Dependence on a variable residue limits the breadth of an HIV MPER neutralizing antibody, despite convergent evolution with broadly neutralizing antibodies

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Abstract

Broadly neutralizing antibodies (bNAbs) that target the membrane-proximal external region (MPER) of HIV gp41 envelope, such as 4E10, VRC42.01 and PGZL1, can neutralize >80% of viruses. These three MPER-directed monoclonal antibodies share germline antibody genes (IGHV1-69 and IGKV3-20) and form a bNAb epitope class. Furthermore, convergent evolution within these two lineages towards a 111.2GW111.3 motif in the CDRH3 is known to enhance neutralization potency. We have previously isolated an MPER neutralizing antibody, CAP206-CH12, that uses these same germline heavy and light chain genes but lacks breadth (neutralizing only 6% of heterologous viruses). Longitudinal sequencing of the CAP206-CH12 lineage over three years revealed similar convergent evolution towards 111.2GW111.3 among some lineage members. Mutagenesis of CAP206-CH12 from 111.2GL111.3 to 111.2GW111.3 and the introduction of the double GWGW motif into CAP206-CH12 modestly improved neutralization potency (2.5–3-fold) but did not reach the levels of potency of VRC42.01, 4E10 or PGZL1. To explore the lack of potency/breadth, viral mutagenesis was performed to map the CAP206-CH12 epitope. This indicated that CAP206-CH12 is dependent on D674, a highly variable residue at the solvent-exposed elbow of MPER. In contrast, VRC42.01, PGZL1 and 4E10 were dependent on highly conserved residues (W672, F673, T676, and W680) facing the hydrophobic patch of the MPER. Therefore, while CAP206-CH12, VRC42.01, PGZL1 and 4E10 share germline genes and show some evidence of convergent evolution, their dependence on different amino acids, which impacts
The 4E10 bNAb class, including 4E10, PGZL1 and VRC42.01, are amongst the broadest antibodies described to date (neutralizing >80% of multi-subtype virus panels) [14,20]. These bNAbs target the membrane proximal external region (MPER) of the HIV-1 gp41 envelope glycoprotein [14,20,21]. 4E10, PGZL1 and VRC42.01 use the same heavy and light chain germline genes: IGHV1-69 and IGKV3-20. While 4E10 and VRC42.01 have modest SHM (heavy chains: 8.3 and 11.5%, respectively; light chains: 5.3 and 5.7%, respectively), PGZL1 has high levels of SHM in both the heavy (20.9%) and light (12.6%) respectively. In addition to shared germline gene usage, 4E10 and VRC42.01 show convergent evolution within the CDRH1 involving the 25SGGSFS30 motif that is crucial for binding [21,22]. This motif is encoded in all germline IGHV1-69 alleles, with only the S28 being mutated within 4E10 and VRC42.01, since all IGHV1-69 germline alleles contain a T28 (www.imgt.org). Within the CDRH3, all three
bNAbs, PGZL1, VRC42.01 and 4E10 contain a $^{111.2}\text{GW}^{111.3}$ motif (IMGT numbering), with 4E10 having a double $^{111}\text{GWGW}^{111.3}$ motif, which is crucial for its neutralization [14,21].

We have previously reported the isolation of mAbs from donor CAP206 who developed broadly neutralizing plasma responses to the MPER [23]. CAP206-CH12 was isolated at 120 weeks post-infection (wpi) and an early intermediate of the same lineage, CAP206-CH12.2, from 17 wpi [24,25]. Like 4E10, PGZL1 and VRC42.01, the CAP206-CH12 lineage makes use of the $\text{IGHV1-69}$ and $\text{IGKV3-20}$ germline genes and targets the MPER [24]. However, despite identical germline variable gene usage and an overlapping epitope, CAP206-CH12 shows limited neutralization breadth (~6% of heterologous viruses) compared to the three bNAds [14]. Here, we use longitudinal antibody deep sequencing of the CAP206-CH12 lineage over three years to understand the lack of breadth and potency in this lineage compared to 4E10, PGZL1 and VRC42.01.

**Results**

We have previously described the kinetics of the MPER response in donor CAP206, using an HIV-2/HIV-1 MPER chimera involving an HIV-2 backbone (7312A) with an HIV-1 MPER region. We showed that plasma neutralization against the MPER was detected from 25 wpi and seen throughout infection [26] (Fig 1A). To study the evolution of the CAP206-CH12 lineage, we carried out antibody next-generation sequencing using gene-specific heavy and light chain primers ($\text{IGHV1-69}$ and $\text{IGKV3-20}$) from seven-time points (10, 33, 56, 94, 120, 185, and 198 wpi) during infection (Fig 1A).

Clonally related sequences were detected at all time-points sequenced for both heavy (n = 75) and light chains (n = 53). Most of the clonally related reads were obtained at 10 wpi for both heavy and light chains, with fewer sequences detected at later time-points (Fig 1B). Heavy and light chain sequences with no SHM, representing the UCA (light green) of this lineage, were detected at 10 wpi. Somatic hypermutation increased with the duration of infection, with the heavy chain being more mutated than the light chain, reaching an average of 15.8% and 10.3%, respectively (Fig 1B).

**Neutralization is largely mediated by CAP206-CH12 heavy chain**

Though the CAP206-CH12 lineage light chain showed limited evolution, we identified a number of light chain sequences that were more mutated than CAP206-CH12. Two of these were selected for downstream analysis, K1 (observed at 120wpi) and K2 (observed at 198wpi) (Fig 2A and 2B). To test the role of the light chain in CAP206-CH12 neutralization, chimeras of the UCA and CAP206-CH12 heavy and light chains were tested against autologous viruses isolated within the first year of infection and compared to the wild-type UCA and CAP206-CH12 (Fig 2C). Both the UCA and the UCA heavy chain paired with the CAP206-CH12 light chain showed no neutralization against any of the autologous viruses. In contrast, the CAP206-CH12 heavy chain paired with the UCA light chain showed some neutralization of autologous viruses, though with less potency than CAP206-CH12.

In an attempt to improve potency of CAP206-CH12 we paired K1 (6.4% SHM, light purple) and K2 (10.3% SHM, dark purple) with the CAP206-CH12 heavy chain. Neutralization of eight heterologous subtype B and C viruses (CAP85, COT6, Du156, Du422, QHO692, TRO.11, ZM135, and ZM197) was compared to the CAP206-CH12 wild-type (Fig 2D). For the majority of these viruses, very little or no difference was seen between the CAP206-CH12 heavy chain paired with different light chains. For two of the viruses (Du156
and TRO.11), the most mutated light chain K2 showed reduced neutralization compared to CAP206-CH12.

We next swapped out the CAP206-CH12 CDRL1 with the 4E10 CDRL1, previously identified as having key contact residues with the MPER [14,27]. This chimera showed limited improvement in neutralization of CAP85, COT6, Du156, Du422, QHO692, TRO.11, and ZM197 (Fig 2E). However, mutating a single tyrosine (Y) residue at position 38 (32 in Kabat

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**Fig 1. CAP206-CH12 lineage is detected throughout infection.** (A) Time-points at which longitudinal antibody next-generation sequencing (Illumina-MiSeq) were conducted and CAP206-CH12.2 and CAP206-CH12 mAbs were isolated. ID_{50} plasma neutralization titres of C1C, indicative of an MPER response, are shown in grey scale. (B) Percentage of heavy and light chain somatic hypermutation (SHM) obtained from clonally related sequences from each time-point, compared to their germline IGHV1-69 and IGKV3-20 genes. The grey bars at each time point represent the average percentage SHM. Each dot represents a single heavy or light chain clonally related sequence identified by time-point. The total number of sequences per time-point are given on the x-axis. The levels of SHM for the UCA and CAP206-CH12 are shown as green dots on the graph.

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numbering), known to interact with the MPER in 4E10 [27], to a lysine (K), resulted in a significant increase in potency (p = 0.04). Interestingly, replacing the 4E10 CDRL1 with that of CAP206-CH12 as well as two additional light chains (K1 and K2), used for further analysis, are labeled, and shown as black or coloured circles on the tree. (B) Alignment of light chains of the UCA, CAP206-CH12.2, CAP206-CH12, K1, K2, PGZL1, 4E10 and VRC42.01 and 4E10/CAP206-CH12 chimeras against their shared IGKV3-20 germline gene. Sequences are numbered according to the IMGT numbering system (www.imgt.org). (C) Neutralization of autologous viruses from the first year of infection by the UCA, CAP206-CH12, and UCA/CAP206-CH12 heavy and light chain chimeras. Shown are the IC_{50} titres, with the line representing the geometric mean titre of all viruses which is also given under the graph for each mAb. Coloured blocks above each mAb are schematics representing the heavy and light chain usage coloured by mAb, with CAP206-CH12 in green and the UCA of the lineage in grey. (D) Neutralization curves of CAP206-CH12 with its natively paired heavy and light chains (green) in comparison to CAP206-CH12 heavy chain paired with either K1 (light purple) or K2 (dark purple) against eight heterologous viruses. Shown are the average neutralization titres (IC_{50} in μg/mL) with the error bars representing standard deviations. (E) Neutralization of heterologous viruses by CAP206-CH12, 4E10 and CDRL1 chimeric antibodies, as well as CAP206-CH12 with the K^{38} mutation. Shown are the IC_{50} titres with the lines representing geometric mean, which is also given beneath each mAb. Coloured blocks above each mAb are schematics representing the CDRL regions coloured by mAb, with CAP206-CH12 in green and 4E10 in black.

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«111.2GW111.3» motif in the CDRH3 improves neutralization breadth and potency in CAP206-CH12 lineage

We, therefore, focused on the heavy chain maturation and specifically the CDRH3 since VRC42.01, PGZL1 and 4E10 all showed convergent evolution towards a «111.2GW111.3» motif, which is crucial for neutralization [14,20,21,27]. We observed convergent evolution within the CAP206-CH12 lineage towards the same motif, with the sequences seen in the later time-points having mutated away from the ML motif seen in the UCA (grey) to either a «111.2GL111.3» (orange) or «111.2GW111.3» (red) (Fig 3A and 3B). Notably, none of the CAP206-CH12 lineage members contained a double «111GWGW111.3» motif as seen in 4E10, a «111GAGW111.3» as seen in VRC42.01 or a «111GEGW111.3» seen in PGZL1 (motif highlighted in grey, Fig 3B). In addition to the CDRH3 «111.2GW111.3» motif, we observed maturation within other parts of the heavy chain, particularly within the CDRH1, FR2, and CDRH2, with CAP206-CH12.2 sharing the «25SGGSFS30» motif, crucial for binding for VRC42.01 and 4E10, though most CAP206-CH12 intermediates maintained the germline-encoded T28 (Fig 3B).

To test the influence of the «111.2GW111.3» motif within the CAP206-CH12 lineage, we selected six intermediate heavy chain sequences (H1—H6), including those with either «111.2GL111.3» (orange, Fig 3) or «111.2GW111.3» motifs (red). Since CAP206-CH12 has a «111.2GL111.3» motif we also mutated the L111.3 to a W111.3 (referred to as CAP206-CH12-GW) and introduced a double «111GWGW111.3» in CAP206-CH12 (CAP206-CH12-GGWG). We tested the ability of these antibodies including the UCA, CAP206-CH12, and wild-type CAP206-CH12 to neutralize a panel of 23 viruses that we have previously shown to be sensitive to CAP206 plasma [26], and compared these to VRC42.01, PGZL1 and 4E10 (Fig 3C). The UCA of the CAP206-CH12 lineage was unable to neutralize any of the heterologous viruses, while the early intermediate CAP206-CH12.2 showed limited neutralization (6/23 viruses, 26% breadth). As with many antibody lineages, increased SHM resulted in increased neutralization breadth [10–19]. The mutated CAP206-CH12-GW and CAP206-CH12-GGWG showed statistically significantly greater breadth and potency (p = 0.001 and p = 0.04, respectively) compared to the wild-type CAP206-CH12 (with the «111.2GL111.3» motif), with CAP206-CH12-GW having the best breadth and potency of the three antibodies (Fig 3C). Furthermore, antibodies with lower SHM compared to CAP206-CH12 but having the «111.2GW111.3» motif (H2 – H4) showed greater breadth and potency than CAP206-CH12. Similarly, H6, the most mutated mAb (15.8% SHM) but containing the «111.2GL111.3» motif, showed weaker neutralization breadth and potency compared to a less mutated mAb (H5, 13.5% SHM) present at the same time-point but with the «111.2GW111.3» motif. This data suggests that the «111.2GW111.3» motif improves neutralization potency in the CAP206-CH12 lineage, as with VRC42.01 and 4E10 [14].

Despite this, none of the lineage members showed the same degree of neutralization breadth or potency as 4E10, VRC42.01 or PGZL1 (GMT: 4.1, 2.8 and 6.9 ug/mL, respectively). To assess the role of mutations in the CDRHs, CDRH swaps were made between 4E10 and CAP206-CH12. However, these resulted in reduced or no neutralization of the chimeric antibodies compared to the wild-type 4E10 or CAP206-CH12 (Fig 4D), which may represent structural incompatibility between these regions. We noted that the CAP206-CH12 lineage reached a plateau in neutralization at 52% with most mAbs only able to neutralize 48% of viruses sensitive to the CAP206 plasma, from whom this lineage was derived. This suggests additional antibody lineages exist within this donor that account for the remaining plasma neutralization breadth.
Multiple MPER lineages and specificities detected in donor CAP206 plasma

We performed a longitudinal assessment of CAP206 plasma neutralization of all 23 heterologous viruses neutralized by this donor and partitioned the viruses into those sensitive (green) and those resistant (black) to the CAP206-CH12 lineage (Fig 4A). Neutralization of lineage-sensitive viruses was detected from 15 wpi and persisted until 159 wpi. This is consistent with the timing of the CAP206-CH12 lineage, with the UCA detected from 10 wpi, isolation of mAbs
Fig 4. Multiple MPER lineages and specificities in CAP206 plasma. (A) Longitudinal plasma sampled over three years (shown as weeks post-infection) were assayed against a multi-subtype panel of 23 viruses sensitive to CAP206 plasma shown as ID$_{50}$.[23]. Viruses shown in green are sensitive to the CAP206-CH12 lineage, while those in black are resistant. The time-points at which the different lineage mAbs (UCA, H2 –H4, CAP206-CH12, and CAP206-CH12) were detected/isolated are shown by dashed lines. The time-point at which MPER-depletions were conducted (159 wpi) is shown by the dashed/dotted line. (B) Depletion of MPER antibodies from CAP206 serum results in the reduction of neutralization for some but not all viruses. Serum from 159 weeks post-infection was depleted of anti-MPER antibodies using peptide coated beads and tested for neutralization against both lineage-sensitive and lineage-resistant viruses. Results are shown as ID$_{50}$ neutralization with blank bead depleted samples in black and MPER depleted samples in green for the lineage-sensitive viruses (top panel) and grey for lineage-resistant viruses (bottom panel). Viruses that showed >2-fold change decrease in neutralization between the blank and MPER-depleted samples were considered to be neutralized by MPER-directed antibodies. Viruses with fold change differences <2-fold were considered non-MPER antibodies. (C) Alignment of CAP206-CH12 lineage-sensitive (top panel) and lineage-resistant (bottom panel) viruses compared to C1C, a consensus subtype C MPER sequence. Differences at a single position, 674, distinguished lineage-sensitive viruses, all of which contain a D compared from lineage-resistant viruses containing either an N or S, highlighted in grey.

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CAP206-CH12.2, and CAP206-CH12 at 17 and 120 wpi respectively, and the most mutated mAb detected at 198 wpi. Furthermore, CAP206-CH12.2 is capable of neutralizing UG024 and 92RW, which are the first viruses neutralized by the plasma from 15 wpi. The emergence of the \( ^{111-2}GW^{113} \) motif at 33 wpi (detected in H1—H4) enabled the lineage to neutralize additional heterologous viruses, with all the lineage-sensitive viruses neutralized by 80 wpi. Interestingly, with the exception of ZM109, neutralization of all lineage-resistant viruses (black) developed after 80 wpi, suggesting another lineage or specificity emerged at that time-point.

Magnetic beads coated with the MPR.03 peptide were used to adsorb MPER neutralizing antibodies from CAP206 plasma at 159 wpi (~ 3 years post-infection). We tested the ability of the adsorbed and unadsorbed plasma to neutralize both lineage-sensitive and lineage-resistant viruses (Fig 4B). Within the CAP206-CH12 lineage-sensitive viruses (top panel), the majority (7/11) showed reduced neutralization following depletion (green), confirming MPER specificity [24]. However, four of the viruses that are neutralized by mAbs in this lineage (UG024, 92RW, QH0962, and Q23) showed no reduction of neutralization following MPER-depletion, suggesting other, non-MPER, specificities dominate neutralization of these viruses.

Within the CAP206-CH12 lineage-resistant viruses, five (CAP63, WITO, CAP84, CAP244, and AC10) showed no reduction in neutralization following MPER-depletion, but four (CAP45, CAP88, CAP172, and Du151) did show reduced neutralization titres (Fig 4B, bottom panel). This data indicates that at least two antibody specificities, one of which is MPER-directed and the other not, account for the neutralization of lineage-resistant viruses. Overall, this suggests that, in addition to the CAP206-CH12 MPER-directed lineage, donor CAP206 also has another MPER lineage as well as another bNAb with unknown specificity.

An alignment of the MPER sequences between the CAP206-CH12 lineage-sensitive (top panel) and lineage-resistant (bottom) viruses highlights a single amino acid at position 674 (highlighted in grey) that distinguishes these two groups (Fig 4C). Where lineage-sensitive viruses have a negatively charged D\( ^{674} \) compared to the uncharged S\( ^{674} \) or N\( ^{674} \) seen in the lineage-resistant viruses. This confirms [24] that the CAP206-CH12 lineage is dependent on D\( ^{674} \) while the additional MPER lineage within donor CAP206 is not.

**CAP206-CH12 lineage is dependent on a highly variable MPER residue**

In order to compare epitopes, we introduced mutations at position 674 and other sites within the MPER known to affect sensitivity to CAP206-CH12, PGZL1, VRC42.01, and 4E10 [14,20,24,27,28] into the COT6 virus which is sensitive to all four antibodies (Fig 5A). We compared the ability of these antibodies (including CAP206-CH12-GW) to neutralize the different mutant viruses compared to the wild-type (COT6).

Strikingly, CAP206-CH12 had distinct epitope dependencies (highlighted in green) compared to VRC42.01, PGZL1 and 4E10 (highlighted in black) (Fig 5A and 5B). Both CAP206-CH12 and CAP206-CH12-GW were highly dependent on the negatively charged aspartic acid (D) at position 674, with mutation of this residue to either an A\( ^{674} \), S\( ^{674} \), or N\( ^{674} \) resulting in complete knock-out of neutralization. N\( ^{674} \) had no effect on neutralization for VRC42.01, PGZL1 and 4E10. However, the S\( ^{674} \) or A\( ^{674} \) mutations resulted in reduced neutralization, with VRC42.01 and 4E10 most sensitive to S\( ^{674} \) (10- and 16-fold, respectively), and PGZL1 highly sensitive to A\( ^{674} \) (54-fold). Furthermore, VRC42.01, PGZL1 and 4E10 showed a more than 50-fold reduction or complete loss of neutralization when W\( ^{672} \), F\( ^{673} \), T\( ^{676} \), or W\( ^{680} \) were mutated, whereas these mutations resulted in modest effects on CAP206-CH12 and CAP206-CH12-GW.

When comparing the frequencies of these residues among 7,510 MPER sequences, including all HIV group M subtype sequences, from the Los Alamos Database (www.hiv.lanl.gov),
we noted that residues 672, 673, and 680, upon which VRC42.01, PGZL1 and 4E10 are dependent are highly conserved. In contrast, amino acid 674 upon which CAP206-CH12 and CAP206-CH12-GW show dependence is highly variable, with only 53% of global viruses containing an aspartic acid (Fig 5A and 5C). Furthermore, modeling of the residues which form part of the two MPER sub-epitopes recognized by 4E10/VRC42.01/PGZL1 versus CAP206-CH12 suggests that these antibodies likely target different aspects of the MPER. Although CAP206-CH12, VRC42.01, PGZL1 and 4E10 all target the MPER within the turn of the C-terminal helix [14,24,29], VRC42.01, PGZL1 and 4E10 target residues facing the hydrophobic core whereas CAP206-CH12 targets residues on the outer solvent-exposed surface of the helix (Fig 5D). Therefore, CAP206-CH12, VRC42.01, PGZL1 and 4E10 depend on different amino acids, and have different modes of binding to the MPER, likely accounting for the observed differences in breadth and potency.

Fig 5. CAP206-CH12 lineage is dependent on the highly variable D674 residue. (A) MPER sequence alignment of mutations introduced into the MPER of the COT6 virus (sensitive to CAP206-CH12, VRC42.01, PGZL1 and 4E10). Highlighted in black are key amino acid residues for VRC42.01, PGZL1 and 4E10 and in green are key residues for CAP206-CH12 neutralization. (B) Heat map showing the fold change differences in neutralization between COT6 wild-type and mutant viruses (represented by the rows) for VRC42.01, 4E10, PGZL1, CAP206-CH12, and CAP206-CH12-GW. Red represents reduction or loss of neutralization against the mutant, and black represents increased neutralization against the mutant. (C) Pie charts representing the relative frequency of amino acid residues at positions 674 (CAP206-CH12 dependent residue shown in green), 672, 673, 676, and 680 (VRC42.01, PGZL1, and 4E10 dependent residues are shown in black) based on 7,510 MPER sequences, representing all HIV group M subtype sequences from the Los Alamos Database (www.hiv.lanl.gov). (D) Schematic representation of the MPER “turn” and “kink” structure [29] with key residues for the CAP206-CH12 lineage shown in green and those for VRC42.01 and 4E10 shown in black.

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Discussion

Antibodies CAP206-CH12, VRC42.01, PGZL1 and 4E10 all target the C-terminal helix of MPER of gp41 and use the same variable germline genes in both the heavy and light chains (IGHV1-69 and IGKV3-20) [14,20,24,28]. Despite these shared features, VRC42.01, PGZL1 and 4E10 are amongst the broadest antibodies (>80%), whereas CAP206-CH12 only displays ~6% breadth [14]. Here we show, using antibody lineage sequencing, that the CAP206-CH12 lineage undergoes some convergent evolution with VRC42.01, PGZL1 and 4E10, introducing a 111.2GW111.3 motif within the CDRH3 region of the heavy chain. As with VRC42.01 and 4E10 [14], this 111.2GW111.3 motif improves neutralization potency within the CAP206-CH12 lineage, but not to the same levels as seen in VRC42.01, PGZL1 and 4E10. Within VRC42.01 and 4E10 the GW and GWGW motifs are not part of the antibody paratope but have been shown to enable conformational changes within the antibodies. This may contribute to overall neutralization breadth and potency, and these residues may perform a similar role in CAP206-CH12 [14]. We explored the mechanism for limited breadth and showed that CAP206-CH12 is dependent on a highly variable residue on the solvent-exposed surface on the elbow of the MPER whereas VRC42.01, PGZL1 and 4E10 are dependent on multiple conserved residues facing the hydrophobic core of the MPER. CDR1 swaps between CAP206-CH12 and 4E10 resulted in improved neutralization in 4E10 and limited improvement for CAP206-CH12 with the K38 alone significantly improving CAP206-CH12 neutralization potency. However, CDRH1 and CDRH3 swaps, known to be important in lipid viral membrane interactions in 4E10 [21], between 4E10 and CAP206-CH12 resulted in drastic reductions or no neutralization compared to CAP206-CH12. Thus, despite using the same germline genes and demonstrating convergent evolution, the dependence of these lineages on different residues within the same epitope and orientation of binding to the MPER likely determine neutralization breadth and potency.

The CAP206-CH12 lineage contains a string of hydrophobic tyrosine residues next to the 111.2GW111.3 motif which is encoded by IGHJ6. In contrast VRC42.01 and 4E10 use IGHJ4 which is much shorter and doesn’t contain the string of hydrophobic tyrosine residues. The hydrophobic residues in the CAP206-CH12 lineage may cause a steric clash between the antibody heavy chain and the hydrophobic core of the MPER, perhaps driving this lineage to favor the external solvent exposed region. Thus, the pairing of the IGHV1-69 with IGHJ6 instead of IGHJ4 may have resulted in the CAP206-CH12 lineage initially engaging the MPER in a different orientation than that of VRC42.01 and 4E10 and thereafter maturation dependence on D674, leading to narrower neutralization.

We show that donor CAP206 contained multiple bNAb specificities. In addition to the CAP206-CH12 lineage, we have provided evidence for an additional MPER lineage as well as another bNAb specificity to an undefined epitope. We and others have previously described the development of multiple bNAb specificities within a single donor, which contribute towards the overall breadth of these donors [14,30,39,31–38]. An effective vaccine would likely need to induce responses to multiple epitopes [2,40]. Therefore, studying donors that develop multiple bNAb lineages during infection may provide the insight needed for immunogen design for such strategies.

Neutralizing antibody responses to the MPER tend to be rarer than those targeting other epitopes on the HIV envelope [41]. It is therefore intriguing that both CAP206-CH12 and VRC42.01 were identified within donors that had multiple lineages targeting the MPER [14]. This suggests that there may be certain properties within the viral variants that infected the donors CAP206 and RV217.40512 (the donor from whom the VRC42 lineage was isolated) that gave rise to multiple MPER responses. Due to the highly conserved nature of the MPER,
bNAbs targeting this region tend to be among the broadest antibodies isolated to date [42]. Sequential immunogens designed based on variants identified in CAP206 and/or RV217.40512 may enable the development of such responses in a vaccine setting.

Overall, this data helps to explain why the CAP206-CH12 lineage, despite convergent evolution of variable heavy and light chain genes and similar epitope targeting, fails to develop the same level of breadth and potency as PGZL1, VRC42.01 and 4E10. Thus, while shared germ-line genes and convergent evolution within bNAb classes are crucial aspects of germline-targeting, they may not be sufficient to drive neutralization breadth.

Materials and methods

Ethics statement

CAP206 is a participant enrolled in the Centre for the AIDS Programme of Research in South Africa (CAPRISA) 002 Acute Infection study, established in 2004 in Kwa-Zulu Natal, South Africa. The CAPRISA 002 Acute Infection study was reviewed and approved by the research ethics committees of the University of KwaZulu-Natal (E013/04), the University of Cape Town (025/2004) and the University of the Witwatersrand (MM040202). CAP206, an adult, provided written informed consent.

Human samples, viruses, and mAbs

CAP206, a CAPRISA Acute Infection cohort participant, infected with an HIV subtype C virus, developed plasma neutralization breadth [43]. Stored peripheral blood mononuclear cells (PBMCs) taken at 10, 33, 56, 94, 120, 185 and 198 weeks post-infection (wpi) from donor CAP206 were used for CAP206-CH12 lineage tracing using Illumina MiSeq next generation sequencing. Stored serum samples from 159 weeks post-infection (3 years) were used for MPER adsorptions. Stored plasma samples from 8, 12, 15, 22, 33, 40, 56, 68, 81, 94, 107, 120, 133, 146, 159, 185 and 198 wpi were used for longitudinal heterologous neutralization assays. The CAP206-CH12 and CAP206-CH12.2 mAbs [24,25] were obtained as plasmids from Drs Larry Liao and Barton Haynes of Duke University. VRC42.01 and 4E10 were synthesized by GenScript (Hong Kong, China) and subcloned into a SEK vector. PGZL1 was provided by Dr Nicole Doria-Rose of the NIH VRC. Envelope viral clones used in this study were obtained from the NIH AIDS Research and Reference Reagent Program or the Programme EVA Centre for AIDS Reagents, NIBSC, UK. The C1C MPER chimera was obtained from George Shaw (University of Alabama, Birmingham).

CAP206-CH12 lineage next-generation sequencing

Total RNA was extracted from cryopreserved PBMCs at 7 different time-points (10, 33, 56, 94, 120, 185, and 198 wpi) using the AllPrep DNA/RNA mini kit (Qiagen) as per the manufacturer’s specifications. Reverse transcription was carried out using Random Hexamers (Integrated DNA Technologies) and Superscript III RT enzyme (Invitrogen). Primers specific to CAP206-CH12 heavy and light chains (IGHV1-69 and IGKV3-20) were designed such that forward primers bound in the leader sequence (IGHV1-69: 5’-CAGGTSCAGCTGGTGCARTCTGGG-3’ and IGKV3-20: 5’-GAAATTGTGTTGACRCAGTCTCCA-3’). Reverse primers bound to CH1 of the constant region (IgG: 5’-AGGGYGCCAGGGGGAAGAC-3’, Kappa: 5’-GGGAAAGATGAAGATGGTCTT-3’). All primers included Illumina MiSeq barcodes to allow sequencing on the MiSeq. Samples from each time point were amplified seven times for both the heavy and light chains to ensure adequate coverage and minimize PCR bias. PCR conditions were as previously described [16]. Nextera XT unique dual indexing combinations
selected from Illumina Indexing Kit V2 Set B were added to the pooled MiSeq amplicon libraries. All products were checked on an Agilent bioanalyzer High Sensitivity DNA kit (Diagnostech) and Qubit dsDNA HS assay (ThermoFisher Scientific) and cleaned-up using 0.75X Ampure Beads (Beckman-Coulter). A final concentration of 4.5 pM denatured DNA library with 10% PhiX control (Illumina) was run on the Illumina MiSeq, using the MiSeq reagent kit (version 3) with 2 x 300 paired-end reads.

**CAP206-CH12 lineage analysis**

Paired-end MiSeq reads were merged into full-length reads for each time point using PEAR [44]. Paired sequences were then de-replicated using USEARCH [45] resulting in unique reads. The SONAR bioinformatics pipeline [46] was used to identify clonally related reads to CAP206-CH12. In brief, germline V and J genes were assigned, the CDR3 regions were identified, and identity to CAP206-CH12 and level of somatic hypermutation for each of the reads was calculated. Clonally related sequences were selected based on germline gene usage and CDR3 identity ≥80% to CAP206-CH12. To account for sequencing and/or PCR error, all singletons were removed and the remaining sequences were clustered at 99% identity using USEARCH. A representative from clusters with a size of more than 1% of the most abundant read per time-point was selected for downstream analyses. Tools from the immcantation portal were used to reconstruct the antibody lineage and create the phylogenetic tree [47]. Briefly, clonally related heavy chain sequences were submitted to IMGT High V-quest [48,49] for analysis, and the resulting summary files were then parsed into a Change-O database. The UCA of the lineage was used as the germline-defined sequence for the reconstruction. Within R, the Alakazam package [47] was used to read in the Change-O database and reconstruct the lineage creating clones and introducing inferred sequences using buildPhylipLineage. The resulting tree was plotted using igraph [50]. The edge lengths of the tree were further modified to be proportional to the number of mutations between nodes and all nodes with no mutations from the parent node were deleted using Inkscape v 0.91.

**CAP206-CH12 lineage antibody production**

Selected clonally related heavy sequences were ordered codon-optimized from GenScript (Hong Kong, China) and sub-cloned in the CMV/R expression vector [51,52]. All heavy chain clonally related sequences (H1—H6), were paired with the mature CAP206-CH12 light chain (CH12 L). The UCA heavy chain was paired with the UCA light chain (UCA L). CAP206-CH12 was also paired with two additional light chains (K1 and K2). These heavy and light chain pairs were sub-cloned into an IgG1 backbone, co-transfected, and expressed in HEK293F cells (obtained from the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH) grown in FreeStyle 293 Expression Medium (Gibco, Thermo Scientific, MA, USA) at 37˚C, 5% CO2, 70% humidity and 125 rpm. Cultures were harvested after seven days by centrifugation at 4000 x g, and supernatants were filtered through 0.22 μm before purification by protein A chromatography.

**Site-directed mutagenesis**

Specific amino acid changes on envelope clones, CAP206-CH12 heavy chain CDRH3 (GW and GWGW), CAP206-CH12 light chain CDRL1 (K<sup>38</sup>) as well as CAP206-CH12/4E10 chimera were introduced using either the QuikChange Lightning Site-Directed Mutagenesis Kit (catalog #210519, Agilent Technologies, CA, USA) or through Gibson Assembly (NEB Q5 HS 2X MM catalog #M0494S; NEB CutSmart 10X Buffer catalog # B7204S, NEB DpnI catalog
#R0176L and NEBuilder HiFi DNA Assembly Master Mix cat # M552OAA) using primers that were synthesized by Inqaba Biotech. Mutations were confirmed by sequence analysis.

**Neutralization assays**

Neutralization was measured as a reduction in luciferase reporter gene expression after a single round of infection of TZM-bl cells with Env-pseudotyped viruses as previously described [53]. Briefly, monoclonal antibodies (mAbs) or heat-inactivated plasma/serum were incubated with pseudovirus for 1 hour, before the addition of cells for infectivity. Neutralization titres were calculated as the inhibitory antibody concentration (IC$_{50}$) or reciprocal plasma/serum dilution (ID$_{50}$) causing 50% reduction of relative luminescence units (RLU) with respect to the virus control wells (untreated virus). Starting concentrations of mAbs were at 200 μg/mL in order to determine a precise titre.

**Anti-MPER activity**

Streptavidin-coated magnetic beads (Dynal MyOne Streptavidin C1; Invitrogen) were incubated with the biotinylated peptide MPR.03 (KKKNEQELLELDKWASLWNWFDITNW LWYIRKKK-biotin-NH$_2$) (NMI, Reutlingen, Germany) at a ratio of 1 mg of beads per 20 μg peptide at room temperature for 30 min. Sera were diluted 1:20 in Dulbecco’s modified Eagle’s medium (DMEM)-10% fetal bovine serum and incubated with the coated beads for one hour at a ratio of 2.5 mg of coated beads per mL of diluted plasma. This was followed by a second adsorption at a ratio of 1.25 mg of coated beads per mL of diluted sample. After each adsorption, the beads were removed with a magnet, followed by centrifugation, and were stored at 4˚C. The separated immunoglobulin G (IgG) was removed and placed into a separate tube, where the pH was adjusted to between 7.0 and 7.4 with 1 M Tris (pH 9.0) buffer. The same beads were acid eluted twice more. The pooled eluates were then diluted in DMEM, washed over a 10-kDa Centricon plus filter, and resuspended in DMEM. The adsorbed sera were then used in neutralization assays. The reduction (>32-fold) in neutralization by the MPER-adsorbed plasma (green, top panel Fig 4B) compared to unabsorbed plasma (black) against C1C, an HIV-2/HIV-1 MPER chimera involving an HIV-2 backbone (7312A) with an HIV-1 MPER region, demonstrated the efficient removal of anti-MPER antibodies.

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