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Lipophilicity is a key factor to increase the antiviral activity of HIV neutralizing antibodies

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ABSTRACT

The HIV broadly neutralizing antibody 2F5 targets the transiently exposed epitope in the membrane proximal external region (MPER) of HIV-1 gp41, by a two-step mechanism involving the viral membrane and this viral glycoprotein. It was recently shown that 2F5 conjugation with a cholesterol moiety outside of the antibody paratope substantially increases its antiviral activity. Additionally, the antiviral activity of D5, a human antibody that binds to the N-terminal heptad repeat (NHR) of gp41 and lacks membrane binding, was boosted by the same cholesterol conjugation. In this work, we evaluated the membrane affinity of both antibodies towards membranes of different compositions, using surface plasmon resonance. A correlation was found between membrane affinity and antiviral activity against HIV-1. We propose that the conjugation of cholesterol to 2F5 or D5 allows a higher degree of antibody pre-concentration at the viral membrane. This way, the antibodies become more available to bind efficiently to the gp41 epitope, blocking viral fusion faster than the unconjugated antibody. These results set up a relevant strategy to improve the rational design of therapeutic antibodies against HIV.

1. Introduction

Despite intensive research to develop new strategies against HIV, no cure or vaccine has yet been achieved. The development of potent broadly neutralizing antibodies (bnAbs) with the ability to recognize diverse HIV-1 strains has been one of the main focuses for the scientific community. Currently, the availability of bnAbs targeting different epitopes is growing [1–4].

HIV-infected patients usually present strong antibody responses to viral epitopes but, in most cases, they are not neutralizing or strain specific [5–8]. However, in rare occasions, potent antibodies are produced to block the infection of healthy cells by HIV-1 [9–12]. These antibodies are polyreactive against non-HIV-1 proteins and lipids [13–17]. Similarly to HIV fusion inhibitor peptides, such as enfuvirtide and C34 [18], human polyreactive bnAbs 2F5 and 4E10 target the viral glycoprotein gp41. These antibodies target specifically a tryptophan-rich sequence named membrane proximal external region (MPER) [19,20]. It has been reported that the viral lipid bilayer is essential for the successful binding of 2F5 and 4E10 to MPER during the inhibition of viral infection [21,22]. Both antibodies have a long hydrophobic loop in the third complementarity-determining region of the heavy chain (CDRH3), responsible for the natural membrane affinity [21,22].

During viral entry, the two viral glycoproteins gp41 and gp120 play crucial and specific roles, such as the recognition of cell membrane receptors and formation of unique structures favorable to membrane fusion [23,24]. gp41 and gp120 are also important drug targets due to their conserved structure [25]. 2F5 and 4E10 inhibit viral entry by a hypothetical two-step mechanism [21,26,27]. The antibody is pre-concentrated at the viral surface, in a reversible way, through its hydrophobic CDRH3 [21]. After gp120-host cell receptor binding, gp41 inserts into the host cell membrane, forming a bridge between the virus and the target cell [28]. At this point, gp41 MPER is exposed during a short temporal window and able to be targeted [29]. The antibody binds to MPER to block the viral entry. The presence of the antibody close to its target is crucial for an effective inhibition.

Based on this hypothetical mechanism, if one adds a lipophilic moiety to an HIV neutralizing antibody, a higher antibody local concentration at the membrane surface may be achieved, improving HIV neutralization. The conjugation of a cholesterol moiety to HIV-

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1 fusion inhibitor peptides has increased not only their membrane affinity, but also their activity against HIV-1 [30–33]. The same strategy was recently implemented by Lacek et al. for HIV antibodies [34]. 2F5 together with a less potent and non-membrane binding antibody named D5 [35], were conjugated with cholesterol in order to prove this concept. To demonstrate that the membrane anchoring by the cholesterol moiety is the driving force for the increased antiviral potency, an amino acid substitution distant from the antibody paratope was engineered, with residue Ser^{457} or Ser^{444} (for 2F5 or D5, respectively) being substituted by a Cys residue, used for cholesterol conjugation [34].

In this study, four antibodies (Fig. 1a) were tested in order to understand and characterize in real-time their interaction with membranes with different lipid compositions, using surface plasmon resonance (SPR), which has become a widely used technique to study protein-membrane interactions. The wild-type antibody 2F5, D5 [$S^{444}C$], and their conjugated forms 2F5 [$S^{457}C$]-Chol and D5 [$S^{444}C$]-Chol were tested for affinity to membranes of different lipid composition. We found that both cholesterol-conjugated antibodies are able to bind membranes to a greater extent than their unconjugated controls. Higher affinity was determined for both cholesterol-conjugated antibodies in all tested membrane compositions. There is a strong correlation between membrane affinity and antiviral activity against HIV-1.

2. Materials and methods

2.1. Reagents and sample preparation

2F5, 2F5 [$S^{457}C$]-Chol, D5 [$S^{444}C$] and D5 [$S^{444}C$]-Chol were produced as previously described [34]. The molecular weights of the cholesterol-tagged and untagged antibodies are 155 kDa and 153 kDa, respectively. 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and egg sphingomyelin (SM) were purchased from Avanti Polar Lipids (Alabaster, AL, USA), while cholesterol (Chol) was from Sigma (Milwaukee, WI, USA). The working buffer used was phosphate buffered saline (PBS) pH 7.4. L1 Biacore sensor chips were purchased from GE Healthcare (Uppsala, Sweden).

2.2. Surface plasmon resonance

SPR measurements were performed on a Biacore X100 (Biacore, GE Healthcare). L1 sensor chips with an alkyl linker were used to immobilize liposomes, in order to form a lipid surface. The binding of the antibodies to model membranes was monitored in real-time, at 25 °C, with sample injections, by using instrument high-performance parameters.

2.3. Liposome preparation

Small unilamellar vesicles (SUVs) of POPC, POPC:Chol 2:1 or POPC:Chol:SM 1:1:1 were used throughout the studies. Lipids were dissolved in chloroform and dried under a gentle stream of nitrogen. Solvent removal was completed under vacuum overnight. Lipids were suspended in PBS at a final concentration of 1 mM and subjected to 7 freeze/thaw cycles, followed by 41 extrusions through polycarbonate filters (50 nm pore diameter).

2.4. Lipid bilayer formation

SUVs were captured onto the L1 sensor chip surface at a flow rate of 2 μL/min over 40 min. A NaOH pulse was used to remove loosely bound liposomes from the surface (10 mM, 50 μL/min, 36 s), resulting in a stable baseline. The immobilization of liposomes corresponds to an increase of approximately 10^4 RU. A full coverage of the surface of the chip was achieved, in order to enable a proper kinetic fitting. After sample injection, the regeneration of the L1 sensor chip surface was done by the sequential injection of CHAPS (20 mM, 5 μL/min flow rate, 60 s contact time), NaOH:methanol (10 mM NaOH in 20% methanol, 50 μL/min, 36 s) and finally NaOH (10 mM, 50 μL/min, 36 s). Octyl-β-D-glucopyranoside (40 mM, 5 μL/min flow rate, 60 s contact time) was used to ensure the complete cleanness of the L1 surface when cholesterol-tagged antibodies were used.

2.5. Antibody binding to membranes

2F5, 2F5 [$S^{457}C$]-Chol, D5 [$S^{444}C$] and D5 [$S^{444}C$]-Chol binding to membranes in PBS were studied on membrane surfaces prepared from the deposition of POPC, POPC:Chol 2:1 or POPC:Chol:SM 1:1:1 SUVs. The antibody solutions, ranging from 20 nM to 100 nM, were injected by using a contact time of 90 s at a constant flow (5 μL/min), followed by a uniform dissociation time of 10 min at the same flow. After each antibody binding assay, the sensor surface was regenerated as mentioned above. These experiments were repeated at least 3 times.

2.6. Analysis and data fitting

Analysis and fitting were performed using BIAevaluation 2.0 software. A kinetic fit for all sensorgrams was possible with the two-reaction model [36,37], refined for fitting the data by setting R_{max} as a local parameter. The dissociation constant, K_d, was calculated by the software according to the two-reaction model.

The method to analyze protein-lipid binding mentioned above has some flaws. For that reason, a simpler model was developed, in order to improve data analysis. The affinity of the antibodies after the dissociation phase was estimated by determining the fraction of antibody that remains bound to the lipid membrane, R_b, calculated as the ratio between the value of response units (RU) after dissociation (500 s) and the RU maximum before dissociation starts (90 s):

$$R_b = \frac{RU_{dissociation}}{RU_{max}} \quad (1)$$

3. Results and discussion

3.1. Interaction of 2F5-Chol with lipid membranes

2F5 potent neutralization make this an ideal antibody for a therapeutic strategy [38]. However, its elicitation *in vivo* together with other bnAbs may be limited by immune tolerance mechanisms [39]. Moreover, the temporal and spatial windows of opportunity to bind gp41 epitope are restricted. Concentrating the antibody close to the gp41 epitope at the membrane surface level was Lacek et al. strategy to improve HIV neutralization [34]. 2F5 [$S^{457}C$]-Chol resulted from the cholesterol conjugation outside the 2F5 paratope. It is important to note that 2F5 [$S^{457}C$]-Chol CDRH3 residues, associated to its natural membrane affinity, remained intact [34].

An SPR biosensor was used to evaluate the interaction of the HIV-1 antibodies 2F5, D5 and their cholesterol conjugates with biomembrane model systems. Three different membrane compositions were tested, in order to mimic mammalian cell membrane liquid disordered (POPC) and liquid ordered (POPC:Chol) domains, as well as lipid rafts (POPC:Chol:SM). The latter has been reported as the preferred location for HIV fusion [40]. These lipids also close resemble the viral membrane.

2F5 [$S^{457}C$]-Chol sensorgrams showed a stronger interaction for all membranes tested (Fig. 1b), when compared with 2F5 (Table 1). These results show a general increase in binding concomitant with

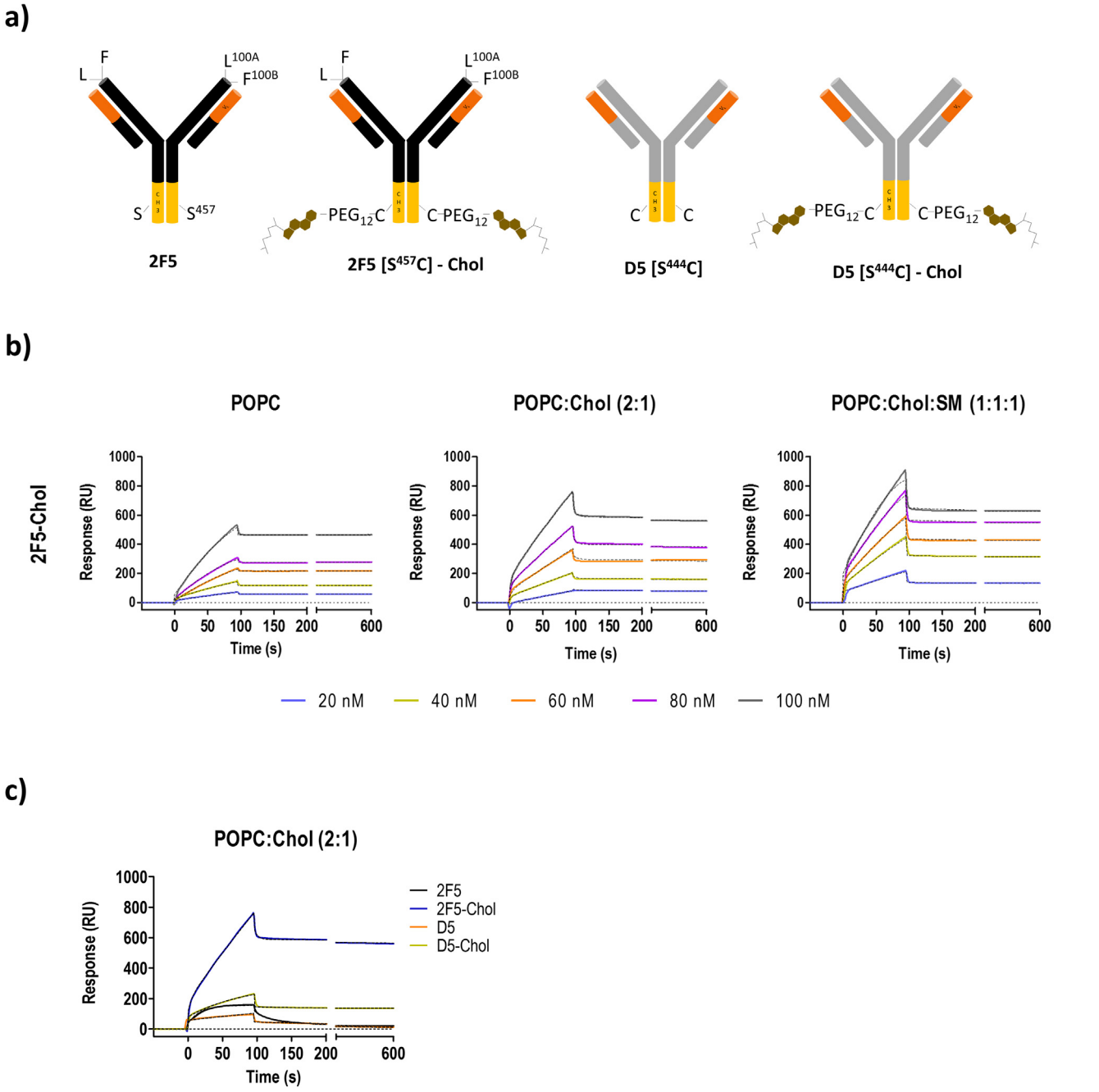

Fig. 1. Evaluation of cholesterol-conjugated antibodies interaction with membranes. (a) Schematic representation of the antibodies, in which the amino acid residues F^{100B} and L^{100A} belong to the CDRH3 loop of 2F5 and are responsible for its natural membrane affinity. C and S stand for the amino acid residues cysteine and serine, respectively, while cholesterol is shown in brown. (b) SPR sensorgrams obtained for 2F5 [S^{457}C]-Chol in the presence of POPC, POPC:Chol 2:1 or POPC:Chol:SM 1:1:1 membranes. (c) SPR sensorgrams obtained for all antibodies at 100 nM in the presence of POPC:Chol 2:1.

Table 1
Antiviral activity (IC_{50}), Dissociation constant (K_d) and fraction of antibody bound to lipid membranes after the dissociation phase (R_b) of unconjugated 2F5, 2F5 [S^{457}C]-Chol, unconjugated D5 [S^{444}C] and D5 [S^{444}C]-Chol binding to POPC, POPC:Chol 2:1 and POPC:Chol:SM 1:1:1 membranes. K_d were determined by numerical integration using the two-state model [36,37]. R_b values were calculated for a 60 nM antibody concentration.

Antibody	POPC	POPC:Chol 2:1	POPC:Chol:SM 1:1:1	POPC	POPC:Chol 2:1	POPC:Chol:SM 1:1:1	IC_{50}
	K_d (nM)			R_b			(μg/mL)
2F5	26.2	2.06	10.5	0.14 ± 0.03	0.17 ± 0.02	0.15 ± 0.01	0.27[b]
2F5 [S^{457}C]-Chol	0.0023	0.846	0.0027	0.88 ± 0.05	0.69 ± 0.10	0.72 ± 0.02	0.03[b]
D5 [S^{444}C]	–[a]	–[a]	–[a]	0.02 ± 0.03	0	0.14 ± 0.01	>100[b]
D5 [S^{444}C]-Chol	7.26	0.134	99.5	0.52 ± 0.07	0.52 ± 0.11	0.44 ± 0.03	0.07[b]

[a] The sensorgram profile obtained impairs proper data fitting.
[b] IC_{50} values were obtained from Lacek et al. [34] for the pseudotyped HIV strain JR-CSF.

increasing concentrations of antibody. In addition, during the dissociation phase, both 2F5 antibodies present distinct dissociation levels. 2F5 [$S^{457}C$]-Chol remains highly bound to the membrane (Fig. 1b), while 2F5 returns almost to the baseline (Fig. 1c). 2F5 is classified as a weak membrane-binding antibody, being the presence of its epitope at the membrane extremely important to drastically change its binding nature [16,17]. Cholesterol-tagging to 2F5 seems to increase both the affinity and the strength of the binding.

In order to calculate the dissociation constant, K_d, the two-reaction model was used to fit the experimental data. The results presented in Table 1 show a high membrane affinity for 2F5 [$S^{457}C$]-Chol in all tested membranes, in comparison to its untagged version. 2F5 [$S^{457}C$]-Chol binding preference shifts from POPC:Chol towards pure POPC and POPC:Chol:SM membranes, which indicates a preferential localization of this antibody either in target cell membranes, including lipid rafts, or on the HIV surface during viral infection. In a recent study, Hardy et al. indicated that 2F5 seems to localize preferentially in liquid disordered lipid membranes [41], compromising the development of vaccine liposome strategies. However, our study shows that 2F5 [$S^{457}C$]-Chol interacts with different membranes, pointing out this antibody as a good candidate to be included in a vaccine strategy.

3.2. Cholesterol-tagging improves antibody-membrane interaction

In Lacek et al. study [34] the cholesterol-conjugated antibodies achieved a dramatic increase in the antiviral activity against all strains (*e.g.*, 10-fold and 10^3-fold increases in antiviral activity against the JR-CSF strain for 2F5 and D5, respectively). Even a weak HIV neutralizing antibody as D5 was able to neutralize all strains after being conjugated with cholesterol. D5 is the ideal candidate to test membrane affinity after cholesterol conjugation, due to the absence of interaction with membranes in the wild-type form. D5 [$S^{444}C$], the D5 variant used in this study, retains its natural affinity to gp41, without implications in its neutralizing ability [34].

D5 [$S^{444}C$] sensorgrams showed no interaction with membranes, while its cholesterol conjugate counterpart D5 [$S^{444}C$]-Chol exhibits affinity for lipid membrane (Fig. 1c). The conjugation of cholesterol to D5 allows the antibody to remain bound to the membrane while the untagged version is washed away during the dissociation phase.

It should be noticed that despite the fact that the study by Lacek et al. [34] shows by flow cytometry that these antibodies bind to cells, it was not shown that this interaction occurs exclusively with lipid membranes, as an eventual binding to proteins or glycoproteins present on the cell surface was not ruled out. Therefore, our approach using lipid system with different known composition demonstrates for the first time the nature of the interaction. To our best knowledge, apart from unconjugated 2F5 [16,17], these antibodies were never tested for their lipid membrane affinity before, which makes this study important to understand how these broad spectrum antibodies act at the membrane level.

3.3. Analysis of the antibody-membrane affinity

The calculated dissociation constants for 2F5 and D5 [$S^{444}C$]-Chol are of the same order of magnitude for all tested lipids and do not truly represent the sensorgrams data (Table 1). The latter remains highly bound to membranes, while 2F5 is washed away (Fig. 1c). This discrepancy may be due to the complexity of the interaction of the antibodies with the lipid membranes, where the kinetic fit involved steady-state approximations and, in most cases, the sensorgrams did not reach a plateau (Fig. 1b–c).

To better distinguish the affinity among the antibodies tested, the fraction of the antibody that remains bound to the lipid membranes after dissociation was calculated using Eq. (1). In our assay, RU_{max} and $RU_{dissociation}$ were taken at 90 s and 500 s after sample injection, respectively. RU_{max} corresponds to the highest RU value obtained before the dissociation starts, despite the absence of a plateau at 90 s. $RU_{dissociation}$ corresponds to the antibody bound to the lipid membrane at 500 s, where the signal is constant.

According to Table 1, 2F5 [$S^{457}C$]-Chol is the antibody that remains more bound to membranes regardless of their composition (>60%), followed by D5 [$S^{444}C$]-Chol (>40%) and 2F5 (>10%). D5 [$S^{444}C$] only remains bound when used in large concentrations, which may be indicative of unspecific binding. Comparing the R_b values between 2F5 and D5 [$S^{444}C$]-Chol, we can observe that the cholesterol-conjugated molecule remains highly bound to all membranes tested, in comparison to 2F5, which was not clear taking the K_d values provided by BIAevalution (Table 1). Our model was able to improve the analysis of the membrane binding ability of these antibodies. This new parameter, R_b, complements the analysis of the sensorgrams, and helps to clarify misinterpretations of the K_d data.

4. Conclusions

The conjugation of cholesterol to 2F5 and D5 improved the membrane affinity and antiviral activity of these HIV antibodies. The increase in membrane affinity allows a higher concentration of the antibody either on the viral lipid bilayer or at the cell membrane level. This is crucial for the binding of the antibodies to gp41, knowing that this viral target is exposed during the beginning of the viral entry process, but before the merging of viral and cellular membranes. A reduction in the spatial restriction of the cholesterol-tagged antibodies is expected due to the presence of a PEG spacer, which gives a certain degree of freedom to the antibody molecule. This question was not addressed here, but we think it may contribute to an increase in flexibility and binding of the antibodies to gp41.

Altogether, this study confirms the cholesterol-conjugation strategy as a good option to overcome membrane affinity problems, as well as to improve the antiviral activity of antibodies against HIV (and eventually other enveloped viruses), without the need to change their amino acid residues sequence.

Author contributions

The experiments were conceived and designed by N.C.S., M.T.A., A.H. and A.S.V. Cholesterol-antibody conjugation was engineered by A.P. and F.T. Experiments and data analysis were performed by M.T.A. The manuscript was written by M.T.A and N.C.S. All authors discussed the results and commented on the manuscript.

Transparency declaration

The authors have declared that no competing interests exist.

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