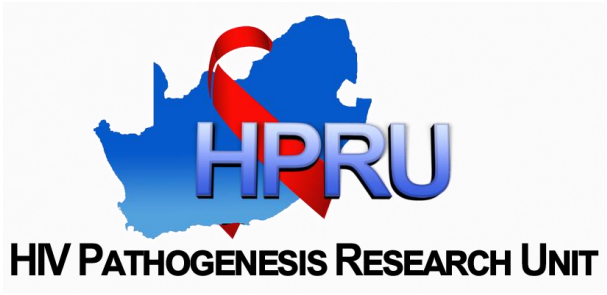
Engineering bispecific antibodies targeting HIV-1 subtype C

Tumelo Moshoette



A thesis submitted to the School of Pathology, Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Doctor of Philosophy

Johannesburg, November 2023

Declaration

I, **Tumelo Moshoette** (student number: 547906), hereby declare that the work encompassed in this Ph.D. thesis was undertaken by myself. Where others' work, thoughts and ideas have been mentioned, I have used conventional referencing to acknowledge their sources. A Turnitin plagiarism report indicating the calculated level of plagiarism according to an internet-based software has been included in the appendix section.

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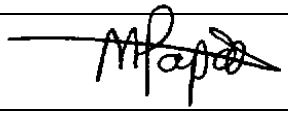
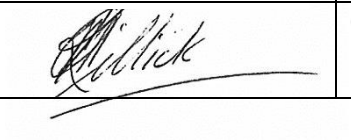
3rd author	Maria Papathanasopoulos		06 Nov 2023
4th author	Mark Killick		06 Nov 2023

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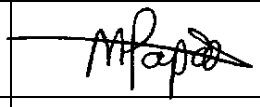
2nd author	Maria Papathanasopoulos		06 Nov 2023
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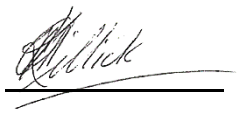
Signature of Primary Supervisor:  Date: 06 November 2023

Dedication

This work is dedicated to:

My mother (*Nomhle Moshoette*) who has been a great source of inspiration and has sacrificed a lot to give us the best life possible.

My late father (*Makabe Moshoette)*, may his soul continue to rest in peace.

My sisters (*Mpho Nobuswana, Masego Moshoette*), nieces (*Tshepo Nobuswana, Tlhalefo Moshoette, and Bophelo Moshoette*) and nephew (*Atlegang Moshoette*).

Publications arising from this thesis

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2. Moshoette, T., Papathanasopoulos, M. A., & Killick, M. A. (2022). HIV-1 bispecific antibody iMab-N6 exhibits enhanced breadth but not potency over its parental antibodies iMab and N6. *Virology Journal*, *19*(1), 143. https://doi.org/10.1186/s12985-022-01876-1

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-Motho ke motho ka batho-

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List of abbreviations

ABBREVIATION	FULL NAME
2dCD4	Two-domain CD4
AAV	Adeno-associated virus
ADCC	Antibody dependent cell cytotoxicity
AIDS	Acquired immunodeficiency syndrome
ART	Antiretroviral therapy
BCA	Bicinchoninic acid assay
bibNAbs	Bispecific broadly neutralising antibodies
bNAbs	Broadly neutralising antibodies
CAP256	CAP256-VRC26.25 monoclonal antibody
CCR5	C-C chemokine receptor type 5
CD4	Cluster of differentiation 4
CD4bs	CD4 binding site
CD4i	CD4 induced site
cDNA	Complementary DNA
CDR H3	Heavy-chain complementary-determining region 3 loops
CFRs	Circulating recombinant forms
CH3	Heavy chain 3rd domain constant region
CHO	Chinese hamster ovary
CXCR4	C-X-C chemokine receptor type 4
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
Env	HIV envelope glycoprotein

Fab	Antigen binding fragment region
Fc	Fragment crystallizable region
FCS	Foetal calf serum
FDA	Food and Drug Administration
FcγRIIIa	Fc Gamma Receptor IIIa
Gag	Group associated antigen
GALT	Gut-associated lymphoid tissue
GMP	Good manufacturing practices
gp41	Envelope glycoprotein 41
gp120	Envelope glycoprotein 120
HEK293	human embryonic kidney
HIV-1	Human immunodeficiency virus type 1
HIV-2	Human immunodeficiency virus type 2
IC_{50}	50% inhibitory concentration
IC_{80}	80% inhibitory concentration
IgG	Immunoglobulin G
iMab	Ibalizumab
INSTIs	Integrase strand transfer inhibitors
kDa	Kilodalton
LA-ART	long-acting antiretroviral therapy
LEDGF	Lens epithelium-derived growth factor
mAb	Monoclonal antibody
MPER	Membrane proximal external region
NAbs	Neutralising antibodies
FcRn	Neonatal Fc receptor

NNRTIs	Non-nucleoside reverse transcriptase inhibitors
NHP	Non-human primate
NRTIs	Nucleoside/tide reverse transcriptase inhibitors
PEI	Polyethylenimine
PI	Protease inhibitors
PIC	Pre integration complex
PMTCT	Prevention of mother to child transmission
PEP	Post exposure prophylaxis
PrEP	Pre-exposure prophylaxis
RNA	Ribonucleic Acid
RT	Reverse transcriptase
SAHPRA	South African Health Products Regulatory Authority
SDS-PAGE	Sodium dodecyl-sulphate polyacrylamide gel electrophoresis
SEC	Size exclusion chromatography
SHIV	Simian–human immunodeficiency chimeric virus
SHM	Somatic hyper-mutations
SIV	Simian immunodeficiency virus
STIs	Sexually transmitted infections
TasP	Treatment as prevention strategies
triNAbs	Trispecific neutralising antibodies
V1/V2 and V3	HIV Env 1st, 2nd, and 3rd variable loop structures
VHC	Heavy chain variable region
VLC	Light chain variable region
Vpr	Viral protein R
WHO	World Health Organisation

WT	Wild type

Abstract

The introduction of antiretroviral therapy has revolutionised the management of HIV-1 infection, effectively reducing morbidity and restoring life expectancy of HIV-1 patients. Continual improvements into the tolerability, increasing the genetic resistance barrier and reduction in antiretroviral drug dosing frequency have improved patient adherence and outcomes, but nonetheless require life-long therapy. By contrast, the development of an effective HIV-1 prophylactic vaccine or practical cure strategy remains elusive. Ongoing development of novel therapies and preventative strategies will be crucial in bringing an end to the global HIV-1 pandemic.

Several immune-based therapeutic and preventative strategies using combinations of isolated HIV-1 broadly neutralising antibodies are currently undergoing clinical development. However, the high cost of combination immunotherapy/immunoprophylaxis in comparison to antiretroviral drugs remains a major obstacle in wide-spread clinical implementation, especially in resource-limited settings like Africa. Specifically engineered antibodies comprising dual- (bispecific) or tri-paratopes (trispecific) have been developed as an alternative to combination-based therapy. This study aimed to design and evaluate novel bispecific neutralising antibodies that outperform (i.e., exhibit improved breadth and potency) combinations of antibodies in preventing HIV-1 infection *in vitro*. A range of bispecific constructs were designed and incorporated the knob-in-a-hole and CrossMab mutations to promote preferential assembly of heterologous heavy and light antibody polypeptide chains into a native-like IgG configuration. Antibody sequences used in the bispecific constructs included known broadly neutralizing antibodies against HIV-1 as well as

ibalizumab (iMab), which targets human CD4. The bispecific antibodies and parental monoclonal antibodies were produced by transient transfection of HEK293T cells and purified by protein-A affinity chromatography followed by size-exclusion chromatography. Purified antibodies were evaluated by SDS-PAGE under reducing and non-reducing conditions and confirmed heterologous assembly of the dual heavy and light chain constituents in the bispecific antibody configurations. The bispecificity of the antibodies was confirmed by binding ELISA to HIV-1 Env and two-domain/four-domain human CD4 immobilized proteins. A diverse panel of 21 pseudoviruses inclusive of the global HIV-1 panel, was used to evaluate the breadth and potency of the bispecific antibodies in an *in vitro* HIV-1 neutralization assay, and compared to their monoclonal parental antibodies, alone or in combination, and selected bispecific antibodies such as 10E08-iMab and PG9-iMab.

We successfully engineered and characterised two novel bispecific antibodies, iMab-Cap256 (Chapter 2; Publication 1) and iMab-N6 (Chapter 3; Publication 2). Our data demonstrate that both antibodies exhibited greater breadth over their respective parental antibodies when evaluated individually and in combination. Impressively, iMab-Cap256 exhibited remarkable potency over its parental antibodies (on average 4× and 59× more potent than CAP25 and iMab, respectively). Although iMab-N6 showed no enhancement in potency over its parental antibodies, it is calculated to have an exceptional IC_{80} coverage at < 1 μg/ml of 90% of global circulating HIV-1 strains, versus 86% and 48% for the N6 and iMab parental antibodies, respectively. These findings are significant as an IC_{80} < 1μg/ml may predict real-world clinical outcomes in protecting against HIV-1 acquisition in humans. In summary, the described bispecific antibodies may be particularly important in the context of HIV-1

subtype C infection, which predominates global infections, and requires uniquely tailored treatment and prevention strategies to overcome the resource-limited challenges facing these endemic regions. These novel bispecific antibodies show great potential for further clinical development given their breadth and potency, but understandably require further testing and development before entering human clinical trials.

Chapter 1: Introduction

1.1. The scale of the HIV-1 pandemic

According to the World Health Organization (WHO), there is an estimated 38.4 million people currently living with human immunodeficiency virus type 1 (HIV-1) infection globally (1). To date, 40.1 million people have died from HIV-1 related causes globally, with an estimated 650 000 of these deaths occurring in 2021 alone (1). Africa remains the region most affected by the HIV-1 pandemic accounting for two thirds of the global cases (25.6 million cases in Africa) (1).

South Africa accounts for majority of the HIV-1 cases worldwide. Currently, the HIV-1 prevalence rate within South Africa is 13.94% (8.45 million people) of the total population, representing an increase of 75.35% (5.99 percentage points) since 2002 (2). South African HIV-1 related deaths have declined significantly since the 2006 peak when they numbered at 282 904 (39.7% of all deaths) and currently stand at 85 796 or 12.9% of total deaths in the country (2). Since 2006, the number of people living with HIV-1 has steadily increased as a result of consistent year on year decreases in HIV-1 related deaths (i.e., improved life expectancy of HIV-1 positive people) driven by the availability of antiretroviral therapy (ART) (2,3). Unfortunately, a noticeable increase in the total number of HIV-1 related deaths was observed in 2021 (87 915 deaths in 2021 versus 80 199 deaths in 2020) and was attributed to ART rollout disruptions due to the COVID-19 pandemic (2). Encouragingly, HIV-1 related deaths have decreased in 2022 as COVID-19 disruptions subside (2). This highlights the fragility of the developing countries healthcare systems and the potential devastation that may result if the HIV-1 pandemic in not controlled. Development of novel therapies and prevention strategies is crucial in bringing an end to the global HIV-1 pandemic.

1.2. HIV taxonomy and diversity

HIV is a member of the retroviridae family within the lentivirus genus (4–6). HIV possesses two copies of a plus strand RNA genome that requires a virally encoded reverse transcriptase enzyme for replication through a DNA intermediary during the replication cycle (5). As a lentivirus, HIV results in chronic infection within humans through integration of the double stranded DNA intermediary into the host genome, establishing a latent viral reservoir (5,7,8).

HIV is broadly classified into two types, HIV-1, and HIV-2, based upon phylogenetic and antigenic differences (4–6). Whilst both types (HIV-1 and HIV-2) result in acquired immunodeficiency syndrome (AIDS) within humans, HIV-2 is reported to have lower plasma viral load levels, slower disease progression and reduced transmission efficiency (9–12). HIV-2 is thought to have originated from a sooty mangabey (*Cercocebus atys*) simian immunodeficiency virus (SIV) (SIVsmm) progenitor and is geographically constrained to West Africa, accounting for a minority of global infections (5,10,12).

HIV-1 is thought to have originated from SIVcpz and SIVgor progenitors which naturally infect chimpanzee (*Pan troglodytes troglodytes*) and gorilla (*Gorilla gorilla)* within Africa (13–19). HIV-1 is further divided into four groups, M. N, O and P, representing four distinctive zoonotic transmission events, with group M responsible for the existing HIV-1 pandemic (5,15,16,20). Group M is further subdivided into nine genetically distinct subtypes/clades (A-D, F-H, J and K) (20,21). Recombination between viral isolates and subtypes has led to further increased diversity amongst viral

strains circulating worldwide, with over a hundred circulating recombinant forms (CFRs) identified to date (21,22).

The extraordinary genetic diversity of HIV-1 is largely driven by the error-prone HIV-1 reverse transcriptase (RT) enzyme, which lacks exonucleases proof-reading ability resulting in many genomic mutations (23). Combined with this, the high replication rate, the ability of the virus to undergo major genetic recombination, and insertion and deletion events in response to host immunological pressure all contribute to the observed global diversity (21,24–26). HIV-1genetic variance within the same subtype may varying between 15-20% and the inter subtype genetic differences are usually between 25-35% (21,27). The greatest genetic diversity is found in West Central Africa where all the major subtypes are found including several CRFs (28). Geographical distribution of HIV-1 amongst other regions is more homogeneous, where a single subtype usually predominates. Subtype B isolates are found to dominate in Latin America, Western and Central Europe and North America, with subtype A predominating in Eastern Europe and Central Asia and East Africa (excluding Ethiopia), CRF01_AE predominates in Southeast and East Asia and Subtype C has overwhelming dominance in Southern Africa, Ethiopia, and India (21). Overall subtype C accounts for approximately 46% of the total number of the HIV-1 infections globally (21,29,30). Given the severity of the HIV-1 pandemic in Sub-Saharan Africa, targeted therapeutics or prevention strategies tailored specially to Subtype C are likely to be pivotal in turning the tide against the HIV-1 pandemic.

1.3. HIV-1 replication cycle and pathogenesis

The HIV-1 replication cycle (Fig. 1.1) is initiated by the viral gp120 component of surface exposed trimeric envelope glycoprotein (Env) engaging the host cell CD4 receptor (23,31). Once bound the Env trimer undergoes a conformational change, where rearrangements in the inner, outer, and bridging sheet domains of gp120 result in the re-positioning of the 1st, 2nd, and 3rd variable loop structures (V1/V2 and V3) and engagement of the co-receptors, C-C chemokine receptor type 5 (CCR5) or C-X-C chemokine receptor type 4 (CXCR4) (23,31–33). Following co-receptor binding, viral-host membrane fusion is initiated with the N-terminal fusion peptide of the pre-hairpin gp41 inserting into the host membrane, anchoring the two membranes (23,31,34). During this phase shedding of the gp120 surface subunit likely occurs as the gp41 subunit transitions from the pre-hairpin intermediate into the post fusion, six-helical bundle conformation, driving the viral-host cell membrane fusion and release of the viral p24 capsid into the interior of the host cell (23,31,34). As the capsid migrates to the nucleus, HIV-1 reverse transcriptase enzyme transcribes the single stranded viral RNAs into double stranded complementary DNA (cDNA) (23,31,35). Once inside the nucleus and near the site of integration, the capsid degrades releasing viral proteins and cDNA (36). The newly formed cDNA is assembled into the pre integration complex (PIC) that includes the viral RT and integrase enzymes, viral protein R (Vpr) and several host derived factors (37–41). The PIC facilitates irreversible integration of the proviral genome into a transcriptionally active genomic region through the interaction with host Lens epithelium-derived growth factor (LEDGF/p75) (35,42,43). Full-length proviral DNA is transcribed by host RNA polymerase II and includes both singly and multi-spliced transcripts that are exported out of the nucleus for translation of the various viral components within the cell cytoplasm (23,44). The Gag (Pr55^{Gag}), Gal-Pro-Pol

(Pr160^{GagPol}) and gp160 (Env) polypeptide precursors are processed into their functional, smaller protein subunits by the viral protease or host protease (e.g., furin) (45,46). The gp160 precursor is expressed on the rough endoplasmic reticulum where it undergoes extensive glycosylation as it transits through the trans-Golgi network, and it assembled into trimeric conformation before being cleaved by the human furin protease into gp120-g41 subunits that remain non-covalently bound (47–50). The functional Env trimeric spikes arrange on the cell surface awaiting virus budding. The precursor Gag protein, Pr55^{Gag}, interacts with unspliced HIV-1 genomic RNA initiating the viral assembly at the host cell membrane and the inclusion of the viral protease and RT enzymes before acquiring the outer lipid membrane with embedded Env spike proteins as the immature virion buds from the host cell (51–53). Viral maturation of the newly formed virions results from the viral protease cleavage of the Gag precursor into the structural proteins; Matrix (p17), Capsid (p24) and nucleocapsid (p7), resulting in fully infectious viral particles capable of subsequent infection (23,31).

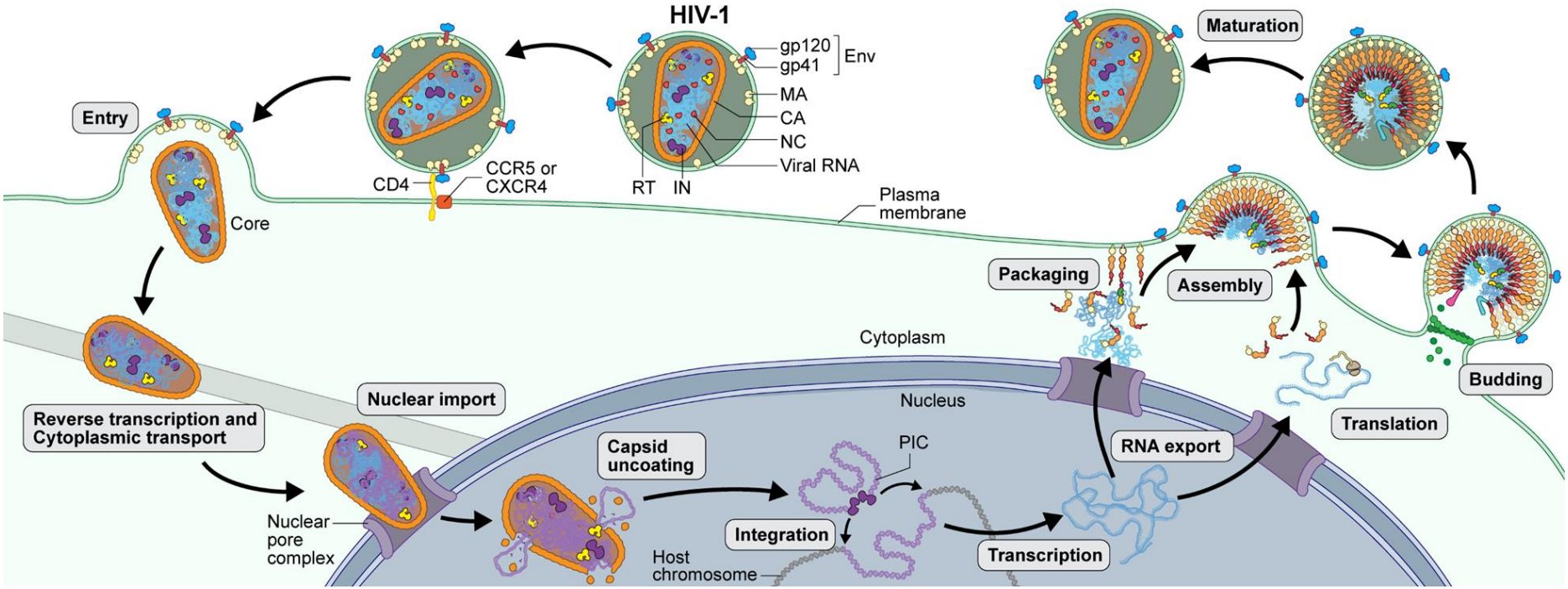

Figure 1. 1: HIV Life Cycle. HIV entry into the host cell is initiated by the binding of viral gp120 to the host CD4 and CCR5 or CXCR4 coreceptors followed by gp41 mediated membrane fusion. Fusion of the viral envelope to the host membrane results in the release of the viral capsid into the host cell cytoplasm. The Capsid protein then migrates to the nucleus. Reverse transcription of the viral RNA to cDNA is initiated in the capsid during this migration. Capsid uncoating happens in the nucleus and the viral cDNA is irreversibly integrated into the host DNA. The integrated viral DNA is transcribed, and production of viral proteins is achieved using the host machinery. Viral proteins together with viral RNA are packaged to form new viral particles. Post budding modifications to the viral proteins driven by proteas results in a mature viral particle ready to infect other host cells. [Image sourced from The Science of HIV project (54)]

Sexual transmission (homo- and heterosexual) is the predominant mode of HIV-1 transmission amongst humans (55). Although reported to be a largely inefficient process, transmission rate increases during the acute stage of infection (of the infected partner) and in the presence of other sexually transmitted infections (STIs) (within the uninfected partner) (56). To establish infection, HIV-1 must cross the mucosal barrier gaining access to the viable target cell population beneath the epithelial layer or the deeper submucosa (57,58). The single columnar epithelial cell layer lining the endocervix represents a site of increased vulnerability for transmission, although physical abrasion of vaginal epithelial barrier may also increase the risk of transmission in females (59–63). The single layer of epithelial cells lining the rectal mucosa, combined with a higher risk of micro trauma during anal receptive sex, account for the ten times higher risk of HIV-1 transmission compared to vaginal transmission (64). The tissue located below the inner foreskin and urethra are established target sites in male transmission (65,66). Once the mucosal barrier has been breached, the virus establishes small foci of infected cell populations (dendritic cells, macrophage and $CD4^+$ T lymphocytes), fuelled by the recruitment of additional immune target cell populations by innate immune system activation (65,66). Subsequent drainage of the infected cell populations to the lymphoid tissues results in rapid expansion of HIV-1 due to the increased availability of target cell populations. This period coincides with the peak viral loads in the peripheral blood and other tissues and severely damages the host immune system through devastating loss in CD4+ T cell populations within the gut-associated lymphoid tissue (GALT) (67–70). It is during this acute phase of infection that the virus establishes latent viral reservoirs. Peripheral blood viral load declines with the activation of the adaptive immune response, specifically the CD8+ cytotoxic T Lymphocytes, establishing the viral load set point,

and representing the start of the clinical latency and the asymptomatic phase (71). During this phase there is generally a recovery in the infected individual's CD4+ T cell numbers (72,73). B lymphocyte activation and maturation result in the emergence of autologous neutralizing responses against the founder virus strain driving viral escape and is thus not associated with viral control. If left untreated, the patients CD4+ T cells will decline over time, resulting in immune dysfunction and dysregulation, further resulting in increased opportunistic infections (including Tuberculosis, pneumonia, and certain malignancies) defining the acquired immunodeficiency syndrome (AIDS) stage of pathogenesis (74). If ART is not initiated, these AIDS patients will succumb to these secondary infections within a relatively short time frame (year).

1.4. Antiretroviral therapy (ART)

Antiretroviral therapy (ART) remains the cornerstone for the treatment of HIV-1 positive patients. The first clinical trials of ART were initiated towards the end of the 1980s and were swiftly followed by combination-based therapy to enhance their antiviral efficacy (1990s) (75,76). The ongoing discovery of novel compounds has resulted in the current ART regimes boasting an extraordinary array of antiretroviral options, with varying HIV-1 targeting modalities that have been clinically proven to decrease HIV-1 patient viral loads below detection, using standard molecular biology diagnostic tests (75,76). Further improvements and refinements have resulted in fixed-dose combinations with improved potency and pharmacokinetics, reduction in toxicity and overall enhancement of patient adherence to treatment (75,76). ART allows for the restoration of the HIV-1 patients' immune system reducing HIV-1 infection to an easily managed, chronic condition with HIV-1 patients exhibiting similar life-expectancies to that of the general population within their region. There are currently eight classes of

ART which include nucleoside/tide reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs), integrase (strand transfer) inhibitors (INSTIs), fusion inhibitors, entry inhibitors, maturation inhibitors and post-attachment inhibitors (77).

ART is not only critical for maintaining the health of HIV-1 positive patients but also forms the backbone of highly successful treatment as prevention strategies [TasP, sometimes referred to as undetectable = untransmittable (U=U)]. Large scale clinical trials have demonstrated ART viral suppression below detectable limits effectively eliminates transmission from the HIV-1 positive partner to their uninfected partner (78). Similarly, timeous initiation of the ART in HIV-1 positive mothers is effective in prevention of mother to child transmission (PMTCT) of HIV-1 during delivery or post-natal breastfeeding (79). ART has also successfully been deployed in Pre-exposure prophylaxis (PrEP) and post exposure prophylaxis (PEP) in preventing HIV-1 acquisition.

1.4.1. Advancements in ART

Major advances in ART includes the development of a new class of drugs that are better tolerated and have minimal side effects, leading to overall improvement in patient adherence (80,81). Moreover, new drugs like dolutegravir have a high genetic resistance barrier because mutations result in viruses with significantly reduced replication capabilities and integrase activity (81).

In the most recent advancements, two long-acting ARTs (LA-ART) (cabotegravir and rilpivirine, injected monthly) have received Food and Drug Administration (FDA) approval for use as HIV-1 combination therapy in individuals with a viral load of < 50 RNA copies/ml (82–86)**.** Furthermore, the long-acting injectable cabotegravir (injected every two months) has received FDA approval for use as PrEP (87–89). These LA-ART were developed to address poor patient adherence associated with the need to take pills daily to retain efficacy**.** Finally, the South African Health Products Regulatory Authority (SAHPRA) has approved the use of a dapivirine vaginal ring which was shown to be 56% to 90% efficacious (efficacy depends on adherence) in preventing HIV-1 acquisition (90–93). These innovations are key as they provide women, in particular, additional options for protecting themselves against HIV-1 acquisition (90,91,93,94). There is no doubt the success of ART in both the treatment and prevention of HIV-1. Continuous innovation within this field will no doubt have a positive impact in the fight against HIV-1.

1.4.2. ART and broadly neutralising antibodies in the fight against HIV-1

HIV-1 viral diversity necessitates continuous improvement of ART and the development of novel anti-HIV-1 agents. Broadly neutralising antibodies (bNAbs) isolated from a subset of HIV-1 positive individuals are one such novel anti-HIV-1 agent. These bNAbs show great potential as both immunoprophylaxis and immunotherapy agents and could have a significant impact on the HIV-1 pandemic alongside ART (95). BNAbs have an added benefit of attacking the virus on two fronts, primarily through neutralisation and secondarily through effector functions (95). Moreover, their low toxicity and long-acting pharmacokinetics makes them promising candidates for clinical application (95).

1.5. Broadly neutralising antibodies

1.5.1. Overview

Broadly neutralising antibodies (bNAbs) develop in approximately 15% of HIV-1 positive individuals (96–98). BNAbs require several years to develop in HIV-1 infected individuals, where continued antigen exposure results in accumulation of high levels of somatic hyper-mutations (SHM) and single point mutations (indels) (99,100). Numerous, bNAbs reported in the literature have been shown to develop characteristically long heavy-chain complementary-determining region 3 loops (CDR H3) which are crucial for accessing several conserved epitopes on the glycan covered HIV-1 Env (99,101,102). Env is an important viral protein that is responsible for initiating and facilitating viral entry into $CD4^+$ T-cells and is the sole viral encoded protein exposed on the virion surface (103). bNAbs target highly conserved regions on the HIV-1 Env thus giving them impressive neutralisation breadth in comparison to other autologous HIV-1 neutralising antibodies (NAbs) that exhibit patient-specific strain neutralization (Fig. 1.2) (102).

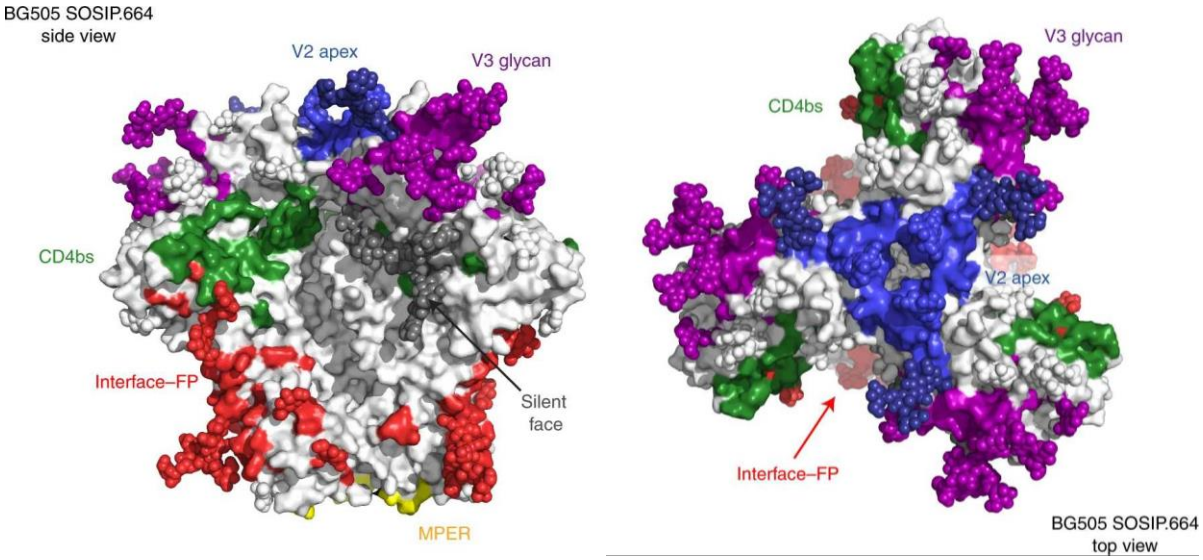

Figure 1. 2: HIV-1 Envelope glycoprotein (BG505 SOSIP.664) with currently identified bNAb binding sites shown. bNAbs target highly conserved regions on the HIV-1 spike namely the V2 apex, V3 glycan, the CD4 binding site (CD4bs), interface-FP, the silent face, and the membrane proximal external region (MPER). The CD4 induced site (CD4i) (not shown) epitope appears following Env conformational changes triggered by CD4 binding. [Image sourced from Sok and Burton (102)]

BNAb targeted regions include the V2 apex, V3 glycan, the CD4 binding site (CD4bs), interface-FP, the silent face, the membrane proximal external region (MPER), and the CD4 induced site (CD4i) (Fig. 1.2) (102,104–106). Generally, V2 apex and V3 glycan targeting bNAbs display long CDR H3 (≥ 20 residues) whereas the CD4bs bNAbs have the highest rate of SHM (102). The V2 glycan bNAbs typically display enhanced potency with moderate breadth, likely a result of their glycan dependency [e.g., CAP256-VRC26.25: geometric mean IC_{50} of 0.002 µg/ml and 59% breadth (107)]. In contrast, MPER bNAbs display enhanced breadth with moderate potency [e.g., 10E08: geometric mean IC_{50} of 0.299 µg/ml and 98% breadth (108)] (102). CD4bs bNAbs [e.g., N6: geometric mean IC_{50} of 0.058 µg/ml and 98% breadth (109)] tend to have the best combination of breadth and potency (102). Although first generation bNAbs (110) [e.g., b12, 447-52D, 2G12, 2F5, and 4E10] exhibited poor efficacy against HIV-1 due to their poor breadth and potency, second generation bNAbs [e.g., CAP256-VRC26.25 (107),

10E08 (108), N6 (109), 1-18 (87), VRC01 (111), and 3BNC117 (112) to name but a few] show promise as potential immunotherapy and immunoprophylaxis agents (113).

1.5.2. bNAb isolation

First generation bNAbs were isolated starting in the early 1990s using either phage libraries or immortalised B-cells (Fig. 1.3A and B) (114). The process of isolating bNAbs using phage libraries starts with the preparation of Fab-phage (Fab = fragment antigen binding region) libraries using samples from HIV-1 positive donors (Fig. 1.3A). These libraries are screened against HIV immunogens in an ELISA followed by Pseudovirus neutralisation assay; positive clones are then sequenced and further characterised (115).

Immortalised B-cells are generated to overcome the short life span of B-cells and thus allow for the screening of, and generation of monoclonal antibodies (Fig. 1.3B). These long-lived cells are generated by fusing B-cells with myeloid cells using electrofusion techniques (114,116–118). Alternatively, long lived B-cells are generated by transforming B-cells with Epstein-Barr virus (EBV) (115,117,119). In both cases, the immortalised B-cells are plated as single cells in a 96 well plate and cultured. Following clonal expansion, the supernatant from each well is harvested and screened using both an ELISA and pseudovirus neutralisation assay to identify bNAb producing B-cells. Positive B-cells are then sequenced and antibody encoding regions cloned for further characterisation (115–117).

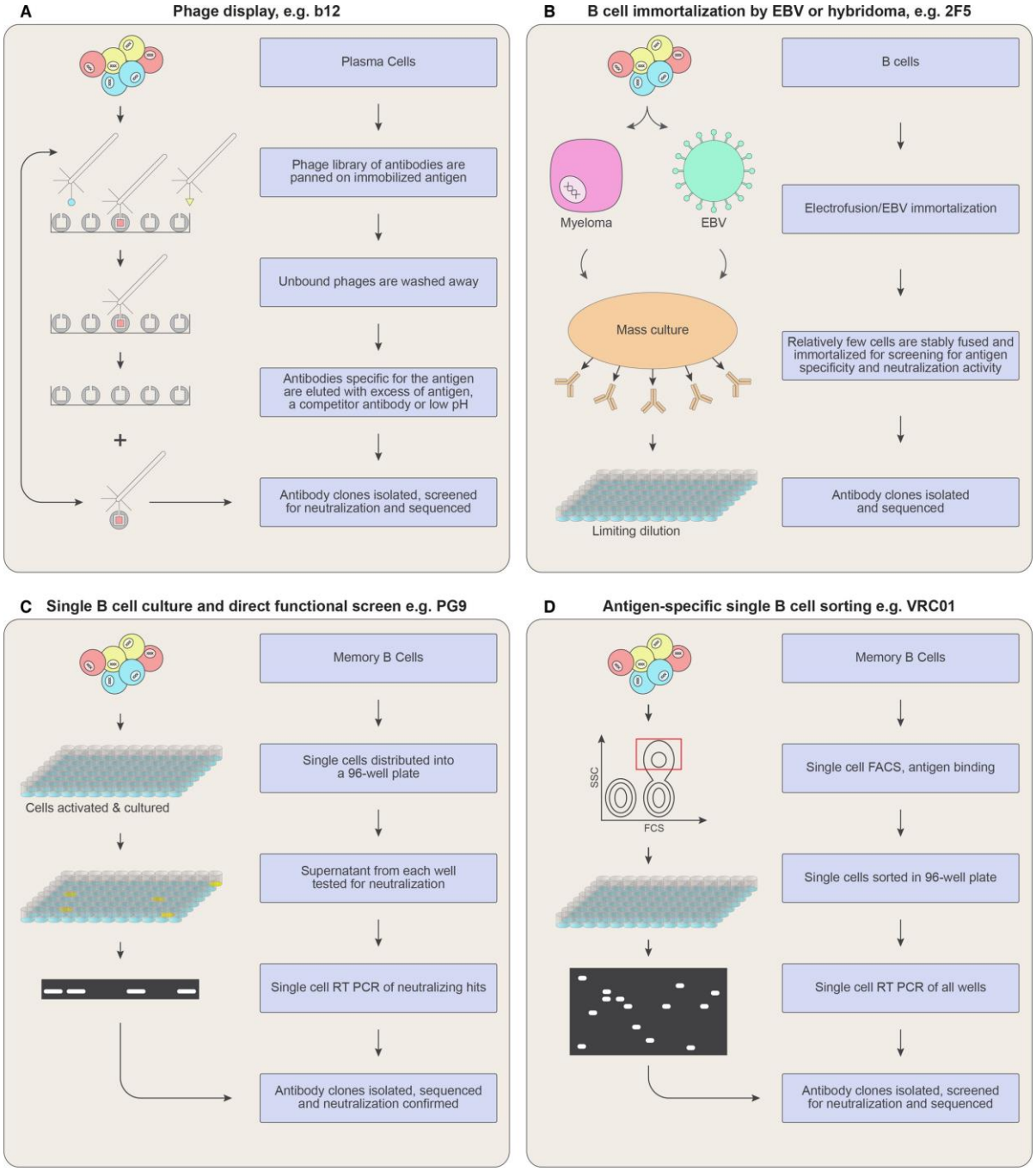

Figure 1. 3: Evolution of bNAb isolation techniques. A and B First generation bNAbs were isolated using phage display or immortalised B-cells. C and D Advancements in molecular biology techniques allowed for the use of single B-cell culture or antigen directed B-cell sorting; this led to the isolation of previously unknown, secondary generation bNAbs. [Image sourced from McCoy and Burton (114)

Second generation bNAbs were isolated using either direct B-cell cultures or flow cytometry. Advancements in molecular biology techniques saw the development of high throughput ELISA and pseudovirus neutralisation assay techniques thus allowing

for the screening of large patient cohorts through direct B-cell cultures (Fig. 1.3C) (114,120). Supernatant from these single cell 96 well B-cell cultures is harvested and screened using high-throughput ELISA and pseudovirus neutralisation assays to identify bNAb producing clones (114,120). Positive B-cell clones are sequenced and cloned for further characterisation. Several second generation bNAbs have been identified from this method, this includes PG9, PG6, CAP256-VRC26, and 10E08 to name a few (114).

Improved understanding of bNAb epitopes and binding specificity lead to the use of Fluorescence-Activated Cell Sorting (FACS) to isolate specific anti-HIV B-cells from donor samples using HIV envelop as a "bait" molecule (Fig. 1.3D) (114,121,122). The sorted B-cells are then sequenced, cloned, and expressed recombinantly. The resultant Ab clones are screened for breadth and potency using pseudovirus neutralisation assay. Notable bNAbs isolated through this technique include PGDM1400, 3BNC177, and 10-1074 (114).

1.5.3. bNAb immunoprophylaxis

BNAb immunoprophylaxis refers to passive immunity strategies that encompasses direct infusion of purified bNAbs *in vivo* or through vector-mediated *in vivo* expression of bNAbs to achieve protection from HIV-1 (95,113,123–138). bNAb immunoprophylaxis strategies have been in preclinical development for over a decade and have recently entered human clinical trials (95,113,123–138). A summary of the prominent work of bNAb immunoprophylaxis is provided below.

Julg *et al*. (136), demonstrated that CAP256-VRC26.25-LS (LS antibodies are described in section 1.8.2.1) protected all macaques from infection following SHIV-325c intrarectal challenge 24 hours post antibody infusion. This protection occurred at an impressive serum concentration of <0.75 µg/ml, consistent with exceptional *in vitro* potency of CAP256-VRC26.25 (136). In a separate study, VRC01 efficacy was determined by challenging macaques intrarectally with BaIP4 two weeks after antibody infusion (123); a significantly longer period between antibody infusion and challenge than in the CAP256-VRC26.25 study. Impressively, VRC01 conferred full protection at serum concentrations of 6.4 – 3.5 µg/ml despite the increased interval between infusion and challenge (123). Against intravaginal SF162P3 challenge (24 hours post antibody infusion), PGT121 protected all macaques with serum concentrations ≤ 95 µg/ml and ≤ 15 µg/ml but offered partial protection (protected 3/5 macaques) at 1.8 µg/ml (139). Taken together, these studies show that bNAbs are effective in protecting against both rectal and vaginal (two common routes of transmission) challenge routes within the non-human primate (NHP) challenge models (123,136,139). Moreover, protection can occur two weeks after infusion owing to the intrinsic, long-acting nature of antibodies (123). Finally, the varying protective serum concentrations points to the importance of bNAb potency in ultimately determining bNAb success as a PrEP strategy.

Single bNAb PrEP strategies may be limited [as seen with PDGM1400's (136)] as no single bNAb has demonstrated 100% neutralization coverage of clinically relevant, HIV-1 Env-pseudotyped panels *in vitro* (127,129). This limitation was recently accentuated in a major phase IIb clinical trial (HVTN 703/HPTN 081 and HVTN704/HPTN 085; AMP) where VRC01 (CD4bs antibody) offered no overall

protection against HIV-1 acquisition compared to the placebo control group (saline infusion) (128). Analysis of the data revealed that VRC01 was effective against viruses with an *in vitro* sensitivity of IC_{80} < 1 µg/ml (IC_{80} or 80% inhibitory concentration refers to the concentration of antibody required to inhibit 80% of viral replication relative to a negative control sample) but not those with *in vitro* IC_{80} sensitivity > 1 µg/ml, equating to only 30% of circulating isolates (128). In retrospect VRC01 performed below expectation based on preclinical data generated in the NHP-challenge model. Nonetheless, this trial served as a crucial proof of concept as VRC01 exhibited 75% efficacy at preventing HIV-1 acquisition, against the sensitive viruses (IC_{80} < 1 µg/ml). These human clinical trial data are consistent with previous macaque studies, where VRC01 didn't offer 100% efficacy due to the emergence of new resistance mutations (128). Overall, this trial confirms the importance of breadth, potency and bNAb selection if high efficacy in the prevention of HIV-1 acquisition is to be met and may require specific regional tailoring dependent on prominent HIV-1 strains in circulation. Moreover, this trial has established the *in vitro* IC_{80} < 1 µg/ml as an early threshold for bNAb PrEP selection.

1.5.4. bNAb immunotherapy

BNAb immunotherapy refers to the use of infused or vector-mediated *in vivo* expressed antibodies to treat HIV-1 infection (129,140–152). Preclinical investigations of bNAb immunotherapy using PGT121 and 3BNC117 (142), N6-LS (146), and 1-18 (87) have been shown to suppress HIV-1 viral load in either humanised mice or NHP. However, bNAb monotherapy eventually failed due to the emergence of 3BNC117 resistance mutations and the decline of N6-LC and PGT121 below effective serum

neutralisation levels (142,146). Interestingly, 1-18 therapy did not select for resistance mutations, likely due to the high viral fitness cost associated with 1-18 resistance (87).

In a number of small human clinical trials, 3BNC117 (phase 1: NCT02018510 and phase IIa: NCT02446847), VRC01 (phase I: NCT01950325 and NCT02463227), and 10-1074 (phase I: NCT02511990) were shown to suppress viral load in viraemic individuals (143,145,150) or prevent viral rebound during treatment interruption (140,153). Importantly, these therapies were found to be safe and well tolerated. However, these monotherapies failed due to pre-existing resistant viruses and/or the emergence of new resistant viruses (140,143,145,150,153). As with bNAb PrEP, these results once again underscore the importance of antibody breadth.

1.5.5. bNAb combination immunotherapy and immunoprophylaxis

bNAb combinations (i.e., a cocktail of two or more bNAbs) is preferred over single bNAb strategies because of the improved neutralization coverage and potency as demonstrated through computational modelling, and empirical *in vitro* and *in vivo* studies (134,154–156). 3BNC117+10-1074 combination therapy was shown to be more effective than 3BNC117 monotherapy at preventing viral rebound following treatment interruption (median viral rebound of 21 weeks and 6 – 10 weeks, respectively) (phase Ib, NCT02825797) (151). In viraemic individuals, 3BNC117+10-1074 combination therapy was shown to suppress viral loads for significantly longer period than either 3BNC117 or 10-1074 monotherapy alone (phase Ib, NCT02825797) (141,143,145). However, differences in bNAb half-life (3BNC117: 17.6 or 12.3 days, respectively, and 10-1074: 23.2 or 12.7 days, respectively) meant 3BNC117 levels dropped faster than

10-1074 effectively reducing this combination to monotherapy over time; this in turn resulted in rapid viral rebound driven by 10-1074 resistance (141,151). Variations in bNAb pharmacokinetics, the requirement for bNAb combination and cost implications thereof, threatens the wide-scale clinical implementation of bNAbs as PrEP/therapy strategies.

1.6. Bispecific broadly neutralizing antibodies

Bispecific bNAbs (bibNAbs) have been developed as possible replacements for bNAb combinations. These bibNAbs are specifically engineered to incorporate two bNAbs into a single antibody structure thus retaining the benefits of bNAb combinations without any of the associated challenges. Multiple bibNAb configurations have been engineered to date as described extensively by *Padte et al* (157), *Sedykh et al* (158), and *Fabozzi et al* (159). BibNAbs can be categorised as host-virus or virus targeting. Host-virus targeting bibNAbs are engineered using a host-targeting bNAb [e.g., iMab: targets CD4 or PRO140: targets CCR5] and an Env targeting bNAb [e.g., V2 glycan CAP256 or MPER 10E08 to create iMab-CAP256 (160) or 10E08-PRO140 (161)] whereas virus targeting bNAbs comprise of two different Env-directed bNAbs [e.g., CD4bs targeting; VRC07, and V2 glycan targeting; PG9-16 to create, VRC07xPG9-16 (162)]. Some host-virus bibNAbs [10E08$_{v2}$-iMab, PGT123-iMab, PG9-iMab, and iMab-CAP256 which was developed as part of this work)] exhibit enhanced potency (28×, 12×, and 38× more potent, respectively) and breadth over the most active parental bNAb (161,163,164). Likewise, some virus targeting bibNAbs (e.g., VRC07 x PG9-16 and 3BNC117-PGT135) exhibit enhanced potency and breadth over their parental bNAbs (162,165).

This enhancement in bibNAb potency over the parental bNAbs is due to the simultaneous binding of the paratopes resulting in increased avidity (161,163). For the iMab-based bibNAbs, the localisation/concentration of the bibNAbs at the site of entry (surface of the $CD4^+$ T-cells) by the iMab arm, avails the corresponding Env targeting arm to neutralise incoming virus thus contributing to potency enhancement (161,163). When bibNAb $10E08_{v2}$-iMab was tested in humanised mice, it conferred protection against repeated viral challenges (HIV-1 JR-CSF) even at serum concentrations as low as < 0.11 µg/ml (161). These data highlight the potential of bibNAbs.

1.7. Trispecific antibodies

To further enhance breadth and potency, trispecific bNAbs (tribNAbs) [e.g., N6/PGDM1400-10E8v4 and VRC01/PGDM1400-10E8v4 (166), 10E08/Bi-ScFv$_{dVRC01\text{-}5X\text{-}PGT121}$ (167), 10E8Fab-PGDM1400fv-PRO140fv (168), and PGDM1400-N49P7-10E8v4 (169)] incorporating three bNAbs have been successfully engineered. triNAbs exhibit by far the best combination of breadth and potency of any engineered HIV-1 antibody characterised to date (166–169). Unsurprisingly, tribNAb VRC01/PGDM1400-10E8v4 exhibits 100% efficacy (vs 75% and 62% efficacy for VRC01 and PGDM1400, respectively) in protecting NHPs from a combination of SHIV BaLP4 and SHIV 325c mucosal challenge five days post antibody infusion (166). tribNAbs benefit from greater avidity (triple epitope engagement) and better overlapping coverage (virus sensitivity to each bNAb in the tribNAb confirmation) in comparison to bibNAbs (166–169). Bi/tribNAbs have greater clinical potential than single bNAbs and clinical trials are currently underway to evaluate the safety, tolerability, pharmacokinetics, and anti-viral

activity of bibNAb 10E08$_{v4}$-iMab (NCT03875209) and tribNAb VRC01/PGDM1400-10E8v4 (NCT03705169).

Table 1. 1: A selection of bNAbs, bibNAbs and triNAbs referred to in this thesis.

bNAb	Epitope	IC_{50} (µg/ml)	Breadth (%)	Human Clinical Trial development
1-18	CD4bs	0.05	97	None to date
3BNC117	CD4bs	0.12	84	https://clinicalinfo.hiv.gov/en/drugs/3bnc117/patient
10-1074	V3	0.03	60.5	https://clinicalinfo.hiv.gov/en/drugs/10-1074/patient
10E08	MPER	0.30	98	https://clinicaltrials.gov/study/NCT03565315
CAP256-VRC26.25	V1/V2	0.002	59	PACTR202003767867253
N6	CD4bs	0.058	98	NCT04871113 and NCT03538626
PG9	V3	0.30	79	https://clinicaltrials.gov/ct2/show/NCT01937455
PDGM1400	V1/V2	0.003	83	NCT03205917 and NCT04983030
PGT121	V3	0.07	70	https://clinicaltrials.gov/ct2/show/NCT02960581
VRC01	CD4bs	0.22	89	HVTN 703/HPTN 081 and HVTN 704/HPTN 085 https://clinicalinfo.hiv.gov/en/drugs/vrc01/patient
VRC07	CD4bs	0.11	93	https://clinicalinfo.hiv.gov/en/drugs/vrc07-523ls/patient
bibNAb	**Epitopes**	**IC_{50} (µg/ml)**	**Breadth (%)**	**Human Clinical Trial development**
$10E08_{V2}$-iMab	MPER and CD4	0.002	99	NCT05890963 and NCT03875209
10E08-PRO140	MPER and CCR5	0.001	99	None to date
PG9-iMab	V3 and CD4	0.004	100	None to date
PGT123-iMab	V3 and CD4	0.006	100	None to date
VRC07xPG9-16	CD4 and V3	0.048	97	None to date
3BNC117-PGT135	CD4bs and V3	0.036	93	None to date
tribNAb	**Epitopes**	**IC_{50} (µg/ml)**	**Breadth (%)**	**Human Clinical Trial development**
10E08/Bi-$ScFv_{dVRC01\text{-}5X\text{-}PGT121}$	MPER, CD4bs, and V3	0.071	99.5	None to date
10E8Fab-PGDM1400fv-PRO140fv	MPER, V1/V2, and CCR5	0.01	98	None to date
N6/PGDM1400-10E8v4	CD4bs, V1/V2 and MPER	0,02	99.5	None to date
PGDM1400-N49P7-10E8v4	V1/V2, CD4bs, and MPER	0.0009*	100	None to date
VRC01/PGDM1400-10E8v4	CD4bs, V1/V2 and MPER	0.039	98	NCT03705169

IC_{50} = geometric mean. *Median IC_{50}, geometric mean IC_{50} not published.

1.8. bNAb cost reduction strategies

The large-scale rollout of immunotherapy and PrEP will be hindered by their prohibitive cost especially in comparison to current ART regimes. To overcome this challenge, numerous cost reduction strategies are under investigation and include alternative recombinant protein expression platforms, antibody engineering to extend half-lives and or delivery systems that provide sustained antibody serum concentrations at clinically relevant levels, thereby reducing the antibody dosage required for good clinical outcomes. A summary of these strategies is provided below.

1.8.1. Plant expression system

1.8.1.1. Benefits and challenges

Production of antibodies in plants offers numerous advantages over traditional mammalian expression, including low manufacturing costs, minimal technical expertise, minimal initial investment, and minimal equipment needs (170,171). Nandi *et al* (172) predict that plant production could cost ≥50% less than the current mammalian cell culture system for the same Ab yield (plant production: $33 million operating cost and $131/g unit production cost vs mammalian cell production: $65 million operating cost and $260/g unit production cost). In addition to lower costs, plant production systems allow for the transfer of manufacturing knowledge and localisation of production in low-to medium-income countries leading to the availability of antibody-based therapies in these historically disadvantaged regions (170,171).

The biggest challenge with plant-based antibody expression is the presence of non-mammalian N-glycans (β1,2-xylosyltransferase and α1,3-fucosyltransferase) which

have been shown to be immunogenic. Strasser *et al* (173) incorporated RNAi technology to downregulate β1,2-xylosyltransferase and α1,3-fucosyltransferase genes in *Nicotiana benthamiana* (close relative of tobacco plants) plants to generate antibodies with human-like N-glycan structure. The resultant plant expressed 2G12 antibody (p2G12) with human-like N-glycan structures and exhibited similar binding kinetics and neutralisation potency as its Chinese hamster ovary (CHO)-derived 2G12 counterpart (CHO cell line is used to produce 2G12 for clinical trials) (173). Concerns over the manufacturing process were addressed with the establishment of good manufacturing practices (GMP) for plant antibody expression (174). Subsequently, GMP compliant p2G12 was shown to be safe and well tolerated in humans (phase I clinical trial, Eudract No. 2009-015609-38) (174). However, challenges with achieving consistent Ab yields for different bNAbs have been observed (175) and need to be addressed for this system to become a viable alternative.

1.8.1.2. Second generation pbNAbs

Second generation bNAbs [N6 and VRC01] have been produced using plant-based systems [*Nicotiana benthamiana* (N6 and VRC01) or *Nicotiana tabacum* (tobacco plant) (VRC01)] in proof-of-concept studies (176,177). Both pN6 and pVRC01 exhibited similar binding kinetics and neutralisation potency when compared to their HEK293 (human embryonic kidney cells) cell line expressed counterparts (176,177). Crucially, antibody yields were 49 mg/kg and 80 – 100 mg/kg for pN6 and pVRC01, respectively, a positive outcome given the informal 30 mg/kg manufacturing feasibility lower limit (176,177). pN6 also exhibited better antibody dependent cell cytotoxicity (ADCC) activity [due to its greater affinity to FcγRIIIa (Fc Gamma receptor IIIa, located on the surface of select leukocytes through which ADCC is initiated), 8× higher affinity than

mammalian expressed N6] in comparison to the mammalian expressed N6 (176). Overall, pbNAbs show great potential as an alternative antibody manufacturing platform and are likely to be crucial in making immunotherapy and immunoprophylaxis affordable.

1.8.2. Increasing antibody *in vivo* half-life

Multiple techniques have been developed to improve the serum half-life of bNAbs *in vivo*. This includes the introduction of mutations to the FC region thereby preventing the early degradation of bNAbs as explained in 1.8.2.1 below. Examples of these mutations are the LS mutation (M428L/N434S), YTE mutations (M252Y/S254T/T256E), T307A/E380A/N434A mutations, and the N434A mutation to name a few (178–180). In this section, we focus on LS-antibodies as they represent the most advanced clinical candidates currently under development.

1.8.2.1. LS mutations improve antibody affinity to the neonatal Fc receptor (FcRn)

A second strategy is the engineering of antibodies with a longer half-life thereby reducing the required frequency of antibody administration to maintain serum neutralization titres. Antibody half-lives are controlled by the FcRn receptor through the endolysosomal pathway (181,182). During endocytosis, FcRn binds to IgG (immunoglobulin g), unbound IgG are directed to the lysosome for degradation and FcRn bound IgG are released back into circulation thereby improving their *in vivo* half-lives (181,182). FcRn-IgG binding and dissociation occurs in a pH dependant manner with binding occurring at a pH < 6.5 (within the endosome) and subsequent

disassociation at pH 7.4 (physiological pH: intracellular space, blood, mucosa, etc) (181,182). IgG affinity for FcRn can be significantly improved through the introduction of M428L and N434S mutations (referred to as LS mutations) into the Fc (fragment crystallizable) region of the antibody (123,136,146,183). The resultant increase in affinity translates to an overall increased half-life and has been reported in the literature with various bNAbs (Table 1.2) (123,136,146,183).

Table 1. 2: Enhancement in antibody half-life. Antibody half-life improvement was achieved through the introduction of LS mutations (M428L and N434S) into the Fc region of the antibody.

Antibody	Half-life (in days)	Reference
VRC01-WT VRC01-LS	4.7 11.8	Ko *et al* (183)
VRC01-WT VRCO1-LS	7.3 11.8	Saunders *et al* (123)
CAP256- VRC26.25-WT CAP256-VRC26.25-LS	4.4 8.8	Julg *et al* (136)
N6-WT N6-LS	3 8	Julg *et al* (146)
10E08-WT 10E08-LS	5 10	Kwon *et al* (184)

Notes: These mutations promote IgG-FcRn binding thereby preventing degradation in the lysosome thus improving antibody half-life. Half-lives as observed in Non-Human Primates. WT: Wild type.

The introduction of the LS mutations has been shown to not affect IgG binding to FcγRIIIa and by extension FcγRIIIa-mediated ADCC activity (183). Moreover, LS-antibodies exhibit the identical Env binding kinetics and *in vitro* neutralisation potency as their wild type (WT) counterparts (146).

1.8.2.2. LS-antibodies exhibit better efficacy over their WT counterparts *in vivo*

To determine the efficacy of LS-antibodies in comparison to WT-antibodies, Ko *et al* (183) intravenously infused three groups of NHP (n = 12 per group) with either human IgG (control group), VRC01-WT (half-life 4.7 days) and VRC01-LS (half-life 11.8 days) antibodies. The NHPs were then challenged intrarectally with SHIV-BaLP4 five days later. VRC01-LS exhibited superior efficacy protecting 7/12 NHP in comparison to 2/12 and 0/12 for VRC01-WT and human IgG, respectively (183). This superior efficacy was due to the improved half-life (resulting in increased serum concentration for a longer duration following a single antibody infusion) and most importantly, the localisation of VRC01-LS at higher concentrations than VRC01-WT, within the rectal mucosa, the site of infection (183). The localisation and persistence of VRC01-LS in the rectal mucosa (VRC01-LS > 100 ng/ml to < 10 ng/ml over 70 days vs VRC01-WT > 100 ng/ml to 0 ng/ml in 28 days) was driven by the FcRn receptor transporting VRC0-LS from the blood to the vaginal and rectal mucosa sites (183).

In a follow up comparison study, Saunders *et al* (123) assessed the long-term performance of VRC01-LS in comparison to VRC01-WT. Three groups (control IgG, n = 4; VRC01-WT, and VRC01-LS; n = 3 for both) of NHP were infused with four doses (week 0, 2, 5, and 8) of antibody and intrarectally challenged with SHIV-BaLP4 52 days (week 15.5) after the final dose (123). VRC01-LS exhibited 2.5-fold higher trough serum concentration in comparison to VRC01-WT (123). Moreover, VRC01-LS serum concentrations dropped below 0.1 µg/ml at week 22 (i.e., maintained protective serum concentrations for ~5 months) compared to week 15.5 for VRCO1-WT (123). Unsurprisingly, VRC01-LS protected all three NHP (12.5-week observation period

post challenge) from infection, with VRC01-WT managing to protect 2 of the 3 NHP (all control group NHP were infected) (123). Consistent with the earlier study, VRC01-LS persisted longer in the rectal tissue than VRC01-WT (3.5-fold higher concentration than VRC01-WT at day 54, final day of observation) (123).

In summary, LS mutations have been shown to improve antibody half-lives thereby reducing the required frequency of infusion in a clinical setting. Moreover, increased FcRn affinity results in both increased antibody concentrations within the serum and at the relevant mucosal sites (Rectal and vaginal mucosa) both of which correlate with protection against HIV-1. LS-antibodies in combination with plant production could have a significant impact in reducing the overall cost of immunotherapy and immunoprophylaxis. Whilst LS-antibodies have been successfully produced using the plant production platform, the resultant yield and in vivo half-life were disappointing, indicating that further refinement of this system is required (175).

1.8.3. Adeno-associated virus (AAV) antibody expression

This technique employs AAV-viral vectors to deliver bNAb genomic material into the host thereby using the host machinery for *in vivo* production of bNAbs (185,186). These antibody genes are expressed episomally without the need for integrating into the host genome, thus making AVV-vectors safe to use (185,186). The selection of AAV serotypes not currently circulating within the target population is crucial for the success of this approach given the potential for immunological neutralisation (185,186). Overall, AAV-mediated antibody expression may contribute significantly towards an affordable, real-word application of HIV immunotherapy/immunoprophylaxis strategies.

1.8.3.1. AAV-antibodies exhibit sustained expression and confers protection against viral challenges.

AAV-mediated antibody expression may be preferred over actual antibody infusion as sustained high antibody levels can be achieved through fewer dosages. Balazs *et al.* (138) demonstrated in humanised mice that b12 and VRC01 expressed from AAV vectors maintained serum concentration as high as 100 µg/ml for the duration of the study (7 to 12 weeks). Moreover, AAV expressed b12 and VRC01 offered full protection at 34 µg/ml and 8.3 µg/ml, respectively, against intravenous challenge with the NL4-3 strain (138). Crucially, due to high AAV-b12 serum levels, protection from infection was observed even at viral inoculum levels100× higher than is necessary to establish infection (138). In a follow up study, Balazs *et al.* (124) showed (in humanised mice) that high serum concentration of AAV-VRC01 translated to full or partial protection after intravenous challenge with two clinically relevant HIV-1 strains (JR-CSF and REJO.c). Furthermore, AAV-VRC01 protected against low-dose-repeated (weekly challenge for 15 weeks) JR-CSF intravaginal challenge (to mimic heterosexual HIV-1 transmission) for all but two humanised mice (vs no protection in the AAV-luciferase control group) (124).

The more potent AAV-VRC07 also exhibited sustained high serum concentrations (as high as 100 µg/ml) which translated to full protection despite low-dose-repeated (weekly for 21 weeks) intravaginal challenges with REJO.c virus (124). In another study, Van den Berg *et al.* (187) showed that AAV-CAP256 resulted in sustained antibody expression (with a serum concentration of 8 µg/ml – 60 µg/ml) in humanised mice for

as long as 24 weeks (study limit); juxtaposed with the AMP trial (128) antibody infusion schedule of once every eight weeks, the potential of AAV-antibodies is unmatched. Taken together, these studies demonstrate that AAV-mediated immunoprophylaxis strategies result in sustained antibody expression for periods longer than can be achieved with using a single antibody infusion and that concentrations of *in vivo* expressed bNAbs are higher than necessary to confer protection despite repeated viral challenges from diverse HIV-1 strains.

In addition to achieving sustained, high antibody expression that translates to protection, AAV-vector-mediated expression of bNAbs [e.g., AAV-CAP256 (187)] have been shown to have similar *in vitro* potency as their mammalian expressed counterparts. This indicates no loss in potency with the change of expression system. However, as with antibody infusion, AAV-antibody monotherapy and single-bNAb-PrEP has limited use due to pre-existing or *de novo* mutations (124,138). Li *et* al (188) demonstrated that bispecific antibodies (iMab-PG9, iMab-PGT123 and iMab-NIH45-46) can be expressed in humanised mice using AAV vectors and achieve sustained antibody expression (up to 25.83 ± 6.97 μg/ml, 20.12 ± 5.06 μg/ml, and 22.28 ± 6.41 μg/ml, respectively) for the duration of the study (24 weeks) with similar potency as their mammalian expressed counterparts (188).

Further preclinical testing in NHPs, which have similar mucosal tissue and immune responses to humans, has been used to further validate these AAV immunoprophylaxis strategies (189). Saunders *et al* (190) tested AAV-VRC07 in six NHP and observed increasing VRC07 titres for four weeks in all six macaques with mean

peak serum concentrations of 38.2 μg/ml. Subsequently, anti-VRC07 responses resulted in the rapid clearance in three macaques with the other three exhibiting sustained expression (maximum serum concentration ≤ 10 μg/ml) for a further 13 weeks (limit of observation) (190). All six macaques were challenged intrarectally with SHIV-BaLP4 five and half weeks after AAV-VRC07 administration; two developed systemic infection and the other four were protected for the six-week observation period (all six-control group AAV-non HIV IgG macaques got infected) (190). One of the two infected macaques had non-detectable VRC07 serum titres days before the challenge while the other macaque exhibited the lowest serum concentration on day of challenge, possibly explaining the lack of protection in these instances (190). Overall, AAV-VRC07 exhibited sustained antibody serum concentrations which translated to protection against SHIV acquisition and paved the way for human clinical evaluation.

1.8.3.2. AAV- mediated delivery of antibody encoding sequences is safe and well tolerated in healthy individuals

Results of the first ever AAV vector mediated-antibody phase I human clinical trial (NCT01937455) were published in 2019 showing that AAV-PG9 is safe to use and was well tolerated (191). Unfortunately, serum PG9 was undetectable by ELISA (Enzyme-linked immunosorbent assay) but detectable indirectly by serum-HIV-1 pseudovirus neutralisation assay, suggesting low expression (191). The minimum ELISA detection limit was 2.5 μg/ml indicating that exceptionally potent antibodies like CAP256 [median IC_{50} 0.75 μg/ml (136)] could have gone undetected but would still offer robust protection against HIV-1 infection. Nonetheless, Priddy *et al* (191) suggest that future clinical trials should consider using alternative AAV serotypes as the low antibody concentrations are likely due to pre-existing immunity to the AAV serotypes

used in the trial. Moreover, they suggest the use of other gene expression cassettes in addition to testing at higher dosages. A second phase I clinical trial (NCT03374202) testing the more potent VRC07 is on-going with results expected in 2027. Early results show that AAV-VRC07 was well tolerated, and expression was detectable even a year after administration (192). Although the maximum AAV-VRC07 expression (< 2 µg/ml) is still low in comparison to levels attained through antibody infusion methods [e.g., levels as high as 50 µg/ml were achieved through intravenous VRC01 administration (140)], this study serves as an important proof of concept (192,193).

1.9. Study rationale and summary

1.9.1 Study rationale

Sub-Saharan Africa and particularly South Africa is the region most affected by the HIV-1 pandemic. Within this region, HIV-1 subtype C is the most predominant subtype. Therefore, novel anti-HIV-1 agents should be formulated to target this subtype for maximal impact on the HIV-1 pandemic. Bi/tribNAbs could potentially have a major impact in the fight against HIV-1 especially when used to complement the current ART regimens. Here we report the development of two novel bispecific antibodies, iMab-CAP256 (paper 1) and iMab-N6 (paper 2), specifically engineered to improve the neutralization breath and/or potency over the individual parental monoclonal bNAbs with a particular interest in subtype C enhanced neutralization coverage and/or potency. These two bispecific antibodies comprise a host CD4 targeting antibody ibalizumab (iMab) and a Env targeting antibody CAP256-VRC26.25 (CAP256, targets the V2 glycan) or N6 (CD4bs antibody).

1.9.1.1. Inclusion of iMab in the bibNAb configuration

Ibalizumab is humanised monoclonal antibody that exhibits exemplary broadly neutralizing coverage (92% neutralisation coverage against a panel of diverse HIV-1 subtypes) and moderate potency (median IC_{50} of 0.03 µg/ml) (194). Moreover, iMab is the only FDA approved monoclonal antibody (under the name Trogarzo®) for use as HIV-1 salvage therapy (195). iMab was included in the bibNAb configuration to take advantage of its binding to the host CD4 receptor, thereby concentrating the bibNAbs at the site of infection. The concentration of bibNAbs at the site of infection by iMab has been shown by Pace *et al* (163) and Huang *et al* (161) to contribute to the enhancement in potency of the bibNAb over the parental antibodies. Additionally, iMab was incorporated into the iMab-CAP256 bibNAb conformation to compensate for CAP256's moderate breadth [57% breadth against diverse subtypes and 70% against subtype C (107)].

1.9.1.2. Inclusion of CAP256 in the bibNAb configuration

CAP256 is an exceptionally potent (median IC_{50} of 0.001 µg/ml) bNAb with moderate breadth (57% breadth against diverse subtypes and 70% against subtype C) and was isolated from an HIV-1 subtype C infected individual in South Africa (107). It targets a quaternary, glycan dependent epitope of the Env V1/V2 apical loop and is the only bNAb shown to protect macaques at very low serum concentrations of <0.75 µg/ml against SHIV challenge (136). Therefore, CAP256 was selected due to its exceptional potency especially against HIV-1 subtype C. Furthermore, CAP256-LS is currently under clinical development (safety and pharmacokinetics) in a phase I clinical trial

(PACTR202003767867253) in both HIV-1 negative and positive women in South Africa (196,197).

1.9.1.3 Inclusion of N6 in the bibNAb configuration

The N6 bNAb, which targets the CD4bs, was chosen due to its impressive combination of breadth and potency (98% breadth with a median IC_{50} of 0.066 µg/ml) against HIV-1 subtype C (109). This high performance against subtype C was also observed by Wagh *et al* (156) were N6 exhibited an IC_{80} coverage of 95-96%, outperforming seven other bNAbs. Like CAP256, N6-LS is also under clinical development, entering a phase I clinical trial (NCT03538626) to evaluate the safety, tolerability, and pharmacokinetics.

1.9.2. Engineering iMab-CAP256 and iMab-N6

iMab-N6 and iMab-CAP256 were designed based on the conventional antibody architecture to avoid potential unfavourable pharmacokinetics and manufacturing difficulties that may emerge with designs that deviate from the typical IgG structure (Fig. 1.4) (161,198) [different bibNAb designs are reviewed in (199–202)]. To optimise the production of the bibNAbs, knob-in-a-hole (162) and CrossMab$^{CH1\text{-}CL}$ (203) mutations were introduced *in silico* (Fig. 1.4). The knob-in-a-hole (162) mutations (Fig. 1.5) ensures the exclusive dimerisation of the CAP256/N6 heavy chain with the iMab heavy chain whereas the CrossMab$^{CH1\text{-}CL}$ (203) domain-swopping (Fig. 1.5) ensures that the iMab light chain pairs exclusively with the corresponding iMab heavy chain. Together, these mutations eliminate the assembly of the unwanted antibody by-products and allow for the preferentially assembly of the bispecific configurations depicted in Fig

1.5. Crucially, the addition of the CrossMab$^{CH1\text{-}CL}$ mutations does not alter the binding affinity and specificity of the bibNAbs (203).

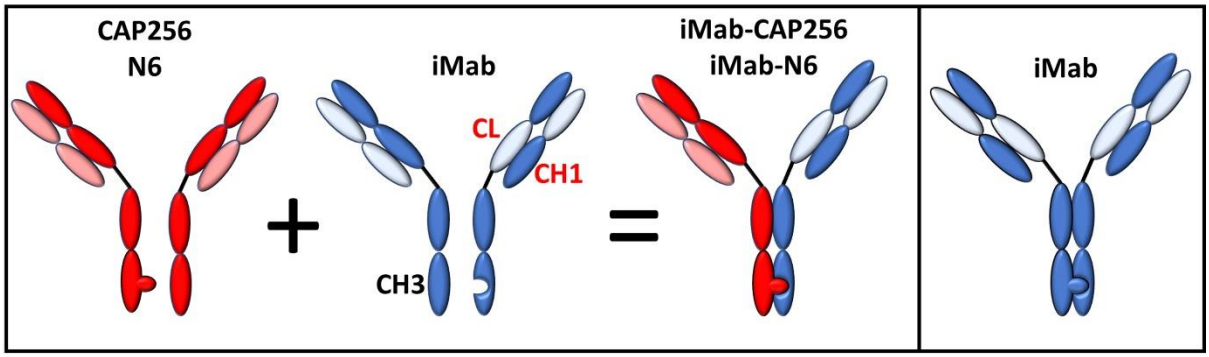

Figure 1. 4: Schematic of iMab-CAP256, iMab-N6 and iMab. Left panel bibNAbs were designed based on the traditional IgG "Y" shaped structure to avoid potential unfavourable pharmacokinetics and manufacturing difficulties. CH3 "Knob" mutations were introduced. CAP256/N6 was paired with hole – CrossMab$^{CH1\text{-}CL}$ iMab to produce iMab-CAP256/N6. Right panel iMab knob-in-a-hole – CrossMab$^{CH1\text{-}CL}$ was used as an assembly control. Key: CL: light chain constant region, CH1/3: Heavy chain constant region domain 1/3.

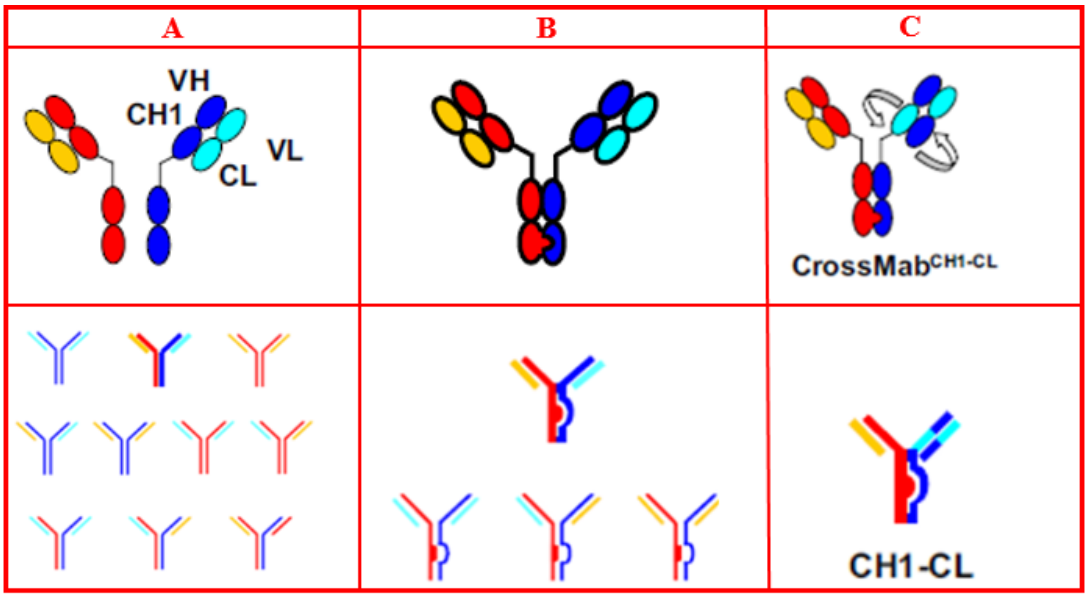

Figure 1. 5: Schematic showing the elimination of possible antibody by-products with the addition of knob-in-a-hole and CrossMabCH1-CL mutations. A Unmodified heavy and light chains of two independent antibodies (two light chains and two heavy chains) may merge to form ten different antibody configurations with the desired bibNAb product indicated in bold (nine side products and one bibNAb). B Introduction of knob-in-hole mutations into the CH3 region of the respective heavy chains reduces unwanted side products, favouring a 25 % chance in the formation of the desired bibNAb. C Domain swapping of one of the heavy chain (CH1) region with that of the light chain constant region (CL) (CrossMab$^{CH1\text{-}CL}$) combined with the knob-in-hole mutations results in the desired bibNAb without any of the side products. The binding affinity and specificity of bispecific antibodies engineered using the CrossMab$^{CH1\text{-}CL}$ mutations has been shown to be equivalent to the wildtype parental monoclonal antibodies. [Image sourced from (203)]

1.9.3. Study summary

In this study we described the design, expression, purification, and characterization of two novel bibNAbs, iMab-Cap256 (Chapter 2) and iMab-N6 (Chapter 3). The *in vitro* activity of the antibodies was evaluated against a diverse panel of HIV-1 pseudoviruses (n = 20 and 21 for iMab-CAP256 and iMab-N6, respectively, inclusive of the global HIV-1 pseudovirus panel (204)) in an *in vitro* neutralisation assay. We report that both bibNAbs exhibited better neutralisation coverage (iMab-CAP256: 20/20 vs iMab: 18/20 vs CAP256: 16/20 and iMab-N6: 21/21 vs N6: 20/21 vs iMab: 19/21) than their parental bNAbs, neutralising both dual and single sensitive (i.e., resistant to one parental bNAb) viruses within the pseudovirus panel tested. The relatively small pseudovirus panel utilized in this study limits our ability to draw definitive conclusions regarding the breadth of both bibNAbs. However, both iMab-CAP256 and iMab-N6 will likely have an impressive neutralisation coverage of (>92% and >98%, respectively) driven by the broadest parental bNAb, iMab and N6 in the respective bibNAb combinations.

Remarkably, the iMab-CAP256 was shown to be on average 4× and 59× more potent than CAP256 and iMab, respectively, and 10× more potent than the parental combination (iMab+CAP6) against the dual sensitive pseudoviruses within the evaluated panel. We have not done any studies to elucidate the mechanism underlying iMab-CAP256's potency but this impressive enhancement in potency (especially over the combination) suggests that there is dual engagement of the iMab and CAP256 moieties resulting in improved avidity. Moreover, Pace *et al* (163) and Huang *et al* (161) studies suggest that the localisation of the bibNAb at the cell surface by the iMab moiety contributes to the enhancement in potency. In comparison to other potent

bibNAbs reported in the literature, iMab-CAP256 exhibited 3× and 10× better potency than 10E08-iMab (161) and PG9-iMab (163) respectively against the same pseudovirus panel. Overall, these findings suggest iMab-CAP256 may be one of the most potent bibNAbs engineered to date and should be considered for future clinical development.

Interestingly, iMab-N6 exhibited no enhancement in potency over both parental bNAbs and the parental bNAb combination suggesting a lack of synergy between the two Fab (antigen binding fragment region) moieties. However, iMab-N6 still has potential for clinical development due to its exceptional IC_{80} coverage at < 1 µg/ml (90% vs 86% for N6 and 48% for iMab). The AMP trial established an *in vitro* IC_{80} coverage at < 1 µg/ml as a potential predictor of clinical success. To determine if this impressive IC_{80} coverage at < 1 µg/ml holds against a broader panel of pseudoviruses (n = 97), we generated Bliss-Hill model IC_{80} predictions for iMab+N6 combination. Subject to actual iMab-N6 testing, the iMab+N6 combination Bliss-Hill predicted data (median IC_{80} of 0.05 µg/ml and 98% IC_{80} < 1 µg/ml neutralisation coverage) suggests that a IC_{80} coverage of 98% at < 1 µg/ml will be achievable by this bibNAb. Therefore, despite the lack of potency enhancement, iMab-N6 does show potential for further clinical development.

1.9.4. Failed bibNAbs

In addition to iMab-CAP256 and iMab-N6, we sought to engineer additional bibNAbs (iMab-PRO140, CAP256-N6, and PRO140-CAP256) specifically intended on improving neutralization breadth and/or potency, particularly against HIV-1 subtype C strains. Unfortunately, we encountered several challenges in producing these

bibNAbs. A summary of the challenges and the attempts to overcome these are provided in Appendix A.

1.9.5. Future work and conclusions

Passive immunization strategies, including vector-mediated immunoprophylaxis, and antibody based-immunotherapies may be thwarted by the emergence of, or circulation of naturally occurring HIV-1 resistant strains. Incorporating the use of antibody combinations is the logical progression to improve the clinical potential of these strategies, however this raises concerns regarding manufacturing cost and regulatory requirements that may be come by bibNAb/TribNAb configurations. We demonstrated that both iMab-CAP256 (Chapter 2) and iMab-N6 (Chapter 3) exhibit great potential for further development. However, these bibNAbs will require evaluation against an expanded panel of pseudoviruses to elucidate their breadth and potency in comparison to other antibodies and to formally assess the IC_{80} criteria and indicator of clinical potential (AMP trial IC80 <1µg/ml). Moreover, *in vivo* studies in NHP will need to be carried out to determine the efficacy of these bibNAbs as either immunoprophylaxis and/or immunotherapy. The re-engineering of iMab-N6 to improve its potency is under consideration, as will be determining the mechanism of iMab-CAP256 potency and potential modification that may result in further improvement.

In summary, the future of HIV-1 PrEP, and therapy will likely involve bi/tribNAbs. Our research findings advance the field in that we were the first group to engineer and characterize the two novel bibNAbs iMab-CAP256 and iMab-N9 that exhibit excellent neutralization coverage, with iMab-CAP256 exhibiting further favourable potency

enhancement. These two bibNAbs show great potential for further clinical development and are particularly suitable for use in regions afflicted by HIV-1 Subtype C. Although further development and refinement will likely be required, it is envisaged that the proof-of-concept data provided here will result in the addition of these two bibNAbs to the current antibody repertoire and will favourably impact the trajectory of the HIV-1 pandemic.

Chapter 2: Engineering and characterising a novel, highly potent bispecific antibody iMab-CAP256 that targets HIV-1

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Engineering and characterising a novel, highly potent bispecific antibody iMab-CAP256 that targets HIV-1

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Abstract

The existing repertoire of HIV-1 patient derived broadly neutralising antibodies (bNAbs) that target the HIV-1 envelope glycoprotein (Env) present numerous and exciting opportunities for immune-based therapeutic and preventative strategies against HIV-1. Combination antibody therapy is required to ensure greater neutralization coverage and limit Env mediated escape mutations following treatment pressure. Engineered bispecific bNAbs (bibNAbs) assimilate the advantages of combination therapy into a single antibody molecule with several configurations reporting potency enhancement as a result of the increased avidity and simultaneous engagement of targeted epitopes. We report the engineering of a novel bibNAb (iMab-CAP256) comprising the highly potent, CAP256.VRC26.25 bNAb with anticipated extension in neutralization coverage through pairing with the host directed, anti-CD4 antibody, ibalizumab (iMab). Recombinant expression of parental monoclonal antibodies and the iMab-CAP256 bibNAb was performed in HEK293T (Human embryonic kidney 293 T antigen) cells, purified to homogeneity by Protein-A affinity chromatography followed by size exclusion chromatography. Antibody assembly and binding functionality of Fab moieties was confirmed by SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) and ELISA, respectively. Breadth and potency were evaluated against a geographical diverse HIV-1 pseudovirus panel (n = 20). Overall, iMab-CAP256 demonstrated an expanded neutralizing coverage, neutralizing single, parental antibody resistant pseudovirus strains and an enhanced neutralization potency against all dual sensitive strains (average fold increase over the more potent parental antibody of 11.4 (range 2 to 31.8). Potency enhancement was not observed for the parental antibody combination treatment (iMab + CAP256) suggesting the presence of a synergistic relationship between the CAP256 and iMab paratope combination in this bibNAb configuration. In addition, iMab-CAP256 bibNAbs exhibited comparable efficacy to other bibNAbs PG9-iMab and 10E08-iMab previously reported in the literature. The enhanced neutralization coverage and potency of iMAb-CAP256 over the parental bNAbs should facilitate superior clinical performance as a therapeutic or preventative strategy against HIV-1.

Keywords: HIV-1 prevention, HIV-1 therapy, Broadly neutralizing antibodies, Bispecific antibodies

Introduction

HIV-1 patient-derived broadly neutralising antibodies (bNAbs) develop in approximately 20% of infected patients [1]. The most potent and broadly neutralizing of these show promise in novel HIV-1 prevention and therapeutic strategies. Characterization of these bNAbs has identified five conserved regions of vulnerability on the HIV-1 envelope glycoprotein (Env) spike. These include the membrane-proximal external region of gp41 e.g. 10E8 [2], the CD4 binding site e.g. VRC01 and N6 [3, 4], the apical V1–V2 glycan supersite (e.g. PG9, CAP256-VRC26.25), the V3 glycan (e.g. PGT128) on the outer domain of gp120 [5–7] and the N332 glycan supersite (e.g. 2G12, PGT121, PGT128 and PGT135 [8]). More recently a quaternary, discontinuous epitope spanning

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the gp120-gp41 interface has been identified, targeted by PGT151, 3BC315 and 35022 [9, 10]. Additionally, non-patient derived antibodies targeting the HIV-1 primary or co-receptors on host target cells, display extraordinary neutralization breadth and potency against diverse viral isolates given their obligatory requirement in viral entry. Ibalizumab, a humanized, anti-CD4 antibody binds the second domain of human CD4 and prevents HIV-1 infection in a non-competitive manner [11–13], whereas PRO140 competitively inhibits HIV-1 entry through binding the extracellular loop 2 region of the chemokine receptor 5 [14].

As a preventative strategy, bNAbs have clearly demonstrated utility in both humanised—mouse and rhesus macaque challenge models. Passive antibody transfer studies have shown to protect against intravenous [15, 16] or mucosal challenge routes [intravaginal [17–20], intrarectal [21–25] and oral [26, 27] (MTCT infant macaque challenge model)]. Encouraged by these results, a major clinical trial (HVTN 703/HPTN 081 and HVTN 704/HPTN 085) is underway to evaluate the ability of bNAb VRC01 to protect against HIV-1 acquisition in humans and to establish the bNAb concentration required for protection [28]. In addition, vector mediated immunoprophylaxis may propel such strategies into mainstream, human application. Long-lived in vivo expression of bNAbs has been shown to confer protection against intravenous and mucosal challenge routes in both the humanised mouse and rhesus macaque challenge models [29–31].

As a therapy, bNAbs offer several advantages over existing antiretroviral drug therapies (ART), including; lower toxicity, improved pharmacokinetics [32], Fc-mediated immune effector function [33–35] and extension of treatment options to patients failing conventional ARTs. However, bNAb monotherapy may be limited due to rapid selection of viral escape mutations leading to viremia rebound [36–39]. This may be circumvented with the use of combination bNAb treatment, extending long-term viral suppression and reducing the emergence of viral escape [40–42].

Pertinent to both these strategies is the associated breadth and potency of the selected bNAbs. In the context of prevention, breadth of selected bNAbs must display adequate coverage to protect against a phylogenetically diverse viral swarm challenge [21] and be present in sufficient concentrations at the site of transmission in order to be effective. This may be subject to HIV-1 and host population bias including: the predominant circulating subtype and founder virus, HIV-1 transmission routes, host genetics, etc. Whilst in a therapeutic setting, broader coverage may be required to combat HIV-1 evolution under the selective pressure of the bNAb action. To date, no single bNAb achieves 100% neutralization against the available HIV-1 multi-subtype, Env-pseudotyped virus panels routinely used in the laboratory to represent clinically relevant HIV-1. Potency of bNAbs is also a critical determinant in protection, as lower mean plasma levels of the more potent PGT126 bNAbs demonstrate superior protection when compared to the less potent b12 bNAb in a humanized mouse model [43]. Overall, neutralization breadth and potency against selected HIV-1 pseudovirus panels can be improved with a combination of bNAbs [44, 45]. However, when translated into a clinical setting, the need to produce two or three different antibodies brings additional costs and regulatory considerations, highlighting the need for a better way of incorporating bNAbs as preventative agents [46].

Engineered bispecific (bi-) and trispecific bNAbs (tribNAbs) present a creative alternative to bNAb combination therapy. These antibody configurations comprise dual [47–51] or multi [52, 53] epitope targeting capabilities in a single antibody molecule. Paratope combinations include those that may solely target the HIV-1 Env [50, 52, 53] or combined Env and the relevant host-cell receptor targeting modalities [47–49, 51, 54]. BibNAbs have demonstrated exceptional improvement in neutralization coverage and potency relative to their parental antibodies [49, 50]. In particular, the 10E08-PRO140 and 10E08-iMab bibNAbs achieved 99% and 100% neutralization coverage of a 118 pseudovirus panel, respectively [49].

HIV-1 subtype C continues to dominate the global pandemic, accounting for ~47% human infections. Unsurprisingly, the geographical distribution of subtype C strains overlap with those regions most severely affected by the HIV-1 pandemic, including Eastern and Southern Africa. The CAP256.VRC26.25 bNAb was isolated from an HIV-1 subtype C patient and targets a quaternary, glycan dependent epitope of the V1/V2 apical loop of the HIV-1 Env spike [7, 55]. CAP256.VRC26.25 displays exquisite potency against sensitive viral isolates (median 50% inhibitory concentration (IC_{50}) of 0.001 μg/ml) yet reduced neutralization breadth, achieving 57% neutralization of diverse subtypes and 70% of subtype C isolates [55]. Julg and colleagues [23] demonstrated the superior in vitro potency of CAP256-VRC26.25-LS (LS-incorporation of the LS mutation into the Fc portion to increase in vivo antibody half-life) mediated complete protection at previously unreported, low serum levels (< 0.75 μg/ml) against intrarectal, SHIV challenge in rhesus macaques. Furthermore, in 2016, Wagh and colleagues [45] reported on the optimal bNAb combinations for the treatment and prevention of HIV-1 subtype C infection. The authors concluded that the best-in-class 2, 3 and 4 bNAbs combinations all contained CAP256-VRC26.25, with preference

for the 3 or 4 combinations due to overall coverage and maximal percent inhibition. Taken together, these studies highlight the potential clinical benefit that may be derived from CAP256.VRC26.25, with the possibility of specific tailoring to the treatment and/or prevention of subtype C infections. However, its usage may ultimately be confined to combination immunotherapy due to limited neutralization breadth. To this end, we sought to improve the breadth of CAP256.VRC26.25 through engineering a novel bibNAb (iMab-CAP256) and pairing it with the host cell directed anti-CD4 antibody, ibalizumab (iMab). IMab was selected as it demonstrates an expanded coverage over CAP256.VRC26.25, neutralizing >90% of diverse HIV-1 Env subtypes at a median IC_{50} < 0.1 μg/ml. In addition, iMab has an established safety record in human clinical trials [56–58], receiving FDA (Food and Drug Administration) approval for treatment of multidrug resistant HIV-1 [59] and it exhibits potency and breadth enhancement in bibNAb conformations [47, 49]. Below we describe the engineering of iMab-CAP256 bibNAb, confirm the functionality of individual Fab binding regions in the bispecific antibody configuration and demonstrate an improved breadth and potency relative to the parental monoclonal antibodies or combination treatment, against selected HIV-1 pseudoviruses.

Methodology

Reagents used in this study

The single plasmid, mAb expression vector (pMin) was generously donated by Balazs [30, 31] and modified to remove the CMV promoter and ITR flanking regions for optimal in vitro antibody expression. The following reagents were obtained from the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID (contributor in parentheses): Antibody expression vectors (heavy and light chain) for the 10E8 mAb (Dr. Mark Connors [2]) and VRC01 mAb (Dr. John Mascola [3]). The PG9-ibalizumab (PG9-iMab) bispecific antibody (Dr. David Ho and Dr. Craig Pace [47]).

Reagents for HIV-1 pseudovirus production including: TZM-bl (JC53-bl) reporter cell line (Drs. John C. Kappes, Xiaoyun Wu and Transzyme Inc. [60–64]), $pSG3^{\Delta env}$ (Drs. John C. Kappes and Xiaoyun Wu [64, 65]) and 20 complementing HIV-1 Env plasmids 25710, 246-F3.C10.2, 398f1 and CH119 (Drs. C. Williamson, M. Hoelscher, L. Maboko, and D. Montefiori [66, 67]), CNE8 and CNE55 (Drs. L. Zhang, H. Shang and D. Montefiori [66, 68]), PVO.4 and QH0692.42 (Drs. D. Montefiori, F. Gao and M. Li [69]), CAP210.2.00.E8 and CAP45.2.00.G3 (Drs. L. Morris, K. Mlisana and D. Montefiori [70]), DU156.12 (Drs. D. Montefiori F. Gao, S. Abdool Karim and G. Ramjee [70, 71]), DU422.01 (Drs. D. Montefiori, F. Gao, C. Williamson and S. Abdool Karim [70, 71]), ZM53 M.PB12 and ZM135 M.PL10a (Drs. E. Hunter and C. Derdeyn [70]), X1632 (Dr. D. Montefiori [66, 72]), TRO11 (Drs. F. Gao and D. Montefiori [66, 69]), X2278 and BJOX2000 (Drs. M. Thomson, A. Revilla, E. Delgado, and D. Montefiori [66]), CE0217 and CE1176 (Drs. R. Swanstrom, L. Ping, J. Anderson, and D. Montefiori [66]). The HIV-1 global pseudovirus panel is included in the abovementioned 20 plasmids.

Bispecific antibody construct design and synthesis

Bispecific antibody constructs were generated according to previously published protocols, and include the knob-in-a-hole [50] and $CrossMab^{CH1\text{-}CL}$ [73] technologies, to ensure correct in vitro assembly of bispecific antibodies. The corresponding iMab [12], CAP256 [55] and 10E08 [2] fragment antigen-binding (Fab) regions (PDB: 3O2D, 5DT1 and 4G6F respectively) were assembled in silico into the bispecific antibody conformation using SnapGene. The $CrossMab^{CH1\text{-}CL}$ mutation were added to iMab along with the "hole" mutations; T366S, L368A, Y349C and Y407V [50, 73] (Fig. 1a). The complimenting "Knob" mutations, T366W and S254C were introduced into the HIV-1 Env, targeting bNAbs, CAP256 and 10E08 (left panel, Fig. 1a). The modified amino acid sequences were sent to GeneArt for codon optimisation and gene synthesis. Unmodified amino acid sequences to express the parental monoclonal control antibodies were generated in tandem (CAP256 and 10E08). The iMab parental antibody contained the respective knob-in-a-hole and $CrossMab^{CH1\text{-}CL}$ to serve as a suitable assembly control (right panel, Fig. 1a). Synthesized antibody gene constructs were sub-cloned into a mammalian expression vector provided courtesy of Balazs [30, 31]. Large scale plasmid production was performed in *E. coli* DH5α cells and purified using the Qiagen Maxi plasmid isolation kit.

Antibody expression and purification

Antibodies were transiently expressed in the HEK293T cell line using a 1:3 DNA to PEI (Polysciences Inc.) transfection protocol. Twenty-four hours post transfection, the cell culture media was discarded and replaced with serum free media, SFMII (Thermo Fisher Scientific) supplemented with 2 mM glutamax (Thermo Fisher Scientific). Cell culture supernatants containing the expressed antibodies were harvested and replaced every 48 h for a maximum of 10 days. Harvested culture supernatants were centrifuged and filtered. Antibodies were purified by Protein-A affinity chromatography (Sigma-Aldrich) followed by size exclusion chromatography (SEC) (Fig. 1b) on a Superdex 200 PG 16/600 HiLoad column

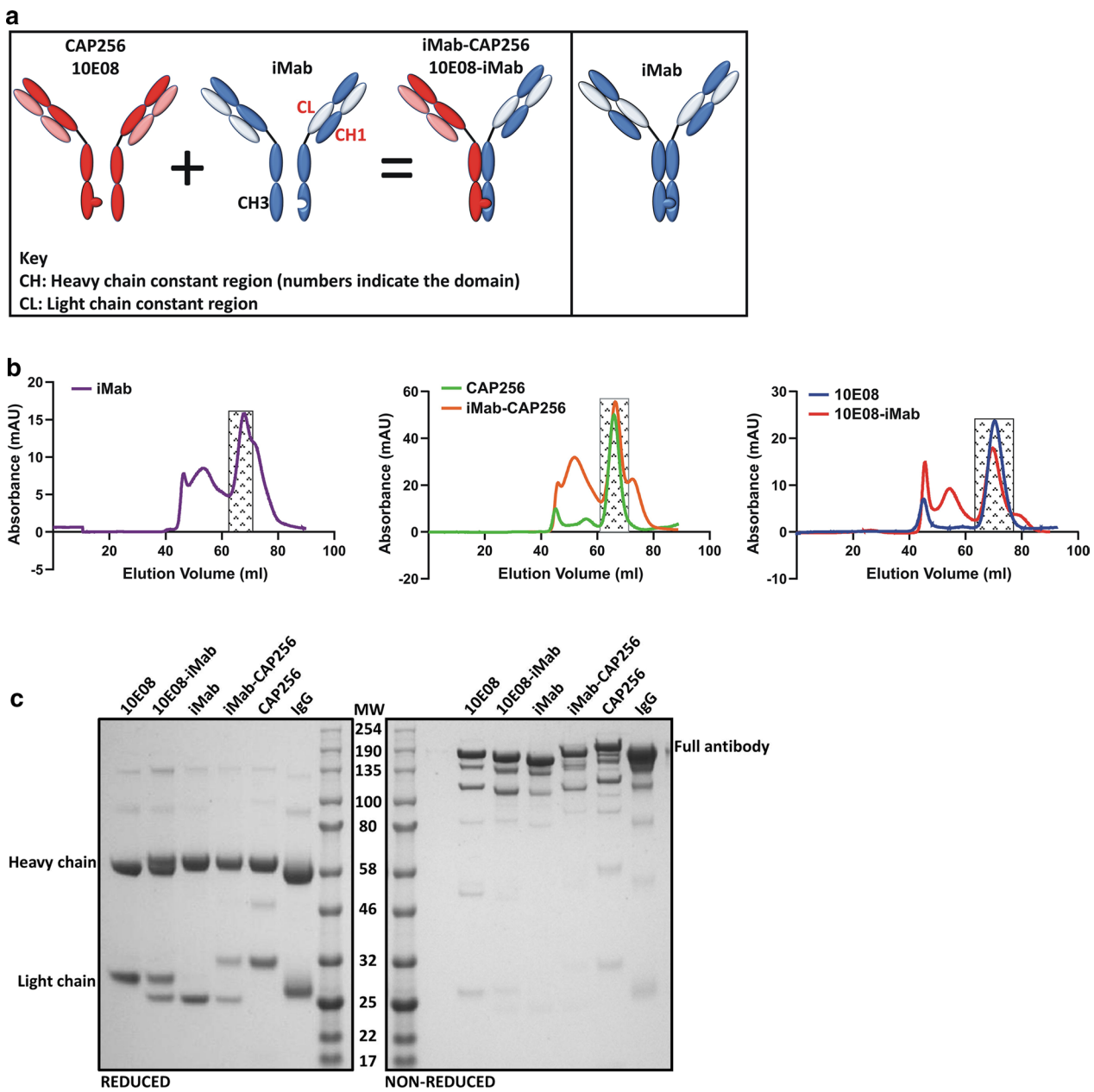

Fig. 1 Bispecific antibody design, purification and SDS-PAGE analysis. **a** Left panel: Schematic of the iMab-CAP256 or 10E08-iMab bibNAb, with 'knob' mutations introduced into the CH3 region of CAP256 or 10E08 and complementing 'hole' mutations introduced into the CH3 region of iMab. Additionally, the CH1 region and the CL region of iMab were interchanged to create a bibNAb with a Crossmab$^{CH1\text{-}CL}$ configuration. **a** Right panel: Schematic of iMab engineering using knob-in-a-hole and CrossMab$^{CH1\text{-}CL}$ to serve as a suitable assembly control. **b** Size exclusion chromatograms of Protein-A purified antibodies. Shaded rectangles represent collected and pooled fractions corresponding to the monomeric antibody conformation. **c** SDS-PAGE analysis of antibodies under reducing and non-reducing conditions post size exclusion chromatography. Purified human IgG was included as a positive control. Molecular weight (MW) is indicated in kDa

(GE Healthcare) to remove protein aggregates and oligomeric forms. Collected fractions were concentrated using an Amicon centrifugal-filtration device with a molecular weight cut-off 50 kDa. The purified proteins were resolved on an SDS-PAGE gel to confirm their purity.

Protein purification

HIV-1 monomeric ($gp120_{FVC}M$) or trimeric ($gp140_{FVC}GCN4$ or $gp140_{FVC}SOSIP$) Env conformations were recombinantly expressed by transient transfection (using PEI) or stably transfected 293F cell lines

[74]. All three constructs comprise the inferred Founder virus consensus C sequence (FVC) described previously [75]. The $gp120_{FVC}$Monomer and $gp140_{FVC}$GCN4 constructs were available in our laboratory and have been described previously [74]. The $gp140_{FVC}$SOSIP trimer was engineered using specific amino acid substitutions as described previously [76] (HxB_2 numbering system): A501C-T605C (gp120-gp41 intra-subunit disulphide bond), I559P (trimer stabilization), enhanced furin cleavage site by replacing REKR with RRRRRR in gp120 and introduction of a stop codon at position 664 to truncate the $gp41_{ECTO}$ domain, increasing solubility and homogeneity. The $gp140_{FVC}$SOSIP were exclusively expressed by transient co-transfection with a furin encoding plasmid (1:4 ratio of furin to $gp140_{FVC}$SOSIP) in HEK293T cells. Twenty-four hours post transfection; culture media was replaced with Freestyle 293 Expression media (Thermo Fisher Scientific). Harvested culture media, containing the expressed Env, was centrifuged and filtered to remove cellular debris and purified using *G. nivalis* lectin affinity chromatography, and SEC, as described previously [74]. Recombinant human two-domain CD4 (2dCD4) was expressed in *E. coli* BL21 (DE3) and purified from inclusion bodies, under denaturing conditions using IMAC, and refolded in a series of glutathione containing phosphate buffers, as described previously [77].

Functional evaluation of bibNAb Fab moieties

The functionality of the Fab moieties was determined by an ELISA. To determine HIV-1 Env binding, decreasing concentrations of HIV-1 Env ($gp140_{FVC}$GCN4, $gp120_{FVC}$Monomer or $gp140_{FVC}$SOSIP) were captured by immobilized *G. nivalis* lectin (Sigma) (200 ng/well) on a 96 well ELISA plate (Maxisorb—Nunc). To assess binding to human CD4, decreasing concentrations of bacterially expressed 2dCD4 was immobilized directly on the 96 well ELISA plate. Plates were blocked for an hour [Dulbecco's phosphate buffered saline (Sigma), 0.05% (v/v) Tween 20 and 1% BSA]. Binding of bibNAbs and parental antibodies was then performed at a concentration of 1 µg/ml, with the exception of VRC01 which was used at a final concentration of 0.5 µg/ml. Bound antibodies were then detected using anti-human, horseradish peroxidase-linked secondary antibody (GE Healthcare) and standard chromogenic methodologies. Absorbance was read at 450 nm using an iMark microplate reader (Bio-Rad). For this assay, $gp120_{FVC}$Monomer was used as a negative control.

HIV-1 in vitro neutralisation assay

Antibody mediated neutralisation was assessed against a panel of 20 geographically diverse pseudoviruses using a single round HIV-1 pseudovirus infectivity assay in the TZM-bl reporter cell line, as described previously [78]. Briefly, antibody concentrations were prepared by tenfold serial dilution and tested at a maximum, final concentration of 4 µg/ml (10E08, 10E08-iMab and PG9-iMab) or 1 µg/ml (CAP256, iMab-CAP256 and iMab) or 0.5 µg/ml per parental antibody for parental combination treatments (total IgG concentration 1 µg/ml). Two hundred $TCID_{50}$ (50% Tissue Culture Infectious Dose) of pseudovirus was added to each well and incubated for an hour at 37 °C in 5% CO_2. Following this, 100 µl of TZM-bl cells [1×10^5 cells/ml in complete DMEM supplemented with DEAE Dextran (Sigma)] were added to each well and the plate was incubated for 48 h at 37 °C in 5% CO_2. The final concentration of DEAE Dextran in the assay was 20 µg/ml. Luciferase was determined using Bright Glo Luciferase reagent (Promega) according to the manufacturer's instructions and quantified using the Promega Glomax Explorer luminometer (Promega). Cell only and virus only control wells were included as appropriate controls. IC_{50} values were calculated using the non-linear regression function in GraphPad Prism 7 and represent the antibody concentration (µg/ml) required to achieve 50% pseudovirus inhibition.

Statistical analysis

Comparisons of median neutralization IC_{50s} between the iMab-CAP256 bibNAb and the parental antibodies (CAP256 or iMab) or parental antibody combination (iMab + CAP256) or additional bibNAbs (10E08-iMab or PG9-iMab) were performed using a non-parametric, *t*-test (Wilcoxon matched-pairs signed rank test) and two-tailed *p*-value. Where multiple comparisons between groups were performed, the level of significance was adjusted using Bonferroni correction (α/the number of comparisons). *p*-values were considered significant when the *p*-value was < 0.0166 using the Bonferroni correction for 2×2 comparison of the iMab-CAP256 bibNAb to each parental antibody (iMab or CAP256) or the parental antibody combination (iMab + CAP256) or when a *p*-value was < 0.025 for 2×2 comparison of iMab-CAP256 with each bibNAbs, 10E08-iMab or PG9-iMab. Statistical analyses were performed using GraphPad Prism 7.

Results

Antibody design and expression

iMab-CAP256 was based on the design of the 10E08-iMab bibNAb described by Huang et al. [49] and was engineered using CrossMab$^{CH1\text{-}CL}$ [73] and knob-in-a-hole [50] mutations (left panel, Fig. 1a). The introduction of these mutations has previously been shown not to affect the antigen binding regions, yet allows for

preferential heterologous heavy and light chain assembly, resulting in the production of functional bispecific antibodies in vitro [50, 73]. Constructs for the expression of 10E08-iMab, as described by Huang et al. [49] were synthesised in parallel and served as an appropriate comparative control for functional assembly, bispecificity and enhanced HIV-1 neutralisation. Sufficient quantities of bibNAbs (iMab-CAP256 and 10E08-iMab) and the parental monoclonal antibodies (CAP256, iMab and 10E08) were expressed in HEK293T cells and purified using Protein-A agarose (Fig. 1b). The SEC chromatograms indicate conformational heterogeneity of the Protein-A purified antibodies particularly for those antibodies expressed from the pMin backbone construct (iMab, CAP256, iMab-CAP256 and 10E08-iMab) compared to the parental 10E08 antibody construct (Fig. 1b). Elution fractions corresponding to the anticipated, correctly assembled antibody conformation (peak retention volume approximately 65 ml) were collected and pooled (shaded boxes, Fig. 1b). Following SEC purification, the antibodies were resolved on an SDS-PAGE under reduced and non-reduced conditions to confirm structural integrity and degree of purity (Fig. 1c). Under reducing conditions, both heavy and light chain resolved at the anticipated molecular weights across all antibodies (Fig. 1c). Two distinct light chain bands were discernible for both bibNAbs (iMab-CAP256 and 10E08-iMab) and correspond to the respective parental monoclonal antibody light chain constituents (left gel, Fig. 1c). Similarly, a discernible separation of the 10E08-iMab bibNAb heavy chains was observed and accurately corresponded to a slight difference in the migration of the iMab and 10E08 parental heavy chains respectively (left gel, Fig. 1c). Under non-reducing conditions, a prominent band was observed at > 150 kDa for bibNAbs and monoclonal antibodies and corresponds to the purified human IgG control (right gel, Fig. 1c). Additional lower molecular weight bands were observed across all purified antibodies; including the human IgG control presumably indicating SDS detergent-sensitive heavy and light chain isoforms.

Testing the functionality of the Fab moieties

Binding functionality of the HIV-1 Env targeting moieties (CAP256 or 10E08) and the host-directed anti-CD4 binding moiety, iMab was confirmed by ELISA (Fig. 2). Bispecific (iMab-CAP256 and 10E08-iMab) and parental (iMab, CAP256 and 10E08) monoclonal antibodies were tested against three different HIV-1 Env configurations based on the identical Env sequence (Consensus FVC sequence). These Env configurations included the gp120 monomeric, and the trimeric $gp140_{FVC}$GCN4 and $gp140_{FVC}$SOSIP configurations (Fig. 2a). As expected, CAP256, 10E08, iMab-CAP256 and 10E08-iMab did not bind to the $gp120_{FVC}$ monomer, as this configuration lacks the gp41ecto domain containing the 10E08 epitope nor does the monomeric configuration recapitulate the quaternary epitope targeted by CAP256 (purple trace, Fig. 2a). CAP256 binding was evaluated using quaternary specific V1/V2 epitope stabilized on the soluble 'native-like' SOSIP trimer. Both the CAP256 parental and iMab-CAP256 bibNAb bound the $gp140_{FVC}$SOSIP configuration confirming the functionality of these CAP256 moieties (green trace, Fig. 2a). The $gp140_{FVC}$SOSIP trimer is truncated at position 664 and therefore lacks the 10E08 epitope. To confirm binding functionality of the 10E08 parental antibody and the 10E08 moiety on the 10E08-iMab bibNAb, antibody binding was therefore assessed using the $gp140_{FVC}$GCN4 Env configuration. Both the parental 10E08 antibody and 10E08-iMab bibNAb bound the $gp140_{FVC}$GCN4 configuration, confirming functionality of the 10E08 moiety on both 10E08 and 10E08-iMab (blue trace, Fig. 2a). The CD4 binding site-targeting bNAb, VRC01, was included as an appropriate positive control and confirms structural integrity of the three HIV-1 envelope configurations tested (Fig. 2a). No binding of the parental iMab antibody was detected against any of the HIV-1 Env configurations tested (Fig. 2a). Bacterially expressed, human 2dCD4 was used to confirm binding of the iMab parental antibody and iMab moiety of the bispecific antibody conformations (10E08-iMab and iMab-CAP256; Fig. 2b). The 10E08-iMab and iMab-CAP256 bispecific demonstrated similar levels of binding to immobilized 2dCD4 compared to the parental iMab antibody and confirms functionality of the iMab moiety in the context of the bispecific configuration (Fig. 2b).

iMab-CAP256 displays enhanced neutralization potency over parental constituents

To determine the potency of iMab-CAP256, a series of neutralisation assays were conducted against a panel of 20 geographically diverse, HIV-1 pseudoviruses (Fig. 3a). Selected pseudoviruses included single sensitive (n = 6; 398f1, CNE55, QH0692.42, TRO11, ZM135.PL10a and X1632) or dual sensitive (n = 14) strains to CAP256 and iMab parental antibodies. We initially compared iMab-CAP256 to its constituent parental antibodies, iMab and CAP256 (Fig. 3a). As expected, iMab-CAP256 showed improved breadth over the parental antibodies, neutralizing all 20 HIV-1 pseudoviruses tested, compared to 16/20 for CAP256 and 18/20 for iMab. Interestingly, iMab-CAP256 was consistently found to be more potent than the parental antibodies CAP256 ($p = 0.0009$) and iMab ($p = 0.0001$) against the dual sensitive pseudoviruses tested, with the exception of CE1176, as observed by the lower IC_{50} value (Fig. 3a, b left panel and Additional file 1: Figure S1). Overall, when the IC_{50} values for

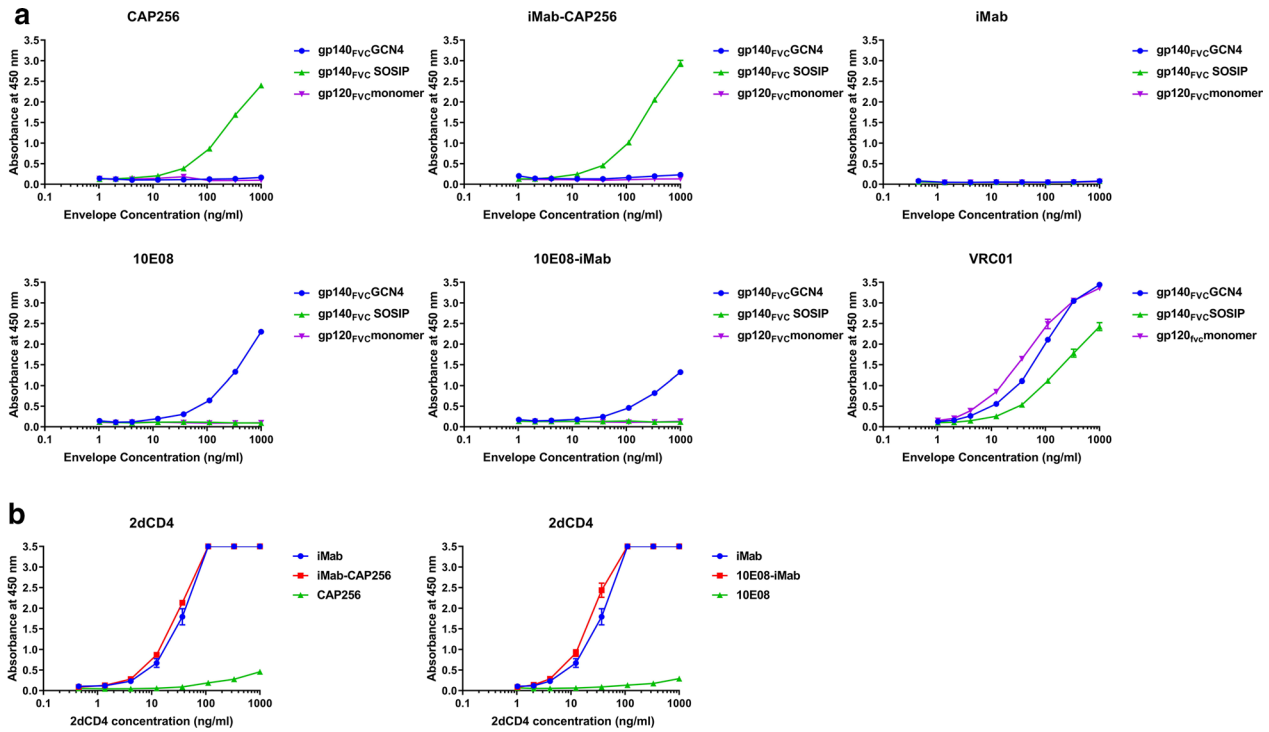

Fig. 2 Binding characterisation of purified bispecific antibodies by ELISA. **a** HIV-1 Env binding of bNAbs and bibNAbs were assessed against different immobilized HIV-1 Env conformations ($gp140_{FVC}$GN4, $gp120_{FVC}$Monomer, $gp140_{FVC}$SOSIP) and **b** the N-terminal two domain fragment of human CD4 (2dCD4) to assess their binding capabilities, specificity and bispecificity or lack thereof. The bNAbs and bibNAbs were tested at a concentration of 1 µg/ml except for VRC01, which was tested at 0.5 µg/ml. Secondary antibody concentration of 1 in 1000 was used for CAP256, iMab-CAP256, 10E08 and 10E08-iMab. For iMab and VRC01, a secondary antibody concentration of 1 in 2000 was used

iMab-CAP256 were compared to the more potent parental antibody (iMab or CAP256) IC_{50} against each dual sensitive pseudovirus, the bibNAb demonstrated a 2 to 31.8 fold increase in potency (Fig. 3a).

In addition, iMab-CAP256 exhibited an 11.4 fold increase in potency compared to the parental CAP256 antibody against the iMab resistant CNE55 pseudovirus (Fig. 3a). This enhancement in potency and breadth of bibNAbs, including 10E08-iMab, has been demonstrated previously [47–50] and is reiterated here. The 10E08-iMab bispecific neutralized all viruses tested (n = 14) and showed a 4 to 290 fold enhancement in potency over the more potent parental antibody (iMab or 10E08) in each instance (Fig. 3a). Since the iMab-CAP256 only demonstrated potency enhancement against the dual sensitive pseudoviruses tested (with the exception of CE1176), subsequent screening of 10E08-iMab and PG9-iMab and the combination iMab + CAP256 focused on these. The relative fold-potency enhancement observed for 10E08-iMab compared to iMab-CAP256 over their respective parental antibodies, is primarily due to the lower potency of the 10E08 parental compared to CAP256 against the viruses tested. Neutralization potency of iMab-CAP256 was compared to the bispecific PG9-iMab [47] which shares antigenic targets on the HIV-1 Env (V1/V2 loop quaternary epitope—PG9 and CAP256) and host-CD4 receptor (iMab). iMab-CAP256 showed enhanced potency compared to PG9-iMAb for 13/14 viruses tested, with a median IC_{50} of 0.00079 compared to 0.0079 µg/ml ($p = 0.0009$, only CAP256/iMAb dual sensitive pseudoviruses were included in this analysis; Fig. 3a, b right panel and Additional file 2: Figure S2).

This observed potency enhancement of iMab-CAP256 over its parental antibody constituents suggests the presence of a synergistic/co-operative neutralization mechanism between these targeted epitopes. To elucidate whether this is a result of dual epitope targeting or simultaneous engagement of these two epitopes, the bibNAb, iMab-CAP256, was compared to a combination treatment of the parental antibodies (iMab + CAP256). This assay was conducted against the dual sensitive HIV-1 pseudoviruses using equivalent molar concentrations of antibody paratope binding regions (i.e. 1 µg/ml of iMab-CAP256 and 0.5 µg/ml final concentration of each parental antibody (total IgG concentration 1 µg/ml).

a

Pseudovirus	Subtype	Antibody potency (IC_{50} = µg/ml)								
		Parental Ab			Combination		bibNAbs			
		CAP256	iMab	10E08	iMab + CAP256		iMab-CAP256		10E08-iMab		PG9-iMab
398F1*	A	>1	0.01292	ND	ND	(-)	0.052841	(0.2×)	ND	(-)	ND
246-F3.C10.2*	AC Recombinant	0.00718	0.01964	0.676	0.00658	(7.3×)	0.0009	(7.9×)	0.00384	(5.1×)	0.00567
CNE8*	CRF01_AE	0.0063	0.02826	0.04946	0.00803	(11.5×)	0.0007	(9×)	0.0025	(11.3×)	0.00854
CNE55*	CRF01_AE	0.0008	>1	ND	ND	(-)	0.00007	(11.4×)	ND	(-)	ND
PVO.4	B	0.08176	0.05668	2	0.05204	(5.4×)	0.00958	(5.9×)	0.01408	(4×)	0.1005
QH0692.42	B	>1	0.24	ND	ND	(-)	0.55	(0.43×)	ND	(-)	ND
TRO11*	B	>1	0.06426	ND	ND	(-)	0.1304	(0.5×)	ND	(-)	ND
X2278*	B	0.00256	0.07674	0.7221	0.00916	(39×)	0.00024	(10.7×)	0.00359	(21.4×)	0.00102
BJOX2000*	CRF07_BC	0.00192	0.60954	2.831	0.00335	(3.4×)	0.00097	(2×)	0.0021	(290×)	0.05235
CH119*	CRF07_BC	0.00954	0.09441	1.796	0.01853	(6.8×)	0.00274	(3.5×)	0.00185	(51×)	0.03389
25710*	C	0.00031	0.3978	0.1126	0.00083	(35.7×)	0.00002	(15.5×)	0.00099	(113.7×)	0.01462
CAP45.2.00.G3	C	0.00071	0.03957	1.39	0.00041	(1.7×)	0.00024	(3×)	0.0014	(28.3×)	0.00078
CAP210.2.00.E8	C	0.0035	0.03812	1.03	0.0082	(71.3×)	0.00012	(29.2×)	0.0045	(8.5×)	0.0074
CE0217*	C	0.00286	0.1201	0.4177	0.0082	(88.6×)	0.00009	(31.8×)	0.00341	(35.2×)	0.00289
CE1176*	C	0.0021	0.05623	1.366	0.00413	(1.2×)	0.00333	(0.6×)	0.00229	(24.6×)	0.00182
DU156.12	C	0.00617	0.01704	0.05273	0.01129	(6.2×)	0.00183	(3.4×)	0.00058	(29.4×)	0.00258
DU422.01	C	0.08207	0.02826	0.2697	0.0132	(9.2×)	0.00143	(19.8×)	0.00071	(39.8×)	0.0299
ZM53M.PB12	C	0.00386	0.08062	3.62	0.0021	(3.3×)	0.00064	(6×)	0.00224	(36×)	0.01374
ZM135M.PL10a	C	>1	0.08874	ND	ND	(-)	0.195	(0.5×)	ND	(-)	ND
X1632*	G	0.00121	>1	ND	ND	(-)	0.00158	(0.8×)	ND	(-)	ND

*Global Panel of HIV-1 Env Reference clones (Cat # 12670), NIH AIDS Reagent Program

KEY: 10, 1, 0.1, 0.01, 0.001, 0.0001, 0.00001

b

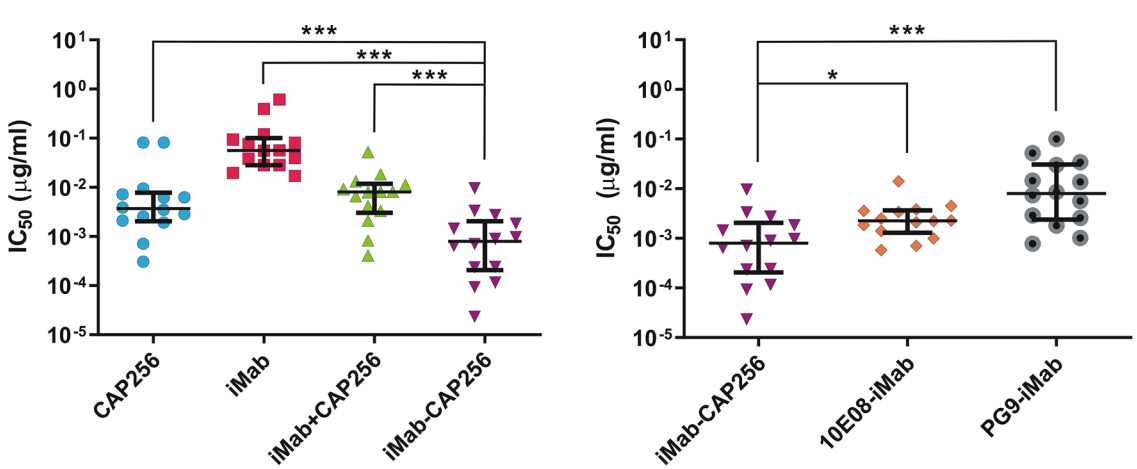

Fig. 3 Differences in potency between bNAbs (individually and in combination) and bibNAbs against 20 geographically diverse HIV-1 pseudoviruses. **a** IC_{50} values were calculated for each parental bNAb, bNAb combination and bibNAb. Values in (red) indicate the fold increase in potency of the bibNAb iMab-CAP256 or 10E08-iMab compared to the more potent parental bNAb (either iMab, CAP256 or 10E08). Values (red) for the iMab + CAP256 combination show the fold increase in potency of the iMab-CAP256 relative to the combination. The smaller the IC_{50} or the darker the red, the more potent the Ab is. CAP256 resistant or iMab resistant viruses were included in the panel to test the neutralisation activity of both the CAP256 and iMab moieties in the bibNAb. **b** Scatter plots representing the median IC_{50} titres of parental antibodies (CAP256 and iMab), parental antibody combination (iMab + CAP256) and iMab-CAP256 bibNAb (left panel) or bibNAbs; iMab-CAP256, 10E08-iMab and PG9-iMab (right panel), against the CAP256/iMab dual sensitive pseudoviruses. Statistical differences in neutralization were determined using non-parametric *t*-test (Wilcoxon matched-pairs signed rank test) with $*p < 0.05$ and $***p < 0.001$ represented. Errors bars indicate the interquartile ranges

iMab-CAP256 demonstrated enhanced potency (ranging from a 1.71 to 88.6 fold increase) over the parental combination treatment against all dual sensitive pseudoviruses tested ($p = 0.0001$, Fig. 3 and Additional file 3: Figure S3). The parental combination treatment demonstrated no improvement over the neutralization titres obtained for the more potent of the two parental antibodies, against the respective viruses tested (Fig. 3a and Additional file 3:

Figure S3). This indicates that the bispecific combination of the CAP256 and iMab moieties reported here are crucial for the observed enhancement in potency.

Discussion

In the absence of an effective, prophylactic HIV-1 vaccine, novel preventative strategies and therapeutics are needed to complement existing options. Technological advancements in the field of antigen specific, B-cell sorting have led to a dramatic increase in the discovery, isolation and characterisation of novel bNAbs from HIV-1 infected individuals [2–8, 55]. Clinical investigation of bNAb immunotherapy in HIV-1 infected patients and the prevention of HIV-1 in non-infected individuals are currently underway [36, 38, 39, 42, 79, 80]. BNAb combinations readily enhance the neutralization breadth and potency against a given HIV-1 pseudovirus panel [44, 45, 81], however these tend to be additive in nature [44]. Synergistic antibody combinations that result in increased potency have been reported e.g. PG9 + 10E8 [44], yet the improvements are limited and seldom exceed a twofold enhancement over the more potent, parental mAb in the combination. In contrast, bi/tribNAb configurations offer several advantages: their "single" molecule conformation supports manufacturing and regulatory registration convenience [53], while more importantly, their enhanced avidity may result in increased breadth and superior potency enhancement against HIV-1 [49, 50, 52–54].

We describe the engineering of a novel bispecific antibody comprising of the host-directed, anti-CD4 antibody iMab and HIV-1, V1/V2 targeting bNAb CAP256-VRC26.25 (CAP256). This bibNAb, iMab-CAP256, was designed using the CrossMab$^{CH1\text{-}CL}$ [73] and knob-in-a-hole [50] mutations (Fig. 1a). Binding ELISA's confirmed functionality of the individual Fab moieties and while this data is unable to confirm bispecificity in the true sense, i.e. simultaneous engagement of two differing antigenic targets, it clearly demonstrates binding functionality of the respective Fab moieties within this bibNAbs configuration (Fig. 2).

Despite the limited number of pseudoviruses tested, the neutralization breadth of the iMab-CAP256 was shown to be improved over the parental antibodies, neutralizing all viral strains tested (20/20) including single resistant strains (Fig. 3a). Neutralizing potency of the iMab-CAP256 bibNAb was also improved relative to the individual parental antibodies and the parental antibody combination, against all dual sensitive viral isolates tested except for CE1176 (Fig. 3a). Against the single resistant strains, iMab-CAP256 exhibited similar IC_{50} values to the parental antibody that demonstrated activity, except for CNE55, where iMab-CAP256 exhibited enhanced potency (Fig. 3a and Additional file 1: Figure S1).

Potency enhancement is highly desirable and a common feature shared amongst bibNAbs and tribNAbs reported in the literature [47, 49, 50, 52–54, 82]. This enhancement is attributed to improved avidity that allows for simultaneous epitope engagement [47, 49, 54], or intra-trimeric crosslinking [53, 82]. Interestingly, this enhancement in potency appears to be more pronounced when combining an Env and host-cell receptor targeting strategy [47, 49, 54]. The proposed mechanism is that anchoring of the bibNAbs to the cell surface via the iMab moiety effectively concentrates and allows for correct spatial positioning of the corresponding anti-Env paratope within the virus–host cell synapse, thereby enhancing the potency [47, 49].

Our iMab-CAP256 bibNAb clearly demonstrates enhanced potency that could not be reproduced by the parental combination (CAP256 + iMab) (Fig. 3 and Additional file 3: Figure S3). This suggests dual engagement of the targeted epitopes (CD4 and quaternary V1/V2 apical loop epitopes) and is in agreement with previously published findings that incorporate iMab into bibNAb configurations and reported potency enhancement [47, 49]. Closer inspection of the CNE55 neutralization curve (Additional file 1: Figure S1) indicates that although iMab did not achieve 50% inhibition over the concentrations tested, retention of iMab sensitivity, albeit to a lower degree, still facilitated the enhancement of neutralizing potency of the iMab-CAP256 bibNAb over the parental antibodies (iMab or CAP256).

IMab-CAP256 compared favourably to the PG9-iMab bibNAb against 15/16 of our selected pseudovirus isolate panel, with an approximate tenfold greater median IC_{50} (μg/ml) potency (Fig. 3b right panel). This was not unexpected given that the CAP256 parental mAb is more potent than PG9 and indeed the most potent within the V1/V2 glycan dependent class isolated to date [45, 55]. Although iMab-CAP256 and PG9-iMab share antigenic targets these bibNAbs configurations are structurally dissimilar. PG9-iMab incorporates the PG9 moiety as a single chain variable fragment via a 15-amino acid, glycine-serine linker peptide to the N-terminus of the iMab heavy chain [47]. This structural configuration provides greater avidity potential and enhanced flexibility over the CrossMab/knob-in-a-hole configuration. Whether further potency refinement of iMab-CAP256 may be achieved through structural optimization remains to be explored. Similarly, Haung et al. [49], reported on the PGT145-iMab bibNAb, CrossMab/knob-in-a-hole configuration which also shares antigenic targets with iMab-CAP256. Neutralization coverage of this bibNAb increased to above 95% yet moderate potency enhancement (< 10 fold enhancement) was observed compared to parental antibodies. Further work was not pursued

on the PGT145-iMab bibNAb given the superior performance of 10E08-iMab reported in the same study [49]. However, their findings corroborate ours; showing potency enhancement was achieved using this epitope targeting combination, in the CrossMab/knob-in-a-hole configuration.

10E08-iMab has been described as the most potent and broadly neutralizing bibNAb characterised to date [49, 81]. It is unlikely iMab-CAP256 will exhibit broader neutralization coverage than 10E08-iMab, or PG9-iMab bibNAbs for that matter, given the reduced coverage of the parental CAP256 (57% of global isolates) in relation to 10E08 (98%) and PG9 (80%) [2, 8, 55]. However, the potency achieved by iMab-CAP256 may be comparable to, or outperform 10E08-iMab against certain HIV-1 subtypes. We observed a slightly lower, yet significant, difference in median IC_{50} values obtained for iMab-CAP256 compared to 10E08-iMab, against the pseudoviruses tested (Fig. 3b right panel). In addition, we present preliminary evidence of complementarity between the iMab-CAP256 and 10E08-iMab bibNAbs, as it was noted that the combined results neutralized 11/16 viral isolates (dual sensitive pseudoviruses only) at an IC_{50} value of < 0.001 µg/ml (Fig. 3a).

Ultimately, iMab-CAP256 will need to be evaluated against an extended HIV-1 pseudovirus panel, to validate the enhanced breath, potency and possible complementarity with 10E08-iMab. Of particular interest would be the inhibitory activity against HIV-1 subtype C viral isolates, given the worldwide predominance of this subtype and its specific affliction in the Sub-Saharan Africa region. In their follow up study, Wagh and colleague's evaluated an updated bNAb panel that included the newly characterized bNAb, N6 [4] and two bibNAbs, 3BNC117-PGT135 [82] and $10E08_{v2.0}$-iMAb [49], against an expanded pseudovirus panel that included subtype A, C and D [81]. Dual antibody combinations comprising CAP256-VRC26.25 paired with N6 or 3BNC117 were shown to be the best-in-class against subtype C and D viral isolates, respectively. However, it should be noted that all dual bNAb combinations were surpassed by the single bibNAb, 10E8-iMAb across subtype A, C and D [81]. Once again, this study highlighted the superior advantage that may be attained through bi- and multi-specific antibody engineering compared to combination therapy. Indeed, further advantage was gained when the bibNAb, 10E08-iMab was used in combination with single bNAb N6 [81].

In conclusion, our results confirm the superiority of bibNAbs as compared to single bNAbs to neutralize HIV-1 in vitro. Moreover, incorporating CAP256.VRC26.235 into proven, multi-specific antibody configurations through class replacement (e.g. N6/PGDM1400-10E8v4 [52]) could provide further benefit. Thus, the iMab-CAP256 bibNAb configuration, and potential derivatives described here provides a unique and exciting opportunity for clinical development, given its improved neutralization breadth and potency.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s12977-019-0493-y.

Additional file 1: Figure S1. Breadth and potency of iMab-CAP256 in comparison to the parental bNAbs ibalizumab (iMab) and CAP256. VRC26.25 (CAP256). iMab-CAP256 shows greater potency than both the parental bNAbs for 13 of the 14 dual sensitive pseudoviruses tested as shown by the left shift of the bibNAb line on the graphs (median IC_{50} in µg/ml: iMab-CAP256 0.00079, iMab 0.056 and CAP256 0.0037). Against the six single resistant pseudoviruses (CAP256 resistant: 398F1, QH0692.4, TRO11 and ZM135.PL10a; iMab resistant: CNE55 and X1632), iMab-CAP256 has a potency similar to that of the active parental bNAb except for CNE55 were iMab-CAP256 exhibits enhanced potency. Unlike the parental bNAbs iMab and CAP256, iMab-CAP256 neutralised all 20 pseudoviruses tested thus demonstrating improved breath. All three Abs were tested at a maximum concentration of 1 µg/ml.

Additional file 2: Figure S2. Potency of iMab-CAP256 in comparison to 10E08-iMab and PG9-iMab. iMab-CAP256 shows on average, a potency on average 10× greater than PG9-iMab across all the 14 dual sensitive pseudoviruses tested except CE1176 (median IC_{50} for the 14 dual sensitive pseudoviruses: iMab-CAP256 0.00079, PG9-iMab 0.0079 µg/ml). Compared to 10E08-iMab, iMab-CAP256 is more potent against 11 of the 14 dual sensitive pseudoviruses tested (median IC_{50} for the 14 dual sensitive pseudoviruses in iMab-CAP256 is 0.00079 µg/ml, 10E08-iMab is 0.00226 µg/ml). iMab-CAP256 was tested at a maximum concentration of 1 µg/ml whilst PG9-iMab and 10E08-iMab were tested at 4 µg/ml.

Additional file 3: Figure S3. Potency of iMab-CAP256 in comparison to the parental bNAb combination (iMab+CAP256). BibNAb iMab-CAP256 shows a potency on average 10× greater than that of the parental combination iMab+CAP256 for all fourteen dual sensitive pseudoviruses tested (median IC_{50} in µg/ml: iMab-CAP256 0.00079, iMab+CAP256 0.0081). iMab-CAP256 was tested at 1 µg/ml and the bNAb combination was tested at 0.5 µg/ml of each parental Ab to achieve a total concentration of 1 µg/ml.

Abbreviations

ART: antiretroviral therapy; bNAbs: broadly neutralising antibodies; bibNAbs: bispecific bNAbs; CAP256: CAP256.VRC26.25; Env: HIV-1 envelope glycoprotein; Fab: fragment antigen-binding; FDA: Food and Drug Administration; HEK293T cells: Human embryonic kidney 293 T antigen cells; IC_{50}: 50% inhibitory concentration; iMab: ibalizumab; iMab-CAP256: ibalizumab-CAP256.VRC26.25; PG9-iMab: PG9-ibalizumab; pMin: mAb expression vector; SDS-PAGE: sodium dodecyl sulphate polyacrylamide gel electrophoresis; SEC: size exclusion chromatography; $TCID_{50}$: 50% Tissue Culture Infectious Dose; tribNAbs: trispecific bNAbs.

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Authors' contributions

MAP, MAK and SA conceptualised the project. MAK and TM designed the experiments and TM performed the experiments. MAK and TM wrote the manuscript and analysed the data. All authors read and approved the final manuscript.

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Availability of data and materials
The datasets used and/or analysed during the current study are available from the corresponding author on request.

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An ethics waiver (ref: W-CBP-180305-02) was applied for and granted by the Human Research Ethics Committee (Medical), Faculty of Health Sciences, University of the Witwatersrand.

Consent for publication
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Competing interests
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Supplementary Information

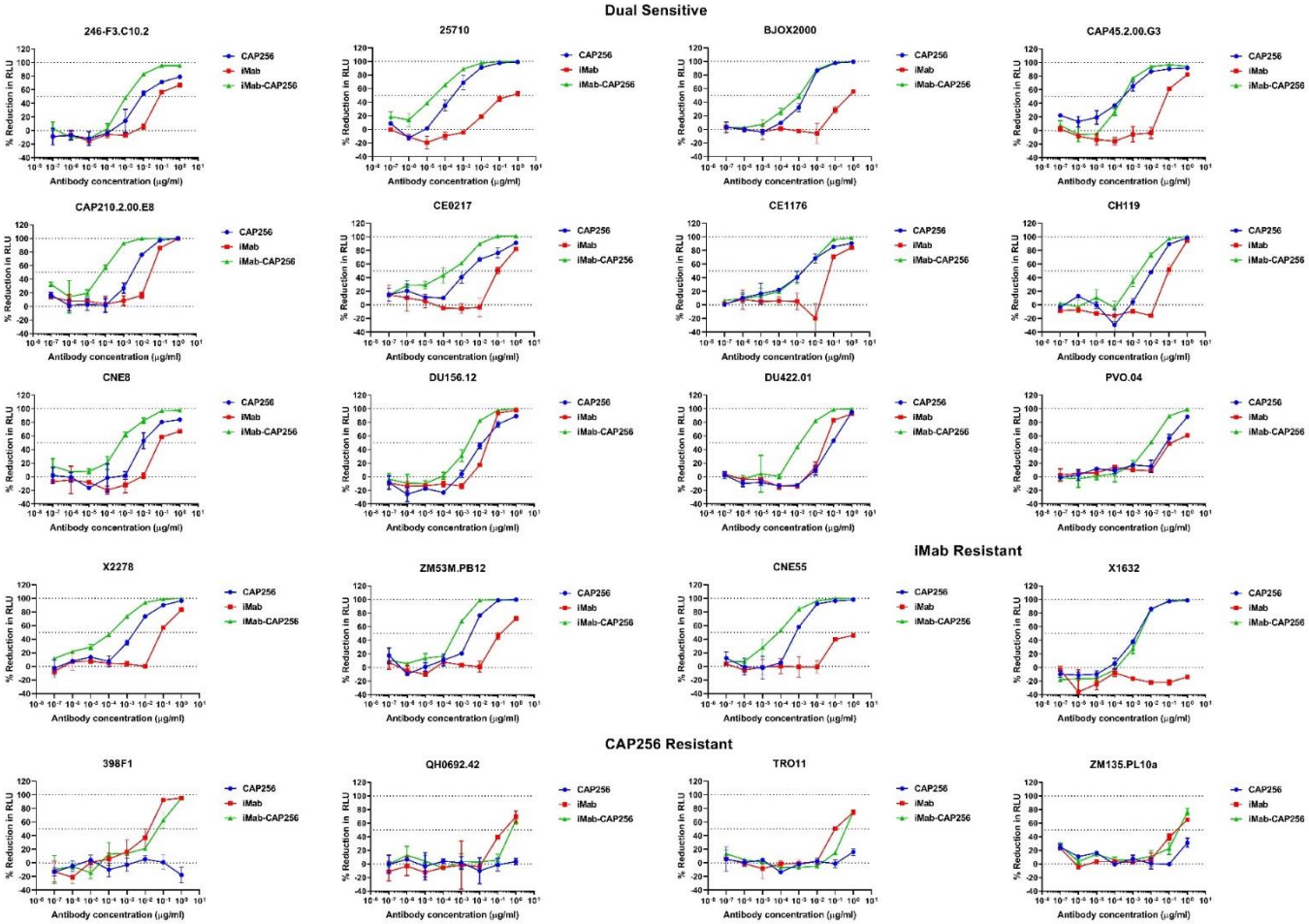

Figure S1: Breadth and potency of iMab-CAP256 in comparison to the parental bNAbs ibalizumab (iMab) and CAP256.VRC26.25 (CAP256). iMab-CAP256 shows greater potency than both the parental bNAbs for 13 of the 14 dual sensitive pseudoviruses tested as shown by the left shift of the bibNAb line on the graphs (median IC50 in µg/ml: iMab-CAP256 0.00079, iMab 0.056 and CAP256 0.0037). Against the six single resistant pseudoviruses (CAP256 resistant: 398F1, QH0692.4, TRO11 and ZM135.PL10a; iMab resistant: CNE55 and X1632), iMab-CAP256 has a potency similar to that of the active parental bNAb except for CNE55 were iMab-CAP256 exhibits enhanced potency. Unlike the parental bNAbs iMab and CAP256, iMab-CAP256 neutralised all 20 pseudoviruses tested thus demonstrating improved breath. All three Abs were tested at a maximum concentration of 1 µg/ml.

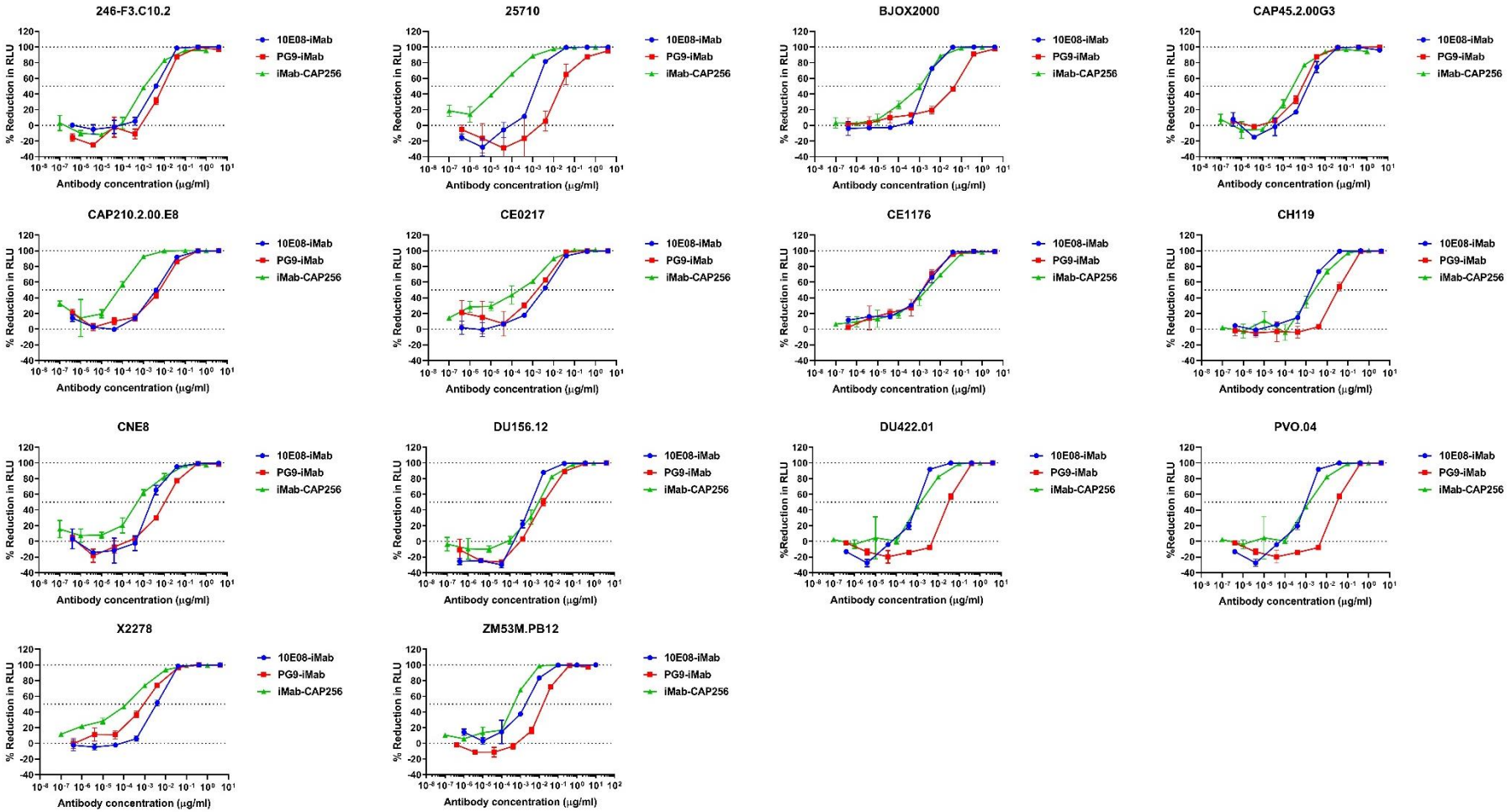

Figure S2: Potency of iMab-CAP256 in comparison to 10E08-iMab and PG9-iMab. iMab-CAP256 shows on average, a potency on average 10× greater than PG9-iMab across all the 14 dual sensitive pseudoviruses tested except CE1176 (median IC50 for the 14 dual sensitive pseudoviruses: iMab-CAP256 0.00079, PG9-iMab 0.0079 µg/ml). Compared to 10E08-iMab, iMab-CAP256 is more potent against 11 of the 14 dual sensitive pseudoviruses tested (median IC50 for the 14 dual sensitive pseudoviruses in iMab-CAP256 is 0.00079 µg/ml, 10E08-iMab is 0.00226 µg/ml). iMab-CAP256 was tested at a maximum concentration of 1 µg/ml whilst PG9-iMab and 10E08-iMab were tested at 4 µg/ml.

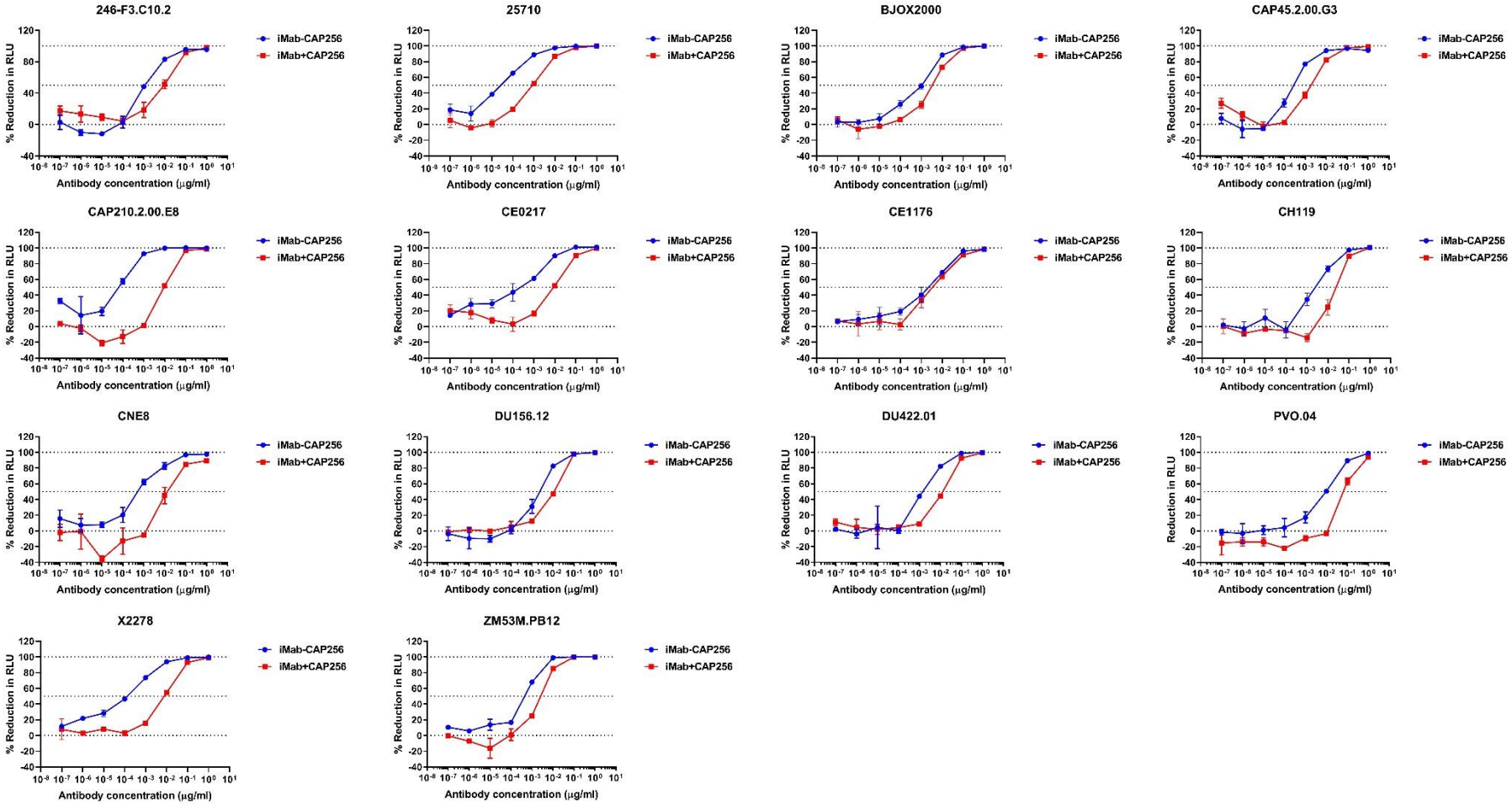

Figure S3: Potency of iMab-CAP256 in comparison to the parental bNAb combination (iMab+CAP256). BibNAb iMab-CAP256 shows a potency on average 10× greater than that of the parental combination iMab+CAP256 for all fourteen dual sensitive pseudoviruses tested (median IC50 in μg/ml: iMab-CAP256 0.00079, iMab+CAP256 0.0081). iMab-CAP256 was tested at 1 μg/ml and the bNAb combination was tested at 0.5 μg/ml of each parental Ab to achieve a total concentration of 1 μg/ml.

Chapter 3: HIV-1 bispecific antibody iMab-N6 exhibits enhanced breadth but not potency over its parental antibodies iMab and N6

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HIV-1 bispecific antibody iMab-N6 exhibits enhanced breadth but not potency over its parental antibodies iMab and N6

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Abstract

The recently published AMP trial (HVTN 703/HPTN 081 and HVTN704/HPTN 085) results have validated broad neutralising antibodies (bNAbs) as potential anti-HIV-1 agents. However, single bNAb preparations are unlikely to cope with the onslaught of existing and de novo resistance mutations, thus necessitating the use of bNAb combinations to achieve clinically relevant results. Specifically engineered antibodies incorporating two bNAbs into a single antibody structure have been developed. These bispecific antibodies (bibNAbs) retain the benefits of bNAb combinations, whilst several conformations exhibit improved neutralisation potency over the parental bNAbs. Here we report on the engineering of a bibNAb comprising of an HIV-1 spike targeting bNAb N6 and a host CD4 targeting antibody ibalizumab (iMab). Antibodies were expressed in HEK293T cells and purified by protein-A affinity chromatography followed by size exclusion chromatography to achieve homogenous, monomeric, bibNAb preparations. Antibody purity was confirmed by SDS-PAGE whilst epitope specificity and binding were confirmed by ELISA. Finally, antibody breadth and potency data were generated by HIV-1 neutralisation assay (n = 21, inclusive of the global panel). iMab-N6 exhibited better neutralisation breadth (100% coverage) in comparison to its parental bNAbs iMab (90%) and N6 (95%). This is encouraging as exceptional neutralisation breadth is necessary for HIV-1 treatment or prevention. Unfortunately, iMab-N6 did not exhibit any enhancement in potency over the most potent parental antibody, iMab ($p = 0.1674$, median IC_{50} of 0.0475 µg/ml, and 0.0665 µg/ml respectively) or the parental combination, iMab + N6 ($p = 0.1964$, median IC_{50}: combination 0.0457 µg/ml). This result may point to a lack of dual engagement of the bibNAb Fab moieties necessary for potency enhancement. Against the previously reported bibNAbs; iMab-CAP256, 10E08-iMab, and PG9-iMab; iMab-N6 was the lowest performing bibNAb. The re-engineering of iMab-N6 to enhance its potency, while retaining breadth, is a worthwhile endeavour due to its clinical potential.

Keywords: HIV-1 prevention, HIV-1 therapy, Broadly neutralizing antibodies, Bispecific antibodies, Bliss-Hill potency prediction

Introduction

Broadly neutralising antibodies (bNAbs) isolated from a subset of HIV-1 positive individuals are ideal candidates for development as novel anti-HIV-1 agents [1]. These antibodies neutralise the virus through targeting highly conserved regions on the HIV-1 spike, namely, the V2 apex, V3 glycan, CD4 binding site (CD4bs), the silent face, interface-FP, and the membrane-proximal external region [2]. It is the targeting of these conserved regions that gives bNAbs improved breadth and potency over HIV-1 neutralising antibodies (NAbs) [3]. The poor neutralization coverage and lower potency of first generation monoclonal neutralizing antibodies (Nabs) [4] (e.g.,

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b12, 447-52D, 2G12, 2F5 and 4E10) against a range of clinically relevant HIV-1 cast doubt on the clinical use of these NAbs as anti-HIV-1 agents. However, the isolation of more contemporary bNAbs (e.g., CAP256-VRC26.25 [5], 10E8 [6], N6 [7], 1–18 [8], VRC01 [9], and 3BNC117 [10] and the N49 antibody lineage [11] to name a few) has reinvigorated the field as many potential clinical candidates have been identified [12]. Generally, CD4bs bNAbs (e.g., N6 [7], VRC07-523 [13], 1–18 [8], and 3BNC117 [10]) offer the best combination of breadth and potency whereas V2 antibodies (e.g., CAP256-VRC26.25 [5], PGDM1400 [14]) offer superior potency coupled with moderate breadth [2].

Broadly neutralising antibodies are currently being investigated for use as HIV-1 pre-exposure prophylaxis (PrEP) and/or therapy. As PrEP, bNAbs such as CAP256-VRC26.25 and PGDM1400 [15], PGT121 [16], and VRC01 [17] were shown to protect non-human primates (NHP) following SHIV challenges. Encouragingly, the highly potent V2 specific bNAbs; PGDM1400 and CAP256-VRC26.25 conferred protection against subtype C SHIV-325c with a very low serum concentration of 2.5 and <0.75 μg/ml, respectively; consistent with the extraordinary potency of these antibodies in vitro [15]. However, these researchers observed breakthrough infections as a result of variability in the V1/V2 loop sequence of the SHIV viral challenge stock that was able to convey resistance against the PGDM1400 antibody [15]. Indeed, the existence and/or emergence of HIV-1 resistance mutations is a major concern in single bNAb PrEP intervention strategies, as no single bNAb has demonstrated 100% neutralization coverage of clinically relevant, HIV-1 Env-pseudotyped panels in vitro. This limitation was recently accentuated in a major phase IIb clinical trial (HVTN 703/HPTN 081 and HVTN704/HPTN 085; AMP) where VRC01 (CD4bs antibody) offered no protection against HIV-1 acquisition compared to the placebo control group (saline infusion) [18]. Closer inspection of the data revealed that only 30% of the circulating viruses within these trial participants were sensitive to VRC01 ($IC_{80}<1$ μg/ml), thereby rendering the bNAb ineffective against the majority of the circulating viruses [comprising of intermediate ($IC_{80}=1–3$ μg/ml) and resistant ($IC_{80}>3$ μg/ml) viruses] [18]. Against the sensitive viruses, VRC01 was 75% effective at preventing HIV-1 acquisition, failing in the other 25% due to the emergence of new resistance mutations [18]. This trial underscores the importance of bNAb breadth and potency, and choosing the correct bNAb, in offering full protection against HIV-1 acquisition within the real world, clinical setting. As disappointing as these results are, the AMP trial has validated bNAbs as potential PrEP candidates subject to a selection that maximises neutralising breadth and potency of the circulating regional/global HIV-1 strains.

As therapeutic agents, bNAbs such as PGT121 and 3BNC117 [19], N6-LS [20], and 1–18 [8] have been shown to suppress HIV-1 viral load in either humanised mice or NHP. However, viral rebound was detected due to the emergence of resistance mutations against 3BNC117 and as a result of declining N6-LS and PGT121 plasma levels [19, 20]. Interesting, 1–18 fully suppressed HIV-1 in humanised mice without selecting for resistance mutations, indicating a high genetic barrier to resistance [8]. BNAb therapy has also been investigated in a number of small clinical trials where 3BNC117 (phase 1: NCT02018510 and phase IIa: NCT02446847), VRC01 (phase I: NCT01950325 and NCT02463227), 10-1074 (phase I: NCT02511990) and PGT121 (NCT02960581) were shown to transiently suppress viral load in viraemic individuals [21–24] or prevent viral rebound during treatment interruption [25, 26]. However, pre-existing resistant viruses coupled with the emergence of new resistant viruses rendered these treatments ineffective overtime [21–23, 25, 26]. As with bNAb PrEP, these results once again underscore the importance of antibody breadth.

To improve neutralization coverage, appropriate bNAb combinations with proven synergistic effects may be used in place of bNAb monotherapy. Modelling, in vitro and in vivo studies have shown that bNAb combinations offer improved neutralisation coverage and potency over bNAb monotherapy [27–30]. Moreover, in a phase Ib clinical trial (NCT02825797), bNAb combination (3BNC117 and 10-1074) was shown to be more effective than bNAb monotherapy (3BNC117) at preventing viral rebound following treatment interruption (median viral rebound of 21 weeks and 6–10 weeks respectively) [31]. In a separate phase Ib clinical trial (NCT02825797), the 3BNC117 and 10-1074 combination was shown to supress viral load in viraemic individuals for a significantly longer period than either 3BNC117 or 10-1074 monotherapy [21, 22, 32]. However, in both studies, differences in bNAb half-life (3BNC117: 17.6 or 12.3 days, respectively, and 10-1074: 23.2 or 12.7 days, respectively) meant 3BNC117 levels dropped faster than 10-1074 effectively causing monotherapy; this in turn resulted in viral rebound driven by 10-1074 resistance [31, 32]. These differences in bNAb pharmacokinetics and the cost implications of bNAb combination PrEP/therapy are what makes this strategy challenging to implement in mainstream clinical settings.

Specifically engineered antibodies incorporating two or three bNAbs into one antibody structure have been developed as possible replacements for bNAb combinations. These bi/tri-specific antibodies (bi/tribNAbs)

retain the benefits of bNAb combinations without any of the associated challenges. Several bi/tribNAbs reported in the literature (10E8v4-iMab [33], PG9-iMab [34], iMab-CAP256 [35], VRC01/PGDM1400-10E8v4 [36], N6/PGDM1400-10E8v4 [36], and 10E08/Bi-ScFv$_{dVRC01\text{-}5X\text{-}PGT121}$ [37]) exhibit greater breadth and potency than their parental bNAb combination. This enhancement in potency is due to the simultaneous binding of the paratopes resulting in increased avidity. For the iMab-based bibNAbs, the localisation/concentration of the bibNAbs at the site of entry (surface of the CD4 T-cells) positively contributes to the potency enhancement [34]. IMab, a humanised mouse antibody exhibits broad-spectrum neutralization of HIV-1 viral isolates through targeting a conformational epitope on the second N-terminal, extracellular domain of the human CD4 receptor [38, 39]. iMab does not compete for HIV-1 gp120 envelope (Env) binding to the CD4+ T cell receptor, but rather inhibits viral infection through sterically hindering the post-binding rearrangement steps required for viral and host target cell membrane fusion [39, 40]. Importantly, iMab displays an excellent safety profile in vivo as its binding does not interfere with CD4-mediated major histocompatibility complex II immune signalling functions [41, 42]. Combined, these properties make iMab a suitable candidate for generating bispecific antibodies in combination with HIV-1 Env targeting bNAbs. Clinical trials are currently underway to evaluate the safety, tolerability, pharmacokinetics, and anti-viral activity of bibNAb 10E8v4-iMab (NCT03875209) and tribNAb VRC01/PGDM1400-10E8v4 (NCT03705169).

Although promising, the prohibitive cost associated with antibody therapy limits their large-scale implementation compared to more traditional ART-based strategies. Alternative plant-based expression systems for the expression of bNAbs are being investigated to reduce the associated manufacturing costs [43]. Moreover, antibody engineering strategies (LS-mutations) to improve serum half-lives and reduce the frequency of antibody administration [15, 20]; and vector-mediated immunoprophylaxis strategies that result in sustained antibody expression in vivo and protection against HIV-1 acquisition in preclinical trials, are also being investigated as cost reduction strategies [44–46]. These strategies may be combined to further reduce the cost of antibody-based PrEP or therapy against HIV-1 and allow for their clinical implementation. Whatever strategy is employed, antibody therapy is promising.

We previously reported on a highly potent and broad bibNAb iMab-CAP256 [35]; specifically engineered with HIV-1 subtype C in mind. HIV-1 subtype C is the dominant subtype in Southern Africa and by extension the world [47]. Overcoming the HIV-1 pandemic therefore requires PrEP or therapeutic agents that encompass this subtype. Here we report on the engineering and characterisation of an additional bibNAb comprising of an HIV-1 Env targeting bNAb N6 [7] and the host CD4 targeting humanised monoclonal antibody ibalizumab (iMab) [38–40]. Huang et al. reported on N6's superior performance against a panel of 181 pseudoviruses, achieving a neutralisation coverage of 98% with a median IC_{50} of 0.038 μg/ml [7]. Crucially, N6 was shown to have an exceptional neutralisation coverage of 98% with a median IC_{50} of 0.066 μg/ml against HIV-1 subtype C [7]. Moreover, N6 was the best performing out of 8 bNAbs against HIV-1 subtype C with a 95–96% IC_{80} coverage [30]. Combined, these data suggest that the incorporation of N6 into combination-based therapies or inclusion into bi/tribNAb configurations would be beneficial. The monoclonal antibody iMab has a neutralisation coverage of 92% against diverse HIV-1 Env subtypes with a median IC_{50} of 0.03 μg/ml [48]. iMab (Trogarzo®) is the only FDA approved monoclonal antibody for use as HIV-1 salvage therapy [49]. By combining N6 with iMab, we hope to produce a bibNAb with exceptional breadth and enhanced potency especially against HIV-1 subtype C. To this end, we describe the engineering of iMab-N6 bibNAb, confirm the functionality of individual Fab binding regions in the bispecific antibody configuration and demonstrate an improved breadth relative to the parental bNAbs against a panel of 21 diverse HIV-1 pseudoviruses.

Methodology

Reagents used in this study

The single plasmid, mAb expression vector (pMin) was generously donated by Balazs [44, 45] and modified to remove the CMV promoter and ITR flanking regions for optimal in vitro antibody expression. The following reagents were obtained from the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID (contributor in parentheses): N6 antibody heavy and light chain expression vectors (Drs. Jinghe Huang and Mark Connors [7]). Reagents for HIV-1 pseudovirus production including: TZM-bl (JC53-bl) reporter cell line (Drs. John C. Kappes, Xiaoyun Wu and Transzyme Inc. [50–54]), pSG3Δenv (Drs. John C. Kappes and Xiaoyun Wu [54, 55]) and 21 complementing HIV-1 Env plasmids 25710, 246-F3.C10.2, 398f1 and CH119 (Drs. C. Williamson, M. Hoelscher, L. Maboko, and D. Montefiori [56, 57]), CNE8 and CNE55 (Drs. L. Zhang, H. Shang, and D. Montefiori [56, 58]), PVO.4 and QH0692.42 (Drs. D. Montefiori, F. Gao and M. Li [59]), CAP210.2.00.E8 and CAP45.2.00.G3 (Drs. L. Morris, K. Mlisana and D. Montefiori [60]), DU156.12 (Drs. D. Montefiori F. Gao, S. Abdool Karim and G. Ramjee [60, 61]), DU422.01

(Drs. D. Montefiori, F. Gao, C. Williamson and S. Abdool Karim [60, 61]), ZM53 M.PB12 and ZM135 M.PL10a (Drs. E. Hunter and C. Derdeyn [60]), X1632 (Dr. D. Montefiori [56, 62]), TRO11 (Drs. F. Gao and D. Montefiori [56, 59]), X2278 and BJOX2000 (Drs. M. Thomson, A. Revilla, E. Delgado, and D. Montefiori [56]), CE0217 and CE1176 (Drs. R. Swanstrom, L. Ping, J. Anderson, and D. Montefiori [56]), RHPA4259 (Drs. B. H. Hahn and Dr. J. F. Salazar-Gonzalez [59]). The HIV-1 global pseudovirus panel (Cat #12670) [56] is included in the above-mentioned 21 plasmids.

Antibody construct design and synthesis

The bispecific antibody expression constructs were engineered as described by Moshoette et al. [35]. Briefly, iMab-N6 was designed in silico using SnapGene by introducing the fragment antigen-binding region (Fab fragment) sequences of iMab [40] and N6 [33] (PDB: 3O2D and 5TE7 respectively) to their respective bibNAb constructs. Additionally, Knob-in-a-hole [63] (KiH) and CrossMab^{CH1_CL} [64] mutations were introduced into the sequences to optimise the correct assembly of the bibNAb (Fig. 1a). CrossMab^{CH1_CL} and "hole" (L368A, Y349C and Y407V) mutations were introduced into the host targeting iMab whilst the corresponding "knob" (T366W and S254C) mutations were incorporated into the HIV-1 Env targeting N6 to create a host-Env dual targeting bibNAb (Fig. 1A). Parental N6 antibody heavy and light chain constructs were sourced from the NIH AIDS Research and Reference Reagent Program (ARP-12966 and ARP-12967, respectively). iMab was engineered using the same bibNAb modifications (CrossMab^{CH1-CL} and KiH) to serve as an assembly control (Fig. 1A, rightmost panel). The Ab designs were sent to GeneArt (ThermoFisher Scientific, Waltham, MA, USA) for codon optimisation and construct synthesis. The synthesised antibody gene constructs were subcloned into a mammalian expression vector provided courtesy of Balazs. Large scale plasmid production was performed in *E. coli* DH5α cells and purified using the Qiagen Maxi plasmid isolation kit (Qiagen, Hilden Germany) according to the manufacturer's protocol.

Antibody expression and purification

Antibody production was performed as described previously [35, 65]. Briefly, 24 h before transfection, HEK293T cells were seeded in a tissue culture flask and allowed to grow to 70% confluency. After 24 h, transient transfection was done using a 1:3 DNA to PEI MAX (Polysciences Inc., PA, USA) ratio and the cells were incubated for a further 18 h. The cell culture media was then replaced with SFMII media (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 2 mM glutamax (Thermo Fisher Scientific, Waltham, MA. USA). The transfected cells produced and secreted the antibodies into the cell culture supernatant which was harvested every 2 days up to a maximum of 10 days. The harvested supernatants were filtered through 0.2 µm filters and stored at 4 °C until further processing. Antibody purification was done by means of Protein-A affinity chromatography (Sigma-Aldrich, St Lois, M0, USA) followed by Size Exclusion Chromatography (SEC) on a Superdex 200 PG 16/600 HiLoad column (GE Healthcare, Chicago, IL, USA) to attain a pure sample comprising of monomer antibodies. SEC fractions containing the pure Ab sample (as shown by the chromatograms) were pooled, filtered with a 0.2 µm filter and concentrated using an Amicon centrifugal-filtration device (Merck-Millipore, Burlington, MA, USA) with a molecular weight cut-off 50 kDa. The purified proteins were resolved on a NuPAGE™ Bis–Tris Mini Gel (Polyacrylamide percentage: 8%, 10%, 12%, and 4–12%, Thermo Fisher, Waltham, MA, USA) to confirm their purity.

gp140$_{FVC}$GCN4 and 4dCD4 protein production and purification

Constructs for the recombinant expression of HIV-1 gp140$_{FVC}$GCN4 trimer or human, wildtype four domain CD4 (4dCD4) were available in our laboratory. The HIV-1 gp140$_{FVC}$GCN4 trimer comprises an inferred Founder virus consensus C sequence (FVC), and was recombinantly expressed by stably transfected 293F cell lines and purified by a combination of lectin-affinity chromatography and SEC, to produce a functional, conformationally intact protein as described previously [66].

The 4dCD4 plasmid construct, codon-optimised for mammalian expression, was designed and obtained from GeneArt (Thermo Fisher Scientific, Waltham, MA. USA) and included an N-terminal, membrane-localisation signal that in the absence of the CD4 transmembrane and cytoplasmic regions, allows for efficient secretion of the expressed proteins. The 4dCD4 coding sequence was PCR amplified using the Q5 High-fidelity 2× Master mix (New England Biolabs, Ipswich, MA, USA) and sub-cloned into pCINeo (Promega, Madison, WI, USA) using the *Xba*I and *Not*I restriction sites. A C-terminal HIS tag (6× histine residues) was included in frame with the 4dCD4 coding sequence for downstream purification using immobilised metal affinity chromatography (IMAC). Large scale plasmid isolation was performed as described above. 4dCD4 was expressed by standard mammalian, cationic lipid transfection protocols in the FreeStyle 293F cell line (Thermo Fischer, Waltham, MA, USA). Briefly, 293F cells were maintained between 0.5 and 3.0×10^6 cells/ml in FreeStyle 293 Expression medium (Thermo Fisher, Waltham, MA, USA) at 37 °C,

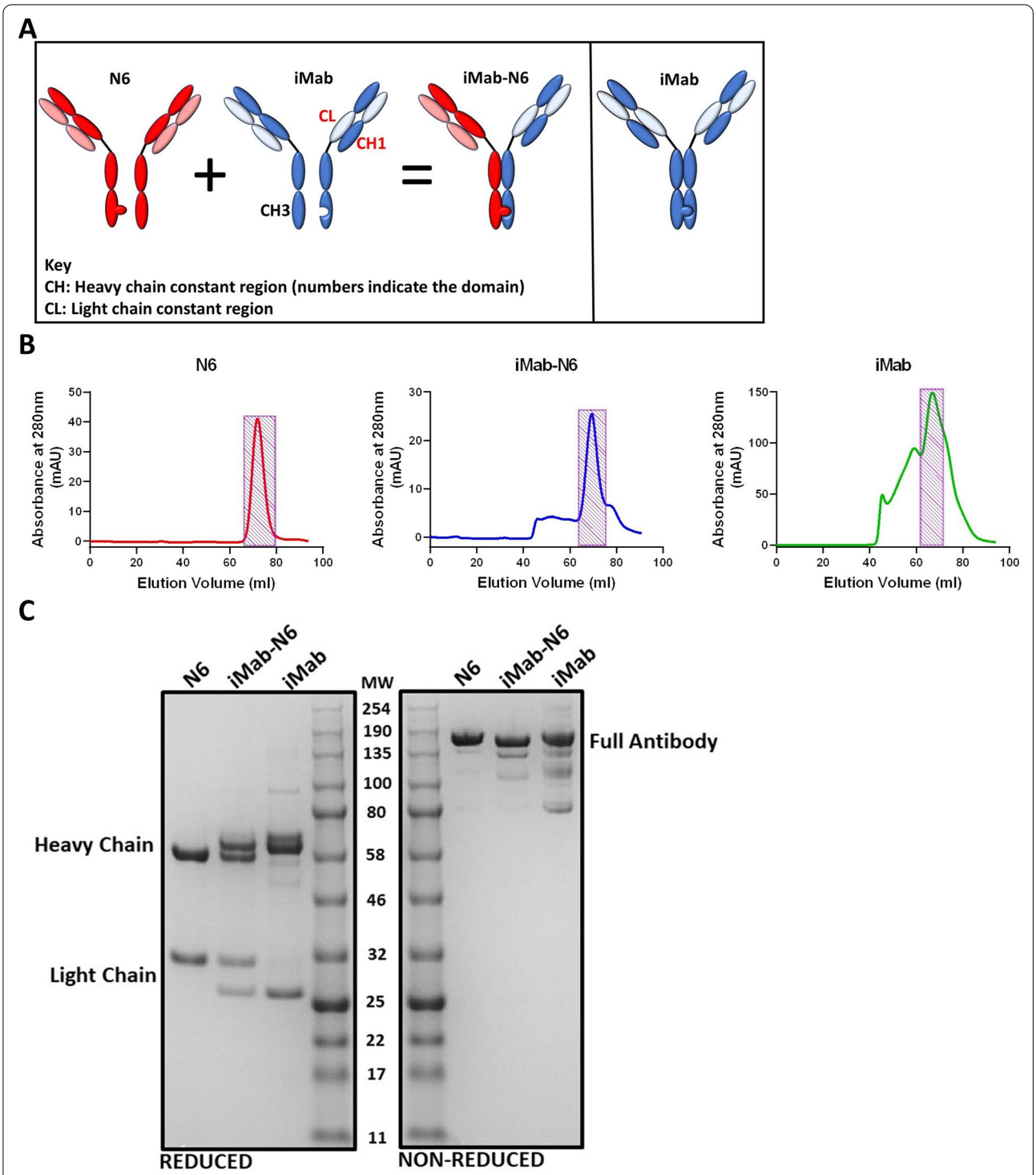

Fig. 1 Bispecific antibody design, purification and SDS-PAGE analysis. **A** Left panel: Schematic of iMab-N6 engineered using knob-in-a-hole mutations in the CH3 region of N6 and CrossMAb^{CH1_CL} mutations in iMab. These mutations promote preferential assembly of iMab-N6 by limiting the formation of by-products. **A** Right panel: To show that these mutations do not affect the function of the antibody, iMab monoclonal antibody was engineered with knob-in-a-hole and CrossMAb^{CH1_CL} mutations. **B** Chromatograms of size exclusion chromatography (SEC) purified antibodies with elution fractions corresponding to the correctly assembled antibody confirmation represented by the shaded rectangles. **C** Antibodies were resolved on a gradient SDS-PAGE post SEC under reduced and non-reduced conditions

in atmosphere supplemented with 5–8% CO_2 on an orbital shaking platform (130 rpm). Cells were transfected at density of $1.5–2.0 \times 10^6$ cell/ml, with a viability > 90% using 1 µg plasmid DNA per ml of culture. DNA:PEI MAX ratios were maintained at 1:3 ratio, and the total transfection reagent volume (including the plasmid DNA, PEI MAX, and Freestyle media) was 10% of the total culture volume. Culture supernatants containing the expressed 4dCD4 protein were harvested every second day and clarified by centrifugation (300×g, 5 min) for a maximum of 7 days. Cultures were re-seeded at 1.5×10^6 cell/ml following the harvesting of the supernatants. IMAC purification of the 4dCD4 was performed using 1 ml of packed, Ni-charged resin incubated per ~ 500 ml volume of culture supernatant overnight at 4 °C with stirring to maintain the resin in suspension. The resin was collected by centrifugation at 1000×*g* for 5 min and washed with wash buffer (25 mM KH_2PO_4, 150 mM KCl, pH 8.0) to disrupt any nonspecific protein-resin interactions. The 4dCD4 was eluted from the resin using the wash buffer containing 250 mM imidazole, concentrated and buffer-exchanged into Dulbecco's PBS (without calcium and magnesium) (Thermo Fisher Scientific, Waltham, MA, USA) using an Amicon centrifugation concentrating device (MW cut off 30 kDa, Merck-Millipore, Burlington, MA, USA). Concentrated protein was quantified using the BCA protein assay (Thermo Fisher Scientific, Waltham, MA, USA), and flash frozen in liquid nitrogen for storage at − 80 °C.

Functional evaluation of bibNAb Fab moieties

To confirm the purified antibodies bind to their respective epitopes, on the recombinant HIV-1 Env $gp140_{FVC}$GCN4 and 4dCD4, an ELISA was performed. N6 and the HIV-1 targeting arm of iMab-N6 were tested against HIV-1 Env $gp140_{FVC}$GCN4 whereas iMab and the host targeting arm of iMab-N6 were tested against 4dCD4. In preparation for the Env ELISA, 96 well Nunc Maxisorb ELISA plates (Fisher Scientific, Waltham, MA, USA) were coated with 100 ng/well *G. nivalis* lectin (Sigma-Aldrich, St Lois, MO, USA) and incubated overnight at 4 °C. For the CD4 ELISA, serial dilutions of 4dCD4 (starting concentration of 1000 ng/ml per well; 3× dilution series) were added to an uncoated 96 well Nunc Maxisorb ELISA plate (Fisher Scientific, Waltham, MA, USA) and likewise incubated overnight at 4 °C. The plates were then blocked for an hour at room temperature (Blocking buffer: Dulbecco's phosphate buffered saline (DPBS) (Sigma), 0.05% (v/v) Tween 20 and 1% BSA). Serial dilutions of $gp140_{FVC}$GCN4 (starting concentration of 1000 ng/ml per well; 3× dilution series) were added to the coated Env ELISA plates followed by an hour incubation at room temperature. With plates ready, N6 was added at a uniform concentration of 0.8 µg/ml and iMab-N6 was added at a uniform concentration of either 0.8 µg/ml or 1.6 µg/ml for the Env ELISA. Both iMab and iMab-N6 were tested at a single uniform concentration of 1 µg/ml for the CD4 ELISA. Bound antibodies were detected using anti-human, horseradish peroxidase linked secondary antibody (GE Healthcare, Chicago, IL, USA) and standard chromogenic methodologies. To avoid contamination by non-bound proteins (i.e., HIV-1 Env or 4dCD4 or 1° antibodies or 2° antibodies), the plate was washed after each step with wash buffer [DPBS (Sigma) containing 0.05% (v/v) Tween 20]. Both iMab and N6 parental antibodies served as appropriate negative controls in the $gp140_{FVC}$GCN4 and 4dCD4 ELISAs, respectively. All samples were run in duplicate.

HIV-1 in vitro neutralisation assay

The breadth and potencies of iMab-N6, parental bNAbs (iMab or N6) and parental bNAb combination (iMab + N6) were determined against a panel of 21 geographically diverse pseudoviruses. This was done using a single round HIV-1 pseudovirus infectivity assay in the TZM-bl reporter cell line, as described previously [65]. Briefly, single-round HIV-1 Env pseudoviruses were produced by co-transfection of HEK293T cells with HIV-1 *rev/env* expression plasmids (Reagents—HIV-1 Env plasmids) and the *env*-deficient HIV-1 back bone plasmid (pSG3ΔEnv) at 1:2 ratio using FuGene HD transfection reagent (Promega, Madison, WI, USA). Culture supernatant containing the pseudoviruses was harvested at 48 h post transfection, filtered through a 0.45 µm Acrodisc filter (Pall Corporation, New York, USA), supplemented to a final concentration of 20% foetal calf serum (Gibco, Thermo Scientific) before being aliquoted into 1 ml volumes and stored at − 80 °C. Tissue culture infectious dose to achieve 50% infection ($TCID_{50}$) was determined in the TZM-Bl cell line as described previously [59]. Neutralization assays were performed by preparing serial dilutions of the antibodies (iMab, N6, and iMab-N6) in a 96 well cell culture plate using a starting concentration of 4 µg/ml and a 5× dilution series. The parental combination (iMab + N6) was prepared using a 50/50 split of each parental Ab to a final starting concentration of 4 µg/ml and a 5× dilution series. Following Ab preparations, 200 $TCID_{50}$ of pseudovirus was added to each well and incubated for an hour at 37 °C in 5% CO_2. Following this, 1×10^4 cells/well of freshly trypsinised TZM-bl cells were added, and the plate was incubated for a further 48 h at 37 °C in 5% CO_2. The TZM-bl cells were prepared in complete DMEM supplemented with DEAE Dextran (Sigma-Aldrich, St Lois, Mo, USA) to a final assay concentration of 20 µg/ml; this was done to enhance the infectivity of the pseudoviruses to the TZM-bl cells. Luciferase

expression was determined using Bright Glo Luciferase reagent (Promega, Madison, WI, USA) according to the manufacturer's instructions and quantified using the Promega Glomax Explorer luminometer (Promega, Madison, WI, USA). Cell only and virus only control wells were included as appropriate controls. Viral inhibition was determined by the reduction in relative luminescence units (RLU) compared to the virus only containing wells after subtracting the background RLU values of the cells only containing wells. IC_{50} and IC_{80} values were calculated using the non-linear regression function (log (agonist) vs. response—Find ECanything) in GraphPad Prism 7 and represent the Ab concentration (μg/ml) required to achieve 50% and 80% pseudovirus inhibition respectively. iMab-CAP256, 10E8-iMab and PG9-iMab IC_{50} data was sourced from Moshoette et al. [35] for comparison to iMab-N6. Only those pseudoviruses sensitive to all parental antibody combinations were included in the analysis (i.e. pseudoviruses sensitive to N6, iMab, 10E8, CAP256-VRC26.25 and PG9). These included: 246-F3.C10.2, CNE8, CNE55, PVO4, X228, BJOX2000, CH119, 25710, CAP45, CE2017, CE1176, DU156, DU422 and ZM53 (n = 13). The Bliss-Hill Model in CombiNAber [29, 67] was used to predict IC_{80} values of the parental combination iMab + N6 against an expanded panel of 97 diverse pseudoviruses. Parental bNAbs (iMab and N6) IC_{80} and IC_{50} values used to generate Bliss-Hill predictions were sourced from the publicly available CATNAP database (http://hiv.lanl.gov/catnap) [68]. A total of 448 pseudoviruses were assessed from the CATNAP database but only 97 pseudoviruses with complete data (i.e., have both IC_{80} and IC_{50} data for both N6 and iMab) were used to generate Bliss-Hill combination predictions (the model requires complete data to generate predictions). The remaining 351 pseudoviruses were excluded.

Statistical analysis

Comparisons of median neutralization IC_{50}s and IC_{80}s between iMab-N6 bibNAb and the parental antibodies (N6 or iMab) or parental antibody combination (iMab + N6) or additional bibNAbs (iMab-CAP256 or 10E8-iMab or PG9-iMab) were performed using a non-parametric, t-test (Wilcoxon matched-pairs signed rank test) and two-tailed *p* value. Where multiple comparisons between groups were performed, the level of significance was adjusted using Bonferroni correction (α/the number of comparisons; α = 0.05). *p* values were considered significant when the *p* value was < 0.01667 using the Bonferroni correction for 2 × 2 comparison of the iMab-N6 bibNAb to each parental antibody (iMab or N6) or the parental antibody combination (iMab + N6) or each bibNAbs (iMab-CAP256 or 10E8-iMab or PG9-iMab). Statistical analyses were performed using GraphPad Prism 7. The breadth-potency curves were generated by plotting the cumulative neutralization coverage against the experimentally determined or predicted IC_{80} values for each antibody using the survival model in GraphPad Prism 7.

Results

Antibody design and expression

iMab-N6 was designed using iMab-CAP256 [35] and 10E8-iMab [33] blueprints. The design incorporated CrossMab^{CH1-CL} and knob-in-a-hole mutations as described by Schaefer et al. [64] and Asokan et al. [63] respectively (left panel, Fig. 1A). These mutations allow for preferential assembly of the bibNAb, limiting unwanted by-products, without structurally compromising the paratope nor affecting epitope binding [63, 64]. iMab-N6 and its parental monoclonal antibodies were successfully expressed in HEK293T cells and purified using Protein-A agarose followed by Size-Exclusion Chromatography (SEC) (Fig. 1B). N6 chromatogram shows a homogenous sample whereas iMab-N6 and iMab (both produced using the pMin plasmid) chromatograms suggest a heterogenous sample (Fig. 1B). Elution fractions corresponding to the correctly assembled antibody confirmation were collected and pooled (shaded rectangles, Fig. 1B). The pooled SEC samples were resolved by a gradient SDS-PAGE under both reduced and non-reduced conditions to confirm purity and the correct assembly of the antibodies (Fig. 1C). Under SDS-PAGE, reduced conditions, both heavy chain and light chain bands resolved at the anticipated positions for all the antibodies suggesting correct assembly of the antibodies (Fig. 1C). Two distinct light chain bands were observed for the iMab-N6 bispecific antibody, each corresponding with the light chain bands of the parental monoclonal antibodies, iMab and N6 (Fig. 1C). Likewise, iMab-N6 heavy chains resolved into two distinct bands, each corresponding with the heavy chain bands of either iMab or N6 (Fig. 1C). Therefore, the heavy and light chain banding pattern observed for the purified iMab-N6 is consistent with parental monoclonal antibodies, suggesting correct assembly following in vitro expression. Under non-reduced conditions, the full antibodies resolved at a position between 135 and 190 kDa (Fig. 1C).

Testing the functionality of the Fab moieties

Antibody binding capabilities and specificity were determined by means of an ELISA (Fig. 2). HIV-1 Env targeting moieties (N6 and iMab-**N6**) and the host-directed CD4 binding moieties (iMab and **iMab**-N6) were tested against $gp140_{FVC}$GCN4 and 4dCD4, respectively. As expected, both N6 (blue trace) and iMab-**N6** (green trace) bound to HIV-1 Env $gp140_{FVC}$GCN4 but iMab (red

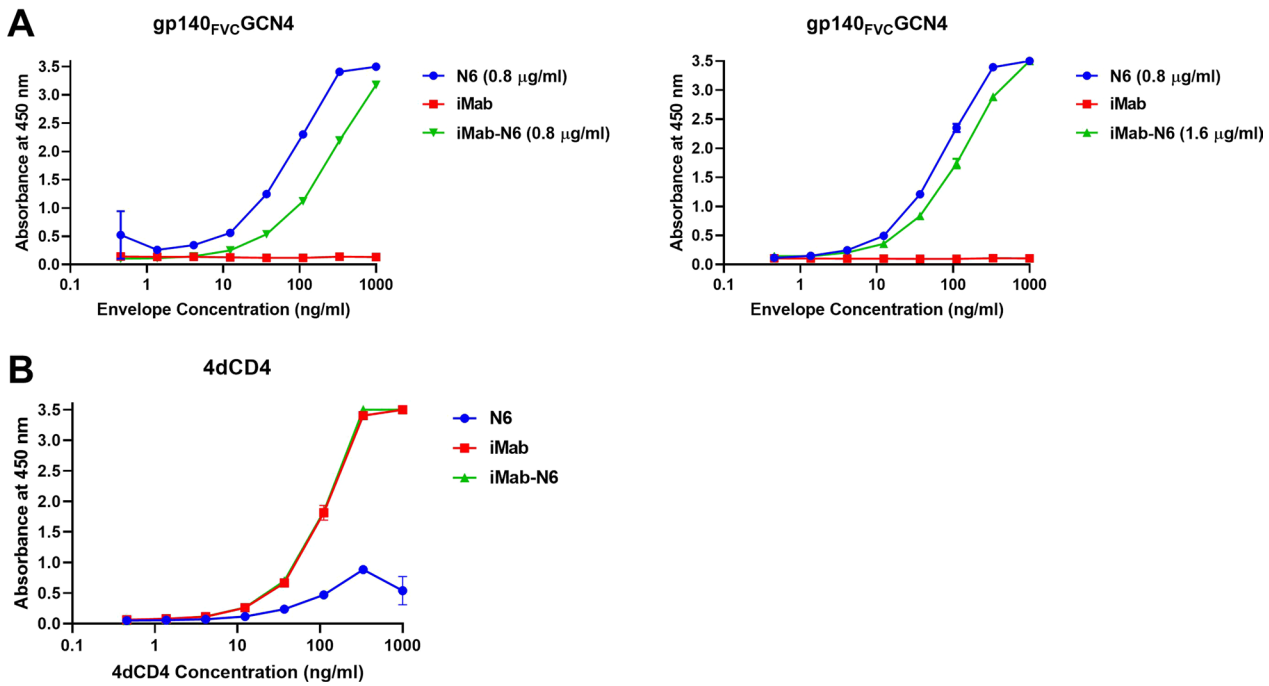

Fig. 2 Binding characterisation of purified antibodies by ELISA. **A** Left panel: HIV-1 Env targeting moieties (N6 and iMab-N6) were tested against $gp140_{FVC}$GCN4 with iMab being used as a negative control. **A** Right panel: iMab-N6 was used at double the concentration of N6 to try and correct for the difference in binding affinity seen in **A** Left panel. **B** CD4 binding moieties (iMab and iMab-N6) were tested against 4dCD4 with N6 serving as an appropriate negative control

trace, CD4 targeting) did not, thus confirming the functionality of the HIV-1 Env targeting moieties (left panel, Fig. 2A). Interestingly, a reduction in the binding titres of iMab-N6 to $gp140_{FVC}$GCN4 compared to N6 was evident (left panel, Fig. 2A). Doubling the concentration of iMab-N6 from 0.8 to 1.6 µg/ml to stoichiometrically match the number of N6 Fab fragments in the parental N6 monoclonal antibody (at 0.8 µg/ml) reduced the difference in the binding levels, although not completely (right panel, Fig. 2A). The CD4 binding moieties in iMab and **iMab**-N6 bound to 4dCD4 whilst N6 (HIV-1 Env targeting), did not. Notably, the binding levels of iMab and iMab-N6 to 4dCD4 are the same unlike N6 and iMab-N6 to $gp140_{FVC}$GCN4 (Fig. 2). Here, we confirm the activity of both Fab moieties of the bispecific antibody.

iMab-N6 displays enhanced breadth over parental constituents

Antibodies were tested against a panel of 21 geographically diverse HIV-1 pseudoviruses in a neutralisation assay to determine breadth and potency. The panel consists of both single sensitive (n = 3, CAP210.2.00.E8, RHPA4259 and X1632) and dual sensitive (n = 18) HIV-1 pseudoviruses. HIV-1 pseudoviruses from the global panel are part of the 21 selected HIV-1 pseudoviruses as they are representative of the globally circulating HIV-1 viruses [56]. When comparing iMab-N6 to its parental monoclonal antibodies, we observed an enhancement in the breadth of the bibNAb, neutralising 21/21 (100%) of the HIV-1 pseudoviruses compared to 20/21 (95%) by N6 and 19/21 (90%) by iMab

(See figure on next page.)

Fig. 3 Comparison of antibody breadth and potency against a panel of 21 HIV-1 pseudoviruses. **A** IC_{50} heat map of the parental monoclonal antibodies alone, iMab and N6; antibody combination iMab + N6; and the bibNAb iMab-N6. The numbers in brackets next to iMab + N6 represent the fold difference in potency (red = enhancement, black = reduction) of iMab-N6 over iMab + N6. The numbers in brackets next to iMab-N6 represent the fold difference in potency (green = no enhancement) of iMab-N6 over parental N6 and iMab antibodies, respectively. Individual cells are colour-coded according to IC_{50} potency (number), with lower numbers and darker red representing greater potency. *ND* not done. **B** Scatter plot depicting IC_{50} values of dual sensitive HIV-1 pseudoviruses for each antibody with the median and IQR shown. Comparisons were done using non-parametric t-test (Wilcoxon matched-pairs signed rank test) with $*p < 0.0167$ and $***p < 0.001$

A.

Pseudovirus	Subtype	Antibody potency (IC50 = µg/ml)					
		Parental Ab		Combination		bibNAb	
		N6	iMab	iMab+N6		iMab-N6	
398F1*	A	0.0385	0.0200	0.0244	(2.0×)	0.0120	(3.2×; 1.7×)
246-F3.C10.2*	AC Recombinant	0.0437	0.0662	0.0319	(2.1×)	0.0153	(2.9×; 4.3×)
CNE8*	CRF01_AE	0.2176	0.0178	0.0361	(0.9×)	0.0409	(5.3×; 0.4×)
CNE55*	CRF01_AE	0.0813	1.5627	0.0679	(1.3×)	0.0505	(1.6×; 30,9×)
PVO4	B	0.1149	0.0268	0.0415	(0.8×)	0.0503	(2.3×; 0.6×)
QH0692.42	B	0.7998	0.0705	0.1392	(0.4×)	0.3116	(2.6×; 0.2×)
RHPA4259	B	0.0296	>4	ND	(-)	0.0708	(0.4×; -)
TRO11*	B	0.0710	0.0237	0.0498	(2.2×)	0.0223	(3.2×; 1.1×)
X2278*	B	0.0395	0.0256	0.0334	(3.8×)	0.0088	(4.5×; 2.9×)
BJOX2000*	CRF07_BC	0.0539	0.0764	0.0398	(0.7×)	0.0535	(1.0×; 1.4×)
CH119*	CRF07_BC	0.0525	0.0400	0.0388	(0.6×)	0.0677	(0,8×; 0.6×)
25710*	C	0.0666	0.7805	0.0619	(3.1×)	0.0201	(3.3×; 38,8×)
CAP45.2.00.G3	C	0.0405	0.0961	0.0518	(1.1×)	0.0457	(0.9×; 2.1×)
CAP210.2.00.E8	C	>4	0.0478	ND	(-)	0.3093	(- ; 0.2×)
CE0217*	C	0.0790	0.4959	0.0854	(1.7×)	0.0492	(1.6×; 10.1×)
CE1176*	C	0.1959	0.0668	0.0775	(0.6×)	0.1221	(1,6×; 0.5×)
DU156.12	C	0.0136	0.0167	0.0134	(4.5×)	0.0030	(4.5×; 5.6×)
DU422.01	C	0.0626	0.0178	0.0317	(0.6×)	0.0566	(1.1×; 0.3×)
ZM53M.PB12	C	0.3812	0.2049	0.1486	(0.7×)	0.2015	(1.9×; 1.0×)
ZM135M.PL10a	C	0.1988	0.1066	0.0872	(2.2×)	0.0396	(5.0×; 2.7×)
X1632*	G	0.0771	>4	ND	(-)	0.1980	(0.4×; -)

* Denotes Global Panel of HIV-1 Env reference clones (Cat# 12670), NIH AIDS Reagent Program

B.

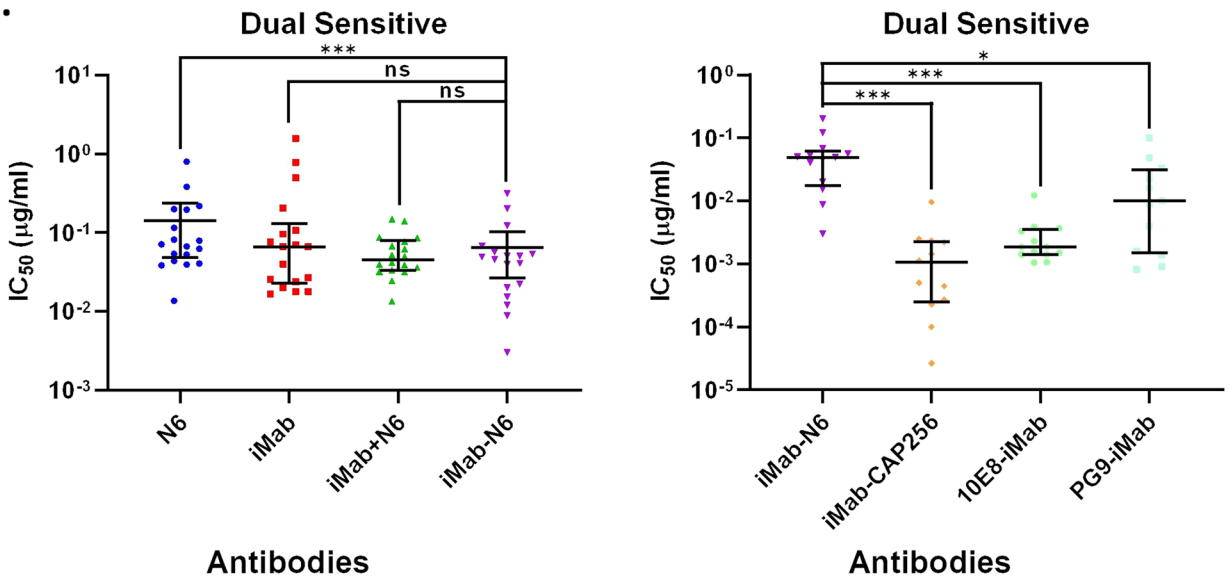

Fig. 3 (See legend on previous page.)

(Fig. 3A and Additional file 1: Fig. S1). iMab-N6 showed no potency enhancement in comparison to iMab or N6 against the 3 single sensitive HIV-1 pseudoviruses (Fig. 3A and Additional file 1: Fig. S1). Examining the dual sensitive HIV-1 pseudoviruses, iMab-N6 in comparison to N6 exhibited a modest enhancement in potency (fold increase ranging from 1.1× to 5.3×) against 15/18 HIV-1 pseudoviruses, with no enhancement against BJOX2000 and a slight reduction in potency against CH119 and CAP45.2.00.G3 (fold reduction of 1.3× and 1.1× respectively) (Fig. 3A and Additional file 1: Fig. S1). An overall comparison of iMab-N6 to N6 showed a statistically significant enhancement in potency ($p=0.0001$), however modest (Fig. 3B). Disappointingly, iMab-N6 did not exhibit an overall statistically significant enhancement in potency compared to iMab ($p=0.1674$) (Fig. 3B). However, iMab-N6 did show noticeable enhancement in potency over iMab against 246-F3.C10.2, CNE55, 25,710, CE217 and DU156 (fold increase of 4.3×, 30.9×, 38.8×, 10.1× and 5.6× respectively) (Fig. 3A and Additional file 1: Fig. S1). Collectively, iMab-N6 only showed an enhancement in potency over both iMab and N6 against 9/18 dual sensitive HIV-1 pseudoviruses (fold increase ranging from 1.1× to 4.5×) (Fig. 3A and Additional file 1: Fig. S1). For completion, we compared iMab-N6 to a combination of the parental monoclonal antibodies (iMab + N6) against the 18 dual sensitive HIV-1 pseudoviruses. Both iMab-N6 and iMab + N6 were tested at a maximum concentration of 4 µg/ml with iMab + N6 prepared by combining 2 µg/ml of each parental monoclonal antibody. iMab-N6 exhibited an enhancement in potency over iMab + N6 against 10/18 dual sensitive HIV-1 pseudoviruses (fold increase ranging from 1.1× to 4. ×) (Fig. 3A and Additional file 2: Fig. S2). Of these ten, nine HIV-1 pseudoviruses (i.e., excluding CAP45.2.00.G3) are the same nine that iMab-N6 exhibited enhancement in potency over both iMab and N6. Unsurprising, an overall comparison of iMab-N6 to iMab + N6 showed no statistically significant difference ($p=0.1964$) (Fig. 3B). Interested in exploring how iMab-N6 compares to other bibNAbs that have shown a statistically significant enhancement in neutralization over both their respective parental monoclonal antibodies, we compared IC_{50} values of iMab-N6 to iMab-CAP256 [35], 10E8-iMab [33] and PG9-iMab [34] against only those pseudoviruses that displayed sensitivity to all four parental antibodies. Disappointingly, all three bibNAbs showed statistically significant enhancement in potency over iMab-N6 (Fig. 3B). IC_{50} data for iMab-CAP256, 10E8-iMab and PG9-iMab were sourced from Moshoette et al.[35].

Predicting the clinical potential of iMab-N6

In the AMP trial, VRC01 was 75% effective at preventing infection against viruses with an IC_{80} of < 1 µg/ml but offered no protection against those with an IC_{80} of > 1 µg/ml [18]. Based on this result, an in vitro IC_{80} cut-off of < 1 µg/ml is seen as a preliminary indicator for clinical PrEP efficacy. Accordingly, the full panel (n = 21) IC_{80} data of the parental bNAbs (N6 and iMab), and the bibNAb iMab-N6 were calculated and used for breadth and potency analysis (Fig. 4A, B). Interestingly, and in contrast to the IC_{50} data (Fig. 3B), iMab-N6 outperformed both parental bNAbs, N6 and iMab ($p=0.001$, and $p=0.0043$ respectively; median IC_{80} of 0.1664 µg/ml, 0.3582 µg/ml, and 2.289 µg/ml respectively) (Fig. 4A). Furthermore, iMab-N6 exhibited 100% IC_{80} coverage of the full panel in comparison to 95% by N6 (20/21) and a disappointing 52% by iMab (11/21) (Fig. 4A, B). Crucially, iMab-N6 showed better neutralisation coverage at $IC_{80} < 1$ µg/ml than both parental bNAbs, N6 and iMab [90% (19/21), 86% (18/21), and 48% (10/21) respectively] (Fig. 4A, B). Against the dual sensitive viruses (as defined by IC_{50} data in Fig. 3A), iMab-N6 showed no significant improvement compared to the parental combination iMab + N6, but outperformed both parental bNAbs N6 and iMab ($p=0.8$, $p=0.0001$, and $p=0.0077$ respectively; median IC_{80} of 0.1534 µg/ml, 0.1263 µg/ml, 0.3601 µg/ml, and 1.349 µg/ml respectively) (Fig. 4C). The parental bNAb combination iMab + N6 achieved 100% (18/18) neutralisation coverage against the dual sensitive viruses at $IC_{80} < 1$ µg/ml, followed by iMab-N6 (94%, 17/18), N6 (89%, 16/18) and iMab (50%, 9/18) (Fig. 4C, D). Overall, iMab-N6 exhibits improved neutralisation coverage and potency over the parental bNAbs iMab and N6, but no significant difference against the iMab + N6 combination when assessing it against the $IC_{80} < 1$ µg/ml criteria. Given the similar neutralisation profile of iMab-N6 and iMab + N6, the Bliss-Hill model [29] was used to predict breadth and potency data of the combination against an expanded HIV-1 panel of 97 diverse pseudoviruses (Fig. 4E, F). This data serves as an indirect indicator of the bibNAb iMab-N6 performance against an expanded panel of pseudoviruses, pending actual in vitro testing. Consistent with our own data, the Bliss-Hill model predicted a median IC_{80} lower than 1 µg/ml for the combination (0.05 µg/ml) with a 98% (95/97) neutralisation coverage at $IC_{80} < 1$ µg/ml (Fig. 4E, F). The parental CATNAP data plotted alongside the Bliss-Hill model predictions also produced median IC_{80} values of less than 1 µg/ml for the parental bNAbs (N6: 0.21 µg/ml and iMab: 0.13 µg/ml) with a neutralisation coverage at $IC_{80} < 1$ µg/ml of 86% (83/97) and 63% (61/97) for N6 and iMab, respectively (Fig. 4E, F). Overall, the iMab + N6 Bliss-Hill combination data suggests that iMab-N6 would

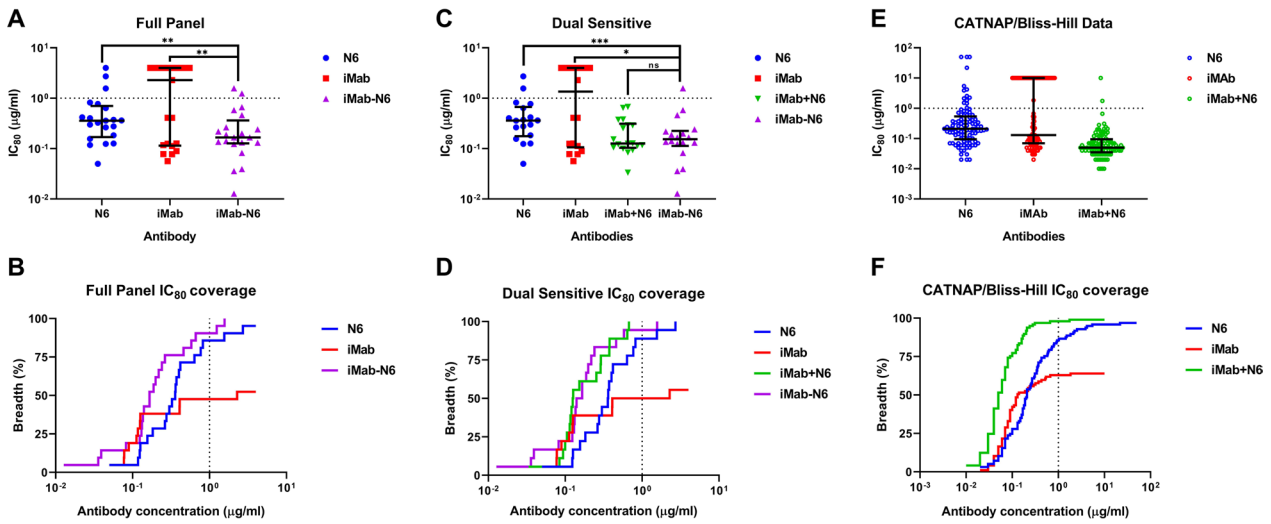

Fig. 4 Predicting the clinical utility of iMab-N6. **A**–**F** Scatter plots depicting IC_{80} values with median and IQR shown (**A**, **C** and **E**), and Breadth-Potency curves (**B**, **D**, and **F**) of each antibody against the full panel (n = 21) (**A** and **B**), dual sensitive viruses (n = 18) (**C** and **D**) and the expanded CATNAP virus panel (n = 97) (**E** and **F**). **A**–**F** Dotted lines represent the $IC_{80} = 1$ μg/ml threshold. A and C Statistical analysis were done using non-parametric t-test (Wilcoxon matched-pairs signed rank test) with $*p < 0.0167$, $**p < 0.005$ and $***p < 0.001$. **A**–**D** bibNAb iMab-N6 exhibited median IC_{80} 6–6.5× lower than 1 μg/ml AMP trial threshold and showed good neutralisation coverage of 90–94% at $IC_{80} < 1$ μg/ml. **E** and **F** N6 and iMab data was sourced from the CATNAP data base and used to generate iMab + N6, Bliss-Hill prediction data

perform well against an expanded panel of pseudoviruses and predicts superior clinical performance compared to VRC01 based on the available IC_{80} data.

Discussion

The isolation of second generation bNAbs with exceptional neutralisation coverage and/or potency has reinvigorated the HIV-1 bNAb PrEP/therapeutic field as many potential clinical candidates have been identified. Moreover, the recently concluded phase IIb clinical trial (AMP Trial: HVTN 703/HPTN 081 and HVTN704/HPTN 085) has validated bNAbs as potential PrEP candidates subject to a selection that maximises neutralising breadth and potency of the circulating regional/global HIV-1 strains. However, pre-existing, and new resistance mutations has necessitated the use of bNAb combinations for antibody PrEP/therapy strategies. Here we report on the engineering of a bibNAb using a host CD4 targeting antibody, iMab [48] paired with an HIV-1 Env CD4bs targeting bNAb, N6 [7]. By combining iMab (92% breadth with a median IC_{50} of 0.03 μg/ml against diverse HIV-1 Env subtypes) with N6 (98% breadth with a median IC_{50} of 0.066 μg/ml against HIV-1 subtype C), we hoped to engineer a bibNAb with exceptional breadth and potency against HIV-1 subtype C, the predominant subtype globally [7, 47, 48]. Like iMab-CAP256 [35] and 10E8-iMab [33], iMab-N6 was designed using the normal antibody architecture and with the addition of Knob-in-a-hole and CrossMab^{CH1-CL} mutations (Fig. 1A). These mutations promote the correct assembly of the bibNAb and do not interfere with its function [63, 64].

Having successfully produced and purified iMab-N6 (Fig. 1), we assessed the binding of the Fab moieties to their respective epitopes (Fig. 2). iMab-N6 bound to both $gp140_{FVC}$GCN4 and 4dCD4 thereby confirming the functionality of both the Fab moieties. Interestingly, iMab-N6 showed lower binding levels to $gp140_{FVC}$GCN4 in comparison to N6, this difference was reduced when the concentration of iMab-N6 was doubled within the assay (Fig. 2A). Whilst the ELISA methodology may not provide sufficient quantitative measure of binding kinetics of the parental N6 compared to the bispecific antibody, these data taken together suggest that the parental N6 antibody displays more favourable binding kinetics. Whether this was due to difference in avidity and/or the bivalent nature of the N6 parental or the immobilized Env glycoprotein that promoted dual engagement remains unknown. Nonetheless, this data does confirm the functionality of both N6 and iMab Fab moieties of the bispecific, albeit with a perhaps reduced Env-affinity.

Having confirmed the functionality of the Fab moieties, we next determined the breadth and potency of iMab-N6 against a panel of 21 (inclusive of the global panel) geographically diverse HIV-1 pseudoviruses (Fig. 3). The selected panel consists of both single sensitive (n = 3, CAP210.2.00.E8, RHPA4259 and X1632) and dual sensitive (n = 18) HIV-1 pseudoviruses. iMab-N6 exhibited an enhancement in neutralisation breadth over its two

parental bNAbs; neutralising 21/21 of the HIV-1 pseudoviruses compared to 20/21 by N6 and 19/21 by iMab (Fig. 3A). This improvement in the neutralization breadth was anticipated given the incorporation of only single resistant viral strains within the assay, and once again confirms the independent, functionality of both Fab moieties of the iMb-N6 bispecific conformation. Against the dual sensitive pseudoviruses, iMab-N6 exhibited a modest, yet statistically significant enhancement in potency in comparison to N6 but not iMab ($p=0.0001$ and $p=0.1674$ respectively, median IC_{50} of 0.0475 μg/ml, 0.0688 μg/ml, and 0.0665 μg/ml respectively) (Fig. 3B). iMab-N6 did show noticeable enhancement in potency over iMab against 246-F3.C10.2, CNE55, 25710, CE217 and DU156 (fold increase of 4.3×, 30.9×, 38.8×, 10.1× and 5.6×, respectively) (Fig. 3A). However, this enhancement in potency can be solely attributed to the improved potency of N6 against these isolates compared to the parental iMab. Consistent with the above findings, no enhancement in potency was noted in relation to the parental bNAb combination iMab + N6 ($p=0.1964$, median IC_{50}: combination 0.0457 μg/ml vs. bibNAb 0.0475 μg/ml) (Fig. 3B). Taken together, these data suggest a lack of synergy (i.e., simultaneous binding to both epitopes) between the bibNAb Fab moieties, albeit in respect to the current bispecific conformation under evaluation. The simultaneous engagement of both epitopes, improves the avidity of these bispecifics which may translate into increased potency [33, 34, 69]. Additionally, for iMab-based bibNAbs, engagement of the host CD4 by the iMab Fab moiety concentrates the bibNAb at the site of viral entry, enabling the other Fab moiety to engage HIV-1 Env thereby neutralising the virus [33, 34]. Potency enhancement of bibNAbs is not guaranteed as has been observed previously for other bibNabs reported in the literature to date; e.g. 3BNC117-PGT135 WT [69], PGT128-iMab [33] and 3BNC117-iMab [33]. Of particular interest, the latter, 3BNC117-iMab shares structural (Cross Mab/KIH assembly) and antigen-targeting (CD4bs HIV-1 spike/domain 2 of host CD4) similarities to the iMab-N6 bispecific described herein, also did not convey significant potency enhancement compared to the parental antibodies [33]. Whether this statement would hold for the expanded class of CD4bs-directed antibodies in combination with iMab, or that further improvements in neutralization potency could be achieved through structural optimization remain to be determined. For example; Bournazos et al. [69] improved the potency of 3BNC117-PGT135 WT by introducing the flexible hinge domain of IgG3 to the bibNAb while retaining the IgG1-Fc region. The increased flexibility allowed 3BNC117-PGT135 to simultaneously engage both its epitopes, thereby enhancing its potency [more potent than WT 3BNC117-PGT135 and the parental bNAbs (3BNC117 and PGT135)] [69]. Alternatively, iMab-N6 potency may be improved by completely switching IgG1 for IgG3 (class switching) instead of only introducing the hinge region to an IgG1 structure. Richardson et al. [70] showed that CAP256 (highly potent bNAb) potency was significantly improved by an IgG1 to IgG3 class switch. Like with 3BNC117-PGT135, the enhancement in potency was attributed to increased flexibility which translates to better epitope accessibility and affinity [70]. Class switching has the added benefit of IgG3 Fc effector functions which are highly desirable in HIV-1 treatment and prevention strategies [70, 71]. In addition, the neutralisation breadth of iMab-N6 may be bolstered further by replacing wild type (WT) iMab with modified iMab LM52 [72] in the bibNAb configuration. Song et al. [72] engineered iMab LM52 by introducing an N-linked glycan to the iMab light chain thereby overcoming iMab resistant pseudoviruses. This single modification to iMab improved its neutralisation breadth from 92% coverage to 100% coverage of the 118 pseudoviruses tested [72]. Combined, these strategies highlight the complexity when optimizing for bispecific conformations for improved potency and breadth against circulating HIV-1.

Recently, the results AMP clinical trials designed as proof-of-concept for bNAbs prophylaxis against HIV-1 acquisition were reported. Overall, VRC01 failed to protect against HIV-1 acquisition relative to the placebo control group. However, VRC01 conferred 75% protective efficacy against VRC01-sensitive viruses ($IC_{80}<1$ μg/ml), accounting for 30% of the viruses circulating within the trial regions compared to the placebo control group. The AMP trial findings highlight the role of neutralization breadth and potency in ultimately determining clinical utility. The trial data does suggest that an in vitro, IC_{80} cut off value < 1 μg/ml may hold real-world application in predicting the success of said strategies. Given the lack of enhancement in potency of iMab-N6 against its parental bNAbs, it came as no surprise that iMab-N6 is the lowest performing bibNAb in comparison to iMab-CAP256 [35], 10E8-iMab [33] (the two bibNAbs that share the same Ab structural architecture as iMab-N6) and PG9-iMab [34] (Fig. 3B). However, we were encouraged to note that that the median IC_{80} data generated (against the dual sensitive pseudoviruses) here was approximately 2.8–7.9-fold lower than the 1 μg/ml AMP trial threshold, for the N6 parental, parental combination and the bispecific (N6 0.358 μg/ml, iMab 2.29 μg/ml, iMab + N6 0.126 μg/ml and iMab-N6 0.166 μg/ml) (Fig. 4A, C). Moreover, iMab-N6 has a 90% IC_{80} coverage at 1 μg/ml in comparison to 86% for N6 and 48% for iMab against the full panel; indicating a significant

improvement over the parental bNAbs (Fig. 4A, B). While confirmation of the iMab-N6 bibNAb median neutralisation potency would require validation against a more comprehensive pseudovirus panel, Bliss-Hill [29] prediction of the iMab + N6 combination suggests an $IC_{80} < 1$ µg/ml would be achievable for the bibNAb iMab-N6 (Bliss-Hill prediction iMab + N6: median IC_{80} of 0.05 µg/ml and a 98% (95/97) neutralisation coverage at $IC_{80} < 1$ µg/ml) (Fig. 4E, F). In conclusion, bibNAb iMab-N6 engineered using the normal Ab architecture with the addition of CrossMab^{CH1-CL} and Knob-in-a-hole mutations, exhibits enhancement in breadth but not potency against its parental bNAbs. Moreover, no enhancement in potency was observed in comparison to the parental bNAb combination suggesting a lack of synergy between the two iMab-N6 Fab moieties. We believe the re-engineering iMab-N6 to further enhance its potency using the strategies described above is a worthwhile endeavour due to the need for a large war chest in the fight against HIV-1.

Abbreviations

ART: Antiretroviral therapy; bNAbs: Broadly neutralising antibodies; bibNAbs: Bispecific bNAbs; tribNAbs: Trispecific bNAbs; Env: HIV-1 envelope glycoprotein; Fab: Fragment antigen-binding; FDA: Food and Drug Administration; HEK293T cells: Human embryonic kidney 293 T antigen cells; IC_{50} or IC_{80}: 50% Or 80% inhibitory concentration; iMab: Ibalizumab; iMab-N6: Ibalizumab-N6; iMab-CAP256: Ibalizumab-CAP256.VRC26.25; 10E8-iMab: 10E8-ibalizumab; PG9-iMab: PG9-ibalizumab; pMin: MAb expression vector; SDSPAGE: Sodium dodecyl sulphate polyacrylamide gel electrophoresis; SEC: Size exclusion chromatography; $TCID_{50}$: 50% tissue culture infectious dose; ELISA: Enzyme-linked immunosorbent assay.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12985-022-01876-1.

Additional file 1: Fig. S1. Neutralisation curves showing breadth of iMab-N6 in comparison to the parental antibodies iMab and N6. iMab-N6 displayed an improvement in breadth by neutralising 21/21 of the HIV-1 pseudovirus compared to 20/21 by N6 and 19/21 by iMab. iMab-N6 showed a reduction in potency (as shown by the right shift of the bibNAb line) over the active parental monoclonal antibody against the three single-sensitive HIV-1 pseudoviruses (RHPA4259.7, X1632 and CAP210.2.00E8). iMab-N6 exhibited enhancement in potency (as shown by the left shift of the bibNAb line) over N6 and iMab against 15/18 and 11/18 dual sensitive HIV-1 pseudoviruses. Collectively, iMab-N6 only showed an enhancement over both iMab and N6 against 9/18 dual sensitive HIV-1 pseudoviruses (fold increase ranging from 1.1× to 4.5×). iMab, N6 and iMab-N6 were tested at a maximum concentration of 4 µg/ml.

Additional file 2: Fig. S2. Neutralisation curves of iMab-N6 in comparison to the parental combination (iMab + N6). iMab-N6 exhibited an enhancement in potency over iMab + N6 against 10/18 dual sensitive HIV-1 pseudoviruses (fold increase ranging from 1.1× to 4.5×). Of these ten, nine HIV-1 pseudoviruses (i.e., excluding CAP45.2.00.G3) are the same nine that iMab-N6 exhibited enhancement in potency over both iMab and N6. Results suggest N6/iMab bispecific structural configuration of these paratopes are crucial for the observed enhancement in potency against these 9 HIV-1 pseudoviruses. Both iMab-N6 and iMab + N6 were tested at a maximum concentration of 4 µg/ml with the iMab + N6 combination prepared by mixing 2 µg/ml of each parental monoclonal antibody.

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Author contributions

MAK and MAP conceptualised the project. MAK and TM designed the experiments and TM performed the experiments. MAK and TM wrote the manuscript and analysed the data. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author upon request. Parental bNAbs (iMab and N6) IC_{80} and IC_{50} values used to generate Bliss-Hill predictions were sourced from the publicly available CATNAP database (http://hiv.lanl.gov/catnap).

Declarations

Ethics approval and consent to participate

An ethics waiver (ref: W-CBP-180305–02) was applied for and granted by the Human Research Ethics Committee (Medical), Faculty of Health Sciences, University of the Witwatersrand.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Supplementary Information

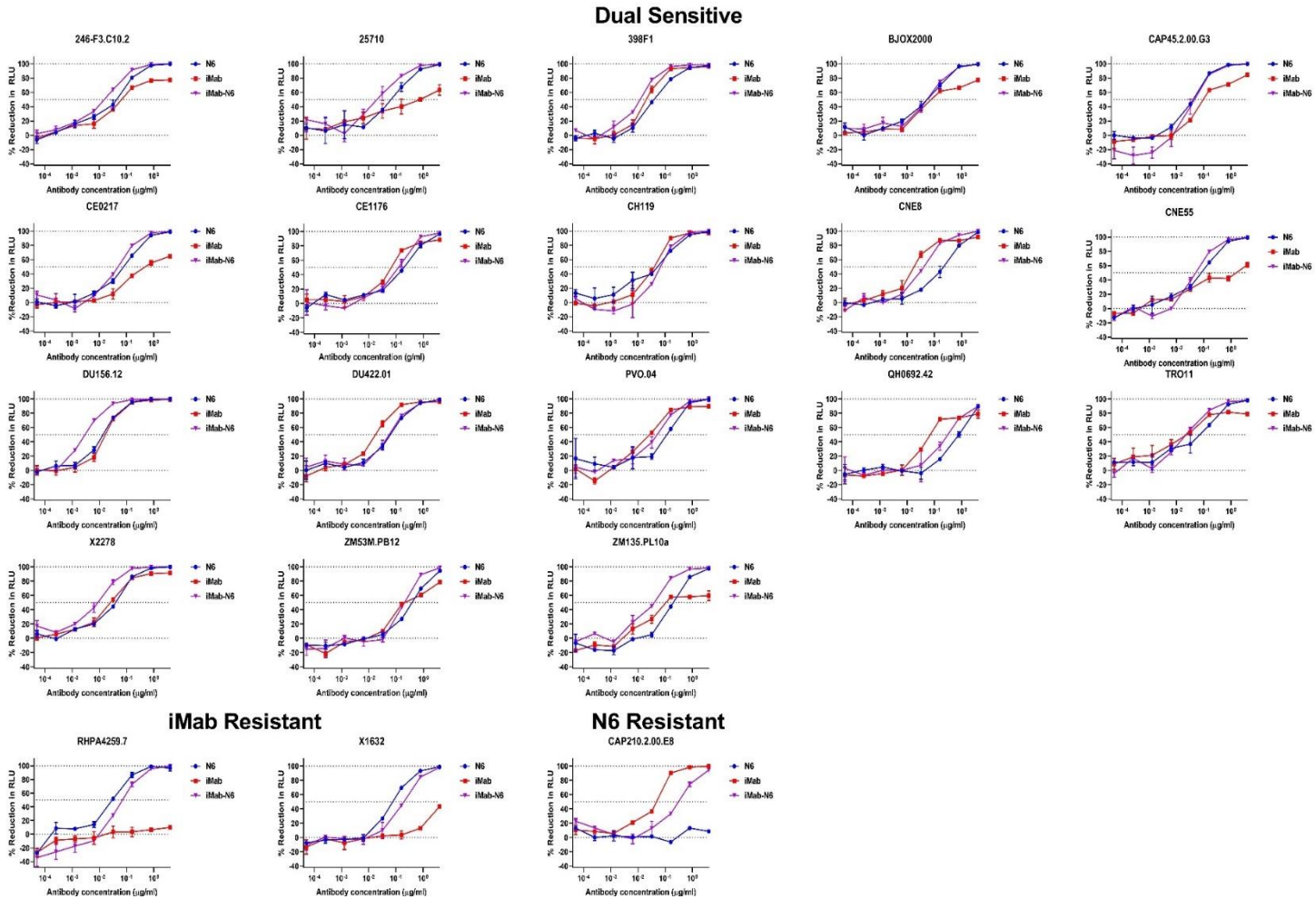

Figure. S1: Neutralisation curves showing breadth of iMab-N6 in comparison to the parental antibodies iMab and N6. iMab-N6 displayed an improvement in breadth by neutralising 21/21 of the HIV-1 pseudovirus compared to 20/21 by N6 and 19/21 by iMab. iMab-N6 showed a reduction in potency (as shown by the right shift of the bibNAb line) over the active parental monoclonal antibody against the three single-sensitive HIV-1 pseudoviruses (RHPA4259.7, X1632 and CAP210.2.00E8). iMab-N6 exhibited enhancement in potency (as shown by the left shift of the bibNAb line) over N6 and iMab against 15/18 and 11/18 dual sensitive HIV-1 pseudoviruses. Collectively, iMab-N6 only showed an enhancement over both iMab and N6 against 9/18 dual sensitive HIV-1 pseudoviruses (fold increase ranging from 1.1× to 4.5×). iMab, N6 and iMab-N6 were tested at a maximum concentration of 4 µg/ml.

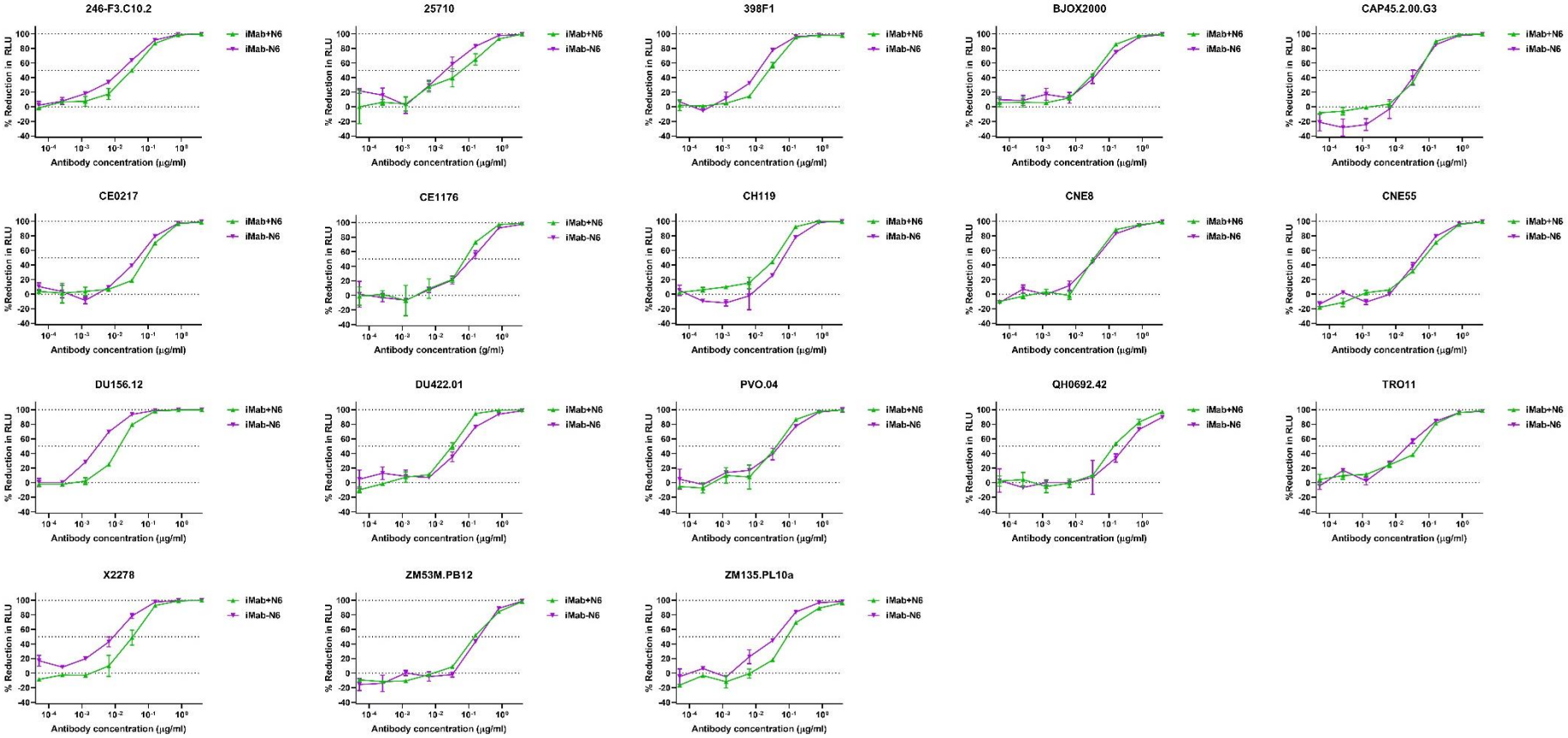

Figure. S2: Neutralisation curves of iMab-N6 in comparison to the parental combination (iMab + N6). iMab-N6 (purple) exhibited an enhancement in potency over iMab + N6 (green) against 10/18 dual sensitive HIV-1 pseudoviruses (fold increase ranging from 1.1× to 4.5×). Of these ten, nine HIV-1 pseudoviruses (i.e., excluding CAP45.2.00.G3) are the same nine that iMab-N6 exhibited enhancement in potency over both iMab and N6. Results suggest N6/iMab bispecific structural configuration of these paratopes are crucial for the observed enhancement in potency against these 9 HIV-1 pseudoviruses. Both iMab-N6 and iMab + N6 were tested at a maximum concentration of 4 µg/ml with the iMab + N6 combination prepared by mixing 2 µg/ml of each parental monoclonal antibody.

Chapter 4: Appendix A

A.1. Engineering additional bibNAbs targeting HIV-1 subtype C.

Additional bibNAbs were conceptualised and engineered with the intention of improving the neutralization breadth and/or potency against HIV-1 subtype C isolates. CAP256-N6, PRO140-CAP256 and iMab-PRO140 were identified as ideal candidates. The CAP256-N6 bibNAb was engineered based on the findings by Wagh *et al* (156) which identified CAP256+N6 as the best dual-antibody combination (twenty dual-antibody combinations were tested) against HIV-1 subtype C (median IC_{80} of 0.041 µg/ml and a 99% IC_{80} coverage). The CAP256+N6 combination could be optimised in a bibNAb configuration with the potential to achieve synergy and overall enhancement in potency compared to the parental combination.

PRO140-CAP256 [PRO140 is a humanised antibody that targets the CCR5 receptor on $CD4^+$ T-cells (205)] was engineered primarily to determine if swapping iMab (in iMab-CAP256) with PRO140 will enhance the potency of the CAP256-bibNAb as observed by Huang *et al* (161) with the 10E08-iMab and 10E08-PRO140. Additionally, the engineering of PRO140-CAP256 would elucidate the importance of the iMab Fab moiety in the bibNAb configuration. Pace *et al* (163) demonstrated that substituting iMab for PRO140 in PG9-iMab (i.e., PG9-iMab to PG9-PRO140) resulted in a loss of bibNAb potency relative to the parental bNAbs (PG9-PRO140 showed similar potency as the most potent parental bNAb, PG9), thus highlighting the importance of the iMab arm in PG9-iMab.

The final conceptualised bibNAb, PRO140-iMab, is unique as it comprises entirely of host targeting antibodies, iMab (targets primary HIV-1, host cell CD4 receptor) and

PRO140 (targets secondary HIV-1 host cell CCR5). This bibNAb, the first of its kind, was engineered to explore the potential of a host-host bibNAb targeting strategy in neutralising a diverse panel of HIV-1 pseudoviruses. Furthermore, this bibNAb would allow for a comparison between host-host (PRO140-iMab), host-virus (iMab-CAP256, iMab-N6 and PRO140-CAP256) and virus-virus (CAP256-N6) targeting bibNAb strategies.

All three bibNAbs (CAP256-N6, PRO140-iMAb and PRO104-CAP256) were engineered using the normal antibody architecture with the addition of Knob-in-a-hole and CrossMab^{CH1_CL} mutations as described previously (Fig. A.1). Despite numerous optimisation attempts, unfortunately, none of these bibNAbs were characterised due to difficulties in obtaining sufficient quantities following mammalian cell expression. Nonetheless, a brief overview of the methodology and attempts to optimize expression is provided below. Hopefully, this documented account may assist in design of future projects emanating from this preliminary body of work.

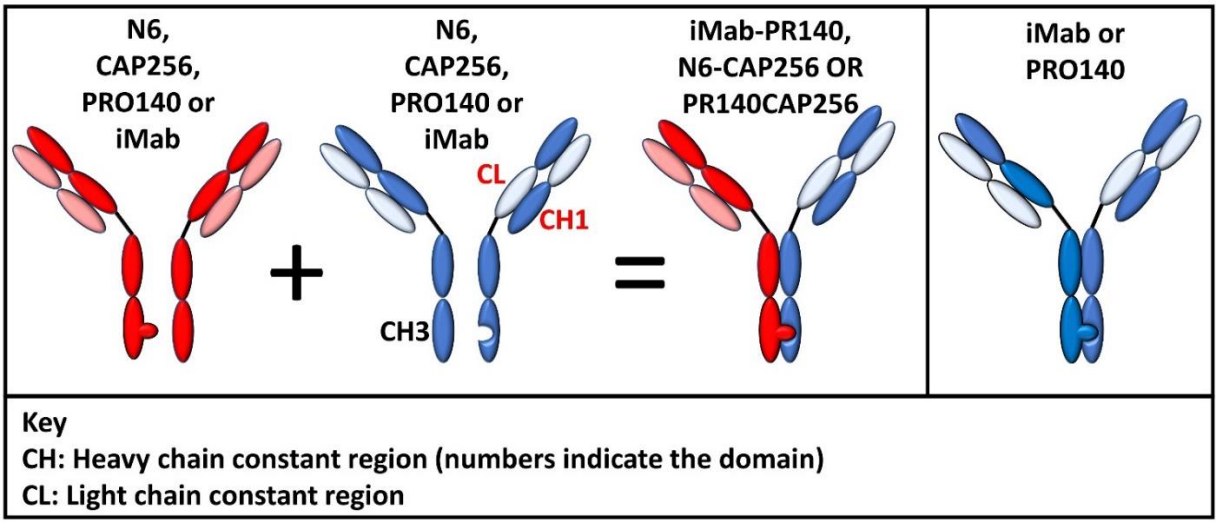

Figure A. 1: Schematic showing the elimination of possible antibody by-products with the addition of knob-in-a-hole and CrossMabCH1-CL mutations. These antibodies were designed in silico using SnapGene and were sent to GeneArt for codon optimisation and construct synthesis. iMab and PRO140 CrossMab^{CH1_CL}were used as assembly controls.

A.2. PRO140, PRO140-CAP256 and PRO140-iMab expression

Antibody production was done through a dual plasmid transfection of HEK293T cells (As described previously in Moshoette *et al* (206)) (Fig. A.2). Unfortunately, PRO140 expression was unsuccessful as confirmed by BCA assay and SDS-PAGE following Protein A purification. To identify the potential challenges, a small-scale transfection of HEK293T (Using the protocol described previously in Moshoette *et al* (206)) was performed in a 6-well tissue culture plate (Nunc, Thermo Fisher Scientific Waltham, MA, USA). This comprised of two PRO140 expressed either from the original/old or a recently purified construct set. The parental N6 (heavy and light chain plasmids) was used as a positive expression control. Antibody expression in tissue culture supernatants was detected using biolayer interferometry performed on the Blitz – ForteBio. Briefly, the tips of disposable Protein-A biosensors were submerged in 4 µl of supernatant and binding (or lack thereof) was recorded (automatically by the ForteBio instrument) over a period of 120 seconds (ForteBio's Dip and Read™ system). As expected, N6 expression was confirmed, however, PRO140 expression was unsuccessful for both construct sets a (Fig. A.3a). The reason for non-expression is unclear but is most likely due to a problem with the PRO140 constructs. This hypothesis was further strengthened by the apparent lack of PRO140 heavy and light chain expression when transfected with the complementing CAP256-knob (PRO140-CAP256) or iMab-hole (PRO140-iMab) bibNAbs (Fig. A.3b). The CAP256-knob and the iMab-Hole plasmids have both previously been shown to be functional during the production of iMab-CAP256 as described in Chapter 2 (Moshoette *et al* (160)).

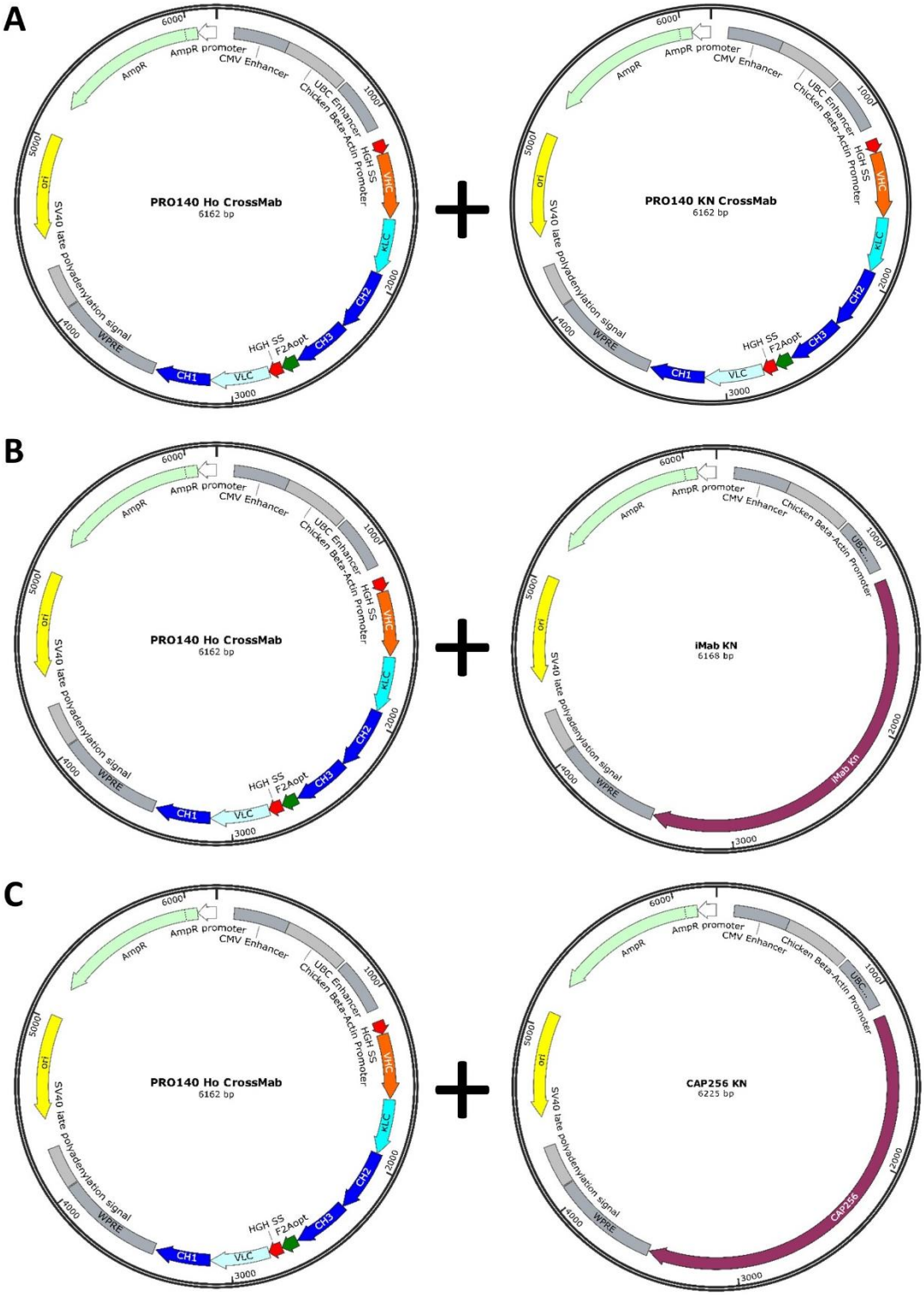

Figure A. 2: Dual plasmid system for the expression of PRO140 parental, and PRO140-iMab and PRO140-CAP256 bibNAbs. The introduction of Knob-in-a-hole and CrossMab mutations meant that antibody expression could only be achieved using a dual plasmid expression system. A PRO140 expressed using PRO140 HO CrossMab + PRO140 KN, B iMab-PRO140 using PRO140 HO CrossMab + iMab KN, and C PRO140-CAP256 using PRO140 HO CrossMab + CAP256 KN. Plasmid maps were generated using SnapGene and the mammalian expression vectors were provided courtesy of Balazs (124,138,160).

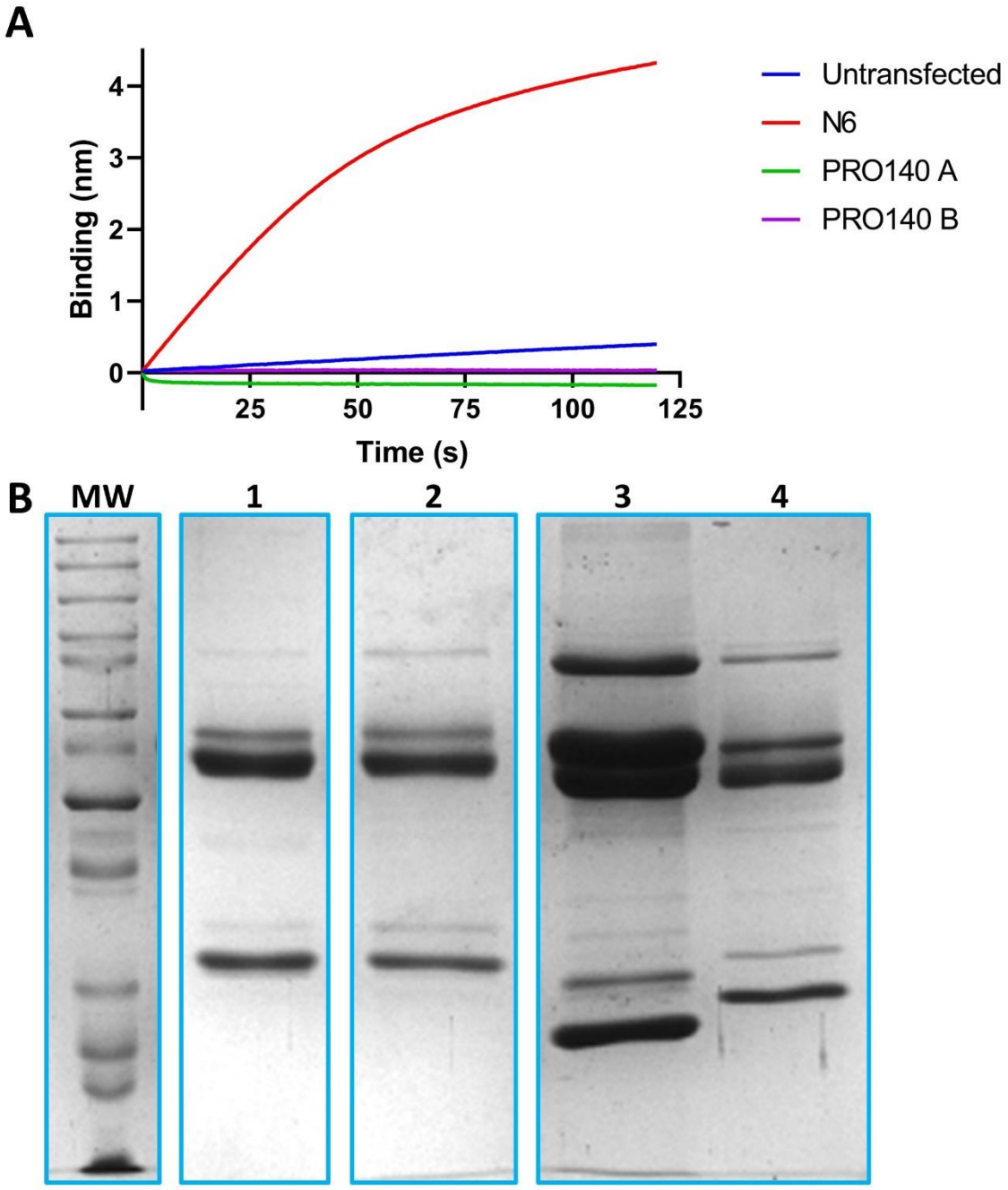

Figure A. 3: PRO140 parental and PRO140-containing bibNAb non-expression in vitro. A Expression of the parental PRO140 was unsuccessful using both the original/old (PRO140 A) and fresh (PRO140 B) DNA construct pair (Figure A.2A). N6 = positive transfection control, untransfected = media from untransfected cells used as a negative transfection control. B 10% SDS-PAGE of Protein A purified antibodies were resolved under reducing conditions. MW = Molecular weight marker, 1 = CAP256, 2 = PRO140-CAP256, 3 = iMab, and 4 = iMab-PRO140. The banding pattern of CAP256 and PRO140-CAP256, and iMab and iMab-PRO140 are similar suggesting the absence of the PRO140 moiety in both bibNAbs, an observation pointing to problems with the PRO140 construct. Uneven heating of the gel caused a “smiling effect” distortion which affected the iMab and iMab-PRO140 lane hence the difference in the light chain positions.

To resolve this issue, the corresponding heavy and light variable regions (labelled VHC and VLC) of PRO140 HO CrossMab plasmid were re-cloned by PCR

amplification (Q5 High-fidelity 2× Master mix, New England Biolabs, Ipswich, MA, USA) and assembled with the iMab vector backbone regions (labelled small and large fragment) by Gibson assembly (New England Biolabs, Ipswich, MA, USA) (Fig. A.4a and Table A.1). iMab construct was used as a backbone template because iMab and PRO140 share the same architecture (Fig. A.1) and the iMab Ho CrossMab was successfully to express and assembled in the CAP256-iMab and 10E08-Imab bibNAbs configurations. Furthermore, no issues with the iMab construct have been identified as evidenced by successful iMab expression. This PRO140Ho Cross mAb was hereafter referred to as version 2 (Fig. A.4).

Similarly, the PRO140 KN CrossMab plasmid re-cloning was done by amplifying (PCR Q5 High-fidelity 2× Master mix, New England Biolabs, Ipswich, MA, USA) the CrossMab and variable regions of PRO140 version 2 plasmid and the CH3 region of N6 KN (containing the knob mutations) then assembling the products by Gibson (New England Biolabs, Ipswich, MA, USA) (Fig. A.5 and Table A.1). Correct plasmid assembly was confirmed by restriction enzyme digestion of plasmids purified from selected bacterial colonies (Data not shown). Disappointingly, the re-cloned plasmids did not solve the PRO140 expression problem (Fig. A.6). Sequencing data identified single nucleotide mutations that may be the reason for non-expression.

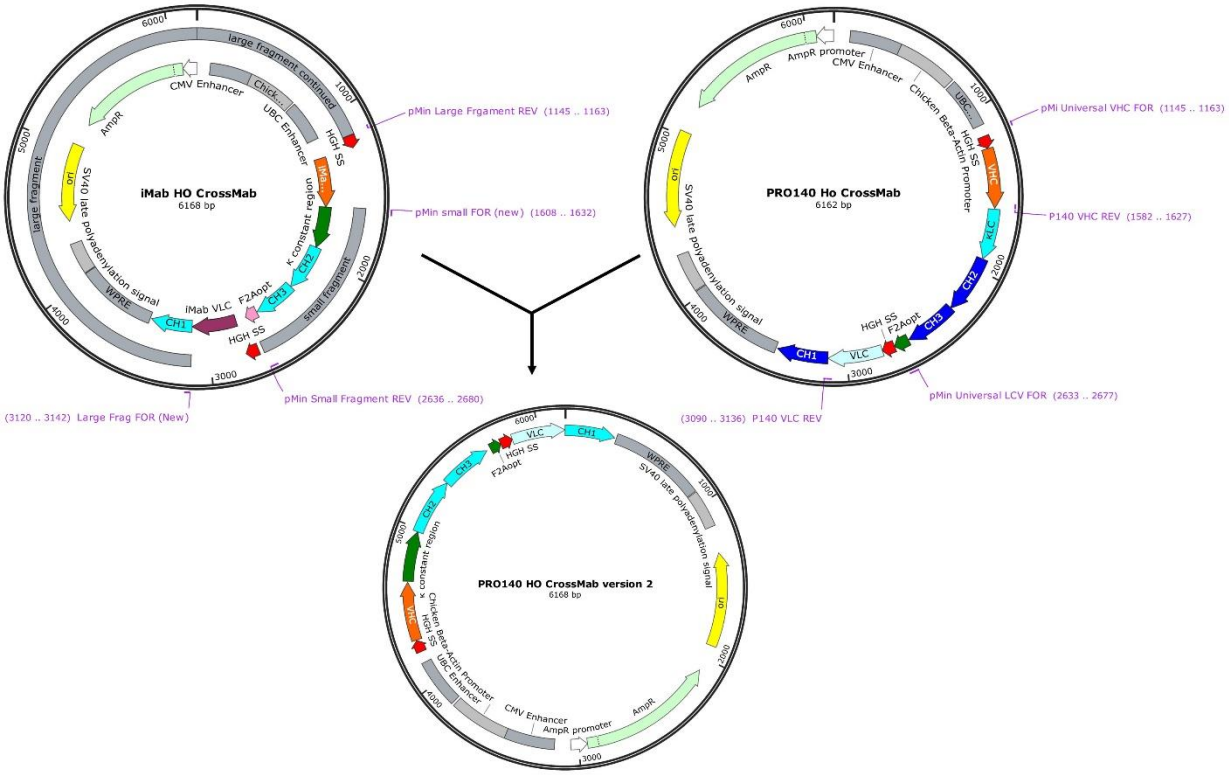

Figure A. 4: PRO140 parental and PRO140-containing bibNAb non-expression in vitro. Small and Large fragments were amplified from the iMab HO CrossMab construct using the primers displayed on the map. PRO140 variable regions (VHC and VLC) were amplified from the original PRO140 HO CrossMab plasmid. The PCR products were assembled by Gibson assembly to generate the new PRO140 HO CrossMab plasmid (version 2 plasmid). Primers were designed in silico using SnapGene and sent to Inqaba Biotec (Pretoria, GP, RSA) for synthesis. Plasmid maps were generated using SnapGene and the mammalian expression vectors were provided courtesy of Dr A. Balazs.

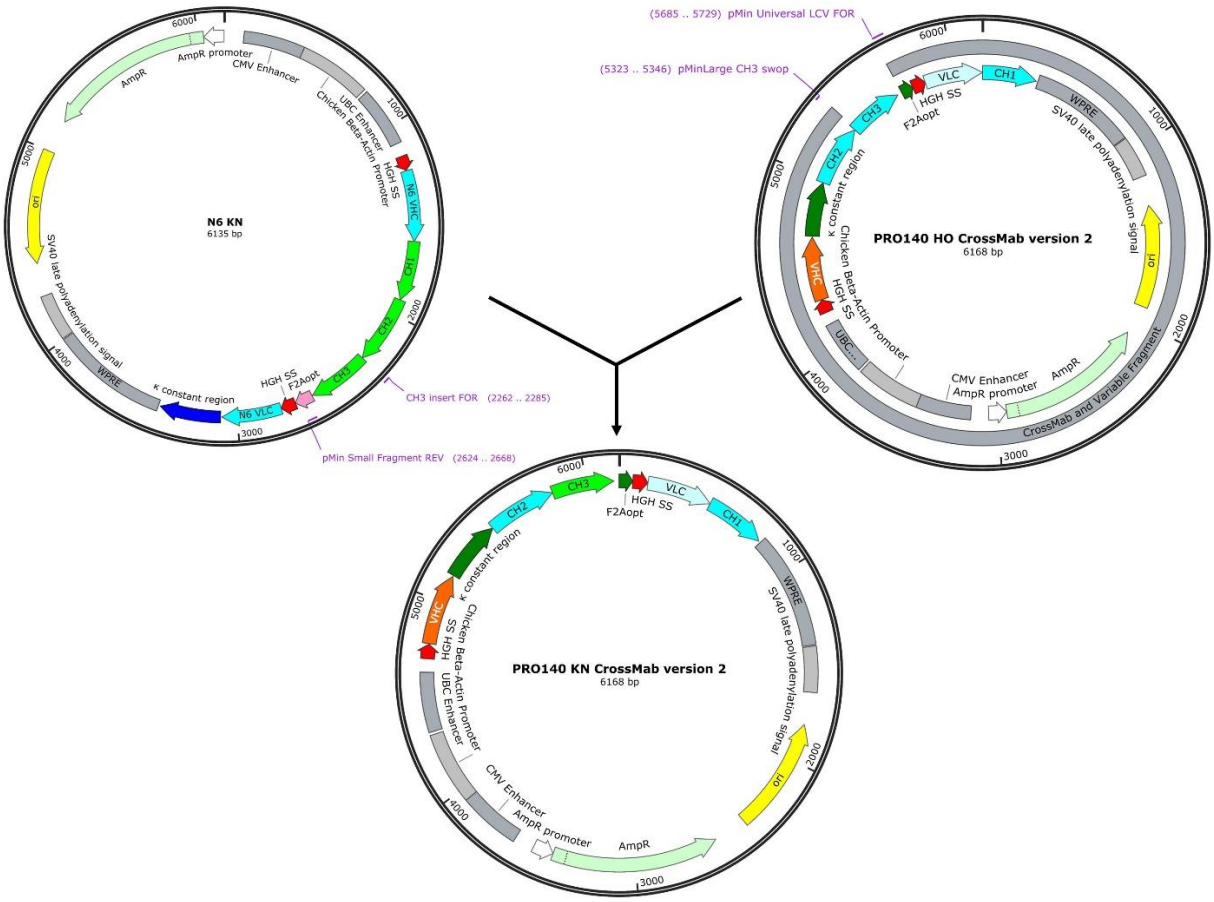

Figure A. 5: PRO140 KN CrossMab version 2 generation by Gibson Assembly. N6 KN plasmid was used as a template for the knob mutations (CH3 region amplification). The PRO140 HO CrossMab and variable fragment were amplified from the PRO140 HO CrossMab version 2 construct. The PCR products were assembled by Gibson to generate the new PRO140 KN CrossMab plasmid (version 2 plasmid). Primers were designed in silico using SnapGene and sent to Inqaba Biotec (Pretoria, GP, RSA) for synthesis. Plasmid maps were generated using SnapGene and the mammalian expression vectors were provided courtesy of Balazs (124,138,160).

Table A. 1: Primers used for PRO140 HO CrossMab and PRO140 KN CrossMab Gibson cloning. A: iMab large fragment, B: iMab small fragment, C: PRO140 Heavy Chain Variable Fragment (VHC), and D: PRO140 Light Chain Variable Fragment (VLC), E: PRO140 version 2 CrossMab and variable fragment and F: N6 KN CH3 region.

PRO 140 HO CrossMab	
Primer Name	**Primer Sequence**
A: Large Frag FOR (New)	tcctctgccagcacaaagggccc
A: pMin Large Fragment REV	ggccgcctgttcgtcaccc
B: Small FOR (new)	agtgcatctgtggccgctcctagcg
B: pMin Small Fragment REV	cctgcaagtttcagcaaatcaaagtttaatgtctgctttactggc
C: pMin Universal VHC FOR	gggtgacgaacaggcggcc
C: P140 VHC REV	aggagcggccacagatgcacttgacactgtaaccagggtgccctg gc
D: pMin Universal LCV FOR	gccagtaaagcagacattaaactttgatttgctgaaacttgcagg

D: P140 VLC REV	ccctttgtgctggcagaggacttgatttccacctttgttccctggcc
PRO 140 KN CrossMab	
Primer Name	**Primer Sequence**
E: pMin Universal LCV FOR	gccagtaaagcagacattaaactttgatttgctgaaacttgcagg
E: pMin Large CH3 swop	ctggcccttggccttgctgatggt
F: CH3 insert FOR	accatcagcaaggccaagggccag
F: pMin Small Fragment REV	cctgcaagtttcagcaaatcaaagtttaatgtctgctttactggc

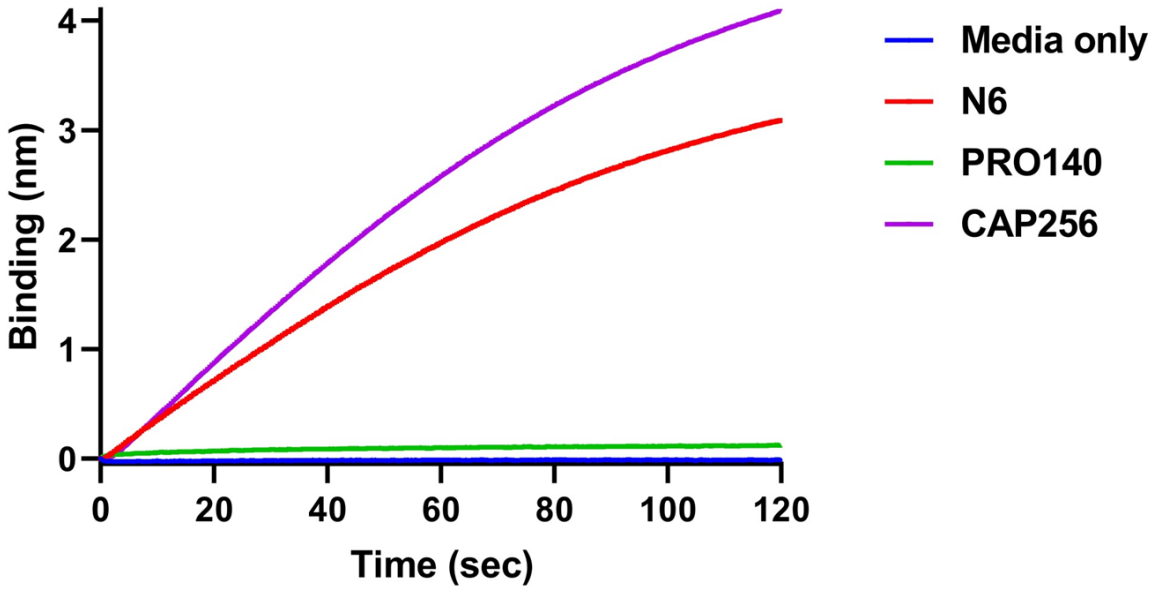

Figure A. 6: PRO140 version 2 in vitro expression evaluation. PRO140 Re-cloning of the constructs failed to solve PRO140 non-expression issues. The parental N6 and CAP256 antibody constructs were included as positive controls. Media only = media from untransfected cells used as a negative transfection control.

The PRO140 version 2 plasmids were unable to express the parental PRO140 antibody. Assembly of the Version 2 PRO140 into bibNAbs configurations CAP256-PRO140 and iMab-PRO140 were not evaluated further because of the failure to produce the PRO140 parental (which would serve as a control for the neutralization breadth and potency comparison to the respective bibNAbs). Sequencing data revealed single nucleotide mutations at a new location. Due to time constraints, the

engineering of PRO140 and its associated bibNAbs (PRO140-CAP256 and iMab-PRO140) was halted.

A.3. N6-CAP256 bibNAb expression

N6-CAP256 expression was performed in HEK293T cells using a dual plasmid expression system as described previously (Fig. A.7a). Culture supernatants were harvested as described previously and recombinantly expressed antibodies were purified by Protein A chromatography according to the methods described Moshoette *et al* (160,206). SDS-PAGE results revealed an N6-CAP256 bibNAb that is missing the CAP256 Fab moiety (Fig. A.7b). The lack of CAP256 in the bibNAb points to a possible issue with the CAP256 CrossMab HO encoding plasmid. As an alternative strategy to producing the N6-CAP256 bibNAb, a new N6 CrossMab HO plasmid was ordered form GeneArt and paired with CAP256 KN plasmid to produce N6-CAP256 bibNAb. The CAP256 KN plasmid had successfully been used to produce the iMabCAP256 bibNAb described by Moshoette *et al* (160). Unfortunately, N6-CAP256 expression was not successful even with the new construct pairing (Data not shown). As was the case with PRO140 and PRO140-bibNAbs, no further work was done to try express N6-CAP256 due to time constraints.

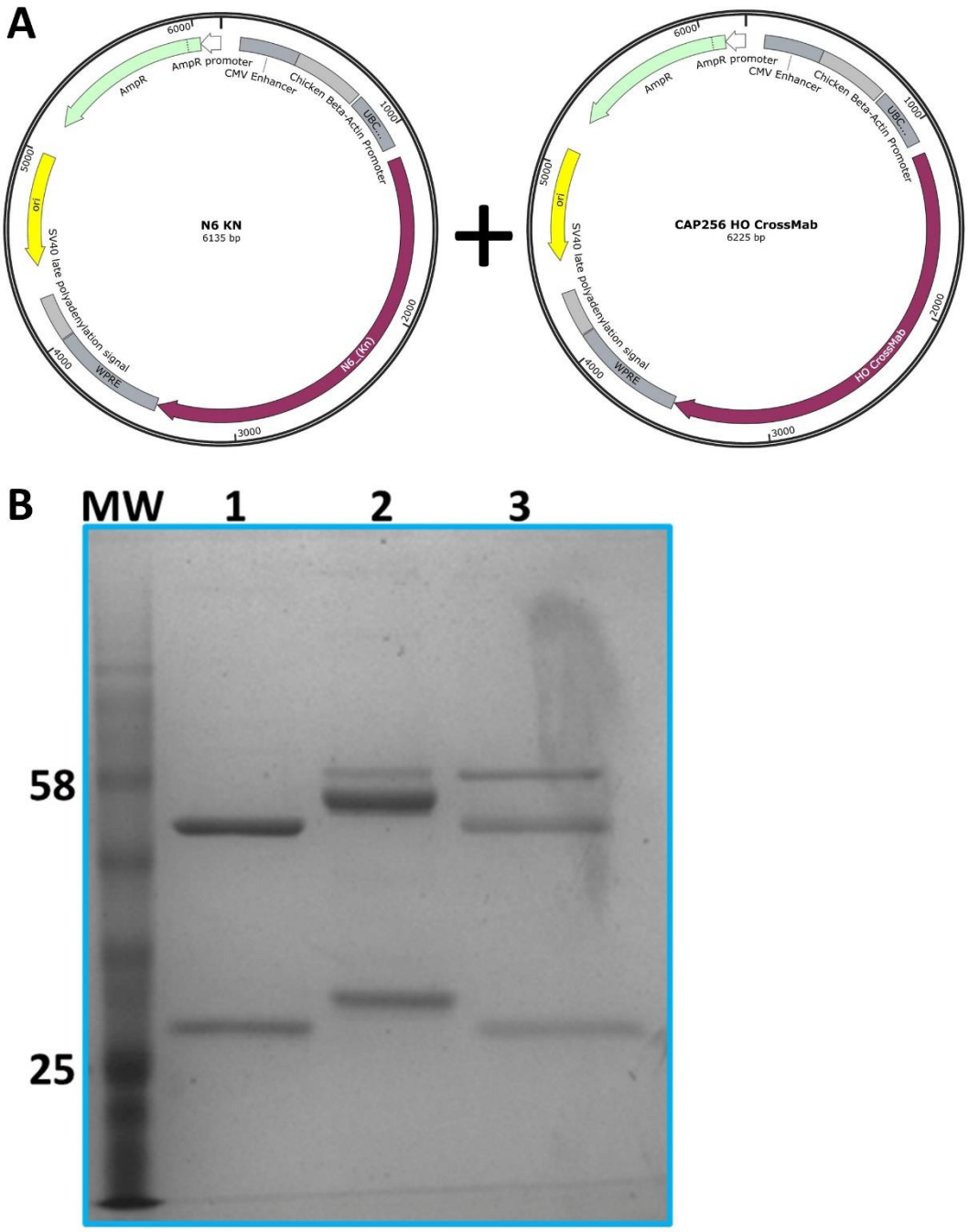

Figure A. 7: N6-CAP256 construct design and in vitro expression analysis. A N6-CAP256 was expressed using N6 KN and CAP256 HO CrossMab plasmids. B Reducing SDS-PAGE following protein A and size exclusion chromatograph showing heavy and light chains of N6 (1) and CAP256 (2) Parental antibodies, resolved at the expected location. Disappointingly, N6-CAP256 (3) lacks a CAP256 light chain and heavy chain as seen by the lack of bands that correspond to the CAP256 bands. The lack of CAP256 in the bibNAb points to a possible issue with the CAP256 CrossMab HO Plasmid. 1: N6 parental antibody, 2: CAP256 parental antibody, and 3: N6-CAP256 bibNAb. MW: Molecular weight marker, numbers in KDa shown on the left.

In summary, the engineering of PRO140-CAP256, iMab-PRO140 and N6-CAP256 would have offered a great opportunity to evaluate a larger variety of bibNAbs targeting HIV-1 subtype C. N6-CAP256 in particular has great potential given the existing data

on the N6+CAP256 combination. Unfortunately, difficulties encountered in expressing these antibodies and time limitations meant that the characterisation of these antibodies could not be included in this current body of work. Future work will focus on further optimizing of the expression of these bibNAbs, allowing for their detailed characterisation.

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Human Ethics Waiver

UNIVERSITY OF THE WITWATERSRAND JOHANNESBURG

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

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DATE: 05/03/2015

REF: *R14/49*

PROTOCOL NO: W-CBP-180305-02 *(This is your ethics application study reference number. Please quote this reference number in all correspondence relating to this study)*

PROJECT TITLE: *Engineering bispecific antibody IMAB-CAP256 targeting HIV-1 sub-type C*

Please find attached the Clearance Certificate for the above project. I hope it goes well and that an article in a recognized publication comes out of it. This will reflect well on your professional standing and contribute to the Government funding of the University.

MSWorks2002/Iain0307/ClearSca1Waiver.wps