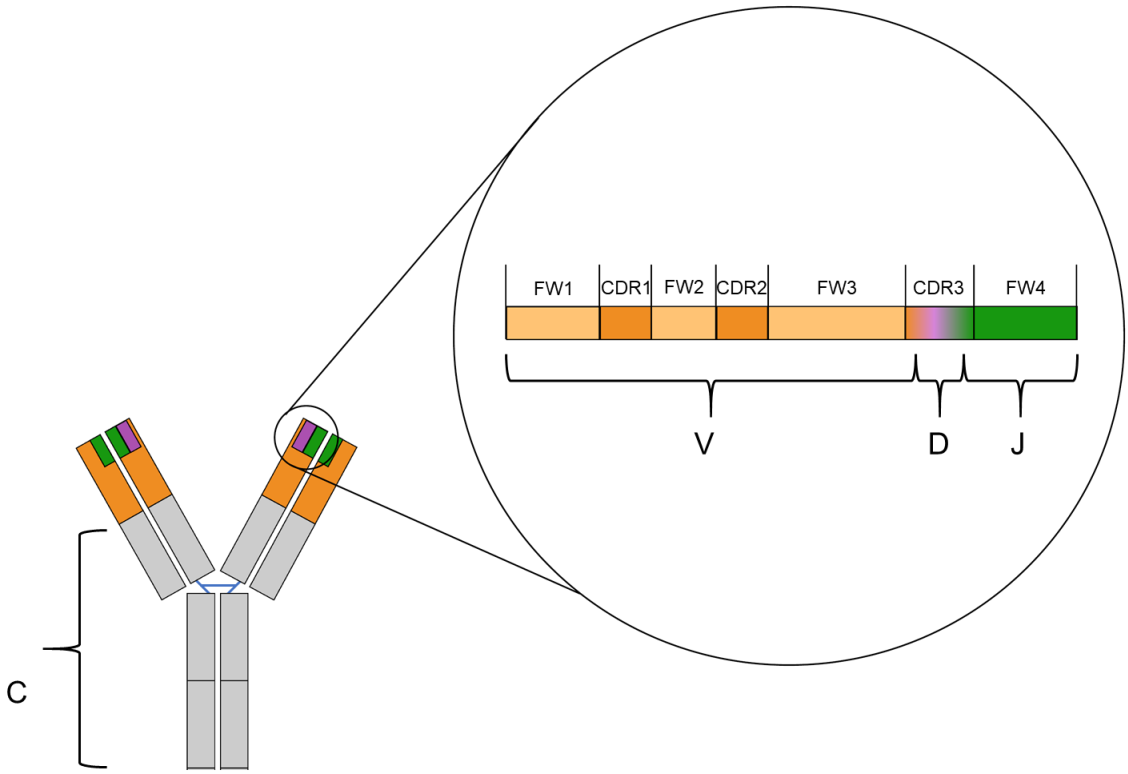


Figure 1. V(D)J Assignments Comprising the Variable Region of the Heavy Chain Immunoglobulin. The variable region is comprised of the V (variable), D (diversity), and J (joining) regions and is linked to the C (constant) region. The V gene codes for framework region 1 (FW1), complementarity determining region 1 (CDR1), framework region 2 (FW2), complementarity determining region 2 (CDR2), and framework region 3 (FW3). Complementarity determining region 3 (CDR3) arises from recombination events from templated (V, D and J genes), and non-templated residues. Framework region 4 (FW4) is encoded by the J gene.

Setting the foundation of the combinatorial diversity in these regions, a B cell integrates one each of 51 different V_H genes, 6 J_H segments, and 25 D_H segments into a heavy chain with a light chain formed by the assembly of one each of 100 V_L genes and 12 J segments in primary lymphoid tissue. Simply the combinatorial diversity of coupling V_H and V_L results in $4.6x10^6$ potential pairs (Rees, 2020). Further diversity is introduced at the junction of segments where they are combined. The activity of terminal deoxynucleotidyl transferase (TdT) adds N-

nucleotides at junctions, hairpin formation during recombination results in palindromic- or p-nucleotide addition, and DNA repair enzymes with exonuclease activity eliminates nucleotides, further increasing combinatorial diversity (Murphy and Weaver, 2016). The theoretical sequence diversity of the naïve repertoire has been liberally postulated to be as high as 3×10^{15} (Briney et al., 2019), while others have experimentally estimated the number of unique B cell receptors to be magnitudes lower at 1.1×10^{7} (Rees, 2020; Soto et al., 2019).

B cell diversity is further amplified after B cell activation. Post-developmental changes to the B cell largely occurs in response to an antigen, after B cells travel from primary lymphoid organs to secondary lymphoid organs. Here, SHM caused by rapid point mutations results in affinity maturation of the B cell population. Mutations that improve binding to antigen are selected over mutations deleterious to antigen recognition, resulting in an antibody-secreting B cell population that more potently recognizes antigen.

Irrespective of the vast potential pool of diverse B cells and given that the number of B cell clones within an individual numbers between 9 and 17 million B cell clonotypes (Soto et al., 2019), individuals have been observed to actually share sequence features. Antibodies that share highly similar sequence features despite originating in different individuals have been dubbed "public" antibodies and have been discovered in a variety of disease states and in healthy individuals.

1.1.3 Diversity in an Individual's Antibody Repertoire does not Preclude Antibodies Shared Within a Population

Despite a large, diverse B cell repertoire, population-level exposure to a pathogen begs the question of whether a convergence of structure or selection of particular sequences in

response to that pathogen exists. Indeed, studies have shown that in response to infection and vaccination, “public” antibody responses have been observed. In fact, shared sequences in the naïve repertoire (Soto et al., 2019) suggest that a pool of public antibodies exist even prior to pathogen exposure.

Parameswaran *et al.* performed high-throughput sequencing on the peripheral blood mononuclear cells (PBMCs) of 60 patients enrolled in a dengue study. They found convergent immunoglobulin sequences in patients with acute dengue that were not present in non-dengue samples (Parameswaran et al., 2013). Their basis for establishing commonalities came from a probability-based approach in identifying V_H sequence features. Moving further, CDR3 signatures were analyzed among samples, and it was discovered that identical CDR3 sequences and sequences with those CDR3 sequences with one mismatch were shared among individuals with acute dengue. Further, frequency of these CDR3 sequences were massively decreased after resolution of infection and not present or were extremely rare in non-dengue samples (Parameswaran et al., 2013).

Public antibodies have also been discovered in the context of influenza vaccination and infection. A *Cell Host and Microbe* study published in 2014 reported on the antibody response of healthy individuals to the trivalent inactivated seasonal influenza vaccine (TIV) and discovered, using a clonality index, that seroconverters had increases in clonality seven days after vaccination (Jackson et al., 2014). The study went on to delineate the antibody response via single-cell sorting of one individual to the single-antigen H1N1 vaccination. Jackson and colleagues identified a heavy chain lineage that utilized *IGHV3-7, IGHJ6* and a CDR3 length of 18 residues (Jackson et al., 2014). This lineage was observed in another individual that likely was not infected with H1N1 but may have had a history of receiving influenza vaccines (Krause

et al., 2011) and another that was infected with H1N1 influenza (Wrammert et al., 2011). The heavy chain from this study was paired with the light chain from the clonally related antibody 4K8 from (Krause et al., 2011), which clarified the antigen specificity of this antibody clonotype and, indeed, that they are public (Jackson et al., 2014).

Public antibodies have also been identified based on non-canonical gene recombination events. Antonio Lanzavecchia and colleagues observed that 5-10% of malaria-exposed individuals contained a translocation of the collagen-binding domain of Leukocyte Associated Immunoglobulin Like Receptor 1 (LAIR1) gene between the V and DJ segments (Pieper et al., 2017). The inserted sequence was observed to be over 100 residues long and contained mutations that abolished collagen binding, but increased binding to malaria-infected erythrocytes (Tan et al., 2016). Prevalence of LAIR1-containing antibodies in European blood donors show far fewer LAIR1 positive antibodies when compared to samples from cohorts in two different malaria-prevalent countries, suggesting the potential for antigen-driven antibody convergence. Lanzavecchia and colleagues observed low affinity binding to diverse tested parasites of LAIR1-containing antibodies. They postulated that inherent LAIR1 binding to infected erythrocytes allows for clonal selection of B cells with antibodies containing this motif, and that this motiff may be responsible for the common binding specificity of these public antibodies (Pieper et al., 2017).

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic beginning in 2019 and extending through 2020 and 2021 galvanized the field into looking at antibody responses to infection and vaccination. Ali Ellebedy's group at Washington University School of Medicine in St. Louis identified a public antibody, 2C08, that targets the receptor binding domain (RBD) of the SARS-CoV-2 spike protein. 2C08 was induced via an mRNA

vaccine and is characterized by neutralization potency against a handful of different SARS-CoV-2 strains and protection in animal challenge studies (Schmitz et al., 2021). Remarkably, they found 20 additional monoclonal antibodies (mAbs) that share heavy and light chain variable region genes as well as highly similar CDRs that are functionally similar in SARS-CoV-2 patients as well as vaccine recipients (Schmitz et al., 2021). Whereas 2C08 targets the RBD, Voss *et al.,* identified public antibodies to the N-terminal domain (NTD) of the SARS-CoV-2 spike protein (Voss et al., 2021). Defined by a heavy chain that possesses the same unique unmutated residues in the first two CDR regions and third framework region but different J_H genes and variable CDRH3 lengths, these public antibodies were found to promiscuously pair with six distinct light chain V_L genes (Voss et al., 2021). Intriguingly, because these antibodies were discovered to target the NTD, a region tolerant to mutations, they were predicted to be particularly susceptible to viral escape.

Public antibodies have also been discovered in the context of HIV-1, which is interesting considering the rapid rate of viral evolution during infection which in turn results in a diverse pool of antibodies. By sequencing PBMCs from six donors from the Centre for the AIDS Programme of Research in South Africa (CAPRISA) cohort Setliff *et al.*, identified a public clonotype shared by three of the donors. Public clonotypes were here defined to include sequences with the same V_H and J_H genes, same junction length, and high CDRH3 amino acid sequence identity. Recombinantly expressing two antibodies from the clonotype helped confirm HIV-1 specificity. Remarkably, in comparing all sequences in each sample with CDRH3 and V-gene assignments against known, HIV-reactive antibodies, antibodies with extra-neutralization function, weak neutralizing activity, putative vaccine-elicited antibody precursors and putative bNAb precursors were identified (Setliff et al., 2018).

Though public antibodies have been suggested to arise in response to antigen, Soto *et al.* identified public clonotypes in healthy individuals. Despite a highly restrictive definition of the same V_H and J_H genes, V_L and J_L genes, and completely identical CDR3 amino acid sequences, clonotype similarities between the three individuals studied were reported to be 0.3%, a number that increased to 1-6% when comparing two of the three subjects (Soto et al., 2019). These shared clonotypes were postulated to have risen in response to common antigens these individuals may have encountered. To determine whether common clonotypes are present even before antigen exposure, umbilical cord white blood cells samples were studied. Astonishingly, percent overlap for pairwise comparisons ranged from 0.4-0.5% and 0.1% for all three cord blood samples studied (Soto et al., 2019), providing evidence of a public naïve repertoire.

The study of public antibodies has implications for immune surveillance either after infection or vaccination (Jackson et al., 2014; Parameswaran et al., 2013), discovering novel mechanisms of the immune system responses to infection (Pieper et al., 2017), potential vaccine templates and therapeutics (Schmitz et al., 2021; Setliff et al., 2018; Voss et al., 2021), and a better understanding of the antibody repertoire (Soto et al., 2019). As such, continued study of public antibodies is highly motivated.

1.2 HIV-1 and HIV-1 Antibody Discovery

Acquired Immunodeficiency Syndrome (AIDS) was first reported on in 1981, when a team of clinicians observed otherwise healthy patients that presented with Kaposi's sarcoma and/or *Pneumocystis carnii* pneumonia – illnesses that were known to be typically identified in immunosuppressed individuals (Centers for Disease Control (CDC), 1981a, 1981b). Two years later, Luc Montagnier from the Pasteur Institute in Paris isolated a retrovirus from a patient that

was distinct from, but related to Human T-cell leukemia virus type 1 (HTLV-1) (Barré-Sinoussi et al., 1983). This retrovirus was then discovered to be the etiological agent of AIDS by Robert Gallo's group at the National Cancer Institute followed by Lyndon Oshiro's group, who were among the first to provide evidence for a causal relationship between infection with HIV-1 and progression to AIDS (Gallo et al., 1984; Levy et al., 1984).

Epidemiologically, little was known about the prevalence of HIV-1. The discovery of HIV-1 antibodies in patient serum (Brun-Vezinet et al., 1984; Safai et al., 1984; Schüpbach et al., 1984) quickly led to the first HIV antibody test (Weiss et al., 1985b). This blood-based ELISA test not only protected blood banks across the country, but also allowed individuals to minimize risk to themselves and others. Serum antibodies were further scrutinized more closely. If antibodies were elicited in response to infection, perhaps they could be used to stem the growing infection rate. The potential for serum antibodies to be elicited in response to a vaccine or act as therapeutics was realized when Robert Gallo reported on the neutralizing capacity of patient sera against HIV-1 (Robert-Guroff et al., 1985; Weiss et al., 1985a). Soon after, monoclonal antibodies to HIV-1 were discovered. Indeed, b12 was the first human monoclonal antibody isolated and one that was directed to the CD4 binding site of the envelope protein trimer (Env) of HIV-1 (Burton et al., 1991; Roben et al., 1994). b12 was discovered to be a broadly neutralizing antibody, capable of neutralizing several different viruses (D'Souza et al., 1997). A decade after its discovery, b12 was found to be protective in macaques challenged with a simian-human immunodeficiency virus hybrid (SHIV) (Parren et al., 2001).

The discovery of the monoclonal antibody b12 was followed closely by the discovery of several others including 4E10, 2F5, 2G12, 4E10 and Z13, all of which are collectively known now as the "first-generation" of anti-HIV-1 antibodies. Like b12, these antibodies displayed

modest neutralization breadth with some being protective in animal studies. However, high concentrations of these antibodies were necessary to achieve these effects, and so due to this impediment, they were deemed undesirable in the field. These antibodies were reviewed in brief at (Shcherbakov et al., 2015). However, these findings were emboldening, and the continued search for effective HIV-1 monoclonal antibodies ushered in the age of "second generation" broadly neutralizing antibodies.

The identification of more potent bNAbs characteristic of the second generation came not only with persistent searching, but also with the advent of more refined technologies. Then-conventional neutralization assays relied on PBMC-generated viral stocks that yielded results that were not particularly accurate and suffered in terms of reproducibility, in part due to the residence of quasispecies of viruses (Mascola and Haynes, 2013). Montefiori and colleagues developed neutralization assays with higher throughput and greater reproducibility (Montefiori, 2004). Further, Montefiori and colleagues optimized a pseudovirus panel designed to more accurately represent the diversity of globally circulating strains (deCamp et al., 2014). Discovered monoclonals could be screened against a standardized panel, using a more reliable neutralization assay. Further breakthroughs include single-cell antibody cloning, high-throughput screening methods and a fundamentally more informed understanding of the envelope glycoprotein.

These breakthroughs contributed to the first high-throughput neutralization screen of B cells from a donor, resulting in the isolation and characterization of the first set of second generation broadly neutralizing antibodies, PG9 and PG16, and the discovery of a then-novel epitope (Walker et al., 2009). Thereafter, Mascola and colleagues leveraged what they knew about the envelope protein to design an antigen that eliminated off-target antibody binding to

specifically search for antibodies that recognize the CD4 binding site epitope. In doing so, they discovered VRC01, a remarkably potent, broadly neutralizing antibody (Wu et al., 2010).

Having shown remarkable breadth in virus neutralization, VRC01 was a prime candidate to test as a prophylactic drug to prevent HIV-1 infection. Regrettably, VRC01 ultimately failed to prevent HIV-1 acquisition, though infection from strains particularly sensitive to VRC01 may have been mitigated (Corey et al., 2021). The study, published in *The New England Journal of Medicine,* suggests that evidence of prohibition of infection from sensitive strains by administration of VRC01 serves as a proof of concept for prophylactic use of bNAbs. As such, bNAbs discovered since VRC01, including 3BNC117 and 10-1074, are currently being used in cocktails to determine efficacy as prophylactics in human clinical trials (ClinicalTrials.gov Identifiers: NCT03928821 and NCT04173819).

Use of VRC01 as a prophylactic did not meet expectations. Nevertheless, efforts to elicit VRC01-class antibodies and other broadly neutralizing antibodies in general via vaccination has long been sought after. Though notoriously difficult to elicit via current empirical methods of vaccine design, innovative tools have improved on these methods. Rational methods of vaccine design have also been explored to elicit broadly neutralizing antibodies.

1.3 Vaccines and the role of Nanoparticle Immunogens as Viable Tools for Hard-to-Vaccinate Pathogens

1.3.1 Empirical Vaccine Development Has Historically Been Efficacious

As early as 430 BC, it was known that smallpox survivors were immune to the disease (Riedel, 2005). It was not until the 18th century, however, that Benjamin Jesty acted on the

observation that dairymaids who contracted cowpox were somehow protected against smallpox. From material resident in lesions on a cowpox-ridden cow, Jesty vaccinated his family with cowpox (Pead, 2006), keeping them immune to smallpox which had devastated his locale, and, indeed, much of the western world. Two decades later, Edward Jenner provided scientific credence to the act of immunization in a published book, where he described a similar procedure of extracting cowpox from a lesion on dairymaid Sarah Helms to then inoculate 8-year-old James Phipps – while Phipps reacted to the initial inoculation, a boost delivered later caused no reaction (Lakhani, 1992; Riedel, 2005). This scientific finding, heralded as one of the greatest in the history of mankind, set the course for modern immunology and vaccinology.

Since then, Louis Pasteur stumbled upon a process of attenuation, or loss of virulence, of a pathogen and subsequently developed the first live attenuated vaccine for anthrax (Barranco, 2020). The development of cell culture of viruses in the 20th century subsequently allowed for the development of live vaccines. Whereas initial live attenuated vaccines held dire side-effects including resurgence of virulence, novel technologies including reverse genetics, gene manipulation and replication fidelity manipulation made this technology far safer and more viable (Plotkin, 2009). Inactivated vaccines, which differ from attenuated vaccines in that they are not alive and cannot replicate and can be whole or fractional, on the other hand, hinged on the 19th century development of extracting and purifying proteins, polysaccharides, and toxoids (Plotkin, 2009). This led to the development of safer protein-based vaccines that, when possible, replaced earlier whole-cell or live vaccines, like the pertussis vaccine (Sato and Sato, 1999) and the anthrax vaccine (Ivins and Welkos, 1988).

To date, classical vaccines stemming from the discoveries mentioned above have been developed against a host of pathogens with immense success (Table 1). However, challenges still

exist in creating vaccines against certain other pathogens. While there currently exists an influenza vaccine, reassortment and genetic drift in strains contribute to high variability that necessitates yearly inoculations; as a result, there is a need for a more optimal vaccine (Crowe, 2018). For other highly mutable pathogens like HIV, at this point in time, no vaccine candidates have been sufficiently protective (Buchbinder et al., 2008; Gray et al., 2021; Haynes et al., 2012; Rerks-Ngarm et al., 2009). Similarly, vaccine candidates against hepatitis C virus have also been ineffective (Page et al., 2021). The field of vaccinology has also been stymied by certain flaviviruses like the four dengue virus serotypes, for which vaccine attempts led to antibody dependent enhancement (ADE) of infection (Shukla et al., 2020). For these pathogens and many others, innovative solutions (DNA/mRNA vaccines, viral vector vaccines, chimeric vaccines, etc.) have been and are currently being tested. One such solution relies on nanoparticle technology.

Vaccine	**Target**
Live-attenuated vaccines	SARS-CoV-2*, measles, mumps, rubella, rotavirus, smallpox, chickenpox, yellow fever, tuberculosis, Ebola
Inactivated vaccines	Hepatitis A, influenza, rabies, polio, pertussis
Subunit, recombinant, polysaccharide, and conjugate vaccines	SARS-CoV-2*, Hib (*Haemophilus influenzae* type b) disease, HPV (human papillomavirus), whooping cough, hepatitis B, shingles, meningococcal disease, pneumococcal disease, anthrax, EBV (Epstein-Barr virus)*
Toxoid vaccines	Diphtheria, tetanus

Vaccine	Target
Viral vector vaccines	SARS-CoV-2*
mRNA vaccines	SARS-CoV-2*

Table 1. Example of Different Types of Vaccines and Vaccine Targets. Asterisks denote developing vaccines.

1.3.2 Nanoparticles are Powerful Medical Tools

In healthcare, nanoparticles, or particles that exist on the nanoscale (between 1 and 100, or even liberally up to 200 nanometers) have been used ubiquitously as drug delivery carriers (Patra et al., 2018), for biological imaging (Daraee et al., 2016) and in vaccines (Singh, 2021). Nanoparticles can be comprised of a whole host of materials, including lipids, metals, and proteins, to name a few.

The fragility of genetic material either as a drug or as a vaccine, or drugs that readily degrade necessitate a protective carrier that is easily taken up by target cells. Lipid nanoparticles have successfully been used as drug-delivery carriers, as evidenced by the development of Doxil, the first FDA approved liposomal drug carrier, encapsulating the drug doxorubicin to treat ovarian and breast cancer (Thi et al., 2021) and Onpattro, an RNA interference therapeutic that has shown long-term efficacy to treat amyloid polyneuropathy (Adams et al., 2021). In addition, Epaxal and Inflexal are both commercially available vaccines comprised of inactivated hepatitis A virus or influenza virus, respectively, adsorbed to liposomes (Brinkkemper and Sliepen, 2019). Finally, lipid nanoparticles for vaccines have seen use in encapsulating mRNA coding for viral antigens as is evidenced by efforts made by Pfizer-BioNTech and Moderna to curb the currently ongoing SARS-CoV-2 pandemic (Tenforde et al., 2021).

Inorganic metal nanoparticles have had impactful use for biological imaging, but they have also acted as carriers to deliver DNA vaccine candidates (Zhou et al., 2008) as well as platforms to deliver viral protein immunogens, like the envelope protein of West Nile virus (Niikura et al., 2013) and viral peptides, like ones specific to foot and mouth disease virus (Chen et al., 2010).

Polymer based nanoparticles, virus-like particles, and even hybrid nanoparticles that marry two different materials have all been used as vaccines to varying levels of efficacy, emphasizing the flexibility in choice and versatility in use of nanoparticle-based platforms (Metz et al., 2016; Shae et al., 2019; Zhao et al., 2020).

1.3.3 Protein Nanoparticle Vaccines are Efficacious

Protein nanoparticles in particular have also shown great efficacy for vaccine development. Protein nanoparticles can act as a scaffold to mount specific antigens in an ordered, repetitive fashion that has been shown to activate B cells through T-cell dependent and independent pathways (Bachmann et al., 1995; He et al., 2016). In fact, the display of multiple antigens can more effectively provide additional binding sites and increase immunogenicity (Figure 2) (Bachmann and Zinkernagel, 1997; Bachmann et al., 1995; Singh, 2021). In a study establishing the potential for a functional cure of hepatitis B infection, Wang *et al.* determined nanoparticles can deliver antigens to specific myeloid cells, including members of the innate immune system that play integral roles in activating B cells and T follicular helper cells (Wang et al., 2020). Further characterizing the innate immune response to nanoparticles, Tokatlian *et al.* determined that, in contrast with soluble protein, nanoparticles travel to the follicular dendritic

cell network in a complement-dependent fashion and localize in germinal centers (Tokatlian et al., 2019).

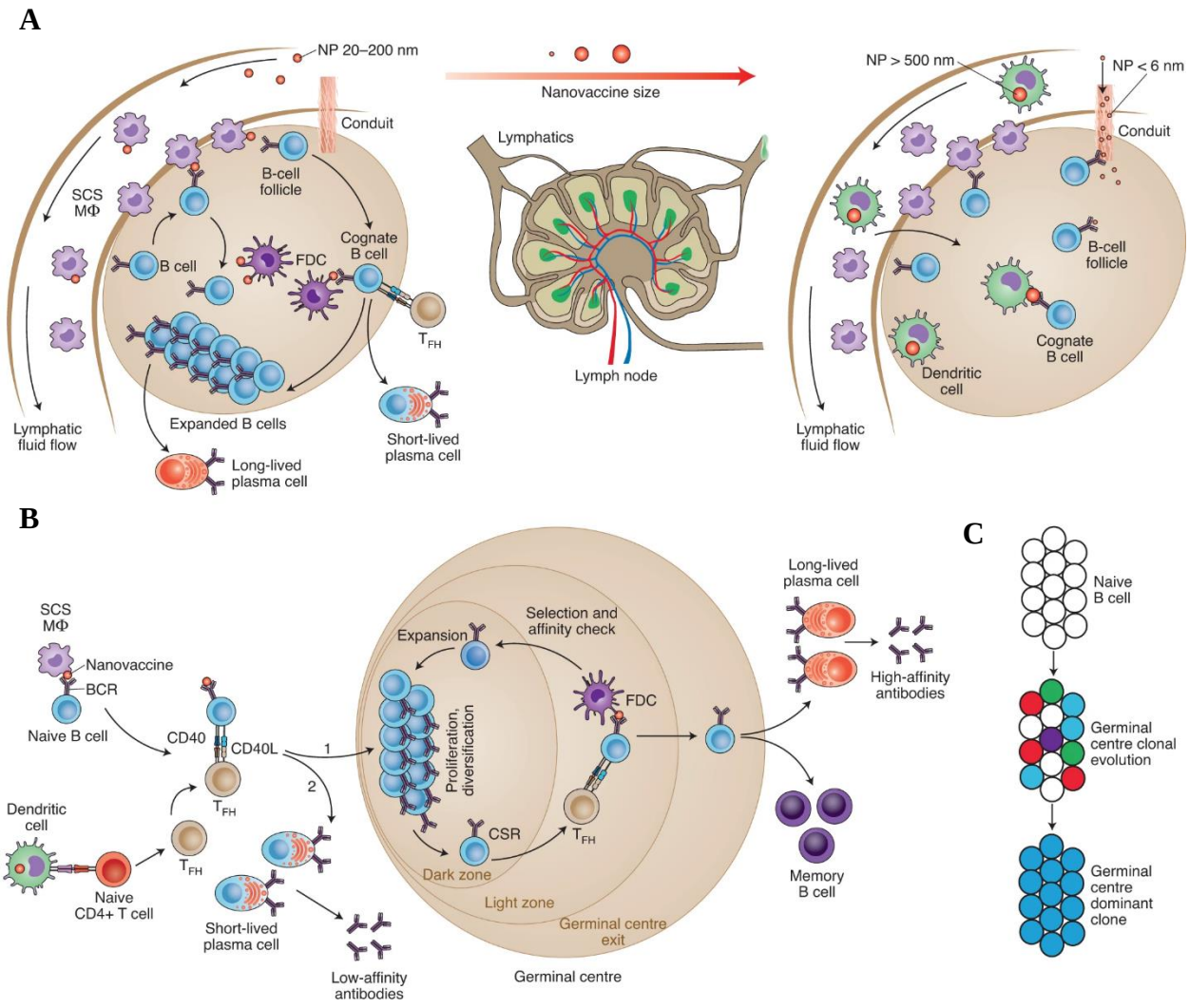


Figure 2. How Nanoavaccines Induce High-Affinity Antibody Response. Reproduced with permission by (Singh, 2021)
(A) Nanovaccines are carried through the lymphatics into the subcapsular sinuses located between the collagenous capsule and the immune cell-rich cortex region of the lymph node. The nanovaccines can localize on subcapsular macrophages overlying B-cell follicles. Nanovaccines are transported to FDCs in B-cell follicles by the relay of complexes from subcapsular sinus macrophages to migrating B cells, which in turn transfer antigen to FDCs, in a complement- and complement receptor-dependent manner. After encountering vaccine antigens, primed B cells decrease their migration velocity and relocate to the border of B-cell follicles (a C–X–C chemokine receptor type 5 (CXCR5) and CC-chemokine receptor 7 (CCR7)-dependent migration), where they encounter a specific subclass of CD4+ T cells, the TFH cells, eventually

leading to germinal centre reactions. Nanovaccine size may regulate transport mechanisms in the lymph nodes, with ~5-nm nanoparticles entering the B-cell follicle through collagen conduits and >500 nm particles transported by dendritic cells.
(B) Naïve B cells differentiate into germinal centre B cells (1) or short-lived plasma cells (2) as two possible outcomes of antigen and T-cell encounter. If successfully induced by nanovaccines, the germinal centre could lead to a high-affinity antibody response through a complex iterative process of somatic hypermutation, affinity maturation and selection.
(C) Multiple distinct B-cell clones seed each germinal centre in a vaccinated individual, and these specialized cells lose clonal diversity at widely disparate rates. Multiple clones can evolve in parallel within the same germinal centre, making it a highly heterogeneous structure, and a fraction of germinal centres become heavily dominated by the substantial expansion of the descendants of a single somatic hypermutation variant arising at or after the onset of germinal centre selection over cells of the same and of different clones. NP, nanoparticles; SCS MΦ, subcapsular sinus macrophage; CSR, class switch recombination.

Protein nanoparticle-based vaccines have been successful in prophylaxis against a number of pathogens. For influenza, a number of vaccine candidates have been developed. Nabel and colleagues designed a self-assembling protein nanoparticle that displayed hemagglutinin (HA) on its surface. Due to the three-fold axes of symmetry of the 24-meric *Heliobacter pylori* ferritin nanoparticle, viral hemagglutinin antigens were able to trimerize into a physiologically relevant state. HA-ferritin nanoparticles not only elicited greater neutralization breadth and potency than the commercial trivalent inactivated influenza vaccine (TIV), they also conferred greater protection in animal challenge studies (Kanekiyo et al., 2013). Building from the success in testing HA-ferritin, Kanekiyo *et al.* successfully designed a "mosaic" nanoparticle incorporating a targeted domain of HA from multiple strains in order to minimize strain-specific antibody responses and instead enhance cross-reactive antibody responses that tolerate antigenic variability (Kanekiyo et al., 2019). In a similar fashion, Georgiev *et al.* utilized ferritin from insect *Trichoplusia ni* (iFerr). iFerr is a non-heterogenous 24-mer comprised of 12 heavy chains and 12 light chains. Georgiev *et al.* used iFerr to express two different influenza HA proteins (dual-flu iFerr), two different HIV-1 Envs (dual-HIV iFerr), and both influenza HA and HIV Env

(flu-HIV iFerr) in a 1:1 ratio on the nanoparticle surface. Though only marginal autologous neutralization against HIV-1 was observed in guinea pigs immunized with dual-HIV iFerr and flu-HIV iFerr, nanoparticles that incorporated influenza HA elicited strong autologous influenza neutralization (Georgiev et al., 2018). The success of these studies and others that also explore nanoparticle platforms for influenza vaccines (Boyoglu-Barnum et al., 2021; Yassine et al., 2015) serve as incentive to continue research into nanoparticle platforms to achieve the highly sought-after universal flu vaccine. As such, there are ongoing clinical trials exploring efficacy of these vaccine platforms.

Nabel and colleagues have also explored nanoparticle platforms in designing vaccines against Epstein-Barr Virus (EBV). Once again, bacterial ferritin was leveraged as a scaffold for vaccine design. In the same manuscript, encapsulin, a 60-meric protein from bacterium *Thermotoga maritima*, was also identified as a potential platform for the viral major glycoprotein, gp350. When challenged with recombinant vaccinia virus expressing gp350, four out of five mice that received soluble gp350 required euthanization. In contrast, four out of five mice that received gp350 on ferritin were protected from challenge (Kanekiyo et al., 2015). The benefits of a gp350-ferritin vaccine are currently being assessed in a human clinical trial (ClinicalTrials.gov Identifier: NCT04645147).

Protein nanoparticle vaccine candidates have been designed in the context of other diseases including, but not limited to (i) respiratory syncytial virus (RSV), in which protein nanoparticles induced stronger neutralizing antibody responses than soluble antigen in both mice and non-human primates (Swanson et al., 2020), (ii) dengue virus, which displayed sterilizing immunity against a single serotype of dengue in non-human primates (McBurney et al., 2016), (iii) rotavirus, in which ferritin nanoparticles that could be expressed in mammary glands and

administered in milk were developed (Li et al., 2019), (iv) hepatitis C virus (HCV), which utilized ferritin nanoparticles to elicit higher neutralizing antibody titers compared to soluble protein in mice (Yan et al., 2020), and (v) human papilloma virus (HPV), for which there currently exists licensed nanoparticle vaccines to combat infection (Group, 2007; Paavonen et al., 2007).

Of all diseases for which nanoparticle vaccines are being developed, arguably few compare to the innovation and breadth of research devoted to developing a vaccine for HIV-1.

1.3.4 Protein Nanoparticles are Promising Tools for HIV-1 Vaccinology

A number of challenges have so far impeded the development of an effective HIV-1 vaccine. For one, HIV-1 is a highly mutable virus with variations in the Env trimer reaching up to 35% (Araújo and Almeida, 2013). As a result, immunization with Env from one strain is not sufficient to generate a protective response to another. The high mutability of HIV-1 necessitates a vaccine that can elicit an antibody response to a diverse array of circulating strains (Corey and McElrath, 2010; McBurney and Ross, 2008; Nabel et al., 2002; Pollara et al., 2017). In addition, Env is notoriously flimsy and conformationally dynamic, causing immune responses to target epitopes not evident on infectious Env proteins (Moore et al., 2006). An understanding of the Env trimer led to improvements to the stability of HIV-1. Such improvements include construction of disulfide bonds between protomers, stabilizing point mutations, trimerization domains, and protein linkers (Georgiev et al., 2015; Do Kwon et al., 2015; Sanders et al., 2013; Yang et al., 2002). As yet Env-based vaccines still have not led to licensed HIV-1 vaccines. This hurdle notwithstanding, Env is still the foundation of all current immunogens in vaccines designed to elicit neutralizing B cell responses.

In line with improved reactions to nanoparticle vaccines for influenza and other diseases, Env-based nanoparticles have been shown to elicit more robust responses compared to soluble Env counterparts. Sleipen *et al.* immunized mice and rabbits with either Env or Env-ferritin and observed higher anti-trimer binding responses in mice immunized with the nanoparticle as well as trends towards greater neuralization potency of pseudoviruses in rabbits (Sliepen et al., 2015).

Recently, two-component nanoparticle immunogens were tested in rabbits; intriguingly, expression of one trimer (based on consensus sequences) onto I53-50 (Bale et al., 2016) nanoparticles were more immunogenic than another on the same nanoparticle (Brouwer et al., 2019), indicating the importance of selecting specific Envs to display on nanoparticles. More recently, I53-50 nanoparticles bearing Envs from virus 16055 elicited greater neutralizing antibody responses against the autologous virus compared to soluble trimer, though increased breadth was not observed. Still, 16055-I53-50 nanoparticles elicited an epitope specific (trimer apex) response, which rationalized by the authors, were due to the geometry and orientation of Envs on the nanoparticle (Brouwer et al., 2021).

While the incorporation of diverse copies of soluble Env in a vaccine formulation, with the thought that exposure to sequence diversity will drive neutralization breadth, so far have largely failed to provide sufficient protection (Haynes et al., 2012; Rerks-Ngarm et al., 2009), mapping the evolution of antibody responses to HIV-1 infection indicate immunizations with antigens that reflect native viral diversity could yield broadly neutralizing antibodies (Liao et al., 2013). This finding is corroborated by *in silico* studies that postulate that increased mutational distance between immunogens (to an extent) yields antibodies with greater breadth (Shaffer et al., 2016; Wang et al., 2015). Similar studies *in vivo* have further suggested that immunization with cross-subtypic Envs may elicit greater breadth compared to that of monovalent Envs

(Bricault et al., 2015; Gao et al., 2004; Julien et al., 2015). This was shown *in vivo* by Georgiev *et al.* with two-component insect ferritin to modest but encouraging results, motivating future studies incorporating diverse Envs on nanoparticle platforms (Georgiev et al., 2018).

Alternatively, some groups have explored epitope-focused vaccine design using nanoparticles. Haigwood and colleagues took a peptide encoding for a specific domain (V3) of HIV Env and expressed it on the surface of the E2 subunit of *Geobacillus stearothermiphilus,* a 60-mer self-assembling protein nanoparticle (V3-E2 nanoparticle). Though broadly neutralizing antibodies were not elicited in animal models tested, serum neutralization of neutralization-sensitive viruses that mapped to the V3 region of the Env trimer were observed (Jaworski et al., 2012). Relatedly, Haigwood and colleagues also looked to another domain of HIV – the membrane proximal external region (MPER) of Env – to display on the E2 surface. They observed modest neutralization breadth in rabbits that was greater than that observed in their earlier publication using V3-E2 nanoparticles (Krebs et al., 2014). Despite the high mutability of HIV-1, some mutations are deleterious to viral fitness. One such region (like MPER) that is characterized by high identity across globally circulating HIV-1 viruses is the fusion peptide of Env, which mediates viral fusion with host cells. The conjugation of the fusion peptide onto keyhole limpet hemocyanin (KLH) remarkably elicited broad, neutralizing antibody responses in mice, guinea pigs and non-human primates (Cheng et al., 2019; Xu et al., 2018).

The success observed in using nanoparticle technology, immense room for future optimization, and abundance of versatility clearly warrants continued exploration into nanoparticle-based platforms.

1.4 Understanding Antibody Responses to HIV-1 Infection is Important for Rational Vaccine Design

Ultimately, understanding and interrogating antibody responses to infection is important for developing a vaccine for HIV-1. And, while there are many tools that are being researched to create an optimal vaccine, nanoparticle technology has shown promise to elicit desired responses. Studies marrying an understanding of antibody responses with nanoparticle technology have been encouraging. Within certain individuals infected with HIV-1, broadly neutralizing antibodies develop capable of neutralizing a vast majority of globally circulating strains. Armed with this knowledge, nanoparticle immunogens have been leveraged to target the germline to elicit specific broadly neutralizing antibodies.

As one of the most potent and broadly neutralizing antibodies, the field has been trying to elicit VRC01 responses through immunization. VRC01 class antibodies utilize *IGHV1-2* and are characterized by short CDRL3 loops, that enable it to avoid intrusive glycans impeding access to the CD4 binding site epitope. Whereas *IGHV1-2* genes are present in the human antibody repertoire, predicted germline precursors to VRC01 do not seem to recognize Env (Zhou et al., 2010). As such, Env itself is not sufficient to elicit VRC01-class antibodies. In a proposed solution to this problem, Jardine *et al.* designed an antigen known as the engineered outer domain (eOD) that could both bind VRC01-class antibodies and engage *IGHV1-2* germline antibodies. To enhance B cell activation and lymph node trafficking, these eODs were displayed as 60 copies on a self-assembling protein nanoparticle known as lumazine synthase, an enzyme found in bacterium *Aquifex aeolicus* (Jardine et al., 2013). To test the efficacy of these germline-targeting nanoparticle immunogens, knock-in mice with the germline-reverted VRC01 heavy chain were immunized with eOD-GT8 (the eighth iteration of the germline targeting eOD).

Understandably, a broad neutralizing antibody response was not observed and could be explained by the fact that eOD-GT8 immunogens that engage the germline do not possess features common to many circulating HIV-1 strains. Nevertheless, B cells expressing antibodies that had features resembling VRC01-class antibodies, like short CDRL3s, were observed in response to these particular immunogens (Jardine et al., 2015). To assess the viability of immunizing humans with germline-targeting nanoparticles, Jardine *et al.* used eOD-GT8 to isolate VRC01-class precursor naïve B cells from healthy donors and concluded that the frequencies of these precursors, their ability to bind eOD-GT8, and their BCR structures were evidence that eOD-GT8 would be a good vaccine candidate (Jardine et al., 2016). As such, eOD-GT8 was tested in a human clinical trial (ClinicalTrials.gov Identifier: NCT03547245). Remarkably, 97% of participants developed VRC01-class B cell precursors (Venkatesan, 2021).

Given the encouraging, albeit early, success of these vaccine designs, a deeper understanding of population level antibody responses coupled with nanoparticle vaccine development may be a powerful tool to once and for all curb the ongoing HIV-1/AIDS pandemic.

1.5 Summary of Thesis Work

This dissertation explores antibody biology in two distinct ways. In one, we characterize antibodies present at the population level in the context of natural infection with HIV-1. In the other, we leverage nanoparticle technology to harness HIV-1 Env diversity to elicit cross-clade antibody responses. In Chapter Two (***Sequence and Functional Characteristics of a Public HIV-Specific Antibody Clonotype***), we build on the initial discovery (Setliff et al., 2018) of clonotype #13905. Specifically, we interrogate evident boundaries by which public antibodies

are classified. In doing so, we discover sequence features that modulate antigen recognition and function. Further, we identify members of the public clonotype capable of modest neutralization breadth, suggesting the potential for vaccine design efforts to target antibodies that may be present at the population level.

In Chapter Three (***Cross-Clade Response to Multimeric HIV-1 Nanoparticle Immunogens***), we utilize a protein nanoparticle presenting multiple, diverse HIV-1 antigens on the surface as vaccine candidates. We select two antigens from different clades in a variety of formulations including single soluble trimer, soluble trimer cocktails, monovalent nanoparticle cocktails in which each nanoparticle bears a single antigen, or mosaic nanoparticles, in which both antigens are presented on each individual nanoparticle surface. We first assess the immunogenicity of our nanoparticles in mice, and then show that nanoparticle vaccines elicit a cross-clade response in guinea pigs.

Finally, in Chapter Four (***Conclusions and Future Directions***), we explore the implications of our findings in the preceding two chapters and speculate on future steps to be taken, innovations to be made, and outstanding questions that need to be answered in order to move ever closer to more efficacious vaccines against HIV-1 and other prevalent diseases.

We believe that this dissertation will serve to provide motivation for continued research into antibody public clonotypes, particularly as potential therapeutics or vaccine templates. Further, we believe that the work presented here on HIV-1 mosaic nanoparticles will pave the way towards future, more efficacious nanoparticle vaccines targeting HIV-1.

CHAPTER 2

SEQUENCE AND FUNCTIONAL CHARACTERISTICS OF A PUBLIC HIV-SPECIFIC ANTIBODY CLONOTYPE

Author contributions to this chapter are indicated in Appendix 1.

2.1 Rationale

Antibody discovery from HIV-infected individuals is a hallmark of HIV-1 research, paving the way towards the development of effective therapeutic and vaccine candidates (Bar et al., 2016; Lynch et al., 2015). These discovery efforts have identified broadly neutralizing antibodies (bNAbs) as potential therapeutic candidates and antibodies as templates for engineering antigens to elicit epitope-specific antibody responses to vaccination (Bricault et al., 2019; Jardine et al., 2015; Xu et al., 2018). Large-scale profiling of human antibody repertoires has shown that the antibody response to infection is vast and complex and, therefore, may contain unexplored avenues for vaccine design (Briney et al., 2019; Galson et al., 2015). One currently under-explored area is vaccine design informed by population-level antibody responses (Davis et al., 2019; Kreer et al., 2020). While the majority of a person's antibody repertoire is unique because of the vast potential diversity generated in part by V (variable), D (diversity), and J (joining) recombination, light chain selection, and somatic hypermutation (SHM) (Briney et al., 2019), individuals can nevertheless possess identical or similar antibodies. Such "public" antibodies have been identified not only for various disease states including HIV-1 infection, SARS-CoV-2 infection, dengue infection, influenza vaccination and others, but also in healthy

individuals (Arentz et al., 2012; Ehrhardt et al., 2019; Jackson et al., 2014; Parameswaran et al., 2013; Setliff et al., 2018; Soto et al., 2019; Voss et al., 2021; Yuan et al., 2020). Yet, very little is currently known about the immunological role of public antibodies and their potential as templates for population-level vaccine design.

To gain a better understanding of the properties of public antibodies, we focused on a clonotype that had been previously identified in samples from multiple HIV-infected individuals from the Centre for the AIDS Programme of Research in South Africa (CAPRISA) cohort (Setliff et al., 2018). In particular, this public clonotype included antibodies from three CAPRISA donors, with publicness defined by the same V_H-gene, J_H gene, and junction length, and CDRH3 amino acid sequences of high identity among donors (Setliff et al., 2018). In that study, two antibody clonotype members with natively paired heavy and light chains from the public clonotype were produced experimentally and confirmed to be HIV-specific. Subsequent analysis of the antibody sequencing data revealed the existence of additional antibody sequences with high CDRH3 identity to the antibodies from the public clonotype but different V_H and/or V_L genes. Therefore, here we sought to build on our previous work (Setliff et al., 2018) by investigating the genetic and phenotypic characteristics that define the members of this public antibody clonotype. The resulting analysis offers insight into the public antibody response in the context of chronic HIV-1 infection and explores the boundaries of antibody "publicness."

2.2 Results

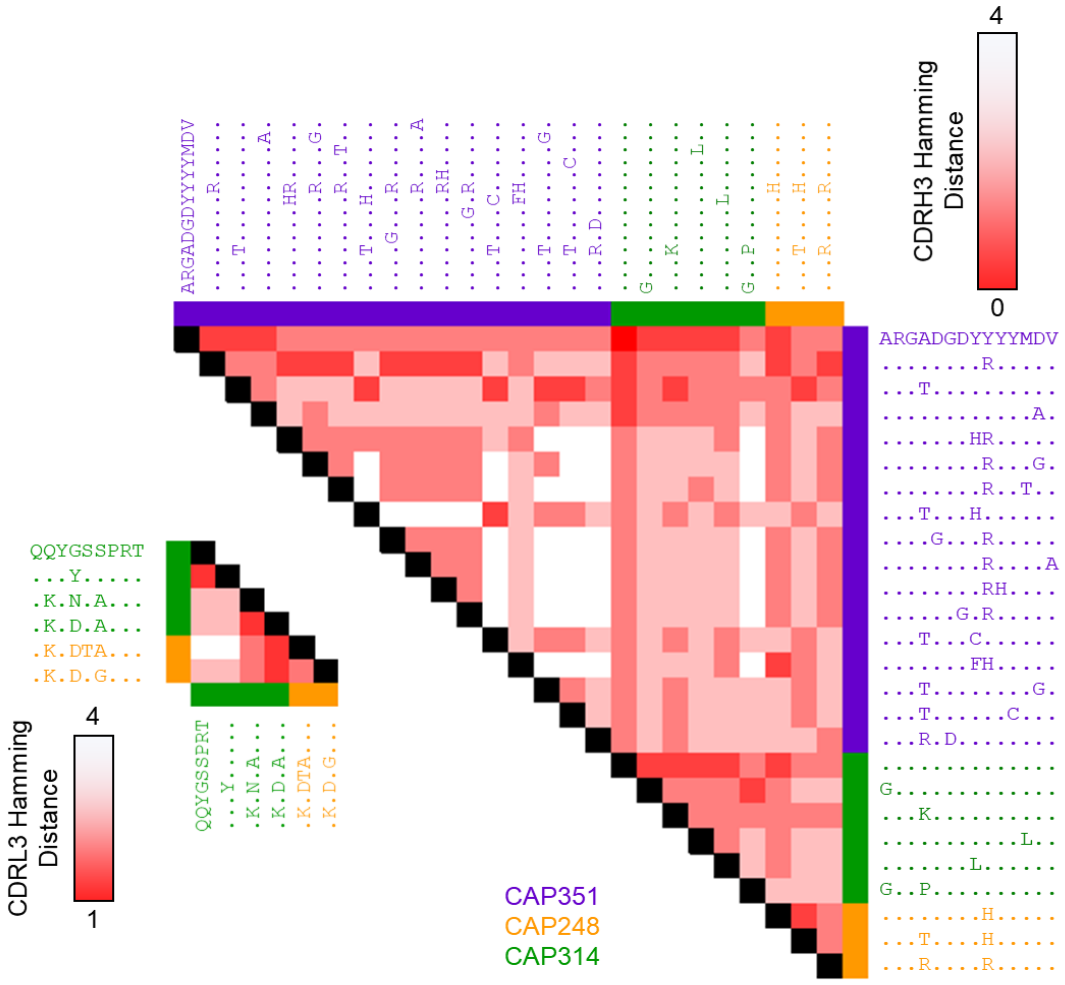
2.2.1 Identification of Antibodies with High CDRH3 Sequence Identity from Multiple HIV-Infected Donors

The antibody sequences from the three CAPRISA donors were combined, and complete linkage clustering was performed to assign clonotype membership for each sequence (Gupta et al., 2015). In contrast to our previously published work (Setliff et al., 2018), we expanded our parameters such that sequences were clustered using the following criteria: CDRH3 amino acid sequence identity of at least 70% with the same CDRH3 and junction length and no consideration for V_H- and J_H-gene usage. This allowed for a more inclusive definition of potential public antibodies that would enable systematic exploration of the boundaries of antibody publicness. Among the 24,218 clonotypes encompassing sequences from one or more of the three donors, clonotype #13905 contained the previously reported public heavy chain sequences (Setliff et al., 2018) and was selected for further analysis. Clonotype #13905 included 171 nucleotide sequences from all three donors (donors CAP314, CAP248 and CAP351), spanning four different V_H-gene assignments (*IGHV1-69*, *IGHV5-51*, *IGHV1-18*, and *IGHV3-23*). Since paired heavy-light chain sequences were available for the datasets for donors CAP314 and CAP248, the corresponding light chain sequences were also retrieved. Two different light chain genes were identified in sequences from clonotype #13905: *IGKV1-27* in both donors CAP314 (20% of light chain sequences from that donor) and CAP248 (100%), and *IGKV3-20* in donor CAP314 (80% of light chain sequences from that donor).

2.2.2 CDR3 Sequences from Different Donors are not Less Similar Than They are Within Donors

To determine whether the CDR3 sequences from the same donor exhibited greater levels of similarity compared to sequences from other donors, we constructed Hamming distance matrices among all the unique CDRH3 and CDRL3 sequences, respectively, from clonotype #13905. The 171 sequences in the public clonotype comprised 25 unique CDRH3s, including 16 from donor CAP351, five from donor CAP314, three from donor CAP248 and one shared between donors CAP314 and CAP351. It also included six unique CDRL3s, with four from donor CAP314 and two from donor CAP248 (Figure 3A). The CDRH3 sequence distance values ranged from zero to four, with lower values corresponding to greater identity between two CDRH3 sequences (Figure 3A). High CDR3 similarity was observed both within donors (mean: 2.57, SEM: 0.08) as well as among donors (mean: 2.46, SEM: 0.06), with some CDR3s exhibiting greater similarity among, as opposed to within, donors (Figure 3B).

A


CDRH3 Hamming Distance
4
0
ARGADGDYYYYMDV
QQYGSSPRT
CDRL3 Hamming Distance
4
1
CAP351
CAP248
CAP314

B

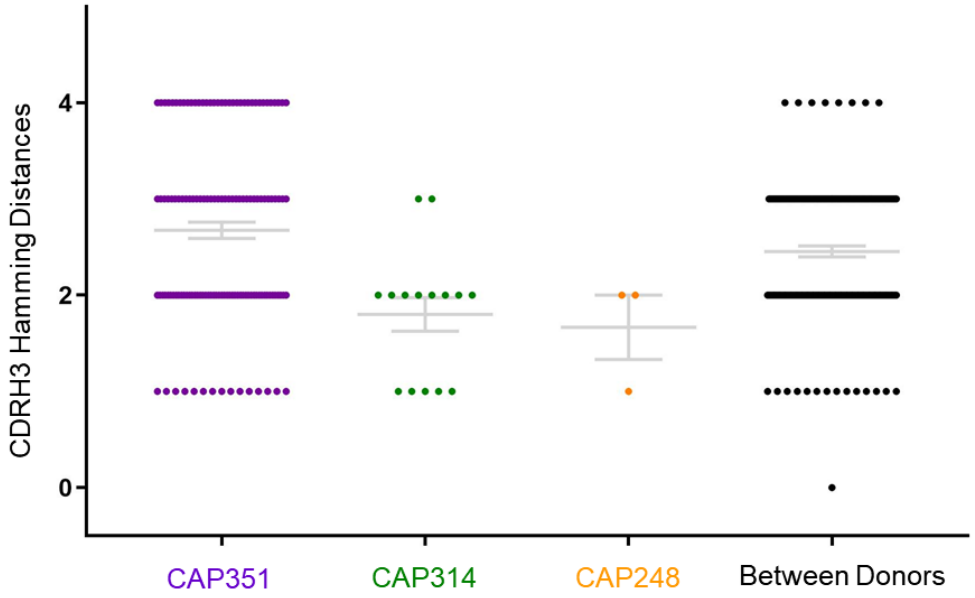
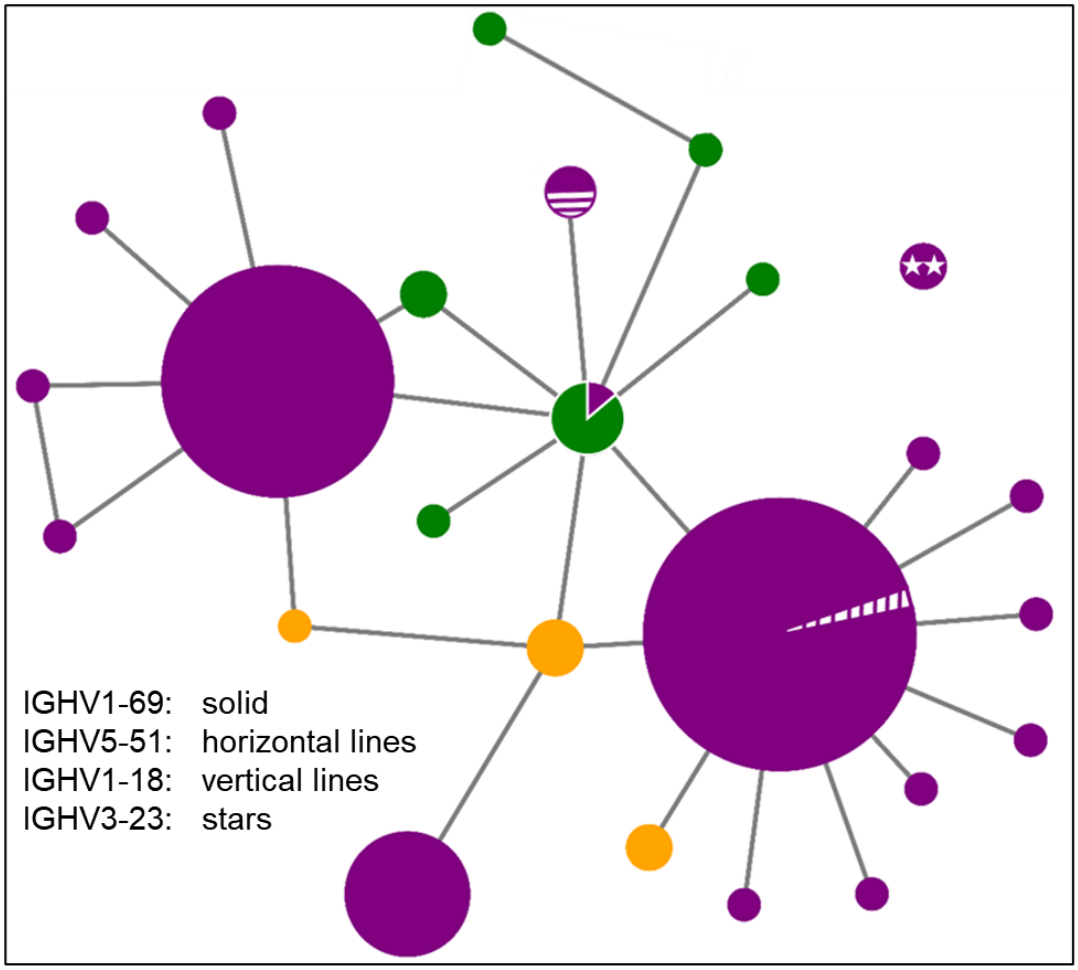

CDRH3 Hamming Distances
4
2
0
CAP351
CAP314
CAP248
Between Donors

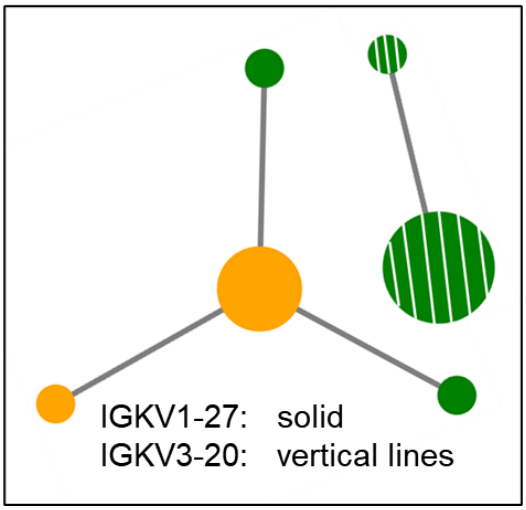
Figure 3. Association of CDR3 Regions Within and Among Members of the Public Clonotype. (A) The Hamming distance values ranged from 0 (red) to 4 (white) for the CDRH3 and from 1 (red) to 4 (white). For heavy and light chains, the Hamming distance between 25 unique CDRH3 and 6 unique CDRL3 are shown, respectively. Each CDR3 is colored based on the respective donor. An arbitrary sequence was used as a template to visualize amino acid identity (dots) or differences (letters) for the different sequences in the alignment.
(B) Grouping of Hamming distance values between pairs of unique CDRH3 of the sequences in clonotype #13905 within each donor and among donors with mean with SEM for each group shown in grey.

2.2.3 Unique CDR3 Sequences can be Associated with Multiple Diverse V Genes

To investigate the patterns of pairing between CDR3 sequences and germline V genes, we constructed a Hamming distance-based network graph for the antibody sequences from clonotype #13905 (Figure 4). The majority (97%) of heavy chain sequences utilized *IGHV1-69*, but antibodies using three other heavy chain germline genes were also observed: *IGHV1-18* (1.2%), *IGHV3-23* (1.2%), and *IGHV5-51* (0.6%). Of note, the antibodies from donors CAP314 and CAP248 only utilized *IGHV1-69*, whereas donor CAP351 used all four of the V_H-genes. We observed two unique CDRH3 sequences from donor CAP351 that each utilized two different V_H-genes: ARGADGDYYYYMAV (*IGHV1-69* and *IGHV5-51*) and ARGADGDYRYYMDV (*IGHV1-69* and *IGHV1-18*). These results suggest that the same CDRH3 sequence can be associated with multiple diverse V_H-genes. With the exception of a single node in the heavy chain portion of the graph, all other antibody sequences from clonotype #13905 were within a Hamming distance of one from at least one other sequence in that clonotype, revealing a tight network of sequence similarity among members of this clonotype, even among different donors (Figure 4). For the light chains, *IGKV1-27* was identified from sequences from both donors CAP248 and CAP314, while donor CAP314 also had antibodies that utilized the *IGKV3-20* germline gene. Overall, a diversity of germline genes was observed for both the heavy and light

chains in clonotype #13905, without any clear patterns of association with specific CDR3 sequences.





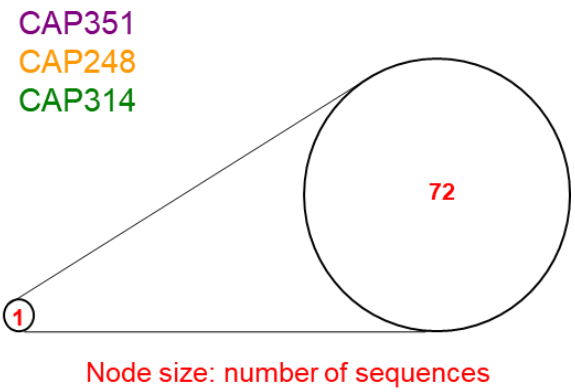


Figure 4. Network Graph Representation of Unique CDR3s in Clonotype #13905. Each node represents a unique CDR3 and the node diameter is proportional to the corresponding number of sequences, which varied from 1 to 72 for heavy chain and 1 to 7 for light chain. Node colors correspond to the respective donor for the given CDR3 sequence, with multiple colors in a single node representing a CDR3 that is shared by multiple donors. The node fill pattern corresponds to the V-genes used for the given set of sequences, with multiple patterns in a single node representing different V-genes associated with the same CDR3 sequence.

2.2.4 Defining the Limits of What Constitutes a Public Antibody Clonotype

The observation that multiple diverse V_H and V_L germline genes can be associated with highly similar CDR3 sequences led us to address the limits of what constitutes a public antibody clonotype. To assess this question, we selected a diverse set of antibody heavy and light chain sequences for experimental validation, making sure sequences from all three donors and all observed V_H and V_L genes were selected. The set included nine heavy chain sequences (six from CAP351, two from CAP314 and one from CAP248) and three light chain sequences (two from CAP314 and one from CAP248). Of these, five, two, one and one sequences were from *IGHV1-69*, *IGHV3-23*, *IGHV1-18*, and *IGHV5-51*, respectively; and two and one were from *IGKV1-27* and *IGKV3-20*, respectively. Among the nine heavy chain sequences, three pairs utilized identical CDRH3s (ARGADGDYRYYMDV, ARGADGDYYYYMDV, ARGRDDDYYYYMDV), and interestingly, sequences in one of these pairs (ARGADGDYRYYMDV) utilized different V_H-genes (*IGHV1-18* and *IGHV1-69*). Thus, the selected set was representative of the diversity of sequences found in clonotype #13905.

All natively paired heavy-light chain sequences, as well as all other non-native heavy-light chain pairs from all three donors (a total of 27 unique antibody heavy-light chain pairs) were successfully expressed as recombinant IgG proteins. Thus, we sought to determine whether and to what extent these antibodies could recognize HIV-1 Env-derived antigens. Two of these antibodies, CAP248_30 and CAP314_30 (native heavy-light chain pairs from donors CAP248

and CAP314), had been previously validated (Setliff et al., 2018) and were also confirmed to be HIV-specific in our experiments (Figure 5, Figure 19). We tested all 27 heavy-light chain pairs against two different HIV Env-derived antigens, clade CRF01_AE 93TH975 gp120 monomer (Figure 5A, Figure 19A) and clade A BG505.SOSIP.664 prefusion-stabilized gp140 trimer (Sanders et al., 2013) ((Figure 5A, Figure 19B). Only antibodies with heavy chains utilizing *IGHV1-69* were able to bind the gp120 monomer (Figure 5A, Figure 19A), suggesting the importance of V_H-gene usage for antigen recognition. Interestingly, the CAP351_04 (*IGHV1-69*) and CAP351_01 (*IGHV1-18*) heavy chains had the same CDRH3 sequence, but different V_H-genes; however, only CAP351_04 (*IGHV1-69*) bound 93TH975 gp120. In contrast, all *IGHV1-69* heavy chains paired with light chains using any of the three V_L genes were able to bind gp120 monomer, albeit to different extents (Figure 5A, Figure 19A). Together, these results suggest potential V_H, but not V_L, germline gene-mediated antigen specificity for this public clonotype.

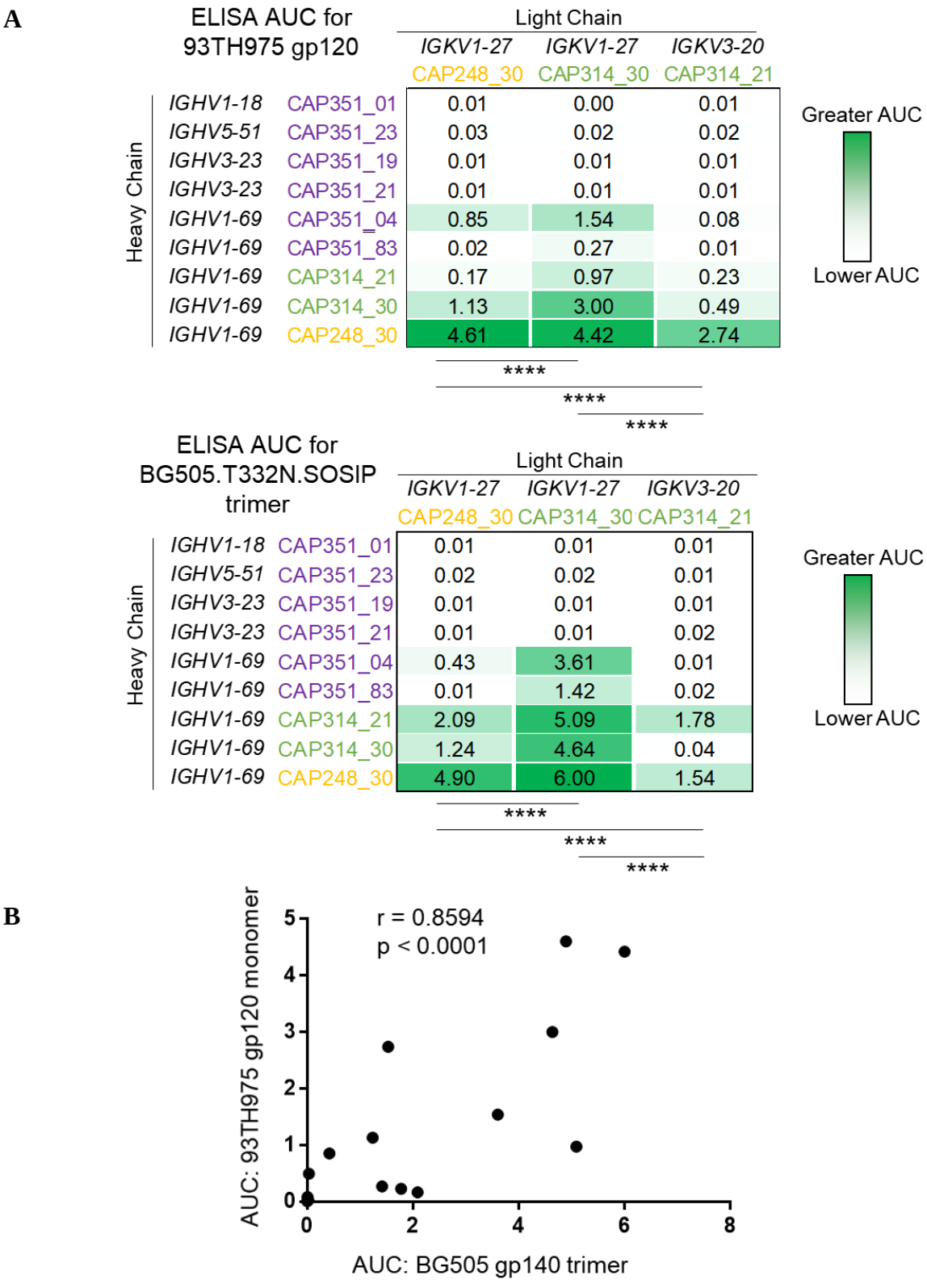


Figure 5. Public Antibody Recognition of HIV-1 Protein.

(A) Binding data for each antibody (both native and non-native heavy-light chain pairs) against 93TH975 gp120 or BG505.T332N.SOSIP.664 are displayed as a heatmap of AUC analysis calculated from the ELISA curves in Figure 19A and 19B, respectively. Statistical significance was determined via 2-way ANOVA p-value ($p < 0.0001$) corrected for multiple comparisons using Tukey's multiple comparisons test for both monomer and trimer.
(B) Spearman correlation between antibody binding to HIV-1 monomer (x-axis) and trimer (y-axis).

The strength of binding to the monomer appeared to be associated with the choice of light chain pairing with a given *IGHV1-69* heavy chain ($p<0.0001$, 2-way ANOVA with p-value corrected for multiple comparisons using Tukey's multiple comparisons test), suggesting that while there appears to be greater promiscuity for the choice of light chain compared to heavy chain, strength of antigen reactivity can be modulated by optimizing heavy chain-light chain pairings. We next determined if the antibodies from this public clonotype recognized gp140 trimer, which is designed to mimic a neutralization-sensitive prefusion conformation of Env (Sanders et al., 2013) (Figure 5A, Figure 19B). For most antibodies, there was markedly increased binding to BG505.T332N.SOSIP.664 single-chain trimer (Georgiev et al., 2015), though the antibody-antigen binding patterns between monomer and trimer showed a significant correlation ($p < 0.0001$, Spearman correlation) (Figure 5B). Binding to different forms of the BG505 trimer for the native CAP248_30 and the non-native CAP248_30$_H$/CAP314_30$_L$ antibody pairs was further validated by surface plasmon resonance (Figure 20).

Overall, the finding that only antibodies using *IGHV1-69* heavy chains were capable of recognizing HIV-1 antigens, despite also testing sequences with highly similar or even identical CDR3 sequences but different V_H gene usage, indicated that the public antibody clonotype may be restricted to only sequences with *IGHV1-69* but with either *IGKV1-27* or *IGKV3-20*. This conclusion was further reinforced by the fact that non-native pairs of heavy and light chains from

either the same or different donors could successfully recognize HIV-1 Env, indicating functional complementation of antibody sequences from the public clonotype.

2.2.5 Public Antibodies Exhibit Consistent Epitope Specificity and Virus Neutralization

We previously determined that binding to HIV-1 Env by CAP248_30 and CAP314_30 was affected by the receptor binding site (CD4bs) epitope knockout, D368R (Setliff et al., 2018). To confirm whether other members of the public clonotype also mapped to CD4bs, we generated epitope knockouts in the context of the BG505.T332N.SOSIP.664 trimer and confirmed that all tested heavy-light chain pairs were indeed affected by CD4bs knockout mutations (D279K and D368R) (Figure 6). These results suggest that non-native pairings of heavy and light chains from the same or different donors did not affect epitope targeting, further corroborating the conclusion that these antibody sequences which were shared among all three donors, are indeed public. Further, in pseudovirus neutralization assays, both native and non-native heavy-light chain pairs exhibited generally consistent ability to neutralize tier 1 HIV-1 strains (Figure 7), in agreement with our previously published work (Setliff et al., 2018) for the native CAP248_30 and CAP314_30 antibodies.

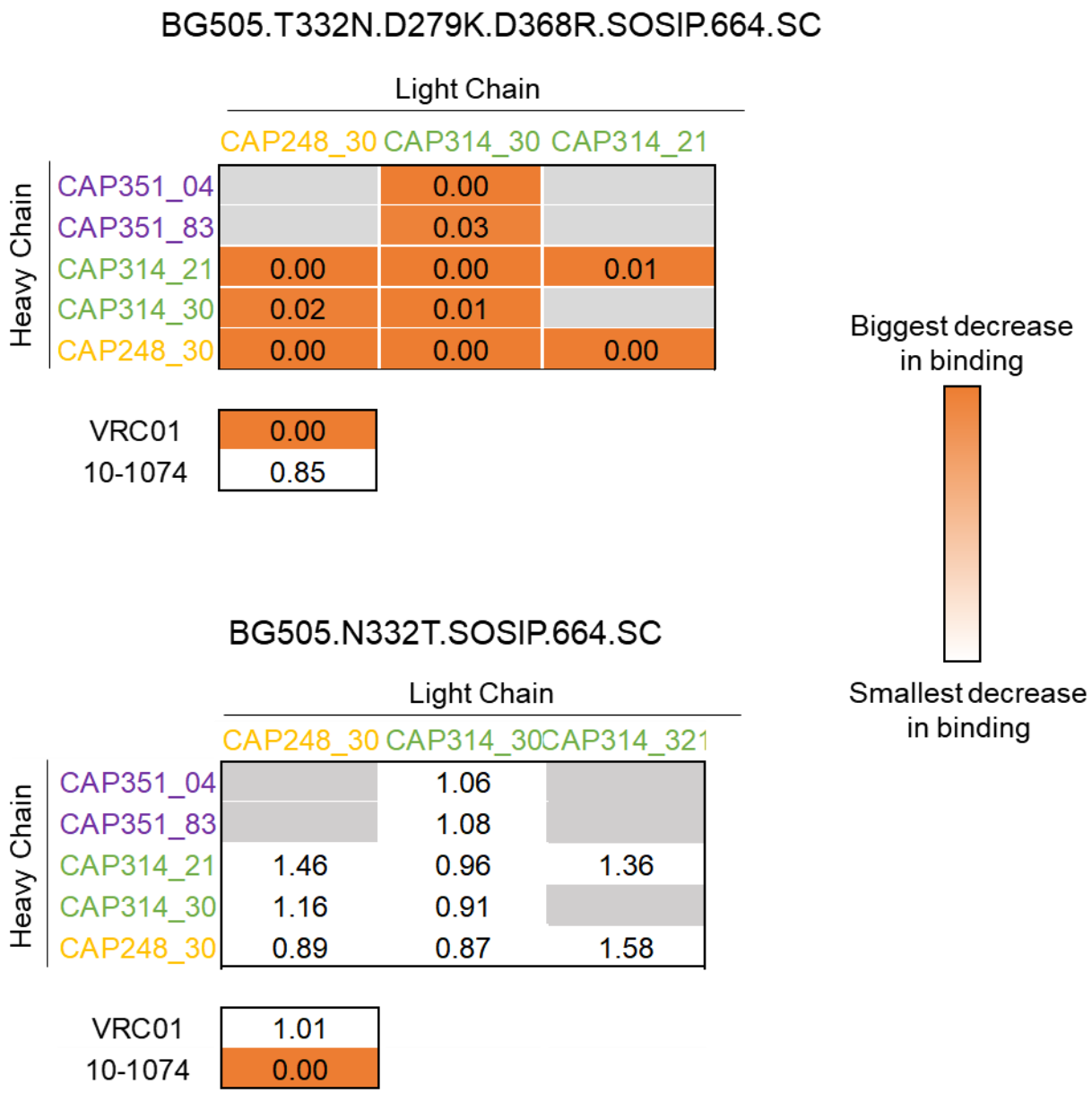


Figure 6. Antibodies from the Public Clonotype Map to the CD4 Binding Site of HIV-1 Env. AUC values from ELISA curves for a given antibody heavy-light chain pair against select epitope-specific point mutants of BG505.SOSIP.664.sc trimers as a percentage of binding to BG505.SOSIP.T332N.664.sc. Level of decrease of binding is shown as a heatmap. Antibodies with AUC values less than 0.5 are shown in grey or are absent. Known HIV-1 antibodies VRC01 and 10-1074 were used as controls. The public antibodies were susceptible to the double epitope knockouts D368R and D279K, suggesting targeting of the CD4 binding site.

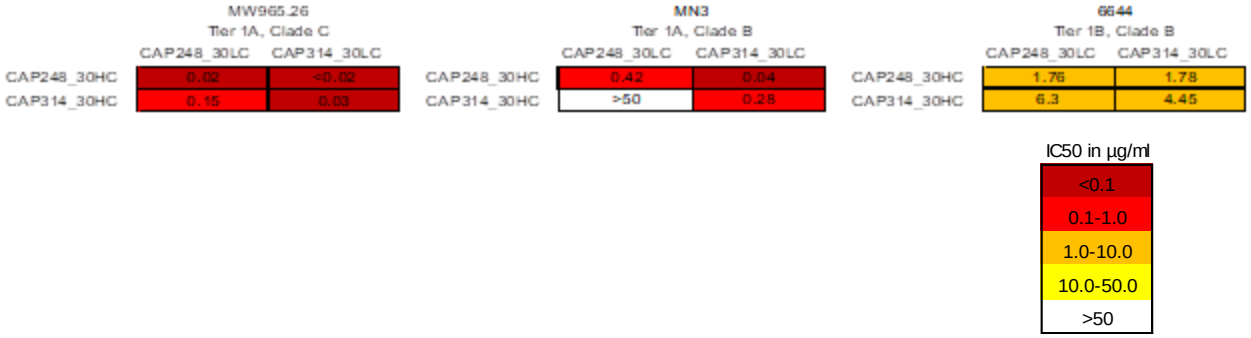


Figure 7. Pseudovirus Neutralization by Public Antibodies. Neutralization IC50 values for different antibody heavy-light chain pairs. IC50 values are shown from high potency (< 0.1 µg/ml, dark red) to no neutralization (> 50 µg/ml, white).

2.2.6 Public Antibody Sequences Exhibit High Similarity Both Within and Among Donors

To visualize the overall sequence similarity among antibodies from different donors, we generated a phylogenetic tree with the sequences from the public antibody clonotype (Figure 8A). Varied levels of somatic hypermutation were observed both in the heavy chains (2.64%-11.16%) and the corresponding light chains (1.4%-4.9% for *IGKV1-27* and 1.04%-5.58% for *IGKV3-20*). Further, multiple clades were observed particularly in the heavy chain tree, suggesting a diversity of antibody evolution within this clonotype (Figure 8A). Of note, however, sequences from different donors were interspersed in the trees. This suggests that in some cases, greater similarity was observed among sequences from different donors, as opposed to sequences from the same donor. This observation of among versus within donor similarities was further supported by the Hamming distances for these sequences (Figure 8B), with distances ranging between 12-22 among donors and 2-19, 2-17, and 6-24 within donors CAP351, CAP248, and CAP314, respectively. Together, these results suggest a diversity of pathways in which

antibodies from this clonotype evolved within each donor, although inter-donor similarities were also observed.

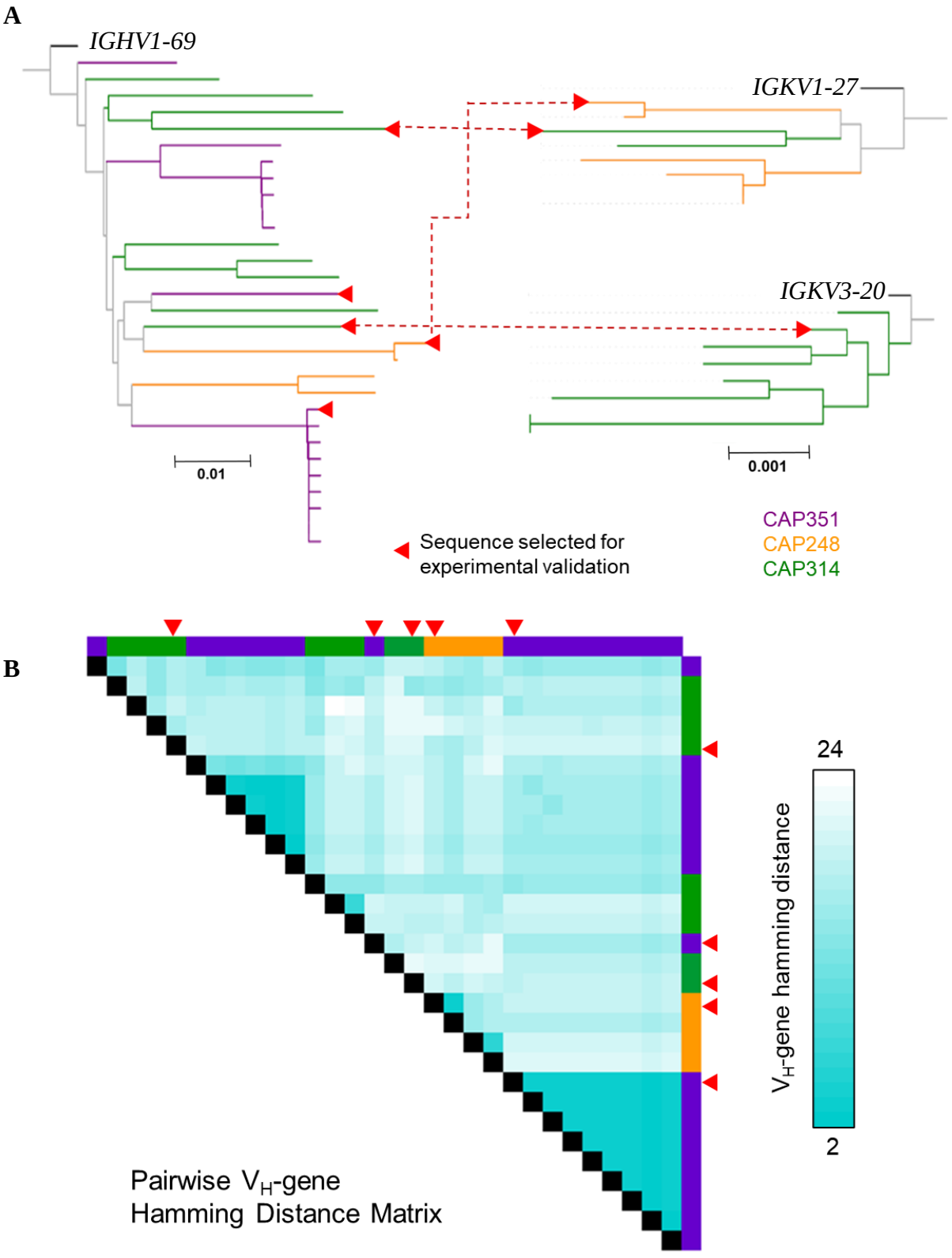


Figure 8. Association of V-Genes Within and Among Members of the Public Clonotype.
(A) Phylogenetic tree representation of heavy and light chain antibody sequences (colored by donor) in the public clonotype, along with the respective germline genes (black). Dotted lines between heavy and light trees represent natively paired heavy and light chains that were selected for experimental validation. Triangle symbols highlight additional sequences selected for experimental validation.
(B) Heatmap of Hamming distance values between the V_H regions of pairs of sequences (row and columns, colored by donor) in the public clonotype. The Hamming distance values ranged from 2 (cyan) to 24 (white). Triangle symbols highlight sequences selected for experimental validation.

2.2.7 Public Antibodies Include Distinctive Somatic Hypermutation Changes

To better understand the types of somatic hypermutation changes that are characteristic of the public antibodies, we analyzed the per-residue frequency of mutations from germline (Figure 9A). While in each donor, a large number of residue positions retained their germline identity in the majority of sequences, a number of residue positions had high frequency of mutations compared to germline (Figure 9A). Of note, several of these residue positions overlapped in multiple donors, with the levels of somatic hypermutation and residue entropy at each position having a significant correlation between all three donors (Spearman correlation test with p-value correction for multiple comparisons using Benjamini-Hochberg method)(Benjamini and Hochberg, 1995). This finding suggests the existence of somatic hypermutation hotspots that are common to all three donors (Figures 9A, B).

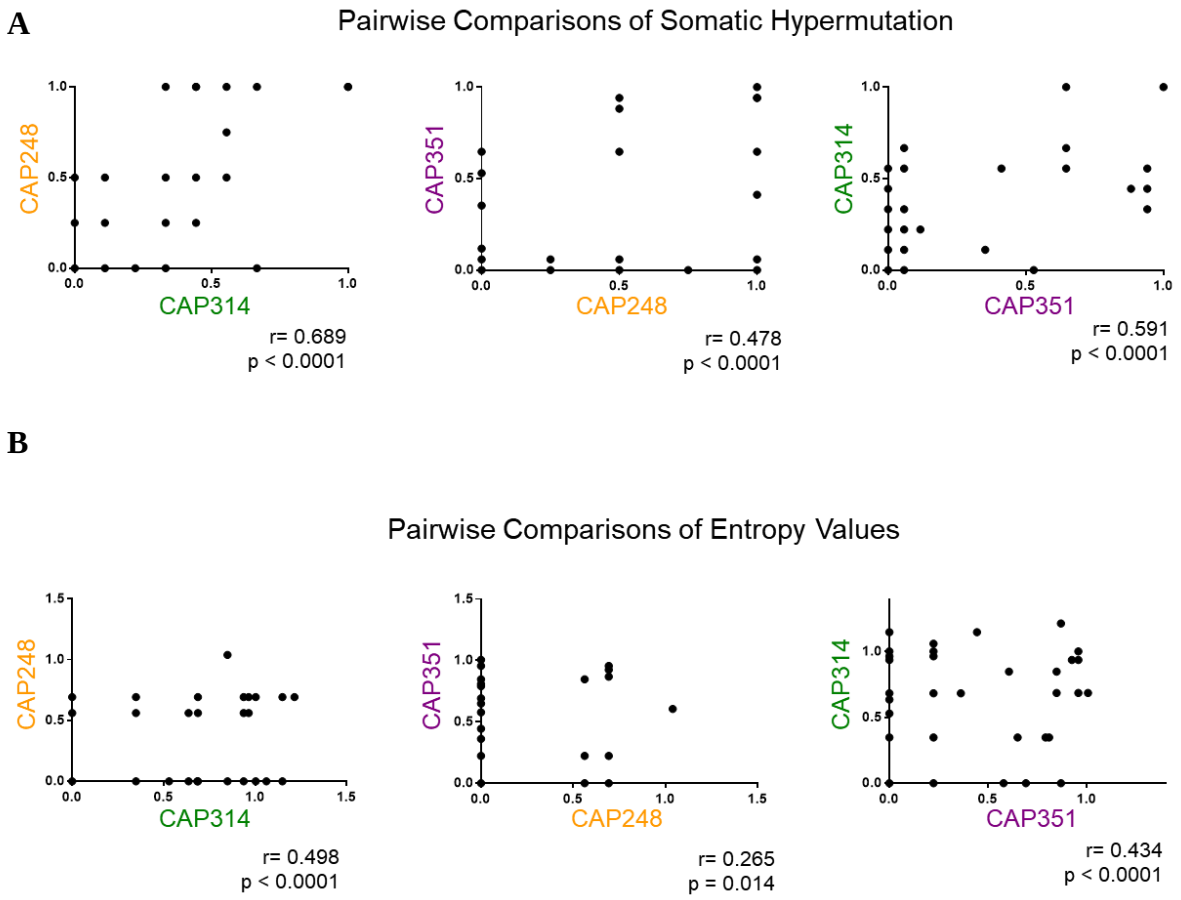


Figure 9. Comparison of Residue-Specific (A) Somatic Hypermutation and (B) Entropy Between Antibodies from Different Donors in the Public Clonotype. Each dot represents a single residue position in the sequence alignment for the public clonotype, showing the somatic hypermutation or entropy value for that residue position in each donor. Spearman correlations between all pairs of donors were computed and p-values corrected for multiple comparisons using the Benjamini-Hochberg method were reported. A total of 71 residue positions (from CDR1 to FR3) were plotted for the somatic hypermutation analysis and 85 residue positions (from CDR1 to CDR3) were plotted for the entropy analysis.

To interrogate whether the somatic hypermutation hotspots in the *IGHV1-69* antibodies are characteristic of the public antibody clonotype, we compared them to a set of unrelated representative antibody sequences that also utilized the *IGHV1-69* germline gene, retrieved from cAb-Rep (Curated Antibody Repertories) (Guo et al., 2019). Although for the majority of residue positions, no major differences among the public antibody sequences and the reference dataset

were observed, there were also several residue positions with >4-fold higher frequencies of non-germline amino acid identities in the public antibody sequences, suggesting the existence of unique *IGHV1-69* mutations that are specific to the public antibody clonotype (Figures 10A, B and Figure 11).

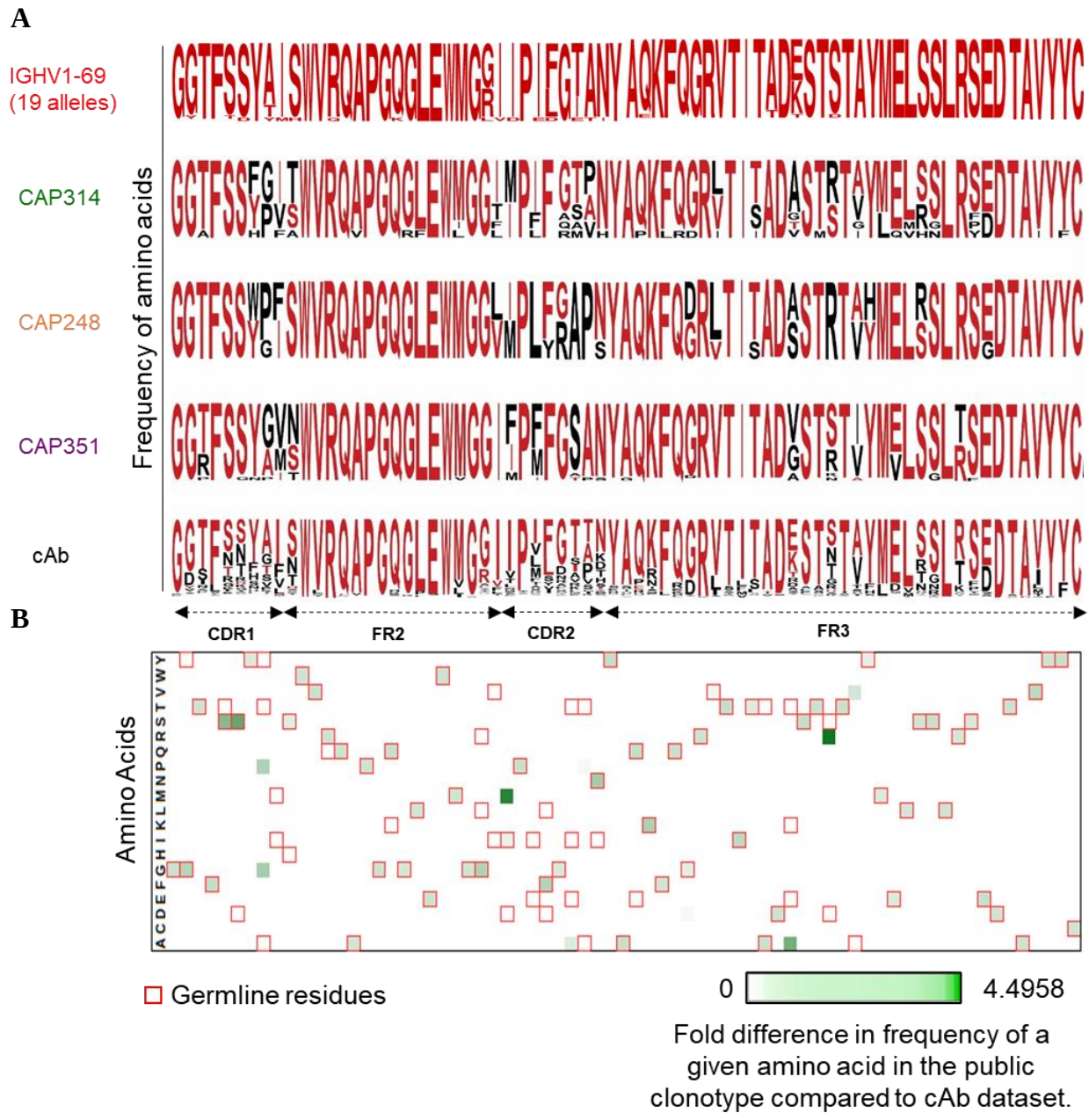


Figure 10. Comparison of Public Antibody Sequences to cAb Dataset.

(A) Conserved sequence features in the public clonotype. Sequence logo plot representation of *IGHV1-69* germline sequence (generated using sequences of 19 different alleles), heavy chain sequences in the public clonotype and a reference (cAb) dataset of *IGHV1-69*-based antibody sequences. Since the NGS data for donor CAP351 only included sequences between CDR1 and FR3 (Setliff et al., 2018), only that region is shown in the alignment. For each of the three donors and the cAb sequences, residues that are identical to the germline sequences are shown in red, while all other amino acids for a given position in the sequence alignment are shown in black. Height of letters corresponds to the frequency with which the respective amino acid is observed for the given position in the sequence alignment.
(B) For each residue position in the sequence alignment, the minimum fold difference between the amino acid frequency for a given residue position in the three donors in the public clonotype and cAb dataset is shown as a heatmap, with a color scale of white (0) to green (4.4958). The *IGHV1-69* germline residues (based on sequences of 19 alleles) in each position are highlighted in red boxes.

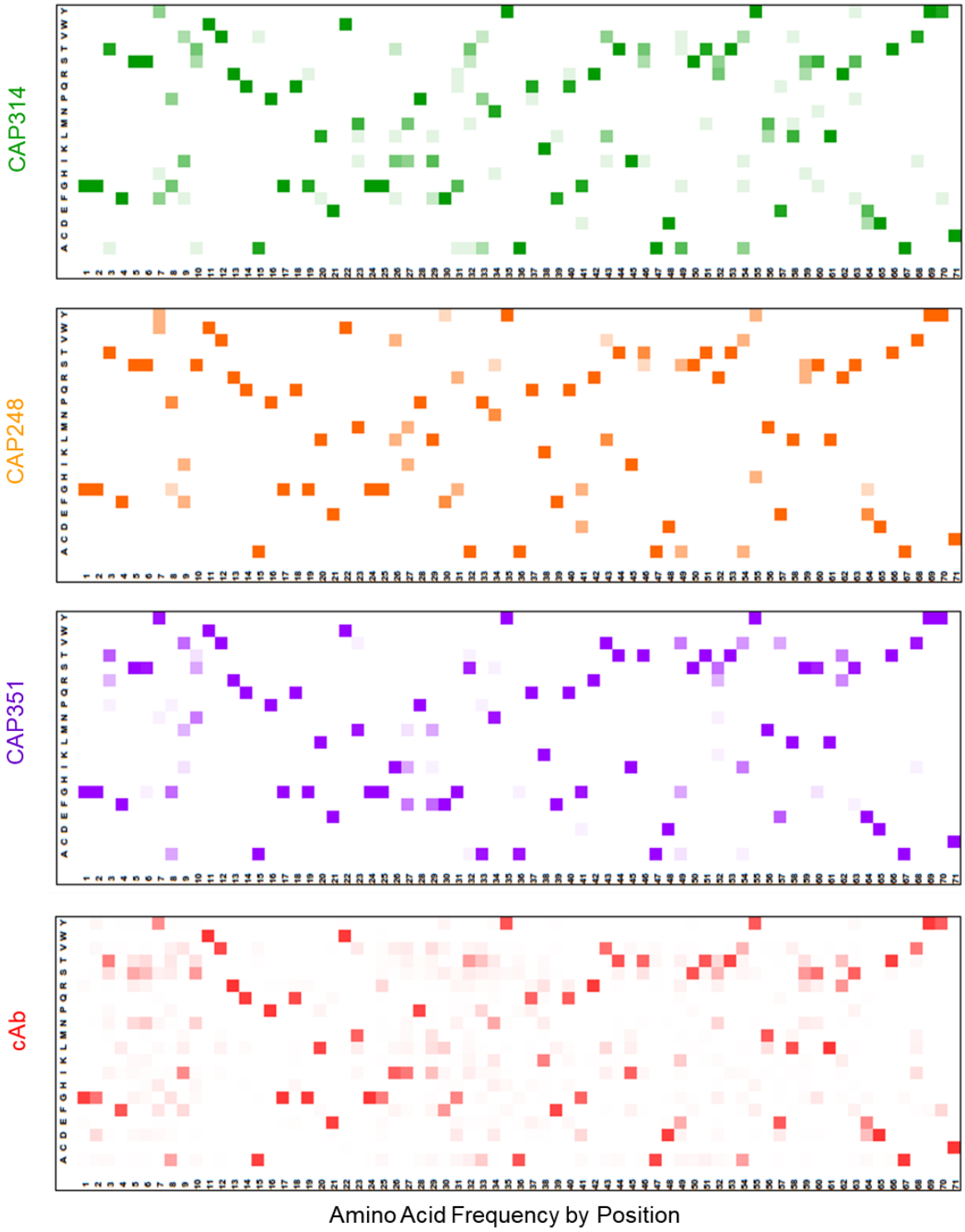


Figure 11. Donor-specific Amino Acid Frequency for Sequences in the Public Clonotype. Heatmap representation of amino acid frequency for the sequences in the public clonotype (shown separately for each donor) and the cAb dataset. Residue positions from CDR1 to FR3 are shown in the heatmap. The frequency values ranged from 0 (white) to 1 (donor-based color).

2.2.9 Heavy Chain Identity to Either Germline or Native Antibody Sequence does not Modulate Env Trimer Recognition

We next examined additional factors that could affect antigen recognition by the different antibody variants from the public clonotype (Figure 5). We first explored the potential role of heavy chain identity to germline sequence. Notably, antibodies at both ends of the V_H germline identity scale showed lower levels of HIV-1 protein binding, whereas some of the strongest binding was observed for antibodies with intermediate V_H germline identity (Figure 12A). We then explored whether antigen binding could be dependent on the sequence identity of the heavy chain in a given antibody pairing to the heavy chain present in the native pair for the given light chain (Figure 12B). For CAP248_30_L, the native heavy-light chain pairing was optimal in terms of binding to both gp120 monomer and to the stabilized trimer; however, high identity to native for the non-native heavy chains was not necessarily associated with improved antigen recognition (Figure 12B). Further, the other two light chains, CAP314_30_L and CAP314_21_L, exhibited better antigen recognition when paired with non-native, compared to their native, heavy chains (Figure 12B). Together, these data indicate that heavy chain identity to germline or native sequence may not be strong determinants of antigen recognition by this public antibody clonotype.

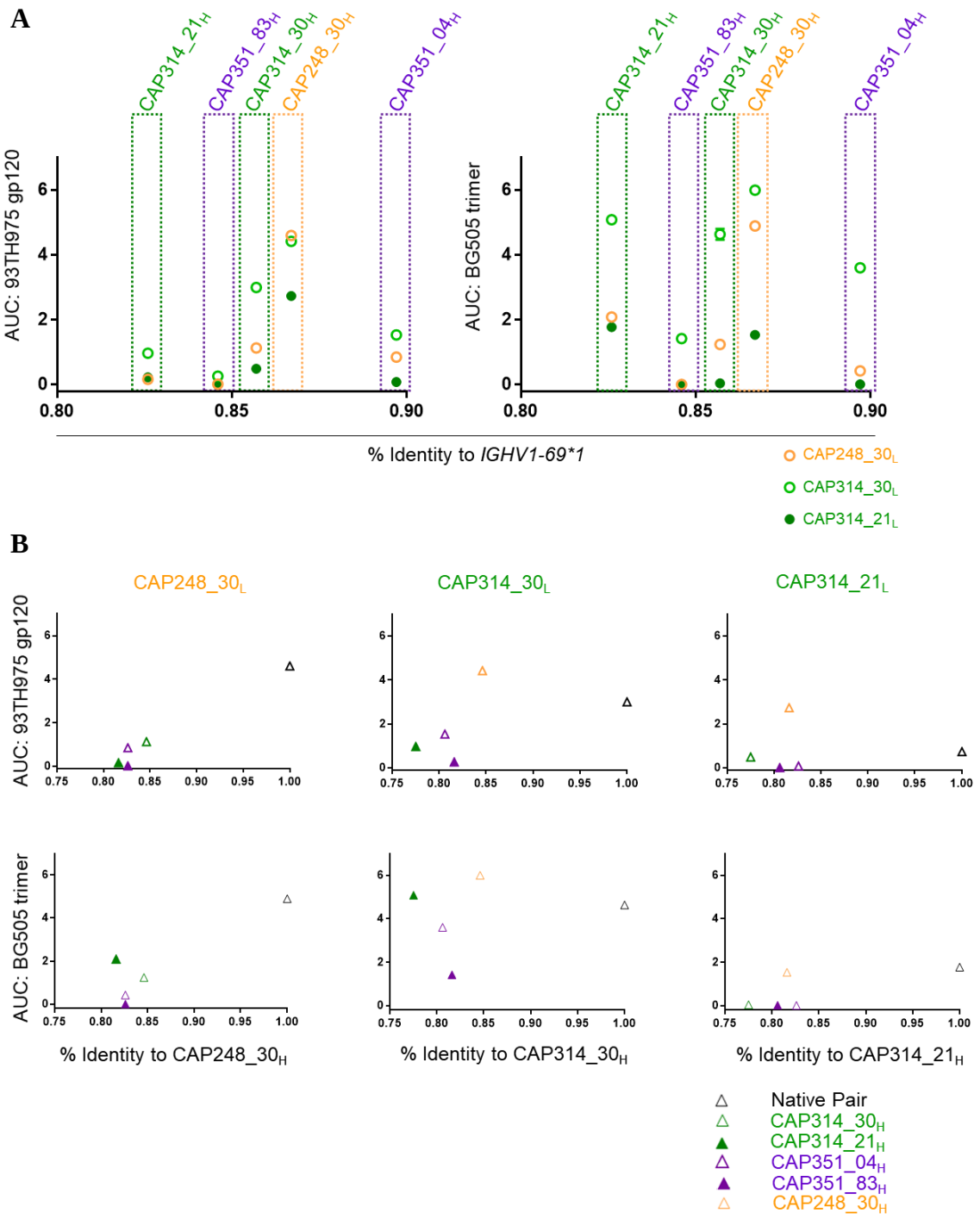


Figure 12. Association Between Antigen Binding and (A) Germline or (B) Native Antibody Sequence Identity.
(A) Antibody ELISA AUCs (y-axis; left – monomer; right – trimer)) were plotted against percent identity to the germline gene *IGHV1-69*01* (x-axis). Dots correspond to the light chain used in each antibody and dotted boxes group antibodies by the respective heavy chain used. Colors show the source donor; for donor CAP314, the two different light chains are shown as open and closed circles, respectively.

(B) For each light chain, antibody ELISA AUCs (y-axis; top – monomer; bottom – trimer) were plotted (triangles) against the sequence identity of the corresponding heavy chain to the natively paired heavy chain (x-axis). Each plot corresponds to the titled light chain paired with each heavy chain, denoted by triangles. Colors correspond to source donor, with multiple heavy chains per donor represented with open and closed triangles, respectively.

2.3 Discussion

Public antibody sequences have become an important research direction for a variety of diseases, including dengue, influenza, HIV-1, and SARS-CoV-2 (Jackson et al., 2014; Parameswaran et al., 2013; Setliff et al., 2018; Yuan et al., 2020). Yet, our understanding of what constitutes antibody publicness has been limited to date. At one extreme, stringent definitions that restrict public clonotypes to only identical antibody sequences have been employed (Soto et al., 2019). Such a stringent approach aims to guarantee complete confidence in the identification of truly public antibodies. However, this approach fails to account for the diversification potential of antibodies undergoing evolution in response to antigen exposure. For example, antibodies from an individual clone from a single donor have been identified with up to 50% or more divergence in CDRH3 sequence (Wu et al., 2015). It is therefore important to understand what levels of sequence-based similarity can be reasonably used for defining public clonotypes among antibodies from multiple individuals.

To that end, here we characterized a previously identified public clonotype found in a cohort of HIV-infected donors by expanding selection criteria and performing phenotypic and genotypic analyses to more carefully define the limits of antibody publicness for this clonotype. To achieve this definition, we explored the choice of V_H genes, the choice of V_L genes, and a range of CDR3 identities for sequences in the putative clonotype. We also explored the potential of heavy and light chains from different antibodies and different donors to produce functional antibodies with unaltered antigen specificity to show functional complementation between non-

native heavy-light chain pairs and further support the phenotypic similarities among different antibodies in this public clonotype. Of course, as expected, the specific choice of heavy and light chain pair appeared to be important for strength of antigen recognition. Whether our findings for the specific clonotype studied here will be generalizable for other public antibodies and in contexts beyond HIV-1 infection will be of significant interest in the antibody field. Additional investigation of public clonotypes may shed light on specific, population-level responses to infection and vaccination. A comprehensive assessment of the publicness of antibody repertoires has the potential to make significant contributions to vaccine development for difficult targets such as HIV-1, influenza, and other diseases.

2.4 Materials and Methods

2.4.1 Reagents

The following reagents were obtained from the AIDS Research and Reference Reagent Program, Division of AIDS (DAIDS), National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health (NIH): HIV-193TH975 gp120 from Steve Showalter, Maria Garcia-Moll, and the DAIDS, NIAID; Anti-HIV-1 gp120 Monoclonal (3BNC117) from Dr. Michel C. Nussenzweig (Shingai et al., 2013); Anti-HIV-1 gp120 Monoclonal (VRC01), from Dr. John Mascola (cat# 12033) (Wu et al., 2010).

2.4.2 Sequencing Data Analysis and Clonal Selection

We used preprocessed B cell repertoire data of donors CAP351, CAP314 and CAP248 from our previously published sequencing data which is available for public access under BioProject PRJNA415492 (Setliff et al., 2018). For donor CAP351, heavy chain variable gene

sequences were available from three time points (pre-infection, six months post-infection and three years post-infection) whereas for donors CAP314 and CAP248, paired heavy-light chain sequences were available for a single time point. Preprocessed sequences were annotated using IgBLAST (Ye et al., 2013) to assign gene information compared to the germline repertoire obtained from IMGT (Lefranc et al., 2015). To find the public clonotype, irrespective of V-gene and J-gene identity, we combined data from all three donors and performed clonal clustering using Change-O (Gupta et al., 2015) with the following criteria: complete linkage, same CDRH3 length and 70% CDRH3 amino acid sequence identity.

2.4.3 Public Clonotype Analysis

For each of the heavy chain and light chain sequence sets, sequences were grouped based on the respective V-genes. Hamming distances were computed between all the pairs of unique CDR3s. Further, sequences were grouped based on CDR3, and a network graph was generated using each unique CDR3 as a node; an edge between two nodes was shown only for Hamming distance value of one. A multiple sequence alignment with phylogenetic analysis was performed using Clustal Omega (Madeira et al., 2019) by also including the respective germline V-gene sequences. Phylogenetic trees were annotated and visualized using iTol (Letunic and Bork, 2019); for better visualization, sequences were clustered based on 98% sequence identity and one (original and/or consensus) or more sequences (if they came from different donors) from each cluster were included.

Residue-wise analysis was performed by computing somatic hypermutation (SHM) and entropy values. SHM was computed using in-house scripts that compared each sequence in the public clonotype to the respective germline sequence. This revealed the frequency of mutation at

each residue position, which can vary from 0 (non-mutated) to 1 (always mutated). Entropy values were calculated by using a log-based formula via Bioedit (Hall, 1999). The entropy of a residue position represents the frequency of different amino acids at that position. Entropy values at each residue position varied from 0 (only one type of amino acid at that position) to 4.322 (different frequencies of multiple amino acids at that position).

Sequences in the public clonotype were also compared to sequences in the cAb-Rep database (Guo et al., 2019). cAb-Rep is a curated database containing, at the time of analysis, antibody sequences from 306 B cell repertoires from 121 donors. Among the 267.9 million heavy chain sequences from cAb-Rep, sequences with gene *IGHV1-69* were extracted, and sequences with less than 98% identity and SHM >10% were included in the "cAb dataset" (30422 sequences). Donor-wise amino acid frequencies were computed for the sequences in the public clonotype the cAb dataset. The ratio of the per-residue amino acid frequencies was calculated with respect to the cAb dataset. The sequence logo plot to visualize residue-wise mutations and amino acid diversity was generated by using Weblogo (Crooks et al., 2004).

2.4.5 Antigen Expression and Purification

BG505 gp140 SOSIP variants were expressed as recombinant soluble antigens. The single-chain variants (Georgiev et al., 2015) of BG505 and selected point mutants, each containing an Avi tag, were expressed in FreeStyle 293F mammalian cells (ThermoFisher) using polyethylenimine (PEI) transfection reagent and cultured for 5-7 days. FreeStyle 293F cells were maintained in FreeStyle 293F medium or FreeStyle F17 expression medium supplemented with 1% of 10% pluronic F-68 and 20% of 200 mM L-Glutamine. These cells were cultured at 37°C with 8% CO_2 saturation and shaking. After transfection and 5-7 days of culture, cell cultures

were centrifuged at 6000 rpm for 20 minutes. Supernatant was 0.45 µm filtered with PES membrane Nalgene Rapid Flow Disposable Filter Units and then run slowly over an affinity column of agarose bound Galanthus nivalis lectin (Vector Laboratories cat no. AL-1243-5) at 4°C. The column was washed with PBS, and proteins were eluted with 30 mL of 1 M methyl-α-D-mannopyranoside. The protein elution was buffer exchanged three times into PBS and concentrated using 30kDa Amicon Ultra centrifugal filter units. Concentrated protein was run on a Superose 6 Increase 10/300 GL or Superdex 200 Increase 10/300 GL sizing column on the AKTA FPLC system, and fractions were collected on an F9-R fraction collector. Fractions corresponding to correctly folded antigen were selected, and antigenicity by ELISA was characterized with known monoclonal antibodies specific for that antigen. Proteins were stored at –80ºC until use.

For producing BG505 DS-SOSIP Env (Do Kwon et al., 2015) expressing high mannose glycans, we produced the Env in GnT1- cells. Env was affinity purified using a PGT145 IgG affinity column (De Taeye et al., 2015), followed by a Superdex 6 Increase 10/300 column.

2.4.6 Antibody Expression and Purification

For each antibody, variable genes were inserted into plasmids encoding the constant region for the heavy chain (pFUSEss-CHIg-hG1, Invivogen) and light chain (pFUSE2ss-CLIg-hl2, Invivogen and pFUSE2ss-CLIg-hk, Invivogen) and synthesized from GenScript. mAbs were expressed in FreeStyle 293F or Expi293F mammalian cells (ThermoFisher) by co-transfecting heavy chain and light chain expressing plasmids using polyethylenimine (PEI) transfection reagent and cultured for 5-7 days. FreeStyle 293F (ThermoFisher) and Expi293F (ThermoFisher) cells were maintained in FreeStyle 293F medium, or FreeStyle F17 expression medium

supplemented with 1% of 10% pluronic F-68 and 20% of 200 mM L-Glutamine. These cells were cultured at 37°C with 8% CO_2 saturation and shaking. After transfection and 5-7 days of culture, cell cultures were centrifuged at 6000 rpm for 20 minutes. Supernatant was 0.45 µm filtered with PES membrane Nalgene Rapid Flow Disposable Filter Units. Filtered supernatant was run over a column containing Protein A agarose resin that had been equilibrated with PBS. The column was washed with PBS, and then antibodies were eluted with 100 mM Glycine HCl at pH 2.7 directly into a 1:10 volume of 1 M Tris-HCl pH 8. Eluted antibodies were buffer exchanged into PBS three times using 10kDa or 30kDa Amicon Ultra centrifugal filter units.

2.4.7 Enzyme Linked Immunosorbent Assay (ELISA)

For gp120 ELISAs, soluble 93TH975 (Aids Reagent Program) protein was plated at 2 µg/ml overnight at 4ºC. The next day, plates were washed three times with PBS supplemented with 0.05% Tween20 (PBS-T) and coated with 5% milk powder in PBS-T. Plates were incubated for one hour at room temperature and then washed three times with PBS-T. Primary antibodies were diluted in 1% milk in PBS-T, starting at 10 µg/ml with a serial 1:5 dilution and then added to the plate. The plates were incubated at room temperature for one hour and then washed three times in PBS-T. The secondary antibody, goat anti-human IgG conjugated to peroxidase, was added at 1:10,000 dilution in 1% milk in PBS-T to the plates, which were incubated for one hour at room temperature. Plates were washed three times with PBS-T and then developed by adding TMB substrate to each well. The plates were incubated at room temperature for 10 minutes, and then 1 N sulfuric acid was added to stop the reaction. Plates were read at 450 nm.

For recombinant single-chain SOSIP trimer ELISAs, 2 μg/ml of recombinant trimer proteins diluted in PBS were added to the plate and incubated overnight at 4ºC. Primary and secondary antibodies, along with substrate and sulfuric acid, were added as described above.

Areas under the ELISA binding curves (AUC) were determined with GraphPad Prism 8.0.0.

2.4.8 Surface Plasmon Resonance (SPR)

The binding of native CAP248_30 to different concentrations of BG505.DS.SOSIP/GnTI- was assessed by surface plasmon resonance on Biacore T-200 (Cytiva) at 25°C with HBS-EP+ (10 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM EDTA, and 0.05% surfactant P-20) as the running buffer. 200nM of CAP248_30 IgG was captured on flow cell of immobilized human Anti-Fc chip (9,000 RU) and it was assayed by flowing over 50, 100, 200, 400nM of BG505.DS.SOSIP/GnTI- in running buffer. The surface was regenerated between injections by flowing over 3M MgCl2 solution for 10 s with flow rate of 100 μl/min. Blank sensorgrams were obtained by injection of the same volume of HBS-EP+ buffer in place of BG505.DS.SOSIP/GnTI- solutions. Sensorgrams of the concentration series were corrected with corresponding blank curves.

The binding of antibody CAP248_30_H/CAP314_30_L to BG505 SOSIP/GnT1- and BG505.DS.SOSIP/GnT1- , and BG505.DS.SOSIP/293F (Do Kwon et al., 2015) was assessed by surface plasmon resonance on Biacore T-200 (GE-Healthcare) at 25°C with HBS-EP+ (10 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM EDTA, and 0.05% surfactant P-20) as the running buffer. The antibody was captured on a CM5 chip by flowing 200 nM of the antibody over a flow cell immobilized with ~9000 RU of anti-human Fc antibody. Binding was measured by flowing over

100, 200, 400 and 800 nM solution of BG505.DS.SOSIP/GnT1- and by flowing over 800 nM solution of BG505.SOSIP/GnT1- and BG505.DS SOSIP/293F in running buffer. The surface was regenerated between injections by flowing over 3M MgCl2 solution for 10 s with flow rate of 100 µl/min. Blank sensorgrams were obtained by injection of same volume of HBS-EP+ buffer in place of trimer solutions. Sensorgrams of the concentration series were corrected with corresponding blank curves.

2.4.9 TZM-bl Neutralization Assays

Antibody neutralization was assessed using the TZM-bl assay as described (Montefiori, 2004; Sarzotti-Kelsoe et al., 2014). This standardized assay measures antibody-mediated inhibition of infection of JC53BL-13 cells (also known as TZM-bl cells) as a reduction in luciferase gene expression after a single round of infection by molecularly cloned Env-pseudoviruses. Murine leukemia virus (MLV) was included as an HIV-specificity control and VRC01 was used as a positive control. Titers (µg/ml) were calculated as the inhibitory concentration (IC_{50}) causing 50% reduction of relative light units (RLU) with respect to the virus control wells (untreated virus). Reported titers represent an average of two or more repeat IC_{50} values with values <0.02 reported as 0.02 and values >50 reported as 50.

2.4.10 Statistical Analysis

Spearman correlation tests were performed using cor.test function in R to obtain p and r values to determine the statistical significance of the differences. For the comparison of multiple independent tests, the p values were adjusted using the Benjamini-Hochberg method (Benjamini and Hochberg, 1995) using the p.adjust function in R. GraphPad Prism 8.0.0 was used to

calculate 2-way ANOVAs as well as p-values adjusted for multiple comparisons via Tukey's multiple comparison test.

CHAPTER 3

ELICITATION OF NEUTRALIZING ANTIBODY RESPONSES TO HIV-1 IMMUNIZATION WITH NANOPARTICLE VACCINE PLATFORMS

Author contributions to this chapter are indicated in Appendix 2.

3.1 Rationale

The extreme genetic diversity of circulating HIV-1 strains has been a barrier to the development of an efficacious vaccine. This is due, in part, to variations in the envelope trimer protein (Env) of HIV-1 (Cuevas et al., 2015; Williamson, 2003). Env mediates virus-host cell fusion and is also the sole target of neutralizing antibodies (Blumenthal et al., 2012). This diversity therefore underscores the need for immunogens that can generate a protective antibody response capable of recognizing a multitude of Envs. Multiple, soluble Env trimers have been used in sequence and/or simultaneously in immunizations to limited effect (Escolano et al., 2016; Klasse et al., 2016; Torrents de la Peña et al., 2018). In addition to soluble trimers, multimerizing Env antigens on the surface of nanoparticles have also been proposed to further improve on the elicited antibody responses (Sliepen et al., 2015; Yassine et al., 2015).

Heterologous nanoparticles are capable of mimicking repetitive multimeric patterns that are recognized by the immune system, engage with antigen presenting cells, and traffic directly to the lymphatic system (Martin et al., 2020; Tokatlian et al., 2019). The arrangement of antigens on a nanoparticle can effectively cross-link B cell receptors, which improves antibody-dependent immune responses (Bachmann and Zinkernagel, 1997; He et al., 2016). Spontaneously self-

assembling protein nanoparticles have been particularly efficient, as their expression in culture circumvents additional steps of nanoparticle-antigen linkage (Kanekiyo et al., 2013; Li et al., 2006; Sliepen et al., 2015). Different-sized protein nanoparticles have been tested against a range of disease states, exhibiting their versatility as a scaffold for antigen presentation (López-Sagaseta et al., 2016). *Heliobacter pylori* ferritin in particular has been used as a platform for many viral antigens, including HIV-1 and influenza (Kanekiyo et al., 2013, 2015; Li et al., 2019; Sliepen et al., 2015; Swanson et al., 2020). These particles are comprised of 24 identical monomers that spontaneously self-assemble into a ~10 nm particle that includes eight three-fold axes of symmetry, making them an optimal candidate for expressing trimeric antigens like the HIV-1 Env. Genetically fusing diverse Env protomers to the N-terminus of self-assembling protein nanoparticles like ferritin also allows individual nanoparticles to display multiple trimers; here, we refer to such particles as "mosaic" nanoparticle immunogens (Kanekiyo et al., 2019). The mosaic nanoparticle strategy aims at generating a cross-reactive B cell response capable of recognizing the different antigens on the surface of the nanoparticle. While mosaic nanoparticles have been successfully developed for various targets including influenza and coronavirus (Cohen et al., 2021; Kanekiyo et al., 2019), in the case of HIV-1, it is currently not well-understood if these particles outperform cocktails of single-antigen nanoparticles or other strategies for incorporating Env diversity. To address this question, we performed immunization experiments with the goal of comparing the elicited antibody responses to mosaic nanoparticles vs. soluble trimer, soluble trimer cocktails, and single-antigen nanoparticle cocktails, with each strategy using the same two, or one of the two, underlying HIV-1 strains. Our results indicate that mosaic nanoparticles can elicit autologous, and in some cases heterologous, HIV-1 Tier 2 neutralizing antibody responses, although significant differences with single-antigen nanoparticles were not

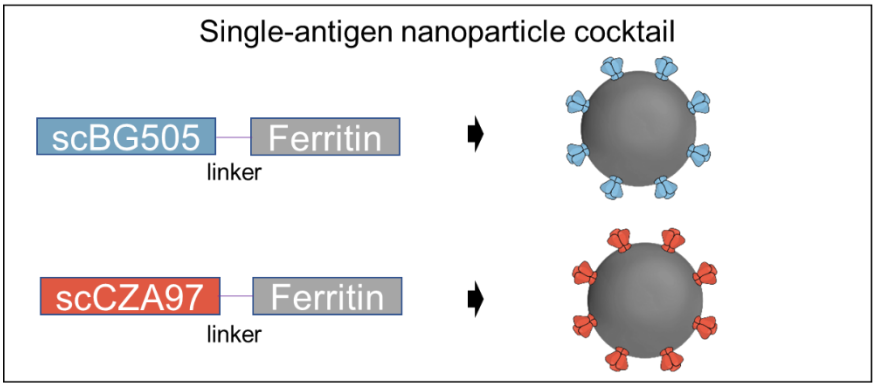
observed for the two specific strains tested here. Overall, our results provide insights into the ability of different vaccine strategies for incorporating Env sequence diversity to elicit neutralizing antibody responses, with implications for the development of broadly protective HIV-1 vaccines.

3.2 Results

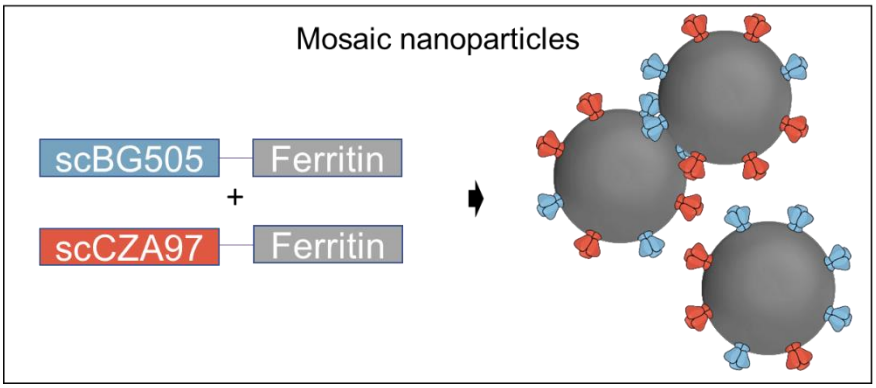
3.2.1 Design and Characterization of HIV-1 Nanoparticle Immunogens

BG505 and CZA97 HIV-1 Env trimers were designed as single chain variants (referred to as scBG505 and scCZA97) with a non-cleavable linker between gp120 and gp41 (Georgiev et al., 2015). These trimers also incorporated SOSIP stabilizing mutations and truncation at residue 664, based on HXB2 numbering (Korber et al.; Sanders et al., 2013). We also designed protein nanoparticles by genetically fusing either scBG505 or scCZA97 to the N-termini of *Heliobacter pylori* ferritin, separated by a 3-residue linker (Sliepen et al., 2015). The expression of the scBG505-ferritin plasmid or the scCZA97-ferritin plasmid in HEK293F cells resulted in particles multimerized with each respective trimer, as reported previously (Sliepen et al., 2015). We will refer to these particles as single-antigen nanoparticle immunogens (Figure 13A). In order to multimerize both scEnvs on the same particle, we cotransfected cells with both plasmids, generating mosaic nanoparticles (Figure 13B). Size exclusion chromatography profiles were used to isolate particles of the correct size (Figure 14). Nanoparticle formation was also confirmed by negative-stain EM, which identified particles approximately 40 nm wide with trimer spikes as expected and previously reported (Sliepen et al., 2015)(Figure 13C).

A


Single-antigen nanoparticle cocktail
scBG505
Ferritin
linker
scCZA97
Ferritin
linker

B


Mosaic nanoparticles
scBG505
Ferritin
+
scCZA97
Ferritin

C

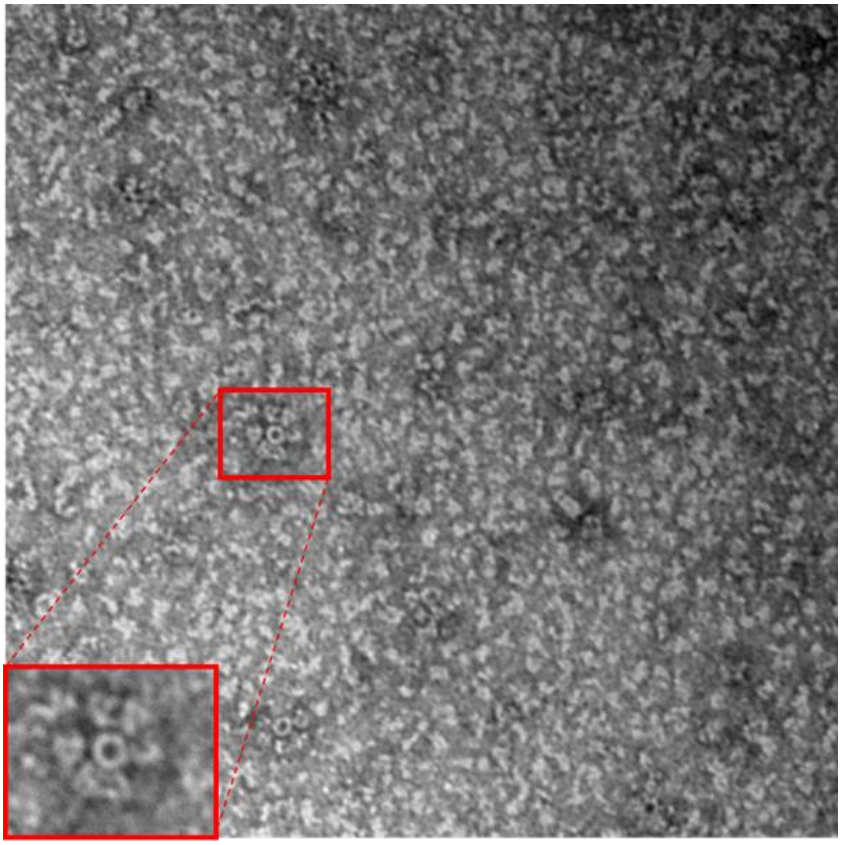


Figure 13. Nanoparticle Immunogens. Schematic depicting the:
(A) Single-antigen nanoparticle immunogens and
(B) Mosaic nanoparticles. Blue and red bars depict the single-chain protomer of envelope proteins from BG505 and CZA97, respectively. The grey bar is representative of one protomer of ferritin.
(C) Representative EM image showing nanoparticle formation.

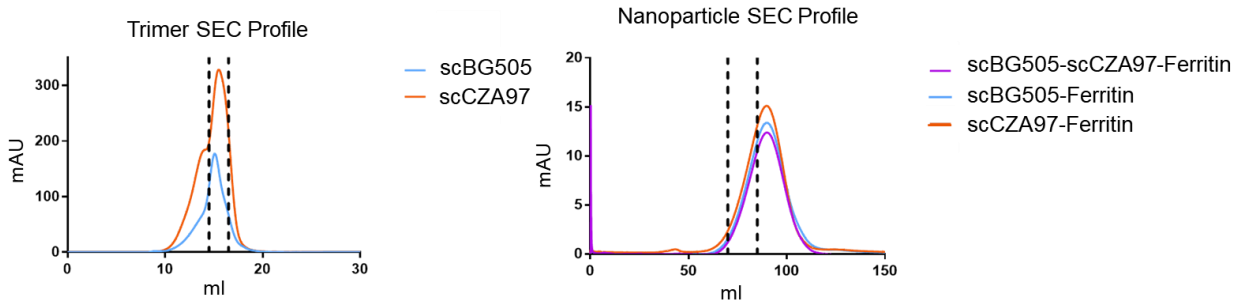


Figure 14. Size-Exclusion Chromatography Profiles of Trimer and Nanoparticle Immunogens. Soluble gp140 trimer immunogens were purified on a Superdex 200 Increase 10/300 GL, and nanoparticle immunogens were purified on 16/60 Sephacryl s500 or Superose 6 columns. Dashed lines denote selected fractions.

3.2.2 Single-Antigen and Mosaic HIV-1 Nanoparticle Immunogens Elicit Comparable Responses in Mice

To determine the immunogenicity of the single-antigen and mosaic nanoparticles, we first immunized mice with either (•) a cocktail of scBG505-ferritin and scCZA97-ferritin single-antigen nanoparticles, and (•) mosaic nanoparticles expressed from the cotransfection of scBG505-ferritin and scCZA97-ferritin. Two additional control groups were also added: (•) scBG505 trimer and (•) a cocktail of scBG505 and scCZA97 trimers. Mice were boosted four and eight weeks post-prime with the same immunogens, for a total of three immunizations per group. Two weeks after the final boost, mice were exsanguinated (Figure 15A). Serum from

mice immunized solely with scBG505 trimer bound scBG505 and scCZA97. While not statistically significant (p=0.222, Mann-Whitney Test), titers to scCZA97 appeared to be lower compared to titers to scBG505 (geometric mean AUC values 7.2±1.5 and 3.6±2.3 for scBG505 and scCZA97, respectively). In contrast, mice immunized with cocktails of trimers (geometric mean AUC values 8.1±1.2, 8.5±1.1), cocktails of single-antigen nanoparticles (8.1±1.4, 6.9±1.3), and mosaic nanoparticles (10.1±1.1, 8.5±1.0), each elicited high titers to both scBG505 and scCZA97 proteins, respectively (Figure 15B). Overall, no significant differences in immunogenicity were observed between the four groups (Figure 15C).

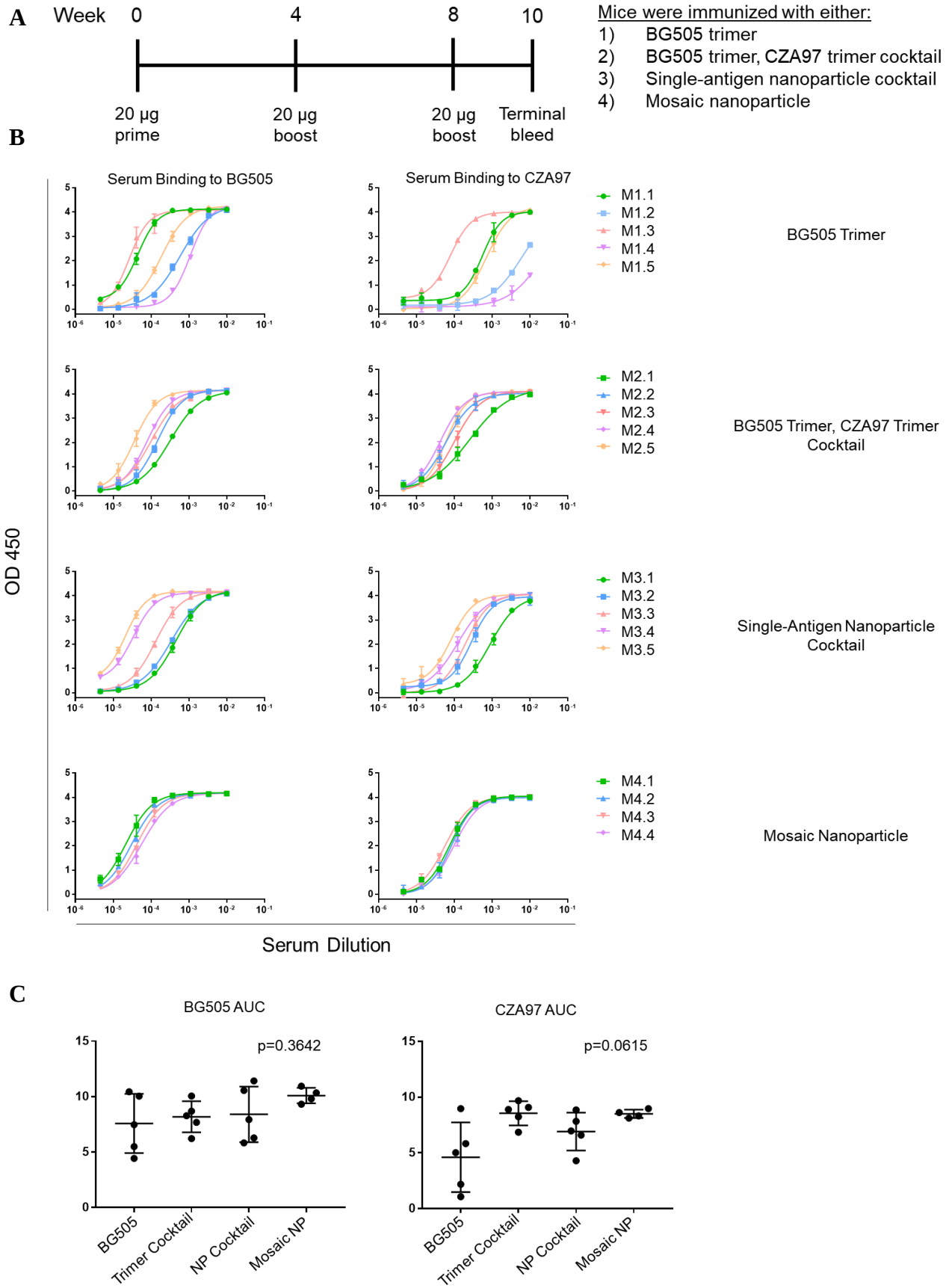

A
Week
0
4
8
10
20 µg prime
20 µg boost
20 µg boost
Terminal bleed
Mice were immunized with either:
1) BG505 trimer
2) BG505 trimer, CZA97 trimer cocktail
3) Single-antigen nanoparticle cocktail
4) Mosaic nanoparticle
B
Serum Binding to BG505
Serum Binding to CZA97
M1.1
M1.2
M1.3
M1.4
M1.5
BG505 Trimer
M2.1
M2.2
M2.3
M2.4
M2.5
BG505 Trimer, CZA97 Trimer Cocktail
M3.1
M3.2
M3.3
M3.4
M3.5
Single-Antigen Nanoparticle Cocktail
M4.1
M4.2
M4.3
M4.4
Mosaic Nanoparticle
OD 450
Serum Dilution
C
BG505 AUC
p=0.3642
CZA97 AUC
p=0.0615
BG505
Trimer Cocktail
NP Cocktail
Mosaic NP

Figure 15. Nanoparticle Immunogens Elicit Strong Responses in Mice.
(A) Mouse immunization scheme. Mice (n=5) were primed at week zero and boosted every 4 weeks. Terminal bleeds were performed 2 weeks after the second (final) boost.
(B) Mice (n=5) were immunized with one of the following: BG505 trimer (M1.1-1.5), cocktail of BG505 and CZA97 trimers (M2.1-2.5), the single-antigen nanoparticle cocktail (M3.1-M3.5), or mosaic nanoparticles (M4.2-4.4). Data for M4.1 are not available due to insufficient volume of serum. Each mouse is color-coded. Curves refer to binding to BG505 (left) or CZA97 (right) trimer.
(C) AUC values from ELISA curves(with arithmetic mean and SD) are shown for each group in (B). P values were calculated using Kruskal-Wallis tests and were adjusted using Dunn's multiple comparisons test.

3.2.3 HIV-1 Neutralization by Vaccine-Elicited Antibody Responses in Mice

Given the robust titers of serum binding to vaccine-matched (autologous) Envs, we then asked whether differences could be observed in the breadth or potency of serum neutralization. Though serum quantities were limited from some mice, we observed autologous neutralization in one mouse solely immunized with BG505 trimer protein. No other vaccinated mice displayed neutralization of the BG505 or CZA97 autologous strains (Figure 16). Intriguingly, serum from one mouse (20%) in the single trimer group, two (40%) from the trimer cocktail group, four (80%) from the nanoparticle cocktail group and five (100%) from the mosaic nanoparticle group neutralized a third, heterologous virus from Clade C, Ce1176, but no other Tier 2 viruses (Figure 16).

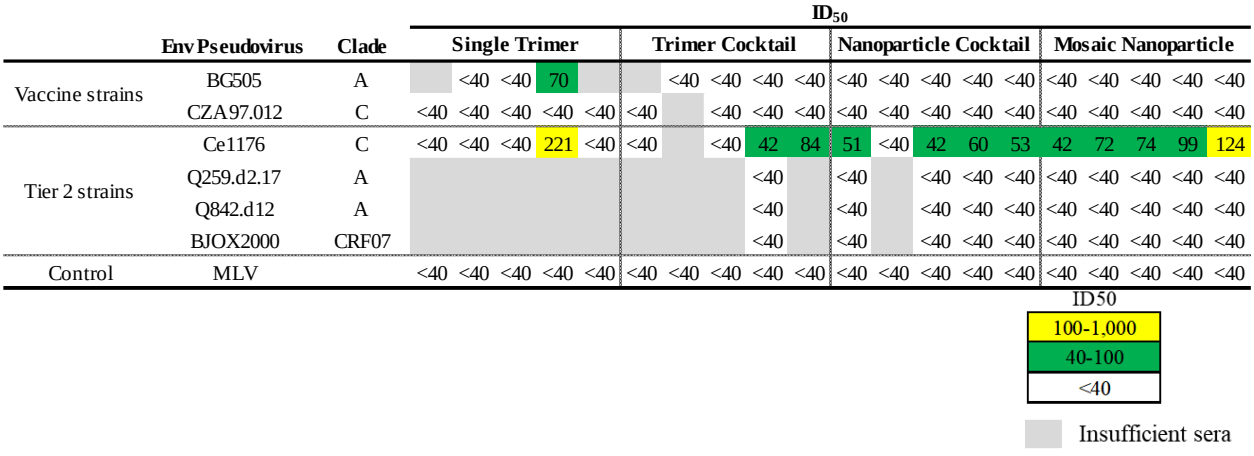


Figure 16. Nanoparticle Immunogens Elicit Neutralizing Antibody Responses in Mice. Plasma samples collected from mice (n = 20) immunized with BG505 trimer, cocktail of BG505 and

CZA97 trimers, monovalent nanoparticle cocktail, or mosaic nanoparticles were tested for neutralization to vaccine-matched and Tier 2 pseudoviruses. Mouse sera neutralization titers were reported as serum dilution required to inhibit 50% of virus infection (ID_{50}). The magnitude of neutralization responses (ID_{50}) is indicated by the color, from moderate potency (yellow) to lower potency (green) and no activity (white). Gray cells denote insufficient sera for testing.

3.2.4 Serum from Guinea Pigs Immunized with Cocktails of Nanoparticles and Cotransfected Nanoparticles Recognize Heterologous gp140 Envs

Because elicitation of broadly HIV-neutralizing antibodies has historically been a challenge in mouse models (Hu et al., 2015), we also immunized guinea pigs after confirming immunogenicity of our two types of nanoparticle immunogens. Guinea pigs have generally shown greater ability to elicit HIV-1 neutralizing antibodies and possess more blood volume, allowing for testing larger virus neutralization panels (Bricault et al., 2019; Xu et al., 2018). Guinea pigs were immunized four times, three weeks apart with either (•) cocktails of scBG505-ferritin and scCZA97-ferritin single-antigen nanoparticles or (•) cotransfected scBG505-ferritin and scCZA97-ferritin mosaic nanoparticles (Figure 17A). Serum from terminal bleeds were tested for binding against vaccine-matched trimers, with both groups generally eliciting high titers against vaccine-matched trimers, with the exception of one animal in the single-antigen cocktail group (Figure 17B, Figure 21A,B,C). To determine whether the two groups resulted in different breadth of HIV-1 Env variant recognition, we tested the vaccine sera against a panel of diverse gp140 Envs (Figure 17C, Figure 21B,C). A similar pattern of antigen binding was observed for both groups against the different antigens in the panel (again, with the exception of the same single animal from the single-antigen cocktail group). Together, these results suggest

that both nanoparticle immunogen formulations can elicit antibodies capable of recognizing Envs from multiple, diverse clades.

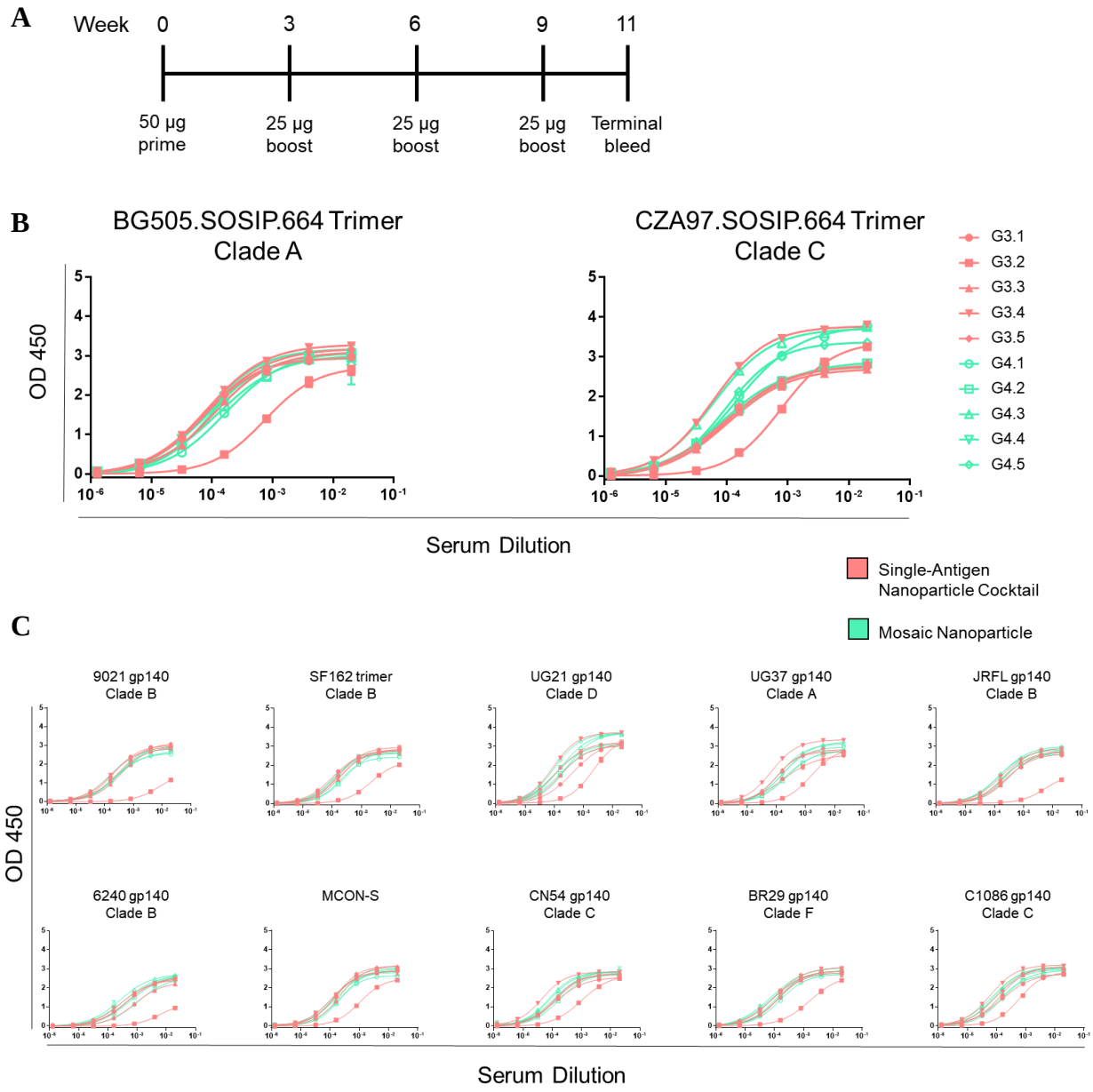


Figure 17. Guinea Pigs Immunized with Nanoparticles Show Breadth of Reactivity against Diverse HIV-1 Envs.
(A) Guinea pig immunization scheme. Guinea pigs were primed at day zero and boosted every 3 weeks. Terminal bleeds were performed 2 weeks after the final boost.
(B) ELISA binding curves against vaccine-matched Envs. Guinea pigs (n=5) were immunized with either the single-antigen nanoparticle cocktail (G3.1-3.5) or mosaic nanoparticles (G4.1-4.5). Each guinea pig is color-coded by immunization group. Curves refer to binding to BG505 (left) or CZA97 (right) trimer.
(C) ELISA binding curves to gp140 Env proteins from diverse clades.

3.2.6 Single-Antigen and Mosaic HIV-1 Nanoparticle Immunogens Elicit Autologous and Heterologous Virus Neutralizing Antibodies.

Sera from guinea pigs from both immunization groups potently neutralized tier 1A and tier 1B viruses (Figure 18), with the exception of one guinea pig from the single-antigen nanoparticle cocktail group that had displayed consistently lower binding to Envs (Figure 17B,C). We next assessed whether guinea pig sera could neutralize the two vaccine-matched (autologous) viruses used for the immunizations. In guinea pigs immunized with the mosaic nanoparticle, 3/5 (60%) neutralized BG505 and 4/5 (80%) neutralized CZA97. Autologous responses to BG505 and CZA97 viruses were observed in 3/5 (60%) guinea pigs immunized with the cocktail. One animal from the mosaic nanoparticle group exhibited some neutralization breadth by neutralizing three of seven heterologous tier 2 viruses from diverse clades at low titers (Figure 18). Low titer neutralization of a single tier 2 heterologous virus was also seen in one animal from the single-antigen nanoparticle group. Another animal from the single-antigen nanoparticle group showed neutralization of the MLV control, and thus the observed neutralization of other heterologous tier 2 viruses likely should be considered as background for that animal (Figure 18).

	Env Pseudovirus	Clade	ID50 Cocktail					ID50 Mosaic				
Tier 1A	MW965.26	C	50,213	>87,480	>87,480	<40	2,407	>87,480	13,809	8,479	>87,480	>87,480
Tier 1B	6644.v2.c33	C	2,025	10,843	6,136	<40	70	1,967	499	715	77	20,073
Vaccine strains	BG505	A	44	<40	46	<40	57	41	<40	40	<40	58
	CZA97	C	47	<40	93	<40	62	123	55	<40	55	60
Tier 2 strains	Du422.1	C	<40	<40	<40	<40	41	<40	<40	<40	<40	<40
	Ce1176	C	<40	<40	<40	<40	<40 *	<40	<40	<40	<40	<40
	Ce0217	C	47	<40	<40	<40	67	<40	<40	<40	<40	72
	CH119	CRF07	<40	<40	<40	<40	47	<40	<40	<40	<40	52
	TR0.11	B	<40	<40	<40	<40	56	<40	<40	<40	<40	49
	Q842.d12	A	<40	<40	<40	<40	<40 *	<40	<40	<40	<40	<40
	KER2018	A	<40	<40	<40	<40	<40 *	<40	<40	<40	<40	<40
Control	MLV		<40	<40	<40	<40	75	<40	<40	<40	<40	<40

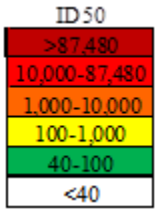


Figure 18. Nanoparticle Immunogens Elicit Vaccine-Matched and Heterologous Neutralization in Guinea Pigs. Plasma samples collected from guinea pigs (n = 10) immunized with either single-antigen nanoparticle cocktails or as mosaic nanoparticles bearing both BG505 and CZA97 were tested for neutralization to vaccine-matched, Tier 1, and Tier 2 pseudoviruses. The magnitude of neutralization responses (ID_{50}) is indicated by the color, from higher potency (dark red) to lower potency (green) and no activity (white). Asterisks indicate titers just below 50% cut-off, indicating potential serum toxicity.

3.3 Discussion

Since antibodies that neutralize one HIV-1 virus do not necessarily confer protection against other circulating strains, heterologous neutralization is an important goal of HIV-1 vaccinology. It is therefore important to design immunogens capable of eliciting such responses. In particular, nanoparticle immunogens have emerged as viable alternatives to soluble trimers due to size, geometry, and the capacity to uniformly display antigens on the surface. Here, we compared cocktails of single-antigen nanoparticles and mosaic nanoparticles simultaneously displaying both Env proteins, showing immunogenicity in mice and both vaccine-matched and heterologous neutralization in guinea pigs.

Our efforts provide preliminary justification for immunizing with multiple envelope proteins from different clades expressed onto nanoparticles. While no autologous responses were observed in mice, as with many other studies (Hu et al., 2015; Sliepen et al., 2015), this animal model has historically been useful to determine immunogenicity. Neutralization of the heterologous Ce1176 virus in mice was unexpected, considering the lack of observed autologous responses in the same mice or analogous Ce1176 neutralization in guinea pigs, although Ce1176 neutralization has been observed in other vaccine studies as well (Pauthner et al., 2017). These results potentially highlight the notable differences in the ability of the immune systems of different animal models to elicit HIV-neutralizing antibody responses.

The lack of significant differences in elicited neutralization breadth between single-antigen and mosaic nanoparticles is notable, given the established advantages of mosaic nanoparticles for a number of other pathogens (Cohen et al., 2021; Kanekiyo et al., 2019). However, a limitation for this study is the lack of comparison of the immunogenicity of nanoparticle formulations against soluble trimers in guinea pigs. More generally, given that high vaccine efficacy is required for an effective HIV-1 vaccine, additional vaccine optimization will be necessary to fully harness the power of mosaic nanoparticles for HIV-1 vaccine design, such as through specific selection of Env antigens based on bNAb epitope availability, glycan coverage, and sequence diversity. Nevertheless, the autologous and, in some cases, heterologous tier 2 neutralization in guinea pigs observed in this study highlights the potential of multimerizing diverse Env variants on self-assembling protein nanoparticles as a viable direction for the design of a broadly protective HIV-1 vaccine.

3.4 Methods

3.4.1 Reagents

The following reagents were obtained from the AIDS Research and Reference Reagent Program, Division of AIDS (DAIDS), National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health (NIH): Anti-HIV-1 gp120 Monoclonal (VRC01), from Dr. John Mascola (cat# 12033) (Wu et al., 2010). The following reagents were obtained through the NIH HIV Reagent Program, Division of AIDS, NIAID, NIH: Human Immunodeficiency Virus 1 (HIV-1) JRFL gp140 Recombinant Protein (B.JRFL gp140CF), ARP-12573, contributed by Drs. Barton F. Haynes and Hua-Xin Liao; Human Immunodeficiency Virus 1 (HIV-1) gp140 Recombinant Protein (B.9021 gp140C), ARP-12575, contributed by Drs. Barton F. Haynes and Hua-Xin Liao; Human Immunodeficiency Virus 1 (HIV-1) gp140 Recombinant Protein (C.1086 gp140C), ARP-12581, contributed by Drs. Barton F. Haynes and Hua-Xin Liao; Human Immunodeficiency Virus 1 (HIV-1) gp100 Recombinant Protein (B.6240 gp140C), ARP-12572, contributed by Drs. Barton F. Haynes and Hua-Xin Liao; Human Immunodeficiency Virus Type 1 BR029 gp140 Protein, Recombinant from CHO Cells, ARP-12066, contributed by DAIDS/NIAID; produced by Polymun Scientific; Human Immunodeficiency Virus Type 1 UG21 gp140 Protein, Recombinant from CHO Cells, ARP-12065, contributed by DAIDS/NIH (Polymun Scientific, Inc); Human Immunodeficiency Virus Type 1 UG037 gp140 Protein, Recombinant from CHO Cells, ARP-12063, contributed by DAIDS/NIAID (Polymun Scientific, Inc); Human Immunodeficiency Virus Type 1 CN54 gp140 Protein, Recombinant from CHO Cells, ARP-12064, contributed by DAIDS/NIAID; produced by Polymun Scientific, Inc; Human Immunodeficiency Virus Type 1 SF162 gp140 Trimer Protein, Recombinant from HEK293T Cells, ARP-12026, contributed by Dr. Leo Stamatatos.

3.4.2 Antigen Expression and Purification

Each of the trimers were expressed in FreeStyle 293F mammalian cells (ThermoFisher) by transfecting plasmids corresponding to either BG505.SOSIP.664sc or CZA97.SOSIP.664sc using polyethylenimine (PEI) transfection reagent and cultured for 5-7 days. These cells were cultured at 37°C with 8% CO_2 saturation and shaking. After transfection and 5-7 days of culture, cell cultures were centrifuged at 6000 rpm for 20 minutes. Supernatant was 0.45 μm filtered with PES membrane Nalgene Rapid Flow Disposable Filter Units. Filtered supernatant was run over a column containing *Galanthus nivalis* snow-drop lectin that had been equilibrated with PBS. The column was washed with PBS, and proteins were eluted with 30 mL of 1 M methyl-α-D-mannopyranoside. The protein elution was buffer exchanged three times into PBS and concentrated using 10 kDa or 30 kDa Amicon Ultra centrifugal filter units. Concentrated protein was run on a Superose 6 Increase 10/300 GL or Superdex 200 Increase 10/300 GL on the AKTA FPLC system, and fractions were collected on an F9-R fraction collector.

CZA97-ferritin and BG505-ferritin were expressed as above. Cotransfected nanoparticles were expressed by transfecting equal parts of CZA97-ferritin and BG505-ferritin DNA, resulting in the assembly of nanoparticles bearing different ratios of each Env in a stochastic fashion, as described in (Kanekiyo et al., 2019) and were purified as above but using a Sephacryl S-500 or Superose 6 sizing column.

3.4.3 Enzyme Linked Immunosorbent Assay (ELISA)

For gp140 ELISAs, soluble proteins (Aids Reagent Program) were plated at 2 μg/ml overnight at 4ºC. The next day, plates were washed three times with PBS supplemented with 0.05% Tween20 (PBS-T) and coated with 5% milk powder in PBS-T. Plates were incubated for

one hour at room temperature and then washed three times with PBS-T. Guinea pig serum were diluted in 1% milk in PBS-T, starting at a 1:50 dilution and was subsequently serially diluted 1:5 and then added to the plate. The plates were incubated at room temperature for one hour and then washed three times in PBS-T. The secondary antibody, goat anti-guinea pig IgG conjugated to peroxidase (abcam), was added at 1:25,000 dilution in 1% milk in PBS-T to the plates, which were incubated for one hour at room temperature. Plates were washed three times with PBS-T and then developed by adding TMB substrate to each well. The plates were incubated at room temperature for 10 minutes, and then 1 N sulfuric acid was added to stop the reaction. Plates were read at 450 nm. Mouse ELISAs were performed the same as the guinea pig ELISAs except serum was diluted 1:100 and the secondary antibody used was goat anti-mouse conjugated to peroxidase (Promega) added at a 1:10,000 dilution. Due to limited amounts of recovered sera, we were unable to perform ELISAs against scEnvs for one mouse in the group that received the mosaic immunogen.

Areas under the ELISA binding curves (AUC) were determined with GraphPad Prism 8.0.0.

3.4.4 Mouse Immunizations

The mouse study was conducted with approval by the Institutional Review Board of Vanderbilt University (IACUC Approved Protocol Number M1700115). 5 Female BALB/c mice 4-6 weeks old (Charles River) were used per group in our immunization studies. Mouse studies included 4 groups for a total number of 20 mice. Mice either received BG505 trimer, BG505 and CZA97 trimer cocktail, BG505-ferritin and CZA97-ferritin cocktail, or BG505 and CZA97 both on ferritin. A prime dose of 20μg of antigen in TiterMax Gold (Sigma-Aldrich) was administered

intramuscularly in the hind leg(s). Boosts were per-formed at 4 and 8 weeks post prime. Pre-immune blood was collected from the submandibular vein before the start of the study and two weeks after each immunization. Two weeks after the final boost, blood was collected from both a submandibular bleed as well as cardiac puncture.

3.4.5 Guinea Pig Immunizations

The guinea pig study was conducted according to the guidelines of Cocalico Biologicals, Inc. Animal Care and Use Committee (The IACUC Approved Protocol Number: 181024CBISTD). Cocalico Biologics administered vaccines by first priming with 50µg of either the single-antigen cocktail of nanoparticles or mosaic nanoparticles in TiterMax Gold (Sigma-Aldrich) and boosting with 25µg of either formulation every 3 weeks for a total of 3 boosts after prime. Immunizations were administered at multiple sites subcutaneously along the back and intramuscularly in the hind limbs as the routes of injection. No more than 0.1ml of the emulsion was used per site. Terminal bleeds were collected by Cocalico after guinea pigs were fully anesthetized.

3.4.6 TZM-bl Neutralization Assays

Serum neutralization was assessed using the TZM-bl assay as described (Sarzotti-Kelsoe et al.,2014). This standardized assay measures antibody-mediated inhibition of infection of TZM-bl cells by molecularly cloned Env-pseudoviruses. Viruses that are highly sensitive to neutralization (Tier 1) and/or those representing circulating strains that are moderately sensitive (Tier 2) were included. Murine leukemia virus (MLV) was included as an HIV-specificity control. Neutralization was measured as a reduction in luciferase gene expression after a single

round infection of TZM--bl cells in a 96 well-plate. Results are presented as the serum/plasma dilution required to inhibit 50% of virus infection (ID_{50}).

3.4.7 Statistical Analysis

GraphPad Prism 8.0.0 was used to calculate Mann-Whitney U tests, Kruskal-Wallis tests with p-values adjusted for multiple comparisons via Dunn's multiple comparison test, and geometric means and geometric standard deviations.

CHAPTER 4

CONCLUSIONS AND FUTURE DIRECTIONS

4.1 Public Antibodies

We characterized and defined a public clonotype from a cohort of HIV-1 infected donors. In doing so, we clarified that members of previously identified clonotype #13905 (Setliff et al., 2018) are restricted in antigen recognition by the V_H-gene, but not the V_L-gene and that functional complementation of heavy and light chain pairs, regardless of donor origin, confers variable degrees of antigen recognition. We further determined that the light chain used, but not identity to germline or identity to the native heavy chain, modulates these degrees of antigen recognition.

Immediate studies to build on this work would include determining the applicability in classifying clonotype #13905 without considering V_H or J_H gene assignments. To do so, it would be prudent to obtain the structure of one of the public antibodies with either the gp120 monomer or an Env trimer. Structures could be obtained via cryo-electron microscopy or x-ray crystallography using the proteins tested (93TH975 gp120 or BG505 trimer). This would put into context the allowed variation for clonotype #13905. While it has been suggested that V, D and J genes pair preferentially (Arnaout et al., 2011; Hoi and Ippolito, 2013), we also determined that antigen recognition of public clonotype #13905, which contains multiple V_H and V_L genes, is reliant on which genes are selected. The exploration of clonotype #13905 identified heavy chain genes that did not recognize antigen, despite sharing features with heavy chains that did.

Specifically, for clonotype #13905, it is not known why some antibodies within this clonotype do not recognize antigens, whereas others possess neutralizing function. Structural

studies can elucidate common features that are important in recognizing the CD4bs epitope, provide a structural basis on which modeling could be performed to determine why other heavy chains do not engage HIV-1 Env, identify clonotype #13905-specific mutations that confer antigen recognition and qualify the role of the light chain in augmenting antigen recognition. Practically, identifying which common features confer antigen recognition may lead to the generalization of these findings into downstream applications including ground-up antibody design. Conversely, determining sequence features that abrogate binding to antigen may act as red flags to avoid in antibody design. To gain a better understanding of the human antibody repertoire and how it functions in the context of infection, continued exploration of other public clonotypes is also warranted in order to identify public antibody recognition elements.

In the same vein, to further contextualize the initial relaxed criteria in selecting putative members of the public clonotype, a search and subsequent analysis of antibody-antigen complexes in the Protein Data Bank using the same selection criteria would provide a better understanding of the novelty of the sequence features in our public antibody clonotype.

Delineating the boundaries of what constitutes public clonotypes has implications for vaccine design focused on the elicitation of population-level responses. Considering the successful vaccine elicitation of potent and neutralizing public antibodies to SARS-CoV-2 (Chen et al., 2021; Schmitz et al., 2021), and the prevalence of putative VRC01 B cell precursors in the human population (Setliff et al., 2018) it is within reason to assume public bNAb responses to HIV-1 vaccines are within reach. As such, researchers are currently attempting to elicit a population-level VRC01-like antibody response. As is evidenced by the identification of clonotype #13905, there may reside more accessible antibody lineages that are broadly neutralizing – a hypothesis worth continued exploration. Of course, understanding determinants

of public clonality is applicable to yet other diseases that heretofore have no licensed or efficacious vaccines like HCV, dengue, and RSV, to name a few. Defining public clonotypes in these contexts can help inform immunogen design that engage productive and available B cell lineages.

As has been previously suggested, public antibodies have the potential to act as tools for immunosurveillance (Jackson et al., 2014; Parameswaran et al., 2013) to clarify previous infection or establish current immunity. This is acutely within the realm of possibility, considering the approval of T cell receptor interrogation as a diagnostic for SARS-CoV-2 infection by Adaptive Biotechnologies (Megha Satyanarayana, 2021). Further, interrogation of public antibody repertoires may also shed light on features of the pathogen infecting a population, with implications for identifying breakthrough pathogens or pathogen variants that can escape antibody response and pathogens that reside in specific populations (Chen et al., 2021).

It also may be possible and necessary to design vaccines based on public clonotype prevalence. One individual may require vaccine “A” because they have public clonotype “1”, where as another individual may benefit from vaccine “B” because they have public clonotype “2.” As such, population-based “personalized” vaccines could be developed to more efficiently elicit immunity.

4.2 Nanoparticle Immunogens

We also developed and tested nanoparticle immunogens designed to elicit cross-neutralizing responses. In choosing two Envs from different clades and using them in various immunogen formulations, we determined that single-antigen nanoparticles and mosaic

nanoparticles can elicit a response against both vaccine-matched viruses. While neutralization breadth was not imminently available consistently, in one animal that received the mosaic nanoparticle vaccine, breadth of neutralizing antibody responses was observed. As broad neutralization is a current goal for HIV-1 vaccine design, this finding was encouraging and should motivate future studies in this direction.

While ferritin nanoparticles have been shown to elicit breadth in the context of other diseases (Yassine et al., 2015), limited neutralization breadth was observed in our study. Assembling HIV-1 Env onto larger nanoparticles that can display more individual trimers may prove to be more efficacious, as larger nanoparticles (50-100nm) have been thought to reside in follicular dendritic cell networks longer and elicit more enhanced responses when compared to smaller nanoparticles (Zhang et al., 2019). As such, it is important to pursue computational modeling and experimentation to identify suitable, larger nanoparticles for which multiple, diverse Envs can be surface-displayed. A future extension of manipulating carrier size would be to compare the breadth of neutralization elicited against larger nanoparticle immunogens to that against ferritin, either in single-antigen cocktail, or mosaic nanoparticle formulations.

Mosaic nanoparticles offer the opportunity to multimerize peptides to not only focus the immune response to a specific epitope (Xu et al., 2018), but also to allow for the presentation of diversity within those epitopes, in order to enable tolerance in immune recognition. While mosaic nanoparticle vaccines have been tested to elicit antibodies against influenza, SARS-CoV-2 (Cohen et al., 2021; Kanekiyo et al., 2019), and now HIV-1, the concept of expressing multiple, diverse antigens on protein nanoparticles should be extensible to other diseases for which diversity precludes a licensed vaccine.

Specifically, to build on these findings, rational selection of Envs may be necessary to elicit breadth, with the hypothesis that immunizing with specific combinations could cover neutralization sensitivity to major bNAb groups. Selection of Envs would be informed by published and available antibody neutralization data as well as an understanding of glycan/glycan-hole differences among circulating viruses. Current efforts have identified six Envs spanning three different clades: KNH1209.18 (Clade A), MB539.2B7 (Clade A), 5768.04 (Clade B), HT593.1 (Clade B), DU172.17 (Clade C), and 286.36 (Clade C) (unpublished data). After validating expression and immunogenicity, two or more Envs can then be expressed on the surface on ferritin as a mosaic nanoparticle in a similar manner described here, or on other nanoparticle platforms described in Chapter 1, to determine if greater breadth can be elicited. Neutralization breadth can then be assessed in guinea pigs or larger animal models to compare mosaic ferritin nanoparticles bearing multiple copies of each Env to single-antigen nanoparticle cocktails. Simultaneously or alternatively, larger nanoparticles, like I53-50, can be leveraged to include up to twenty Envs, in combinations of two through all six selected Envs.

The development of mosaic nanoparticles can then be compared to contemporary vaccine design efforts that similarly seek to present diversity to the immune system. Most notably, comparing mosaic nanoparticle immunogens against efforts to generate an mRNA vaccine incorporating multiple Envs spearheaded by Moderna and the NIH would provide a clear direction for further optimization in order to generate a protective HIV-1 vaccine. A study comparing mosaic nanoparticle and mRNA vaccine formulations, in which mosaic nanoparticle immunogenicity can be predetermined in guinea pig models as explained above, would subsequently benefit from non-human primate vaccine studies.

4.3 The Development of HIV-1 Nanoparticle Immunogens Informed by the Public Antibody Repertoire

Most current HIV-1 vaccine candidates rely on an intimate knowledge of the envelope glycoprotein of HIV. These candidates are optimized for stability between promoters, reliability in mimicking conformationally relevant states, and presenting broadly neutralizing epitopes (Bricault et al., 2019; Chuang et al., 2017; Sanders et al., 2013; Xu et al., 2018) in order to maximize the likelihood of eliciting broadly neutralizing antibodies. On the other hand, others have instead focused on eliciting specific or targeted antibody responses; ones that are already known to be neutralizing. These efforts owe their success in no small part to advances in antibody repertoire sequencing. After the identification of broadly neutralizing antibody VRC01 (Wu et al., 2010), groups explored ways to specifically elicit VRC01-class antibodies. One challenge is that binding of unmutated VRC01 precursors to Env is undetectable using current analytical methods (Jardine et al., 2013; Zhou et al., 2013). To address this challenge, carefully designed immunogens were developed using computational design and protein engineering (Jardine et al., 2013). Nanoparticle-based immunogens in particular were shown to be efficacious in engaging VRC01 B cells (in contrast to trimeric and monomeric Env proteins) and in eliciting VRC01-like antibodies in mice expressing germline-reverted VRC01 heavy chains (Jardine et al., 2013, 2015). Immunogens have also been developed or identified that engage the precursors of trimer apex-directed bNAbs (Alam et al., 2013) and the N332 supersite (Escolano et al., 2016). However, the number of germline-targeting immunogens are currently limited. Furthermore, whereas germline-targeting immunogens have been tested in mouse models engineered to express bNAb precursors, the expression frequency of desired B cells in humans clearly differ from engineered mouse models, and, indeed, among individuals.

Alternatively, there does exist public repertoires of putative bNAb precursors (Setliff et al., 2018), the public antibody clonotype described in chapter 2, and in all probability, other HIV-1-specific public clonotypes. Instead of taking an agnostic approach to eliciting bNAbs, which may yield inconsistent antibody responses, targeting clonotypes that are known to be shared may elicit desirable responses more consistently.

A proposed future study that builds upon the work described here and in the field would be to design nanoparticle immunogens specifically targeting public putative VRC01 precursors. Antibody precursors 02-K, 02-O and 02-S (Yacoob et al., 2016), are known to be public (Setliff et al., 2018). These antibodies can be screened against the eOD. Subsequently, structural studies may elucidate refinements to eOD to increase binding and engagement. Designing immunogens specifically to engage public clonotypes may provide more consistent elicitation of VRC01-like antibodies over current, more agnostic approaches.

It remains to be seen whether the public clonotype characterized in Chapter 2 may be suitable to elicit in response to vaccine (continued exploration of members of this public clonotype, other HIV-1 clonotypes and a comprehensive analysis of clonotype neutralization breadth are all warranted). Regardless, harnessing nanoparticle technology to design vaccines informed by an understanding of the antibody response to infection can overcome many challenges to HIV-1 vaccine design.

Similar to the eOD-GT nanoparticle immunogens being developed to engage VRC01 precursors (Jardine et al., 2016), nanoparticle immunogens can be designed to elicit antibodies of clonotype #13905. It is currently unknown if germline genes in clonotype #13905 recognize Envs. Because the eOD would present an ideal starting point for immunogen design, it would first be prudent to identify whether the inferred germline of this public clonotype binds to the

eOD as clonotype #13905 and VRC01 share the same epitope. If binding is observed to the eOD immunogens by inferred germline antibodies of clonotype #13905, refinements can be made to the eOD to improve clonotype #13905 engagement. If not, diverse Envs can be tested for binding, or iterative computation design and testing of a novel immunogen that similarly eliminates off-target epitopes (glycan at position 276, V1/V2 domains, etc.) can be employed.

BCRs of naïve B cells often have undetectable binding to HIV-1 immunogens, so we therefore propose to multimerize Envs or the refined eOD onto ferritin, lumazine synthase (Jardine et al., 2013), or I53-50 nanoparticles to improve germline engagement, as presentation on nanoparticles provides improved avidity to engage low-affinity BCRs (Abbott et al., 2018; Brinkkemper and Sliepen, 2019; Brouwer et al., 2019). Ideally, engineered mouse models expressing the germline genes for members of the public antibody clonotype would be the animal model of choice to initially test clonotype #13905 nanoparticle immunogens.

As antibodies with multiple light chains were observed in the public clonotype, resulting in a spectrum of antigen recognition, an analysis of antigen engagement with germline and mature public antibodies may result in more than one public clonotype germline-targeting immunogen. As such, the inclusion of multiple germline-targeting antigens on a nanoparticle, either as single antigen cocktails or mosaic nanoparticles, could be used to study the effects of homotypic versus heterotypic immunogens on B cell development.

PUBLICATION LIST

Peer-Reviewed Articles Related to Dissertation

Murji, A.A., Raju, N., Qin, J.S., Kaldine, H., Janowska, K., Fechter, F., Mapengo, R., Scheepers, C., Setliff, I., Acharya, P., et al. (2021). Sequence and functional characterization of a public HIV-specific antibody clonotype 1. *iScience* (in revision).

Murji, A.A., Qin, J.S., Hermanus, T., Morris, L., Georgiev, I.S. (2021). Elicitation of neutralizing antibody responses to HIV-1 immunization with nanoparticle vaccine platforms. *Viruses* (accepted).

Peer-Reviewed and Co-authored Articles

Setliff, I., Shiakolas, A.R., Pilewski, K.A., **Murji, A.A**., Mapengo, R.E., Janowska, K., Richardson, S., Oosthuysen, C., Raju, N., Ronsard, L., et al. (2019). High-Throughput Mapping of B Cell Receptor Sequences to Antigen Specificity. Cell 179, P1636-1646.E15. doi: 10.1016/j.cell.2019.11.003.

Setliff, I., McDonnell, W.J., Raju, N., Bombardi, R.G., **Murji, A.A.**, Scheepers, C., Ziki, R., Mynhardt, C., Shepherd, B.E., Mamchak, A.A., et al. (2018). Multi-Donor Longitudinal Antibody Repertoire Sequencing Reveals the Existence of Public Antibody Clonotypes in HIV-1 Infection. Cell Host Microbe 23, 845-854.e6. doi: 10.1016/j.chom.2018.05.001.

Patents Related to Dissertation

US provisional patent application no. 63/007,989 – “Nanoparticle Immunogens from 2A Constructs”

US provisional patent application no. 63/007,985 – “Design and Development of Empirical and Rational Epitope-Focused HIV-1 Vaccine Candidates”

APPENDIX 1

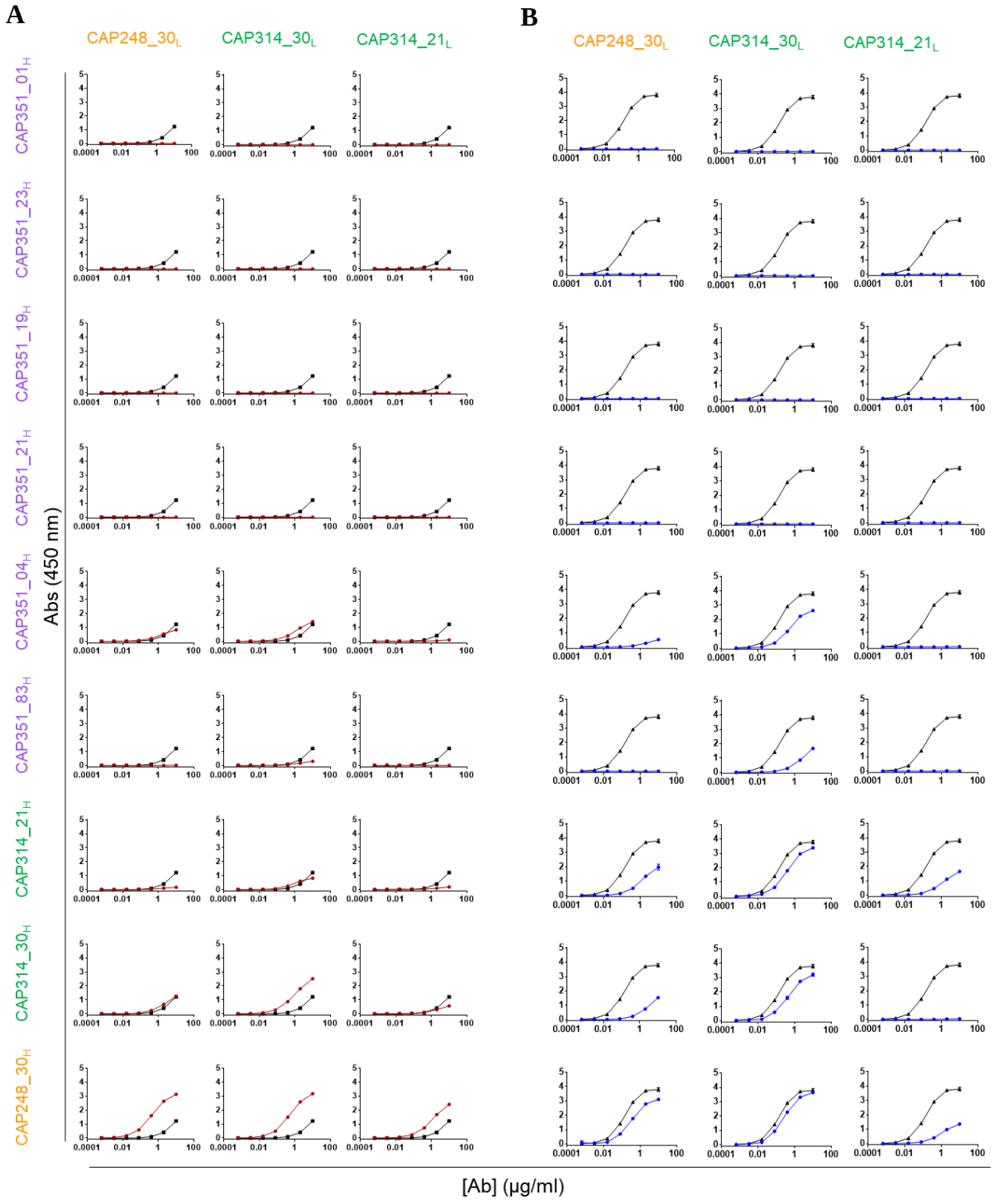


Figure 19. Antibody Binding to (A) Monomer or (B) Trimer. Antigen specificity of each antibody heavy-light chain pair was validated by ELISA against (A) 93TH975 gp120 monomer

and (B) BG505.T332N.SOSIP.664 single-chain trimer. VRC01 or 3BNC117 were used as a positive control and are colored in black.

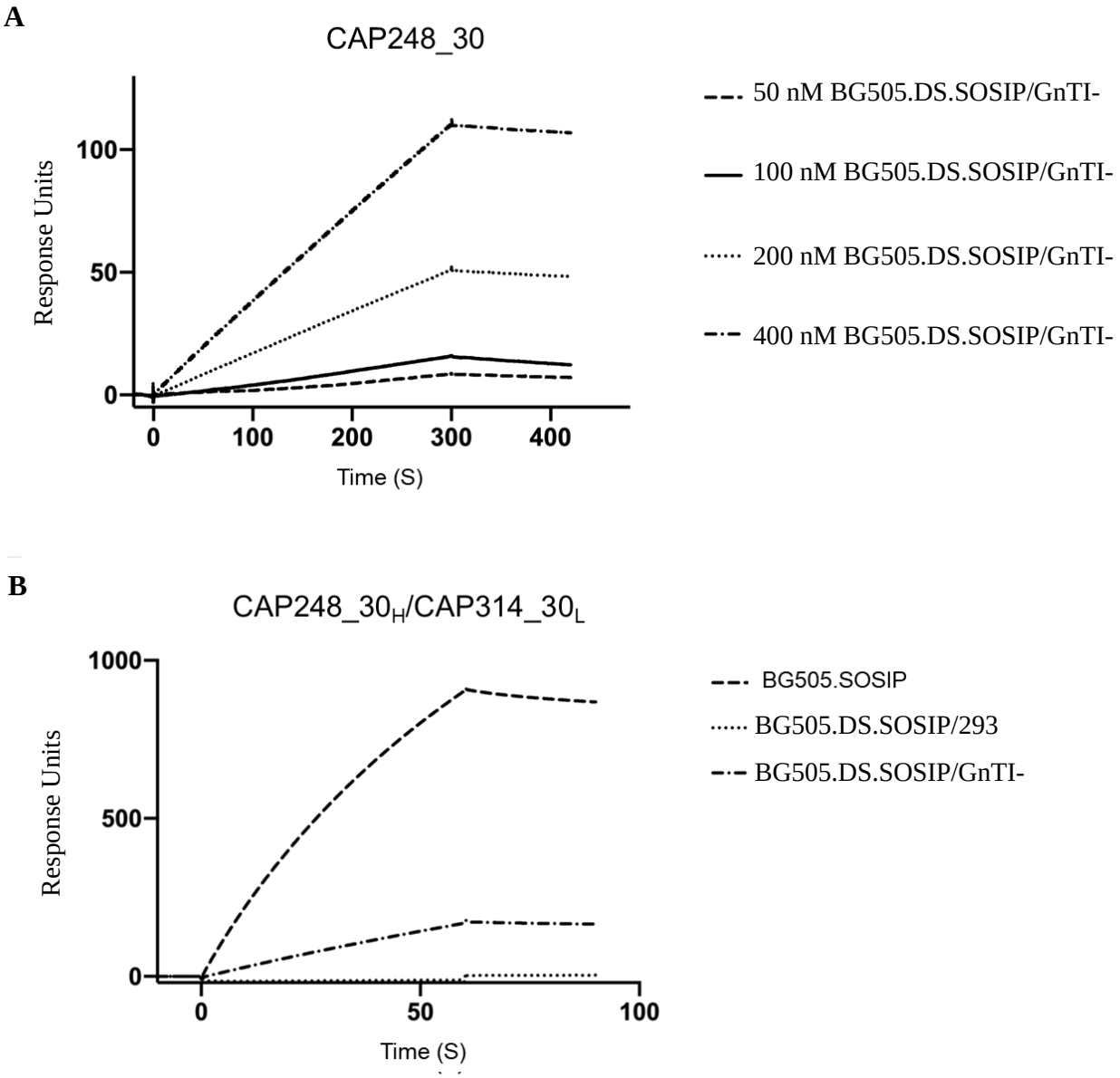


Figure 20. Public Antibody Binding to HIV-1 Trimer by Surface Plasmon Resonance. Binding for (A) native CAP248_30 and (B) non-native CAP248_30$_H$/CAP314_30$_L$ antibodies was assessed against BG505.SOSIP.664 or a SOSIP variant with an additional disulfide stabilization (DS) (Do Kwon et al., 2015) expressed in 293F cells or GnT1- cells.

Contributions to Chapter 2

Conceptualization:	Ivelin Georgiev, Lynn Morris, Priyamvada Acharya, Ian Setliff
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APPENDIX 2

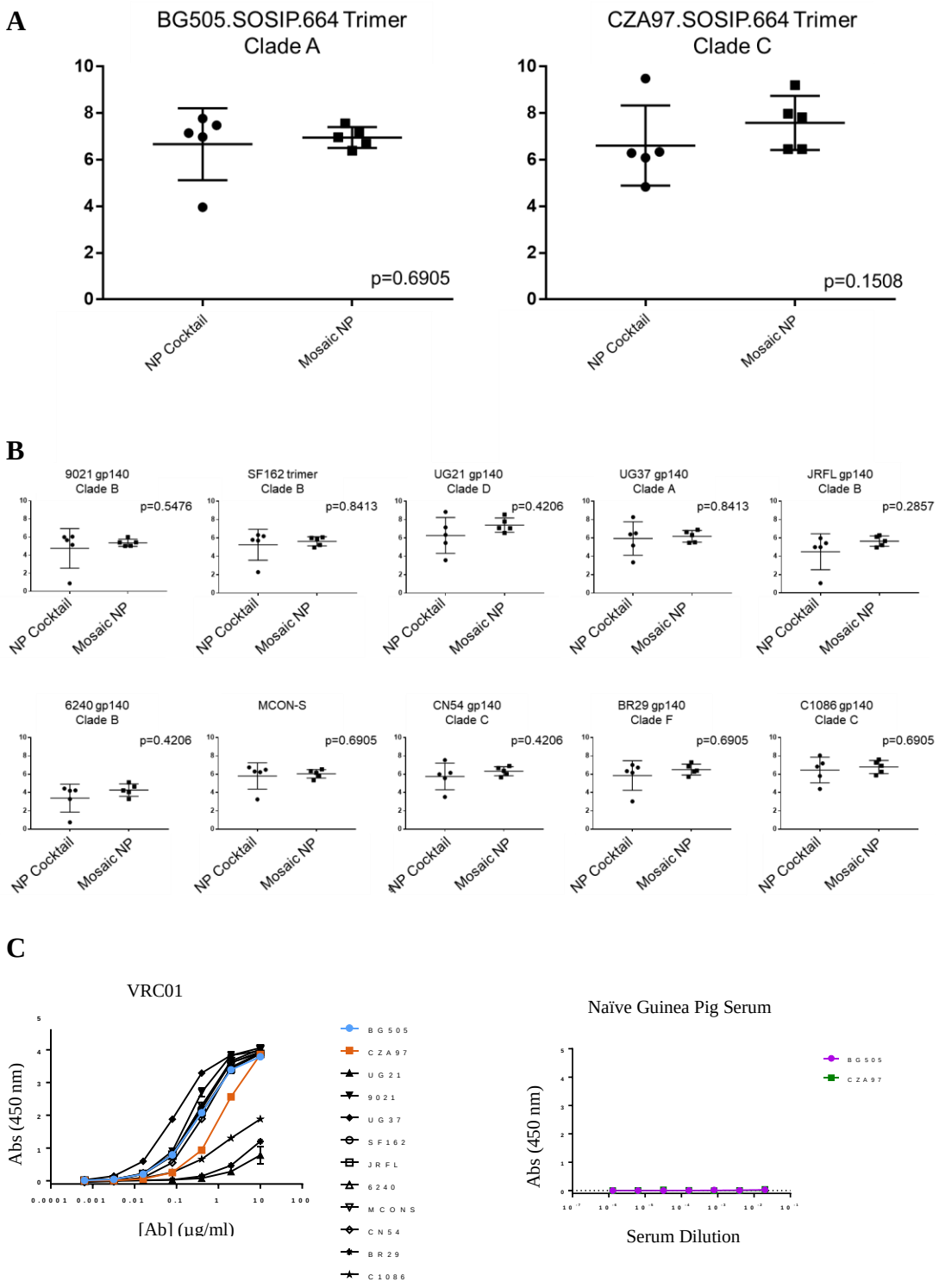

Figure 21. Comparison of AUC values from ELISA Binding Curves of Guinea Pig Serum Against HIV-1 Env Proteins.

(A) AUC values for vaccine-matched Env trimers plotted from ELISA binding curves in Figure 17B (with arithmetic mean and SD)**.**
(B) AUC values for gp140 Env proteins plotted from ELISA binding curves in Figure 17C. Reported are p-values from Mann-Whitney U tests (with arithmetic mean and SD).
(C) VRC01 was used as a positive control (left) and naïve guinea pig serum was used as a negative control (right).

Contributions to Chapter 3

Conceptualization:	Ivelin Georgiev
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