**ABSTRACT**

G-protein coupled receptors (GPCRs) constitute major drug targets due to their involvement in critical biological functions and pathophysiological disorders. The leading challenge in their structural and functional characterization has been the need for a lipid environment to accommodate their hydrophobic cores. Here, we report an antibody scaffold mimetic (ASM) platform where we have recapitulated the extracellular functional domains of the GPCR, C-X-C chemokine receptor 4 (CXCR4) on a soluble antibody framework. The engineered ASM molecule can accommodate the N-terminal loop and all three extracellular loops of CXCR4. These extracellular features are important players in ligand recruitment and interaction for allosteric and signal transduction. Our study shows that ASM\textsubscript{CXCR4} can be recognized by the anti-CXCR4 antibodies, MEDI3185, 2B11, and 12G5, and that ASM\textsubscript{CXCR4} can bind the HIV-1 glycoprotein ligand gp120, and the natural chemokine ligand SDF-1α. Further, we show that ASM\textsubscript{CXCR4} can competitively inhibit the SDF-1α signaling pathway, and be used as an immunogen to generate CXCR4-specific antibodies. This platform will be useful in the study of GPCR biology in a soluble receptor context for evaluating its extracellular ligand interactions.

**Introduction**

CXCR4 is a structurally and functionally characterized\textsuperscript{1–4} alpha-chemokine receptor initially identified for its role in human immunodeficiency virus (HIV) entry of CD4 + T cells through its interaction with the glycoprotein gp120\textsuperscript{5} (Figure 1(a)). However, CXCR4 also interacts specifically with the chemokine SDF-1 (also known as C-X-C motif chemokine 12, CXCL12) and regulates processes that include stem cell function,\textsuperscript{6,7} leukocyte chemotaxis,\textsuperscript{8} and hematopoiesis\textsuperscript{9} (Figure 1(b)). It is overexpressed in over 23 human cancers,\textsuperscript{10} and remains an important therapeutic target.

CXCR4 is part of the G-protein coupled receptor (GPCR) family that has a conserved topology, consisting of a seven-transmembrane hydrophobic helical core that provides structural stability and flexibility, as well as extra – and intra-cellular hydrophilic loops that are responsible for specific interactions with ligands and effector molecules, respectively. The extracellular loops (ECLs) of GPCRs have been identified as important players in initial ligand recognition and binding.\textsuperscript{11} Small molecule inhibitors often include interactions with the ECLs.\textsuperscript{12} Thus, studies to characterize the ECLs are essential to improve therapeutic discovery efforts by gaining a better understanding of the mechanisms behind ligand recruitment and the regulation of signal transduction.

The structural and functional studies of multi-transmembrane proteins have posed substantial challenges due to the inherent difficulties in expression, purification, and analytics outside their native lipid environment. This necessitates protein solubilization that involves extensive optimization under harsh treatment to reduce protein denaturation, aggregation, and inactivation. Transmembrane proteins are a functionally important class of proteins that make up 27% of the human proteome.\textsuperscript{13} There has recently been a steady increase in transmembrane protein structure characterization, yet these proteins are still highly under-represented in the Protein Data Bank. In this regard, our knowledge of these essential players has progressed considerably slower than their soluble counterparts. There have been efforts to generate GPCR synthetic loop peptides to get around dependence on the hydrophobic core. These have been useful in NMR studies\textsuperscript{14} and inhibiting ligand signaling\textsuperscript{15,16} but have limited potential as true GPCR mimetics given that the peptides are not able to capture the three-dimensional conformation of the original receptor. Thus, grafting the ECLs onto a soluble protein scaffold could provide the necessary constraints to retain its native geometries. One effort using a four-helical bundle bacterial protein scaffold, TM1526, showed promise when used as a template that could accommodate a single ECL and the N-terminal loop to identify receptor inhibitors in a soluble format.\textsuperscript{17} Further, β-barrel scaffolds were previously used to graft all three ECLs and the N-terminus of the Y1 receptor,\textsuperscript{18} but the soluble β-barrel scaffold (bacterial lipocalin, Blc) did not refold and the transmembrane β-barrel scaffold (OmpA from *E. coli*) had ligand binding affinities well below that of the natural receptor and still remained dependent on a lipid environment.

**ARTICLE HISTORY**

Received 16 November 2018
Revised 28 February 2019
Accepted 14 March 2019

**KEYWORDS**

G-protein coupled receptor (GPCR); transmembrane proteins; mimetic; C-X-C chemokine receptor 4 (CXCR4); modeling; SDF-1α; gp120; soluble scaffold; extracellular loop (ECL).
We have engineered a novel molecule that uses the antibody as a scaffold to display the CXCR4 ECLs. We propose an antibody scaffold mimetic (ASM) platform that can replace the hydrophobic cores and accommodate the ligand-interacting extracellular portions of CXCR4. In this regard, the antibody represents a natural scaffold for constructing soluble multi-transmembrane mimetics. Uniquely, antibodies have 12 variable loops, the complementarity-determining regions (CDRs), that constitute an interacting surface sufficiently large enough to encompass the extracellular surface area of GPCR proteins. These CDRs are highly flexible in accommodating varying lengths and sequences largely stabilized by its immunoglobulin fold. The overall structure and biophysical characteristics of antibodies are well understood. Thus, they can be engineered for the desired orientation of each ECL, as well as to accommodate the specific charge environment required by the GPCR of interest. In this study, we sought to investigate this platform, using CXCR4 as the GPCR mimetic prototype. We show evidence for the generation of a soluble CXCR4 functional mimic that is recognized by CXCR4-specific antibodies, can bind its protein ligands, can compete for a ligand, and is a suitable immunogen for CXCR4 antibody generation.

Figure 1. Rational design and engineering of ASM\textsuperscript{CXCR4}. (a) Cartoon representation of the co-receptors CXCR4 and CD4 binding distinct sites on the HIV glycoprotein gp120. The b12 antibody blocks the CD4 interaction by binding its epitope on gp120. (b) Cartoon representation of the chemokine SDF-1 binding CXCR4 through its interactions with multiple extracellular loops. (c) Protein modeling of the CXCR4 extracellular and N-terminal loops onto the b12 antibody scaffold (only Fab shown for clarity). Diagram of an antibody molecule with ECLs grafted at the CDRs. One of the Fab arms is left in grey for comparison. Both Fab arms are engineered in ASM molecules. The inset shows the modeled ASM. (d) The CXCR4 receptor and a single Fab of the ASM\textsuperscript{CXCR4} molecule are shown from a top view with the ECLs colored as shown in Figure 1A. The crystal structure of CXCR4 (PDB: 4RWS, left panel) and the refined mimetic model using the b12 crystal structure as a scaffold (PDB: 1HZH, right panel) are shown in cartoon representation. (e) Superimposition of the ECL2 from CXCR4 (orange) and the refined, grafted ECL2 on b12 (blue). The calculated r.m.s.d. is 0.595 for the 13 residues forming the beta-hairpin using the program PyMol.
Results

Antibody scaffold mimic design and construction

Antibody CDRs are highly variable, and coupled with framework mutations can have a significant effect on antibody folding, stability, and antigen binding.\textsuperscript{20} Thus, we mined the protein structure database, both manually and using NCBI/Blat, to identify antibodies with properties amenable to host the CXCR4 ECLs. We determined that the CDRH3 of the broadly neutralizing HIV antibody b12 had sequence homology to the ECL2 of CXCR4 (Figure S1). We posited that this sequence similarity may be advantageous because this antibody would be amenable to ECL2 grafting with the least disruption to its native structure. In addition, its full IgG structure has been crystallographically characterized both alone and in complex with its antigen, the HIV-1 envelope glycoprotein gp120.\textsuperscript{21,22} Given that the structural information for CXCR4 is also known,\textsuperscript{17} we set out to identify the possibility of grafting the CXCR4 N-terminus and ECLs onto b12 at its CDRs.

We initially asked how well the antibody variable surface could accommodate a GPCR extracellular surface both spatially and by sequence graft acceptance. The antibody VH and VL regions combined provide six CDRs on which the ECLs can be placed (Figure 1(c)). When modeling, we set out to constrain the relative distance and orientation of the three CXCR4 ECLs while also keeping ECL2 (residues 176 to 192) positioned at the CDRH3 (residues 95 to 112). Given these constraints, ECL1 (residues 101 to 104) replaced CDRH2 (residues 52 to 55) and ECL3 (residues 269 to 272) replaced CDR1 (residues 29 to 32). This positioning also allowed for the CXCR4 N-terminal loop to be proximal to ECL3, as in its native format, by building it onto the N-terminus of the b12 VL chain (Figure 1(c,d)). The model was manually built in the software COOT and refined in the CCP4 suite. The final fully grafted CXCR4 mimic (ASM\textsuperscript{CXCR4}) model resulted in an ECL2 on the b12 scaffold with an r.m.s.d. of 0.595 when superimposed onto the ECL2 of CXCR4 (Figure 1(e)), and the overall model gave a normalized z-DOE score of \textasciitilde0.7 and a GA341 score of 1.0 indicating a reliable model.

Previous experimental and structural work has demonstrated that ECL2 of CXCR4 alone is necessary and sufficient to bind HIV gp120.\textsuperscript{22,23} Within the ECL2, seven residues have been shown to be essential for gp120 binding by scanning alanine mutation. To test if our mimic constructs could bind gp120 with only this minimal core fragment, we generated constructs where we introduced only the ECL2 in place of CDRH3 (ASM\textsuperscript{ECL2}) and a non-binding multi-alanine mutant (ASM\textsuperscript{ECL2-Ala}) that contains five of the seven amino acids important for binding (Figure S1).

The engineered constructs were expressed at approximately 100 mg/L and at high homogeneity as assessed by gel electrophoresis and size exclusion high-performance liquid chromatography (Figure 2(a) and Table 1). We further tested the purified mimetics for thermostability by differential scanning calorimetry (DSC) to assess profile changes compared to the scaffold antibody b12. All constructs were highly stable in solution with a $T_{m,1}$ of \textasciitilde72°C and a $T_{m,2}$ of \textasciitilde83°C (Figure 2(b) and Table 1). This observation is consistent with previously reported therapeutic human IgG1 molecules.\textsuperscript{24} While the initial $T_m$ transition peak for IgG1 is that of the CH2 domain, the second is for the more stable CH3 domain. The observed increase in specific heat capacity (Cp) in either peak is due to the antigen-binding fragment (Fab) region transition. We observed a profile shift of the Fab in the ASM\textsuperscript{ECL2} and ASM\textsuperscript{ECL2-DL} constructs, moving from $T_{m,2}$ to $T_{m,1}$, yet remaining at the same $T_{m,1}$ level as that of b12 (\textasciitilde73°C). Interestingly, the alanine mutant ASM\textsuperscript{ECL2-Ala} did not present this shift. However, there is no destabilization of the initial $T_m$ transition for any of the molecules, providing evidence for engineering flexibility with this platform.

$\alpha$-CXCR4 antibodies recognize CXCR4 mimetics

To evaluate whether the engineered mimic molecules retain the physiologically relevant extracellular conformation of the native receptor, we probed four constructs (ASM\textsuperscript{CXCR4}, ASM\textsuperscript{ECL2}, ASM\textsuperscript{ECL2-Ala}, and b12) using three antibodies specific to CXCR4 extracellular regions: MEDI3185,\textsuperscript{25} 2B11,\textsuperscript{26} and 12G5.\textsuperscript{27} The epitopes of MEDI3185 and 2B11 were disordered in ASM\textsuperscript{ECL2-Ala} that contains five of the seven amino acids important for binding (Figure S1). Thus, when engineering the ASM\textsuperscript{ECL2} native receptor, we probed four constructs (ASM\textsuperscript{ECL2}, ASM\textsuperscript{ECL2-Ala}, and b12) using three antibodies specific to CXCR4 extracellular regions: MEDI3185,\textsuperscript{25} 2B11,\textsuperscript{26} and 12G5.\textsuperscript{27} The negative control b12 scaffold and the alanine mutant ASM\textsuperscript{ECL2-Ala} showed an increase in binding to 12G5 as compared to the isotype controls, likely due to non-specific interactions by the b12 antibody used as a scaffold. Interestingly, 12G5 has reduced binding to ASM\textsuperscript{ECL2} confirming that the epitope of 12G5 includes the CXCR4 N-terminal loop not present in ASM\textsuperscript{ECL2}.

CXCR4 mimetics bind the viral ligand gp120

Given that CXCR4 is an HIV co-receptor that binds gp120,\textsuperscript{28,29} we wanted to determine if our mimetics could recapitulate this interaction (Figure 1(a)). The antibody scaffold allows for a direct binding ELISA using the b12, ASM\textsuperscript{CXCR4}, ASM\textsuperscript{ECL2} and ASM\textsuperscript{ECL2-Ala} constructs for gp120 recognition. We demonstrate that b12 has an EC$_{50}$ of 0.057 µg/mL $\pm$ 0.004 µg/mL (0.380 nM) (Figure 4(a)), which is in the range of previous reports of less than 1 nM.\textsuperscript{30} We further demonstrate mimetic binding to gp120 at an EC$_{50}$ of 103.0 µg/mL $\pm$ 6.2 µg/mL (0.687 µM) and 138.2 µg/mL $\pm$ 16.4 µg/mL (0.921 µM) for ASM\textsuperscript{CXCR4} and ASM\textsuperscript{ECL2} respectively (Figure 4(a)). CXCR4 alone, in the absence of CD4, is known to have a weak affinity to gp120,\textsuperscript{31} thus these values for the mimic are in its expected lower affinity native range. ASM\textsuperscript{ECL2-Ala} showed no binding, confirming the involvement of the four alanine mutated residues in gp120 interaction.

The ECL2 boundary of CXCR4 ends at residue N192, and leads into its transmembrane helix 5 (TM5) with residues D193 and L194. Interestingly, these two residues are also conserved in sequence homology within the b12 antibody CDRH3 (Figure S1). Thus, when engineering the ASM molecules, we designed constructs that included these two residues in the ECL2 (ASM\textsuperscript{ECL2-DL}). Remarkably, the extension of the
ECL2 loop by two native residues, though highly similar to those in native b12 CDRH3, had a severe negative impact on gp120 binding (Figure 4(a)). This data demonstrates the need to adhere to the sequence boundaries of the extracellular loops regardless of sequence homology.

Since our mimetic constructs use the b12 antibody as a scaffold, we next asked if these mimetics were recognizing gp120 at the b12-binding site, which is also the CD4 coreceptor binding site. First, we tested this by generating an A180W point mutant in ECL2. It was demonstrated that W100 in b12 is a critical residue for gp120 binding, and its mutation to an alanine is abrogating.

The corresponding residue in CXCR4 ECL2 is a native alanine (A180), thus we asked if a “back-mutation” to W would enhance affinity, closer to that of b12 for gp120. Instead, we show that the A180W mutation (ASM\textsuperscript{ECL2-AW}) resulted in significantly

| Construct       | Monomer Homogeneity | T\textsubscript{m1} (°C) | T\textsubscript{m2} (°C) |
|-----------------|---------------------|-------------------------|-------------------------|
| b12             | 95%                 | 72.1                    | 83.2                    |
| ASM\textsuperscript{CXCR4} | 89%                 | 72.1                    | 83.9                    |
| ASM\textsuperscript{ECL2} | 96%                 | 73.6                    | 83.8                    |
| ASM\textsuperscript{ECL2-Ala} | 98%                 | 72.1                    | 83.4                    |
| ASM\textsuperscript{ECL2-DL} | 97%                 | 72.1                    | 80.6                    |
| ASM\textsuperscript{ECL2-AW} | 98%                 | 72.2                    | 83.9                    |
reduced binding to gp120 (Figure 4(a)). Second, we performed an epitope binning experiment using bio-layer interferometry to determine if b12 and the CXCR4 mimetics had overlapping binding sites. We loaded his-tagged gp120 onto Ni-NTA probes, and sequentially assayed for b12 and the association of mimetic constructs. ASM\textsuperscript{ECL2} demonstrated robust association while the control construct ASM\textsuperscript{ECL2-Ala} had none (Figure 4(b)). Taken together, our results indicate that the mimetic constructs bind gp120 in a specific manner, away from the b12 and CD4-binding site agreeing with its functional role as an HIV co-receptor.

**ASM\textsuperscript{CXCR4} recognizes native ligand SDF-1\textalpha**

Since the HIV gp120 binding studies described above demonstrated that the mimetics can replicate the CXCR4/gp120 interaction, we sought to further determine whether these mimetics were able to recruit the native chemokine ligand SDF-1\textalpha. The interaction of CXCR4 with SDF-1\textalpha regulates neuronal development, cell survival, and proliferation involving multiple signaling cascades.\textsuperscript{33} It has previously been demonstrated that SDF-1\textalpha binds CXCR4 through residues on the N-terminal loop, ECL2, and ECL3\textsuperscript{29} (Figure 1(b)). Thus, we asked if our fully grafted construct ASM\textsuperscript{CXCR4} sufficiently mimics the three-dimensional surface conformation of CXCR4 to recognize a native protein chemokine ligand. Using a bio-layer interferometry binding assay, we captured his-tagged SDF-1\textalpha on Ni-NTA probes and assayed for a direct mimetic association. We found that, while b12 and the ECL2-only constructs were not able to bind SDF-1\textalpha, the ASM\textsuperscript{CXCR4} mimetic had substantial binding characterized by a significant association rate coupled with a slow dissociation (Figure 5(a)). Measuring the binding kinetics by bio-layer interferometry resulted in a K\textsubscript{d} of 45.4 nM (Figure 5(b)), consistent with SDF-1\textalpha recognition by cell surface CXCR4.\textsuperscript{34,35} These results suggest that ASM\textsuperscript{CXCR4} is capable of native CXCR4 ligand recognition as a functional mimetic.

**ASM\textsuperscript{CXCR4} inhibits SDF-1\textalpha signaling**

The CXCR4/SDF-1\textalpha pathway has previously also been shown to activate the extracellular signal-regulated kinases (ERK) 1/2.\textsuperscript{36,37} We, therefore, tested whether ASM\textsuperscript{CXCR4} could inhibit this signaling pathway by competing with native CXCR4 on the cell surface. We used Jurkat T lymphocyte cells expressing high levels of native CXCR4 and compared ERK activation by SDF-1\textalpha in the presence or absence of saturating levels of ASM\textsuperscript{CXCR4}. We found that cells...
incubated with SDF-1α or SDF-1α and phosphate-buffered saline (PBS) showed detectable levels of p-ERK. However, when cells were exposed to SDF-1α in the presence of ASM CXCR4, the levels of activated p-ERK were inhibited (Figure 5(c)), indicating that the mimetic inhibited the activity of SDF-1α. This data demonstrates that ASM CXCR4 can successfully compete for ligand binding in the presence of its native cell surface CXCR4 receptor.

**ASM CXCR4 as an immunogen generates α-cxcr4 antibodies**

Finally, given that ASM constructs can be generated in large quantities as stable, soluble proteins that include a native immunogenic antibody Fc region, we asked if we could use ASM CXCR4 as an antigen to generate relevant CXCR4-specific antibodies. To test this, we immunized mice with ASM CXCR4 following a standard immunization protocol over four weeks and collected terminal bleeds. Serum from four mice were pooled and total antibodies were purified over a HiTrap protein G column. The resulting ASM CXCR4 polyclonal antibody (pAb) was used to determine native and recombinant CXCR4 recognition. We screened against HEK293 cells stably transfected with human CXCR4, as well as native CXCR4 expressed on Jurkat cells. Using a cell-based ELISA to probe for direct binding, we observed specific recognition of CXCR4 on the cell surface of both cell lines (Figure 6(a,b)). In both screens, the pAb had a significant signal over human IgG negative control.

---

**Figure 5.** ASM CXCR4 binds CXCR4 native chemokine ligand SDF-1α. (a) His-tagged SDF-1α was captured onto Ni-NTA probes and assayed for direct binding by the indicated constructs using bio-layer interferometry. Association (left of red dotted line) and dissociation (right of the red dotted line) is shown. (b) Kinetics assay of the serially diluted SDF-1α binding to ASM CXCR4 using bio-layer interferometry. (c) Western blot analysis of SDF-1 induced ERK activation in Jurkat cells and its competitive inhibition by ASM CXCR4.
Discussion

Here, we demonstrated the modeling, engineering, and assessment of the ASM platform, which allows the full extracellular regions of the GPCR CXCR4 to be studied on a soluble antibody framework. Collectively, our data suggest that the grafting of all four GPCR extracellular loops can be accommodated among the antibody CDRs and retain the original receptor’s ligand interactions. The ASM molecule confers many of the desirable qualities of antibodies, such as stability, solubility, and flexibility while also allowing for avidity through dual-arm display, and an Fc region for possible immune recruitment. Further, we show the utility of such a molecule in generating receptor-specific antibodies.

In the work presented here, we determined three criteria by which to select a specific antibody as a scaffold and facilitate engineering. First, the full Ig or Fab should be structurally characterized to guide rational design. In the absence of a structure, adherence to the ECL sequence boundaries should be followed. Second, the scaffold antibody or Fab should be generated at high yield and purity. This will allow monitoring of each construct’s overall quality as its effects on general ASM biophysical properties can be assessed during production. Third, the scaffold antibody should have testable ligand binding, allowing for control experimentation.

Our work builds on previous efforts to recapitulate the transmembrane protein extracellular loop functions on a soluble scaffold. While it would be unlikely that any one scaffold could mimic the exact three-dimensional geometries of GPCR extracellular loops, the variable domain surface at each of the two antibody arms is large enough to accommodate the entire surface area of structurally characterized GPCRs. In addition to the surface area, there are 8 possible locations (6 CDRs per Fab (3 in VH and 3 in VL) and the N-terminus of the heavy and light chains) amenable to variable sequences without disruption to the overall immunoglobulin fold. This flexibility allows for comparative analysis when modeling before final selection of ECL grafting locations. The inherent flexibility of CDRs can accommodate potential variances in loop anchor points, but it is also feasible to increase the reach of the loops by adding linkers extending them out from the antibody variable surface. This design could enhance inter-ECL interactions with less antibody variable region interference.

Although we focused our studies on the full IgG (~150 kDa) as a scaffold, some applications may require a smaller molecule where the Fc would not be advantageous. In such instances, the antibody platform allows for multiple truncated versions where: 1) protease digestion of full IgG can provide a Fab (50 kDa) with the mimetic loop ends intact; and 2) engineering can yield a single-chain variable fragment (25 kDa) that could be used alone or in multi-specific antibody formats.

Cell-surface receptor functional inhibition often relies on small molecule inhibitors or, more recently, epitope-specific antibodies. The challenge is, however, that each approach is limited to only one aspect of the receptor, and cannot accommodate alternate binding sites and interactions. In fact, increasing drug tolerance within GPCRs is becoming an area of greater interest. One recent study demonstrated that the use of a small CXCR4-derived peptide from its TM2 and ECL1 was able to act as an antagonist by interacting with SDF-1 without effecting native CXCR4 accumulation or tolerance. We posit that ASM can be used as a potent receptor antagonist to block the full complement of receptor–ligand interactions. In this regard, the ASM model’s use as an inhibitor or agonist would have enhanced pharmacokinetics due to its Fc domain and its retention mechanism.
Further, mimicking the full GPCR extracellular surface allows ASM to be used as a reliable, soluble antigen for immunization purposes in a vaccine format or in antibody therapeutics generation against the transmembrane extracellular domains. GPCRs make up a large superfamily of more than 800 members, 150 of which have yet to be characterized functionally. They act in nearly all organs, making them valuable therapeutic targets in areas that include diabetes, inflammation, cancer, and the nervous system. Consequently, 40% of all marketed drugs target GPCR family members. However, most GPCRs are extremely difficult to express for further characterization. This approach sets the groundwork to generate GPCR surrogates that have antibody-like biochemical and biophysical characteristics, and offers an alternative for presenting ECLs for generation of novel therapeutics. This should help in understanding the structure/function relationship of multi-transmembrane proteins of interest, and in the design of a novel class of drugs targeting the pathways controlled by these proteins.

Materials and methods

**ASM\(^{CXCR4}\) mimetic protein structural modeling**

For initial sequence analysis screens, we searched the NCBI/BLAST protein data bank proteins and non-redundant protein sequence databases with the sequences from human CXCR4 (UniProtKB P61073) extracellular loops 1, 2, 3, and the N-terminal loop. We excluded GPCR sequences and used the PSI-BLAST algorithm. The results were then analyzed manually and the CDRH3 sequence of the monoclonal antibody b12 was followed up due to its high sequence similarity with ECL2, as well as the presence of a crystal structure (PDB: 1HZH). Manual building of the structural mimetic model was performed using the program COOT and refined using CCP4 with structure idealization in Refmac5. Model quality was assessed using the program MODELLER, which uses the z-DOPE and GA341 scoring models.

**Expression and purification**

All CXCR4 mimetics were designed and constructed on the backbone of an in-house IgG1 expression vector pOE. The resulting plasmids were verified by sequencing and transfected into 293X cells for transient IgG production as previously described. The expression level of all IgG variants was evaluated in a ForteBio Octet QK384 instrument using protein A sensors. Constructs were purified over a HiTrap protein G column (GE 17–0405-01) per manufacturer’s protocol. The final products were dialyzed into PBS pH 7.2 buffer for all assays. The monoclonal antibody MEDI3185 is an anti-CXCR4 ECL2 specific antibody and was described in detail in a previous study. Expression and purification of all constructs were done a minimum of four separate times with a representative characterization profile of the purified samples presented in this study.

**Thermostability measurements by differential scanning calorimetry**

DSC experiments were carried out using a Microcal VP-DSC scanning microcalorimeter (Microcal) with an auto-sampler. All samples were in 1xPBS buffer and heated from 20°C to 95°C at a scan rate of 90°C/hr. Samples were assayed at a concentration of 5 μM in 400 μL. Signals from experiments using buffer only were subtracted as the baseline. Assays were done in duplicate and the software Origin (version 7) was used for data analysis to determine apparent melting temperatures (Tm) and graph representative datasets.

**ELISA binding assays**

ASM recognition by α-CXCR4 antibodies: The ASM constructs and b12 scaffold control antibody were coated onto Nunc 96-well ELISA plates at 2 μg/ml in PBS pH 7.4 overnight at 4°C. Plates were blocked in 5% milk, PBS pH 7.2 for 1 hour at room temperature. The detecting antibodies b12, MEDI3185 (in-house sources) and their isotype controls were biotinylated using EZ-Link sulfo-NHS-LC-biotin (Life Technologies #A39257) following the manufacturer’s protocol. b12, MEDI3185, 2B11 (ThermoFisher #12–9991-82), 12G5 (ThermoFisher #35–8800) and the isotype controls (human IgG1x for b12 and MEDI3185, Rat IgG2b for 2B11, and Mouse IgG2ak for 12G5) were tested at 5 μg/ml in 5% milk, PBS pH 7.2 for 1 hour at room temperature. The peroxidase-streptavidin and secondary antibodies against mouse IgG Fcy (Jackson ImmunoResearch #315–035-046), and rat IgG Fcy (Jackson ImmunoResearch #112–035-071) were used at 1:6000 to detect b12/Medi3185, 12G5, and 2B11, respectively. Signal from the horseradish peroxidase (HRP)-conjugates were detected by TMB substrate (KPL #52–00–03). The assay was done in triplicate and the data was plotted and analyzed in GraphPad Prism 8. The statistical significance was calculated by one-way analysis of variance using Dunnett’s multiple comparisons test to compare the ASM\(^{ECL2-Ala}\) control to the test constructs.

gp120 binding by ASM: The his-gp120 antigen was coated onto Nunc 96-well ELISA plates at 4 μg/ml in PBS pH 7.4 overnight at 4°C. The various ASM constructs (ECL2, ECL2-Ala, ECL2-DL, ECL2-AW) and b12 were serially diluted and incubated at room temperature for 1 hour. For detection, goat anti-huFc-HRP (Jackson ImmunoResearch, #109–001-008) was used as the secondary antibody.

**Mimetic polyclonal antibodies binding cell surface CXCR4**

CXCR4 expressing HEK293 stable cells and Jurkat cells were plated onto Nunc 96-well ELISA plates at 5 × 10⁴ cells/well in DMEM with 10% fetal bovine serum (FBS) overnight at 37°C, 5% CO₂. The plates were washed in PBST and blocked with 5% milk, PBS pH 7.2 for 1 hour at room temperature. The control antibody 2B11, the negative control whole human IgG (Jackson ImmunoResearch, #009–000–003), and the ASM\(^{CXCR4}\) pAb at varying concentrations were incubated at room temperature for 1 hour. After two washes, the cells were probed at a dilution of 1:6000 with anti-ratlG-Fcy-HRP for 2B11 and anti-huligG-Fc γ-HRP for the negative control and pAbs. Signal from
peroxidase was detected by TMB substrate. Plates were centrifuged to separate lysed cell debris, and the supernatant was transferred to new 96-well plates for reading the signal. The assay was done in triplicate, and the data was plotted and analyzed in GraphPad Prism 8. The statistical significance was calculated by one-way analysis of variance using Dunnett’s multiple comparisons test to compare the negative control to the ASM<sup>CXCR4</sup> pAb at different concentrations.

**Bio-layer interferometry kinetics and binning**

A ForteBio Octet QK384 instrument was used to study the kinetics of ASM<sup>CXCR4</sup> and ASM<sup>ECL2</sup>, and ASM<sup>ECL2-Ala</sup> in binning experiments competing with b12 for gp120<sub>HI12C2</sub> binding. All the assays were done at 200 μL/well in ForteBio kinetic buffer at 30°C. 0.2 μg/ml of his-tagged gp120 was loaded on the surface of Ni-NTA biosensors for 200 s, reaching capture levels between 1.0 and 1.5 nm, followed by a 60 s biosensor washing step, then coated with b12 at a saturating concentration of 200 nM for 600 seconds. The epitopes of the mimetic constructs were probed in relation to b12 by assaying the bis-GP12-b12 coated biosensors in 100 nM each of CXCR4, ECL2, and ECL2-Ala separately for 500 s, while continuously in the presence of 200 nM b12. All graphs were overlaid and aligned at the baseline.

Affinity measurements: ASM<sup>CXCR4</sup> was loaded onto anti-human IgG Fc biosensors at 5 μg/mL. SDF-1α was then loaded at concentrations of 375, 187.5, 93.8, 46.9, and 23.4 nM in PBS pH7.2. Data processing and Kd measurements were done in the Octet Software package v7.1.

**Competition assay by western blotting**

Jurkat cells were maintained in DMEM supplemented with 10% FBS. The day prior to use, cells were resuspended to 1.5x10<sup>6</sup>/mL in DMEM supplemented with 0.5% FBS. Cells were incubated with 1 ng/ml SDF-1 (Life Technologies, cat# PHC1346) for 10 minutes at room temperature in the presence or absence of 190 μg/mL CXCR4 mimetic. After incubation, cells were pelleted, resuspended in RIPA lysis buffer (150 mM NaCl, 1% TritonX100, 0.5% sodium deoxycholic acid, 0.1% SDS) supplemented with 1X Halt™ Protease and Phosphatase Inhibitor Cocktail (ThermoScientific, cat#1861281), and incubated for 30 minutes. Lysates were run on SDS-PAGE gel, transferred to a nitrocellulose membrane, then probed with anti-p-ERK, anti-total-ERK (Cell Signaling Technology), or anti-actin (Sigma) as a loading control. Li-cor secondary antibodies were used and membranes were analyzed using the Li-cor Odyssey scanner.

**Immunizations and polyclonal antibody generation**

Four female Balb/c mice (6–8 weeks) were injected intraperitoneally with 20 μg of ASM<sup>CXCR4</sup> in Freund’s complete adjuvant (Sigma, #F5881). The following three boosts of 20 μg ASM<sup>CXCR4</sup> in Freund’s incomplete adjuvant (Sigma, #F5506) were given intraperitoneally on days 4, 8, 12, and 22 post-initial prime immunization. Sera collected at day 15 were tested by direct binding ELISA. Three days after the last boost terminal bleeds were collected. Serum was recovered by centrifugation using serum separator tubes (Microvette). All serum was pooled and passed over a HiTrap Protein G column and total polyclonal IgGs were eluted per manufacturer’s protocol (GE Healthcare). All procedures were performed in accordance with federal, state and Institutional guidelines in an AAALAC-accredited facility and were approved by the MedImmune Institutional Animal Care and Use Committee.

**Acknowledgments**

We would like to thank Zack Britton and Joanne Ayriss for the CXCR4 expressing HEK293 cells, Neil Mody for assistance with DSC measurements, Keith Rickert for assistance with data analysis, and Alex Alfaro for mice immunizations.

**Abbreviations**

| Abbreviation | Description |
|--------------|-------------|
| ASM          | antibody scaffold mimic |
| CDR          | complementarity-determining region |
| CXCR4        | C-X-C chemokine receptor 4 |
| ECL          | extracellular loop |
| GPCR         | G-protein coupled receptor |
| mAbs         | monoclonal antibodies |

**References**

1. Zhou N, Luo Z, Luo J, Liu D, Hall JW, Pomerantz RJ, Huang Z. Structural and functional characterization of human CXCR4 as a chemokine receptor and HIV-1 co-receptor by mutagenesis and molecular modeling studies. J Biol Chem. 2001;276:42826–33. doi:10.1074/jbc.M106582200.
2. Busillo JM, Benovic JL. Regulation of CXCR4 signaling. Biochim Biophys Acta. 2007;1768:952–63. doi:10.1016/j.bbamem.2006.11.002.
3. Doranz BJ, Orsini MJ, Turner JD, Hoffman TL, Berson JF, Hoxie JA, Peiper SC, Brass LF, Doms RW. Identification of CXCR4 domains that support coreceptor and chemokine receptor functions. J Virol. 1999;73:2752–61.
4. Ahr B, Denizot M, Robert-Hebmann V, Brelot A, Biard-Piechaczek M. Identification of the cytoplasmic domains of CXCR4 involved in Jak2 and STAT3 phosphorylation. J Biol Chem. 2005;280:6692–700. doi:10.1074/jbc.M408481200.
5. Feng Y, Broder CC, Kennedy PE, Berger EA. HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor. Science. 1996;272:872–77.
6. Mohle R, Bautz R, Rafii S, Moore MA, Brugger W, Kanz L. The chemokine receptor CXCR4 is expressed on CD34+ hematopoietic progenitors and leukemic cells and mediates transendothelial migration induced by stromal cell-derived factor-1. Blood. 1998;91:4523–30.
7. Ponomaryov T, Peled A, Petit I, Taichman RS, Habler L, Sandbank J, Arenzana-Seisdedos F, Magerus A, Caruz A, Fujii N, et al. Induction of the chemokine stromal-derived factor-1 (SDF-1) is regulated by a highly efficacious lymphocyte chemoattractant, stromal cell-derived factor 1 (SDF-1). J Exp Med. 1996;184:1101–09.
8. Bleul CC, Fuhlbrigge RC, Casasnovas JM, Aiuti A, Springer TA. A highly efficacious lymphocyte chemoattractant, stromal cell-derived factor 1 (SDF-1). J Exp Med. 1999;184:1101–09.
9. Zou YR, Kottmann AH, Kuroda M, Taniuchi I, Littman DR. Function of the chemokine receptor CXCR4 in haematopoiesis and in cerebellar development. Nature. 1998;393:595–99. doi:10.1038/31269.
10. Chatterjee S, Behnam Azad B, Nimmagadda S. The intrinsic role of CXCR4 in cancer. Adv Cancer Res. 2014;124:31–82. doi:10.1016/B978-0-12-411638-2.00002-1.
11. Moro S, Hoffmann C, Jacobson KA. Role of the extracellular loops of G protein-coupled receptors in ligand recognition: a molecular modeling study of the human P2Y1 receptor. Biochemistry. 1999;38:3498–507. doi:10.1021/bi982369v.

12. Wu B, Chien EYT, Mol CD, Fenali G, Liu W, Katrich V, Abagyan R, Broou A, Wdls P, Bi FC, et al. Structures of the CXCR4 chemokine GPCR with small-molecule and cyclic peptide antagonists. Science. 2010;330:1066–71. doi:10.1126/science.1194396.

13. Almen MS, Nordstrom KJ, Fredriksrson R, Schioth HB. Mapping the human membrane proteome: a majority of the human membrane proteins can be classified according to function and evolutionary origin. BMC Biol. 2009;7:50. doi:10.1186/1741-7007-7-1.

14. Pham TC, Kriwacki RW, Parrll AL. Peptide design and structural characterization of a GPCR loop mimetic. Biopolymers. 2007;86:298–310. doi:10.1002/bip.20745.

15. Gross A, Brox R, Damm D, Tschammer N, Schmidt B, Eichler J. Ligand selectivity of a synthetic CXCR4 mimetic peptide. Bioorg Med Chem. 2015;23:4050–55. doi:10.1016/j.bmcc.2015.03.003.

16. Chevigne A, Fievez V, Szpakowska M, Fischer A, Counson M, Blum A, Schaller E, Baique P, Viret J, et al. Structure of a neutralizing human IgG against HIV-1: a template for vaccine design. Science. 2006;310:734–738. doi:10.1126/science.1139492.

17. Zhang M-Y, Zwick MB, Arthos J, et al. Structural definition of parathyroid hormone GPCR mimetic. Proteins. 2006;6:343–51. doi:10.1002/prot.204503.

18. Walser R, Kleinschmidt JH, Skerra A, Zerbe O. beta-Barrel scaffolds for the grafting of extracellular loops from G-protein-coupled receptors. Biol Chem. 2012;393:1341–55. doi:10.1515/hsz-2012-0234.

19. Carter PJ. Potent antibody therapeutics by design. Nat Rev. 2006;9:343–57. doi:10.1038/nri1837.

20. Koenig P, Lee CV, Walters BT, Janakiram V, Stinson J, Patapoff TW, Fuh G. Mutational landscape of antibody variable domains reveals a switch modulating the interdomain conformational dynamics and antigen binding. Proc Natl Acad Sci U S A. 2017;114:E486–E495. doi:10.1073/pnas.1613231114.

21. Saphire EO, Parren PW, Pantophtel R, Zwick MB, Morris GM, Rudd PM, Dwek RA. Crystal structure of a neutralizing human IgG against HIV-1: a template for vaccine design. Science. 2001;293:1155–59. doi:10.1126/science.1061692.

22. Zhou T, Xu L, Dey B, Hessell AJ, Van RYk D, Xiang SH, Yang X, Zhang M-Y, Zwick MB, Arthos J, et al. Structural definition of parathyroid hormone GPCR mimetic. Proteins. 2006;6:343–51. doi:10.1002/prot.204503.

23. Zwick MB, Parren PW, Saphire EO, Church S, Wang M, Scott JK, Dawson PE, Wilson IA, Burton DR. Molecular features of the human P2Y1 receptor. Biochemistry. 2007;46:7323–32. doi:10.1021/bi070276s.

24. Dawso PE, Wilson IA, Burton DR. Molecular features of the human P2Y1 receptor. Biochemistry. 2007;46:7323–32. doi:10.1021/bi070276s.

25. Peng L, Damschroder MM, Cook KE, Wu H, Dall Acequia WA. The role of N-glycosylation sites on the CXCR4 receptor for CXCL-12 binding and HIV-1 infection. Biochim Biophys Acta. 2006;1763:105–13. doi:10.1016/j.bbamcr.2004.10.017.

26. Huskens D, Princen K, Schreiber M, Schols D. The role of N-glycosylation sites on the CXCR4 receptor for CXCL-12 binding and HIV-1 infection. Biochim Biophys Acta. 2006;1763:105–13. doi:10.1016/j.bbamcr.2004.10.017.

27. Bleul CC, Wu L, Hoxie JA, Springer TA, Mackay CR. The HIV coreceptors CXCR4 and CCR5 are differentially expressed and regulated on human T lymphocytes. Proc Natl Acad Sci U S A. 1997;94:1925–30.

28. Xiao X, Kinter A, Broder CC, Dimitrov DS. Interactions of CCR5 and CXCR4 with CD4 and gp120 in human blood monocyte-derived dendritic cells. Exp Mol Pathol. 2008;89:133–38. doi:10.1016/j.xmol.2008.12.002.