Phage display and hybridoma generation of antibodies to human CXCR2 yields antibodies with distinct mechanisms and epitopes

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Abbreviations: CXCR2, C-X-C Chemokine Receptor 2; GPCR, G-protein coupled receptor; IL-8, Interleukin-8; Gro-α, growth related oncogene- α; Gro-β, growth related oncogene- β; Gro-γ, growth related oncogene- γ; ENA-78, epithelial derived -neutrophil activating protein; GCP-2, granulocyte activating protein; NAP-2, neutrophil activating protein-2, CLIPS, Chemical Linkage of Peptides onto Scaffolds; FMAT, Fluorescence Microvolume Assay Technology; scFv, single chain Fv fragments; Ig, Immunoglobulin; ECL, extracellular loops; CDR, complementarity determining region; PBS, phosphate buffered saline; BSA, bovine serum albumin; FBS, fetal bovine serum.

Generation of functional antibodies against integral membrane proteins such as the G-protein coupled receptor CXCR2 is technically challenging for several reasons, including limited epitope accessibility, the requirement for a lipid environment to maintain structure and their existence in dynamic conformational states. Antibodies to human CXCR2 were generated by immunization in vivo and by in vitro selection methods. Whole cell immunization of transgenic mice and screening of phage display libraries using CXCR2 magnetic proteoliposomes resulted in the isolation of antibodies with distinct modes of action. The hybridoma-derived antibody fully inhibited IL-8 and Gro-α responses in calcium flux and β-arrestin recruitment assays. The phage-display derived antibodies were allosteric antagonists that showed ligand dependent differences in functional assays. The hybridoma and phage display antibodies did not cross-compete in epitope competition assays and mapping using linear and CLIPS peptides confirmed that they recognized distinct epitopes of human CXCR2. This illustrates the benefits of using parallel antibody isolation approaches with different antigen presentation methods to successfully generate functionally and mechanistically diverse antagonistic antibodies to human CXCR2. The method is likely to be broadly applicable to other complex membrane proteins.

Introduction

G-protein coupled receptors (GPCRs) are a superfamily of integral membrane proteins that transduce signals primarily via the activation of associated heterotrimeric G-proteins. They account for approximately 2–4% of all genes encoded by the human genome and, while over half of the known GPCRs are olfactory or sensory receptors, there are at least 350 GPCR’s that are considered as potential drug targets.1,2

The chemokine receptor CXCR2 is a member of the Family A/Rhodopsin subfamily of GPCRs and is activated by the binding of a number of CXC chemokines with the conserved glutamic acid-leucine-arginine (ELR) sequence motif. Agonists include interleukin-8 (IL-8/CXCL8), growth related oncogene (Gro-α/CXCL1, Gro-β/CXCL2 and Gro-γ/CXCL3), epithelial derived -neutrophil activating protein (ENA-78/CXCL5) granulocyte activating protein (GCP-2/CXCL6) and neutrophil activating protein-2 (NAP-2/CXCL7).3,4 While IL-8 and GCP-2 are potent agonists of both the CXCR1 and CXCR2 receptors, which share approximately 77% amino acid identity, the other ligands show selectivity for CXCR2 over CXCR1. CXCR2 is expressed on a number of different cell types, including endothelial cells, neutrophils, macrophages, monocytes and neurons.5,7 Overexpression of the CXCR2 receptor or its ligands has been implicated in inflammatory diseases such as chronic obstructive pulmonary disorder, rheumatoid arthritis and respiratory distress syndrome.8-12 It is also associated with the development and progression of multiple tumor types, including melanoma, glioblastoma, non-small cell lung carcinoma, prostate, pancreatic, hepatocellular and renal carcinomas.13-24 Thus, targeting the

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CXCR2 receptor with a therapeutic antibody would have potential application in multiple diseases.

A number of technical challenges exist when raising antibodies to GPCR targets. The receptors are characterized by seven transmembrane helices with three intracellular loops, three extracellular loops, an extracellular N-terminal domain and an intracellular C-terminus. With the majority of the receptor either intracellular or embedded in the lipid bilayer, the potential epitopes that are accessible for antibody binding are restricted. This is compounded by relatively low expression levels in targets cells, thus limiting the amount of antigen present compared with non-relevant proteins when cells are used as an immunogen or for antibody library selection approaches. Purification of the GPCR to provide a more concentrated source of antigen can result in loss of structural integrity such that the majority of the receptor is in a functionally inactive form. Detergents may be used to maintain folding of the receptor, but this approach presents its own issues as the detergents that may be required to stabilize the receptor could potentially mask accessible epitopes. Heterogeneity in GPCRs presents an additional challenge. The receptors are dynamic structures that exist in multiple conformations unless constrained by a stabilizing ligand. Furthermore, they undergo post-translational modifications, and there is increasing evidence that they may form oligomers, with both homo and hetero- oligomerisation of GPCRs reported.

To address these challenges a variety of different antigen presentation methods have been employed for use in both immunization and antibody library display approaches with varying degrees of success. These include whole cells, purified proteins and linear peptides or constrained peptides that mimic extracellular loops to represent a more native structure. Detergents, lipids or lipoproteins have been used to reconstitute enriched receptors, e.g., into liposomes or nanodisc structures, providing an environment mimicking that of the plasma membrane. Genetic immunization, where antigen is produced within the host, thus overcoming the requirement for purified protein or cells, has also resulted in the generation of functionally active GPCR antibodies.

In the study reported here, we applied in vivo and in vitro methods to generate human monoclonal antibodies to the human CXCR2 receptor and assess whether use of these different approaches can result in the isolation of antibodies that are diverse in both epitope and function. Cell lines overexpressing human CXCR2 have been used as the antigen for whole cell immunization. The mice are genetically modified to generate antibodies with human variable heavy and light regions and mouse constant regions enabling production of a robust, humoral immune response comparable to that observed in a non-modified mouse. In parallel, in vitro selections have been used to efficiently identify antibody fragments that bind to human CXCR2 from naive phage display libraries, derived from unimmunized humans. The antigen used for selections is a CXCR2 magnetic proteoliposome in which detergent solubilized, affinity purified, tagged human CXCR2 receptor derived from an overexpressing cell line is reconstituted into a lipid bilayer surrounding a paramagnetic bead. This provides a concentrated source of conformationally relevant CXCR2 in a form that is readily amenable to the phage display selection process. Using both approaches, antibodies that bind human CXCR2 antibodies have been identified and their activity characterized in functional assays measuring activation of the G-protein coupled signaling pathway and the β-arrestin recruitment pathway. Finally, we mapped the epitope bound by the antibodies using a large array of linear and conformationally constrained CLIPS peptides derived from the extracellular regions of human CXCR2 and determined that the phage display and immunization approaches generated antibodies that targeted separate non-overlapping epitopes.

### Results

**Generation of functionally active monoclonal antibodies to human CXCR2 by immunization**

To generate antibodies to human CXCR2, three groups of mice were immunized with different doses of HEK human CXCR2 cells (1 × 10⁶, 3 × 10⁶ and 5 × 10⁶ cells), and mice with the highest CXCR2 specific antibody titers, as determined by serum ELISA, were sacrificed and used for hybridoma production. Differential screening of the hybridoma supernatants 13 d post-fusion in a Fluorescence Microvolume Assay identified 303 hybridoma well lines producing antibodies with higher levels of binding to a HEK human CXCR2 cell line compared with parental HEK cells (Table 1). The antagonist activity of the hybridoma-derived antibodies was assessed using a competitive binding assay. Only 3 of the 303 hybridoma supernatants with confirmed human CXCR2 specific binding inhibited fluorescently labeled IL-8 binding to the HEK human CXCR2 cell lines by > 50%, thus only a small percentage (< 1%) of the CXCR2 binders identified were likely to be functionally active.

The ability of the hybridoma-derived antibodies to block agonist-induced CXCR2 signaling via G-protein mediated pathways was assessed using HEK cells overexpressing the human CXCR2 receptor and Gq5, a chimeric G-protein containing the five carboxyl-terminal amino acids from the Gq. This modified G-protein subunit enables Gi-coupled receptors such as CXCR2 to generate a rise in the intracellular calcium signal through the Gq Phospholipase C pathway. Purified antibodies from 175 of the hybridoma supernatants with confirmed CXCR2 binding were tested at fixed dilutions (12.5% and 2.5%) for inhibition of IL-8 and Gro-α functional responses in calcium flux assays. Of the 175, only one (ZY05) effectively inhibited the calcium flux response to IL-8 (92% inhibition at 12.5% sample, 82% inhibition at 2.5% sample) and Gro-α (79% inhibition at 12.5% sample, 75% inhibition at 2.5% sample). This well line was one of the three that had been identified as producing an antibody that inhibited IL-8 binding to the HEK human CXCR2 cells. The remaining 174 CXCR2 specific antibodies tested were not functionally active in the calcium flux assay.

The ZY05 well line was cloned to a single IgG1 producing hybridoma with confirmed CXCR2 specific binding in the fluorescence microvolume assay technology (FMAT) cell binding
The ZY05 antibody fully inhibited calcium release stimulated by IL-8 (geomean IC50 4.8 nM), Gro-α (geomean IC50 4.1 nM), and ENA-78 (geomean IC50 2.6 nM) (Fig. 1) in FLIPR assays. A commercially-available mouse anti-human CXCR2 IgG1 control antibody 6C6 (anti-CD182) derived from the serum of immunized mice, fully inhibited Gro-α (geomean IC50 14 nM), and ENA-78 (geomean IC50 8 nM), responses, but showed minimal inhibition of IL-8 induced calcium response (Fig. 1). Ab24963 a second commercially-available antibody raised to the N-terminal peptide amino acids 1–19 of human CXCR2 MEDFNMESDS FEDFWKGED was also tested in the assay (SI Figure 1) and inhibited Gro-α (geomean IC50 9 nM, n = 2), but showed only partial inhibition of IL-8 stimulated calcium responses (mean maximum inhibition 28%, n = 2).

Binding of agonist to the CXCR2 receptor results in recruitment of β-arrestin, an adaptor protein that is important for receptor desensitization, as well as downstream signaling. Inhibition of β-arrestin recruitment to CXCR2 was assessed using the TANGO™ GPCR Assay System. This assay measures recruitment of protease-tagged arrestin protein to an activated receptor, which results in the release of a non-native transcription factor fused to the C terminus of the receptor that is then quantified using a β-lactamase reporter gene readout. The ZY05 antibody inhibited both IL-8 (geomean IC50 2.2 nM, 95% CI 1.6 – 3.1 nM, n = 4) and Gro-α (geomean IC50 2.9 nM, 95% CI 2.0–4.1 nM, n = 4) stimulated β-arrestin recruitment. Subsequent humanization was performed by cloning of the variable regions for the chimeric antibody into vectors containing the human heavy chain constant domains or human light chain (kappa) constant domains. The activity of the humanized antibody, referred to as HY29–1, was confirmed by profiling against a panel of CXCR2 agonists (IL-8, Gro-α, Gro-β, Gro-γ, ENA-78, GCP-2 and NAP-2) in the TANGO™ assay (Table 2). HY29–1 fully inhibited all the CXCR2 agonists tested in this assay.

Table 1. Screening of Hybridoma Supernatants. Hybridoma supernatants derived from mice immunized with varying concentrations of HEK human CXCR2 cells were tested at a final dilution of 12.5% in an FMAT Direct Binding Assay or at 25% in the FMAT receptor: ligand (R:L) competition assay. Data shown summarizes the number of positive binders/inhibitors obtained in the screens with total numbers shown in bold at the bottom of each column. A positive binder to HEK parental cells was defined by a fluorescence signal (FL1) greater than 100. A positive binder to HEK human CXCR2 cells was defined by a fluorescence signal (FL1) of greater than 200.

| Mouse ID  | No. Cells Immunized | HEK parental cells Number of binders (FL1 > 100) | HEK hCXCR2 Cells Number of binders (FL1 > 200) | Number of hCXCR2 specific binders | R:L Competition Number hits (> 50% inhibition) |
|----------|---------------------|-----------------------------------------------|-----------------------------------------------|-----------------------------------|-----------------------------------------------|
| NO13     | 1 × 10⁶             | 72                                            | 66                                            | 19                               | 0                                             |
| NO17     | 3 × 10⁶             | 212                                           | 184                                           | 43                               | 0                                             |
| NO18     | 3 × 10⁶             | 63                                            | 65                                            | 17                               | 0                                             |
| NO19     | 3 × 10⁶             | 295                                           | 235                                           | 47                               | 0                                             |
| NO20     | 3 × 10⁶             | 1238                                          | 667                                           | 36                               | 2                                             |
| NO22     | 5 × 10⁶             | 670                                           | 382                                           | 46                               | 0                                             |
| NO23     | 5 × 10⁶             | 861                                           | 396                                           | 36                               | 0                                             |
| NO24     | 5 × 10⁶             | 445                                           | 294                                           | 59                               | 1                                             |
|          |                     | 3856                                          | 2289                                          | 303                              | 3                                             |

Figure 1. Inhibition of agonist stimulated calcium flux by the hybridoma derived antibody. ZY05, 6C6 and matched isotype control antibodies (mouse IgG1 and Hybridoma control) were pre-incubated with HEK Gqi5 human CXCR2 cells for 30 min at 37 °C prior to stimulation with 7nM IL-8 (A) 7nM Gro-α (B) or 30nM ENA-78 (C) and measurement of calcium responses. Data for IL-8 and Gro-α is the mean ± SD from 3–5 experiments. Data for ENA-78 is the mean from 2 experiments. Geometric mean IC50 values (95% confidence intervals) are shown in the legends.

Generation of functionally active monoclonal antibodies to human CXCR2 using phage display

In parallel to the hybridoma approach, anti-human CXCR2 antibodies were isolated from naive phage display libraries using CXCR2 magnetic proteoliposomes to enrich for CXCR2 specific scFv. Multiple rounds of selection were performed using the proteoliposomes, resulting in the isolation of 1373 scFv that
Ligand 95% confidence interval for $E_{50}$ values. For HY29–1 inhibition, cells were pre-incubated with varying concentrations of HY29–1 prior to stimulation with 7 nM IL-8, 15 nM Gro-α, 189 nM Gro-β, 76 nM Gro-γ, 311 nM ENA-78, 151 nM GCP-2 or 264 nM NAP-2. Values are the Geometric mean of 3–6 experiments with 95% confidence intervals.

| Ligand     | Agonist EC$_{50}$ nM (Geomean) (95% Confidence Interval) | HY29–1 IgG IC$_{50}$ nM (95% Confidence Interval) |
|------------|----------------------------------------------------------|--------------------------------------------------|
| Human IL-8 | 1.6 (1.2–2.0)                                           | 1.3 (1.0–1.7)                                    |
| Human Gro-α| 2.3 (1.3–3.9)                                           | 1.7 (1.5–2.0)                                    |
| Human Gro-β| 25 (11–59)                                               | 7.2 (6.5–7.9)                                    |
| Human Gro-γ| 19 (18–21)                                               | 3.3 (2.8–3.8)                                    |
| Human ENA-78| 37 (24–56)                                             | 0.5 (0.4–0.8)                                    |
| Human GCP-2| 3.7 (2.4–5.8)                                           | 0.7 (0.5–0.9)                                    |
| Human NAP-2| 21 (13–34)                                               | 9.5 (5.3–17)                                    |

Seventy of the unique scFv binders were converted to full-length IgG and tested for their ability to inhibit IL-8 and Gro-α stimulated calcium responses in the FLIPR assay. Sixteen of the antibodies inhibited the Gro-α responses by > 50% when tested at fixed concentrations of 300 nM to 1 μM (depending on the antibody stock concentration), but none of the antibodies tested showed > 50% inhibition of IL-8 responses (Fig. 2B). This difference in inhibition of the two ligands was confirmed by titration of the antibodies in the assay (Fig. 2C and 2D for inhibition profiles of 6 of the antibodies). A panel of 5 antibodies that had demonstrated the greatest inhibition of Gro-α responses in the FLIPR assay were selected for further profiling in the TANGO™ β-arrestin recruitment assay at concentrations of 1.5 nM IL-8 and 3 nM Gro-α. The phage display derived antibodies inhibited IL-8 with maximum inhibition values of 55–96% (Table 3) and completely inhibited Gro-α responses. Under the same conditions the commercial 6C6 antibody showed a similar profile to the phage display antibodies with partial inhibition of IL-8 and full inhibition of Gro-α under the conditions tested, whereas the hybridoma derived HY29–1 antibody fully inhibited both agonists in the assay.

**Mechanism of action of phage display and immunization-derivered monoclonal antibodies to human CXCR2**

The differences in activity vs. IL-8 between the phage display and hybridoma antibodies may indicate different mechanisms of inhibition due to their interaction with distinct epitopes present on human CXCR2. Molecular mechanisms of inhibition can be assessed by observation of the effects of increasing concentration of antagonist on the pattern of displacement of agonist concentration curves. We determined the effects of the phage display and hybridoma antibodies on IL-8 and Gro-α agonist curves in the TANGO™ β-arrestin recruitment assay. X2–753, X2–1194 and X2–856 phage display-derived antibodies and the commercial 6C6 antibody all produced rightward shifts in the IL-8 and Gro-α dose response curves that reached a maximal dextral displacement (Fig. 3A-H). This is consistent with an allosteric mechanism of action with antibody reducing affinity or efficacy of agonist. The equilibrium dissociation constant (Kd) was consistent with values obtained for the antibodies with similar inhibitory activity. The differences in activity vs. IL-8 between the phage display and hybridoma antibodies may indicate different mechanisms of inhibition due to their interaction with distinct epitopes present on human CXCR2. Molecular mechanisms of inhibition can be assessed by observation of the effects of increasing concentration of antagonist on the pattern of displacement of agonist concentration curves. We determined the effects of the phage display and hybridoma antibodies on IL-8 and Gro-α agonist curves in the TANGO™ β-arrestin recruitment assay. X2–753, X2–1194 and X2–856 phage display-derived antibodies and the commercial 6C6 antibody all produced rightward shifts in the IL-8 and Gro-α dose response curves that reached a maximal dextral displacement (Fig. 3A-H). This is consistent with an allosteric mechanism of action with antibody reducing affinity or efficacy of agonist. The equilibrium dissociation constant (Kd) was consistent with values obtained for the antibodies with similar inhibitory activity.

**Figure 2.** Isolation of functionally active antibodies to human CXCR2 using phage display. (A) Binding of scFv in crude periplasmic extracts (black squares) or purified scFv (open circles) to parental HEK 293, R1610 or C2th cell lines and cells overexpressing the CXCR2, CXCR3 or FPR receptors detected by flow cytometry. (B) (C) and (D) Inhibition of IL-8 and Gro-α calcium responses by purified antibodies. The antibodies were pre-incubated with HEK Gqi5 human CXCR2 cells for 30 min at 37 °C prior to stimulation with 7 nM IL-8 or 7 nM Gro-α and measurement of calcium responses in a FLIPR assay. (A) Inhibition of IL-8 and Gro-α responses by single concentrations of antibody (300 nM - 1 μM). Points are mean% inhibition of two experiments. (B) Inhibition of IL-8 and (C) Gro-α calcium responses at varying concentrations of X2–753, X2–379, X2–409, X2–856, X2–1194 and X2–1251 antibodies. Data are the mean% control values from 2 to 3 experiments with duplicate points within each experiment.
Table 3. Inhibition of β-arrestin recruitment in the TANGOTM Assay by monoclonal antibodies to human CXCR2. Antibodies were pre-incubated with TANGOTM U2OS hCXCR2-bla cells then stimulated with 1.5 nM IL-8 or 3 nM Gro-α (IC_{50} and maximum inhibition determination) or with a range of agonist concentrations (K_0 determination). IC_{50} values are the geometric mean (95% confidence interval) of at least 3 separate experiments. For X2–753, X2–1194, X2–856 and 6C6 K_0 and α values were derived from 2–3 experiments with a minimum of 5 antagonist concentrations in each experiment by fitting to an allosteric model^{54} using GraphPad Prism Software.

| Antibody | IC_{50} nM (95% CI) | Max inhibition | K_0 nM (α value) | IC_{50} nM (95% CI) | K_0 nM (α value) |
|----------|---------------------|----------------|------------------|---------------------|------------------|
| X2–379   | 2.2 (1.4–3.4)       | 80–96%         | nd               | 3.8 (2.6–5.5)       | nd               |
| X2–409   | 1.6 (0.7–3.4)       | 72–86%         | nd               | 6.2 (3.1–12)        | nd               |
| X2–753   | 0.5 (0.4–0.7)       | 62–77%         | 0.6 (α = 0.3)    | 1.1 (0.7–1.8)       | 0.5 (α = 0.02)   |
| X2–1194  | 0.08 (0.04–0.13)    | 55–77%         | 0.06 (α = 0.1)   | 0.07 (0.03–0.18)    | 0.1 (α = 0.05)   |
| X2–856   | 0.7 (0.5–0.8)       | 76–90%         | 0.6 (α = 0.1)    | 1.3 (0.8–2.2)       | 0.6 (α = 0.03)   |
| 6C6      | 0.4 (0.3–0.6)       | 55–78%         | 0.4 (α = 0.2)    | 0.5 (0.4–0.8)       | 0.1 (α = 0.02)   |
| HY29–1   | 0.2 (0.1–0.4)       | 94–100%        | nd               | 0.4 (0.3–0.6)       | nd               |

Nd, not determined

(K_0) values and α (or co-operativity) factor describing the magnitude of the change the allosteric modulator on ligand responses was determined for each antibody by fitting of data to an allosteric modulator equation (Table 3).^{54} The difference in co-operativity factors measured with Gro-α agonist (0.1 – 0.3) compared with IL-8 (0.02 - 0.05) implies that the effects of the antibodies are ligand dependent with a greater impact on Gro-α responses. This agrees with the higher maximum% inhibition that was observed for Gro-α in the antibody competition assays at fixed ligand concentrations.

In contrast, increasing concentrations of the HY29–1 antibody resulted in a parallel shift of the agonist concentration curves that did not reach a maximum dextral displacement (Fig. 3I and J). At high antagonist concentrations this was accompanied by a decrease in the maximal agonist response. At low concentrations of antagonist, a decrease in maximum response was not observed, which may be due to receptor reserve in the system. The HY29–1 inhibition did not appear to be ligand dependent as similar patterns of displacement of the agonist concentration curves were observed for both IL-8 and Gro-α.

Epitope mapping of phage display and immunization derived monoclonal antibodies to human CXCR2

To characterize the epitope bound by the anti-human CXCR2 antibodies, cross-competition assays were performed between fluorescently-labeled antibodies and unlabelled antibodies (Fig. 4). Two mouse monoclonal anti-human CXCR2 antibody clone 6C6 and Ab24963 were included in the assays as they bound to known N-terminal sequences. The 6C6 antibody has been mapped to residues within the 11FEDFWK^{15} sequence contributed to the binding epitope for two antibodies. However, X2–1194 did not fully compete with fluorescently labeled X2–753 (Fig. 4C) and X2–753 did not fully compete with fluorescently-labeled X2–1194 (Fig. 4D), which may be due to these antibodies binding partially overlapping epitopes.

Mapping of the binding of site of the antibodies X2–753 and HY29–1 using linear peptides and CLIPS conformationally constrained peptides^{54} derived from the N-terminus, ECL1, ECL2 and ECL3 of CXCR2 confirmed that these two antibodies bound distinct epitopes (Fig. 5A). Analysis of binding of HY29–1 to sequential linear peptides derived from the CXCR2 N-terminus HY29–1 (Fig. 5B) and mutation analysis of peptides consisting of N-terminus and ECL3 (Fig. 5C) showed that the dominant epitope for HY29–1 binding centered around the 31PFLLD^{35} sequence with the second leucine and the aspartic acid key to the binding interaction.

The binding profile observed for the X2–753 was more complex with N-terminal, ECL2 and ECL3 regions potentially contributing to the receptor antibody interaction (Fig. 5A). In contrast to the HY29–1 antibody, residues within the N-terminal part of the CXCR2 N terminus played a prominent role in X2–753 binding (5B). Mutation analysis indicated that an N-terminal epitope centered around the sequence 11FEDFWK^{16} contributed to X2–753 binding, with mutation of residues F, E, W and K within this sequence resulting in reduced antibody binding and mutation of D increasing binding, most likely due to the charge modification (Fig. 5C). Mutations in the ECL3 sequence did not result in reduced X2–753 binding, but it is possible that introduction of single alanine mutations were insufficient to disrupt the antibody interaction due to a ‘buffering’ capacity within the sequence. Mapping of the N-terminal binding interactions was also performed for the X2–1194 antibody (Fig. 5B and C) to investigate whether the partial competition with X2–753 observed in the epitope competition assay was a result of the involvement of different residues within this region. Weaker binding was observed for this antibody to the N-terminal derived peptides and in addition to residues F, E, W, K and D the second phenylalanine within the 11FEDFWK^{16} sequence was required to maintain binding of X2–1194 antibody.
Discussion

We used two different approaches, whole cell immunization and in vitro antibody library selection methods, to generate antagonistic antibodies to the human CXCR2 G-protein coupled receptor. Immunization has been widely used to produce antibodies to GPCRs; however, when wild type animals are used as the host, the resulting antibodies require humanization to reduce potential immunogenicity risks. In this study, we immunized transgenic mice to produce antibodies with human variable and heavy and light chain sequences and mouse constant regions that could be easily converted to fully human format. The mice were immunized with whole cells overexpressing the human CXCR2 receptor. As expected, the predominance of other endogenous cell surface proteins present on the human embryonic kidney cells resulted in the production of a large number of non-specific antibodies. Differential screening did identify over 300 human CXCR2 specific binders, but the success rate for generation of antibodies that could inhibit CXCR2 mediated functional responses was low. Only one of the CXCR2 binders tested inhibited G-protein mediated signaling and β-arrestin recruitment. This antibody was fully humanized to generate HY29–1.

In parallel in vitro selections methods were used to isolate human CXCR2 binders from libraries of filamentous phage displaying antibody fragments (in this case scFv) of up to $10^{11}$ in size derived from non-immunized humans, thus exploiting the natural diversity present in the human antibody repertoire. The CXCR2 paramagnetic proteoliposomes used in the selection process enabled presentation of homogenous orientated native GPCR embedded in a lipid bilayer and similar structures have been used by other groups to isolate antibodies to receptors such as CCR5 and CXCR4. A panel of antibodies to human CXCR2 was successfully generated using this

![Figure 3](image-url)
A rapid translocation of stimulation of the CXCR2 receptors has been shown to result in new equilibrium due to a slow antibody off rate. IL-8 and Gro-β-arrestin response may be the result of insufficient time to re-establish a body at an orthosteric site. The depression of the maximal (probe independent), suggesting potential interaction of the anti-steric modulation profile in the cally distinct. The phage display antibodies demonstrated an allo-diverse range of epitopes.

Animals immunized with cells, proteoliposomes or a combination of success in generating antibodies via phage display, an alternative approach could be to generate a phage display library from animals immunized with cells, proteoliposomes or a combination of both reagents to generate a larger pool of antibodies against a diverse range of epitopes.

HY29–1 and the phage display antibodies were mechanistically distinct. The phage display antibodies demonstrated an allosteric modulation profile in the β-arrin assay with greater inhibition of Gro-α over IL-8. HY29–1 showed insurmountable antagonism, which can be the result of allosteric or orthosteric blockade depending on the kinetics of the interaction of agonist and antagonist with the receptor. In these assays the antibody was pre-equilibrated with the receptor and then agonist added to stimulate the functional response. Under these conditions the HY29–1 antagonism did not appear to reach a maximal dextral displacement and was similar for both IL-8 and Gro-α agonists (probe independent), suggesting potential interaction of the antibody at an orthosteric site. The depression of the maximal response may be the result of insufficient time to re-establish a new equilibrium due to a slow antibody off rate. IL-8 and Gro-α stimulation of the CXCR2 receptors has been shown to result in a rapid translocation of β-arrestin, which would support the observation of a hemi-equilibrium. However, other mechanisms cannot be completely excluded due to the limitations of the assay format. Under conditions of hemi-equilibria, even at the highest concentrations the agonist response would not be predicted to reach zero, but we could not achieve sufficiently high enough concentrations of agonist to confirm this. CXCR2 rapidly internalizes in response to agonist via both β-arrestin dependent and independent mechanisms through a process modulated by N-terminus, ECL2 and cytoplasmic C-terminus structural determinants. Antibodies that directly stimulate GPCR internalization can inhibit signaling responses through depletion of receptor from the cell surface characterized by an effect on maximal signaling responses, but not ligand potency. The decrease in maximal β-arrestin recruitment observed for HY29–1 was combined with a significant shift in agonist potency. In addition, HY29–1 did not stimulate β-arrestin recruitment in the absence of agonist (data not shown). Although we have not assessed β-arrestin independent internalization, it seems unlikely that HY29–1 is acting through this mechanism.

A two-site model for binding of chemokines to their receptors has been proposed where N-terminal residues of the chemokine first interact with site I located in the N-terminal region of the receptor. The ligand ELR+ motif then interacts with sites within the second and third extracellular loops of the receptor. Site I contributes predominantly to receptor selectivity and affinity, whereas site II binding stabilizes the receptor conformation to elicit signaling responses. Regions involved in the high affinity ligand binding and activation of CXCR2 have been mapped using chimeric receptors, peptide competition studies, site directed mutagenesis, dynamic molecular modeling and investigation of residues that may contribute to species differences in ligand affinity. These studies have widely implicated the N-terminus of the receptor in high affinity binding of ligands, but residues across the extracellular loops have also been shown to be involved. The amino acids required for high affinity binding are not necessarily those required for cell activation. Ahuja et al. demonstrated functional responses from CXCR2

![Figure 4. Epitope competition between hybridoma, phage display and commercial anti-human CXCR2 monoclonal antibodies. Binding of fluorescently labeled HY29–1 (A), 6C6 (B), X2–753 (C), and X2–1194 (D) was measured in the presence of varying concentrations of unlabelled X2–753, X2–1194, HY29–1, Ab24963 or 6C6 antibodies using Fluorescence Microvolume Assay Technology. Data shown is the mean% control from duplicate points in one experiment and is representative of the inhibition profiles obtained in at least three experiments.](image-url)
ligands in a CXCR2 chimeric receptor containing a CCR1 N-terminus in the absence of high affinity ligand binding and point mutations in ECL1 have been shown to have no effect on Gro-α binding, but to inhibit calcium signaling. In addition, the domains involved in binding are ligand dependent as demonstrated by mapping of IL-8, Gro-α and NAP-2. Therefore, antibodies to CXCR2 could have ligand dependent effects due to the different interaction sites of the ligands with the receptor and block signaling, but not ligand binding as has been shown for CXCR1.

The minimal epitopes for several neutralizing anti-human CXCR2 monoclonal antibodies generated from immunization of mice with human CXCR2 overexpressing cell lines have been described as the N-terminal sequence ‘FEDFW’.33,75,76 Epitope mapping of the phage display-derived antibodies X2–753 and X2–1194 indicated that they bound similar core epitope 11FEDFWK,16 which was confirmed by their cross competition in epitope competition assays with the 6C6 antibody. It is possible that the interaction of the antibodies with ECL2 and ECL3 as determined for X2–753 by epitope mapping may contribute to the modulation of receptor activation. We did not investigate the role of ECL2, and mutation of residues within ECL3 did not disrupt binding, although in this case the surrounding residues could be masking any potential effects. Thus, while we have confirmed a key epitope in the N-terminus of the receptor, we cannot completely exclude the possible contribution of ECL2 and ECL3 to the antibody interaction.

In contrast, the hybridoma-derived antibody HY29–1 recognized a distinct epitope, confirmed by epitope competition.

Figure 5. Epitope Mapping of hybridoma and phage display derived monoclonal antibodies to human CXCR2 using Linear and CLIPS Peptides. (A) ELISA binding patterns of X2–753 and HY29–1 to 56 overlapping structured peptides derived from CXCR2 ECD sequences. Each bar is the average and SD of the 56 peptides that combine a common 15-mer peptide with each of the other oligomers in a CLIPS T3 structure. The order of the bars is according to the protein sequence of CXCR2. ECDs are separated by vertical red lines and the horizontal green line depicted the median signal over the complete data set. (B) ELISA binding patterns for CXCR2 N-terminal derived linear peptides. The set consists of overlapping 20-mer peptides with each peptide shown as a vertical line, the height of which depicts the observed ELISA readout for that peptide. (C) Alanine replacement studies showing the relevance of each residue within the binding sites for X2–753, X2–1194 and HY29–1. Sets of CLIPS constrained peptides were created with either the sequence shown below each graph or the same sequence with a single position replaced by alanine. Values are expressed as a% of the binding signal of the unmutated peptide (sequence identical to the X axis). Each bar shows the relative binding for the mutated peptide. Bars with a value below 100% indicate residues that are relevant for binding. Positions without bars were not tested by alanine replacement.
and peptide mapping, with the sequence 31PFLLD35 comprising the minimal epitope. Residues in this region have been highlighted by dynamic molecular modeling as having a potential role in IL-8 binding to CXCR2.72 We found that, in addition to IL-8, the HY29–1 antibody blocked functional responses to all CXCR2 ligands tested.

The association of CXCR2 with inflammatory diseases, neurodegenerative disorders and cancers has resulted in significant interest in the development of therapeutics that target either the receptor or its ligands. While a number of small molecule antagonists, peptides and monoclonal antibodies targeting the receptor or ligands have progressed into clinical trials, we are not aware of any approved therapeutic monoclonal antibodies targeting human CXCR2. We have successfully generated antibodies to human CXCR2 using hybridoma and phage display technologies. These antibodies display diversity with respect to their epitope, which, in turn drives different neutralization mechanisms. The HY29–1 antibody presents the possibility of a therapeutic that could block CXCR2 receptor activation by multiple ligands, with minimal potential for redundancy due to compensatory mechanisms involving alternative receptor agonists. In contrast, antibodies such as those derived from the phage display approach could offer more ‘fine tuning’ of pharmacological responses due to the saturability and ligand dependence of their effects. To our knowledge, this is the first direct comparison of two different methodologies that can be applied to successfully generate diverse anti-GPCR antibodies with potential future therapeutic benefit.

Materials and Methods

Proteins and Antibodies

CXCR2 ligands IL-8/CXCL8 (#208/IL-CF), Gro-α/CXCL1 (#275-GR), Gro-β/CXCL2 (#276-GB), Gro-γ/CXCL3 (#277-GG), ENA-78/CXCL5 (#254-XB), GCP-2/CXCL6 (#333-GC) and NAP-2/CXCL7 (#393-NG) were obtained from R&D Systems. Anti-human CXCR2 antibodies were obtained from BD Biosciences (#555932, clone 6C6) and Abcam (#Ab24963).

Phage Display Selections

ScFv were isolated from naïve antibody libraries using standard phage display protocols. Paramagnetic proteoliposomes containing CXCR2 were prepared as described by Mirzabekov et al.46 and the expression of human CXCR2 confirmed by flow cytometry using commercially available anti human CXCR2 antibodies (BD Biosciences clone 6C6 and R&D Systems #MAB331). The proteoliposomes were used as the antigen for multiple rounds of soluble selections. Briefly, 80 μl of CXCR2 MPLs (~5×10^8 beads/ml) were incubated for 1 h with approximately10^12 phage particles in 1 ml of buffer (phosphate buffered saline (PBS), 1% BSA). The MPLs were washed three times with buffer, then bound phage were eluted using 50 mM Glycine buffer, pH2.2. Eluted phage were used to infect E. coli cells and selection applied by plating phage/E. coli suspension on 2TY agar with ampicillin. Three to four rounds of selection were performed prior to screening of the positive clones. Crude bacterial periplasmic extracts containing scFv antibodies from selection outputs were prepared in 50 mM 4-morpholinoepropanesulfonic (MOPS), 0.5 mM EDTA and 500 mM sucrose pH7.4 buffer according to previously described methods.77

Analysis of binding of scFv to cell lines by flow cytometry

Bacterial periplasmic extracts containing scFv or purified scFv were incubated with untransfected canine thymus cells (Cf2Th), human embryonic kidney cells (HEK293), and Chinese hamster fibroblast cells (R1610) or stable cell lines expressing in either CXCR2 (Accession#P25025), CXCR3 (Accession#P49682) or FPR (Accession#P21462) for 45 min on ice. Cells were washed twice with PBS to remove unbound scFv and then incubated with 40 μg/ml anti-Myc antibody (Calbiochem Ab-1 mAB 9E10) for 30 min on ice. Cells were washed again and 10 μl PE conjugated anti-mouse antibody (Jackson ImmunoResearch Laboratories 115–116–146) added for 30 min on ice. The cells were washed a further two times, fixed and analyzed using the GUAVA flow cytometry system.

Preparation of purified scFvs

Purified scFv with C-terminal His and Myc tags were produced by cloning into the phagemid vector pCantab6 and expression in E.coli TG1 cells. Expression was induced with 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) induction and cells harvested by centrifugation. The protein was removed from the periplasm by osmotic shock using 200 mM Tris, 0.5 mM EDTA, 500 mM sucrose (TES) pH 8 buffer and the scFv purified by affinity chromatography using Histrap FF (GE Healthcare #17-5255 or #17-5319) and buffer exchanged using NAP10 columns (GE Healthcare #17–0854), according to the manufacturers’ protocol. The purified scFv were quantified using a bicinchoninic acid (BCA) assay (Pierce #23225) and visualized by SDS PAGE analysis.

Immunization and hybridoma generation

VelocImmune mice were sourced from Regeneron Pharmaceuticals Inc. These transgenic animals were genetically engineered to produce human:mouse hybrid antibodies by replacing mouse Ig heavy and kappa light variable region germ-line gene segments with their human counterparts while leaving the mouse constant regions intact.78,79 All work was undertaken under the 1986 Animals (Scientific Procedures) act in fully Home Office licensed facilities under an approved Home Office Project License.

HEK cells overexpressing the human CXCR2 receptor were routinely cultured in Dulbecco’s modified Minimal Essential Medium (DMEM, Life Technologies #41966) containing 10% FBS (SAFC Biosciences #12076C), 0.5 mg/ml G418 (Life Technologies #10131). The expression of functionally active human CXCR2 on the cell surface was confirmed by assessment of fluorescently labeled IL-8 binding and CXCR2 agonist induced inhibition of adenylate cyclase activity (Supplementary information, SI Figure 2). For immunizations, cells were harvested and resuspended in PBS. Emulsions were prepared by addition of equal volumes of cells and complete Freund’s adjuvant (Sigma #F5881) for the primary immunization or incomplete Freund’s adjuvant (Sigma #F5506) for boost immunizations. Each mouse was injected subcutaneously
on four separate occasions, 7–8 d apart, with 200 µl emulsion containing 1x 10^6, 3x 10^6 or 5x 10^6 cells, using two sites with 100 µl per site. A final boost was injected intraperitoneally with 200 µl (5x 10^6) cells prepared in PBS without any adjuvant four days before sacrifice on day 28.

Pre-bleed sera and sera following the second and third boosts were evaluated by ELISA for the presence of antibodies that bound to HEK human CXCR2 cells. Cells were plated at a density of 2x 10^5 cells/well onto 96-well Poly-D Lysine black plates and grown overnight at 37 °C, 5% CO_2. The media was discarded, cells fixed for 5 min with 3.7% formaldehyde (Aldrich #533998)/PBS solution then blocked with 3% Marvel/PBS for 1 h at room temperature. Serum samples or control antibodies in 3% Marvel/PBS were incubated with the cells for 1 h at room temperature. Plates were washed three times with PBS, then antibody detected by incubation for 1 h at room temperature with 50 µl per well 10 ng/ml anti-mouse IgG-Europium (Perkin Elmer #AD0124–01) prepared in DELFIA buffer (Perkin Elmer #4002–0010). 50 µl of Enhancement Solution (Perkin Elmer #4001–0010) was added and time-resolved fluorescent emission at 615 nm measured following excitation at 340 nm using a Perkin Elmer Envision plate reader.

Lymph nodes and spleens were harvested from mice with the highest specific antibody titer and cells isolated by mechanical disruption. Cells were combined and fused with SP2 myeloma cells using the polyethylene glycol (Roche #0783641) fusion method. The resulting fusion mixture was plated out in 96-well plates at a concentration of 4.45x 10^4 per well and cultured overnight in Hybridoma growth media containing DMEM, 20% FCS, 4 mM Glutamine (Life Technologies #25030), 100 U/100 µg/ml Penicillin/Streptomycin (Life Technologies #15140–122), 10% BM Condimed H1 (Roche #11088947001), 2% oxaloacetate/pyruvate/insulin (OPI) media supplement (Sigma #O5003), 2% Hypoxanthine/Azaserine (Sigma #A9666). Single hybridoma clones were obtained by limiting dilution.

Small scale purification of antibodies from hybridoma supernatants

Well lines were overgrown in HL-1 serum-free medium (Lonza #77201). Supernatants were transferred to 96 well masterblocks (Greiner #780271) and purified on Protein A 20 µl PhyTips (PhyNexus PTP-92–20–01). IgG were eluted with 75 µl buffer containing 100 mM HEPES 140 mM NaCl pH3 and neutralized with an equal volume of 200 mM HEPES pH 8.

Reformatting of chimeric antibodies to human IgG and expression in mammalian cells

Chimeric antibodies derived from the hybridomas were converted to human IgG format essentially as described by Persic et al. Variable regions of light and heavy chain genes were amplified from cDNA using HiFi extensor polymerase (Abgene #AB0794/B) and specific primers to either the heavy or light chain variable region. The V_H and V_L domains were cloned into mammalian cells.

Heavy and light chain IgG expressing vectors were transfected into EBNA-HEK293 mammalian cells. Supernatants were pooled and filtered, then IgG was purified using a column of appropriate size of Ceramic Protein A (Pal #20078–036). The column was washed with 50 mM TRIS-HCl pH 8.0, 250 mM NaCl and IgG eluted using 0.1 M Sodium Citrate (pH 3.0). The eluant was neutralized by the addition of TRIS-HCl (pH 9.0), then buffer exchanged into PBS using Nap10 columns (GE Lifesciences #17–0854–02). The concentration of IgG was determined spectrophotometrically using an extinction coefficient based on the amino acid sequence of the IgG.

FMAT direct binding assay

Cells were harvested and resuspended in FMAT assay buffer (pH 7.4) containing 1X Hanks Balanced Salt Solution (Sigma #H8264), 0.1% Bovine Serum Albumin (Sigma #A9576), 20 mM HEPES and 0.01% Sodium Azide. Samples (5µl) were transferred into assay plates (Costar #3655) containing 15 µl per well 800 ng/ml goat anti-mouse FMAT Blue® (Applied Biosystems #4362494). Cells (3000 per well) were added to give a total assay volume of 40 µl and plates incubated for 5 h at room temperature. Plates were read on the Applied Biosystems Cellular Detection System 8200 and data analyzed using the Velocity algorithm with appropriate gating.

FMAT receptor-ligand competition assay

Purified antibodies or hybridoma supernatants (10 µl) were incubated with 0.8 nM FMAT Blue® labeled IL-8 (Applied Biosystems #4377770) and HEK human CXCR2 cells (3000 cells/well) in a total assay volume of 40 µl in a 384-well plate (Costar #3655) for a minimum of 5 h at room temperature. Total binding and non-specific binding (NSB) controls were defined using FMAT assay buffer or 80 nM final assay concentration of unlabelled human IL-8 respectively. Plates were read on the Applied Biosystems Cellular Detection System 8200 and data analyzed using the Velocity algorithm with appropriate gating. Specific binding% was calculated as a% of the total binding control signal following subtraction of the NSB signal.

FMAT epitope competition assay

Antibodies were fluorescently labeled using DyLight-649/650 labeling kits (Thermo Scientific 84536, 53051 and Innova Biosciences #326–0010) according to the manufacturers’ instructions. Purified antibodies (7.5 µl) were incubated with 1nM DyLight-649 labeled HY29–1, 1 nM DyLight-650 labeled 6C6, 1nM DyLight-650 labeled X2–753 or 0.2 nM DyLight-650 X2–1194 and HEK human CXCR2 cells (3000 cells/well) for 4 h at room temperature in a total assay volume of 40 µl in a 384 well plate (Costar #3655). Total binding and NSB controls were set up using assay buffer and excess unlabelled IgG, respectively. Plates were read on the Applied Biosystems Cellular Detection System 8200 and data analyzed using the Velocity algorithm with appropriate gating parameters.
CXCCL2 FLIPR assay

HEK cells overexpressing human CXCCL2 and Gq5 were harvested, resuspended in DMEM containing 10% FBS, 0.5 mg/ml G418 and 0.35 mg/ml Hygromycin B at a density of 0.28 x 10^6 cells/ml and plated at 50 μl per well into 384-well black walled Poly-D Lysine coated plates (Greiner #781946). Plates were incubated at 37 °C, 5% CO_2 for 18–24 h, then cell media was aspirated and replaced with 25 μl well per well of Fluor-4AM dye loading solution (component C from the Fluor-4NW Calcium Assay kit Invitrogen #F36206) prepared according to manufacturer’s instructions. Cells were loaded with dye by incubation at 37 °C, 5% CO_2 for 30 min followed by 30 min at room temperature. Samples were diluted to the required concentrations in assay buffer containing 25 mM HEPES, 125 mM NaCl, 5 mM KCl, 1 mM MgCl_2, 1.5 mM CaCl_2, 5 mM Glucose and 0.1% BSA. 12.5 μl diluted sample or assay buffer (stimulated and unstimulated controls) was transferred to the dye-loaded cells and incubated at room temperature for 30 min. Agonist (12.5 μl) or assay buffer (unstimulated control wells) was added and the peak fluorescence of the Fluor-4AM dye recorded at 1 s intervals for 80 measurements followed by 4 s intervals for 30 measurements on the FLIPR® (Molecular Devices). The peak response from each well was corrected for baseline fluorescence by subtraction of the mean fluorescence in the unstimulated control wells. Corrected data was expressed as% of the response in the stimulated control wells. IC_{50} values were determined using GraphPad Prism 6.03 software using the equation for a four parameter logistic curve fit.

TANGO™ β-arrestin recruitment assay

TANGO™ U2OS hCXCR2-bla cells (Invitrogen #K1521) were harvested and plated into 384-well Poly-D Lysine plates (Greiner #781946) at a density of 10,000 cells/well in a 30 μl volume. Plates were incubated at 37 °C, 5% CO_2 for 18–24 h. IgG samples (5 μl) prepared in Freestyle Media (Invitrogen #12338) were added to the pre-plated cells and incubated for 30–60 min at 37 °C, 5% CO_2. Cells were stimulated by addition of agonist (5 μl) for 5 h at 37 °C, 5% CO_2. Unstimulated control wells (cells with 10 μl Freestyle media added) and cell-free control wells (media only) were set up on each plate for determination of basal β-lactamase expression and background fluorescence, respectively. β-lactamase activity was measured by addition of 8 μl detection mix containing the CCF4AM/LiveBLAzer™ FRET B/G Substrate (Invitrogen #K1089) prepared using the Beta Lactamase Loading Solutions kit (Invitrogen #K1085) and Solution D (Invitrogen #K1157) according to the manufacturer’s instructions. Plates were incubated for 2 h at room temperature in the dark and fluorescence emission at 460 nm and 535 nm determined following excitation at 490 nm using a Perkin Elmer Envision Plate Reader. Data was corrected for background fluorescence (cell-free control wells) at each emission wavelength and the emission ratio of 460 nm/530 nm calculated. A response ratio was determined by normalizing to the unstimulated control emission ratio and data was expressed as% stimulated control response ratio. EC_{50} and IC_{50} values were determined using GraphPad Prism 6.03 software using the equation for a four parameter logistic curve fit. Fitting to an Allosteric model was performed using the equation Y = Bottom + (Top-Bottom)/(1+10^((LogEC-X) x HillSlope)) where X is the log concentration of agonist, LogEC represents Log(EC_{50} x Antagonist) in which Antagonist = (1+B/KB)/(1+α x B/KB), α = 10^Logalpha, B is the concentration of antagonist, KB is the equilibrium dissociation constant of the antagonist, EC_{50} is the concentration of agonist that gives half maximal response in the absence of modulator. This is based on the equation for an allosteric modulator that results in a change in affinity of the receptor for the agonist.48

Pepsan Epitope Mapping

Extracellular domains of human CXCCL2 were synthesized as peptide arrays of 3640 peptides comprising the N-terminus (MEDFNMESDSFEDFWKGEDLSNYSSTLPPFLLLDAAPCEPESLEINK, ECL1 (ASKVNGWIFGTLCK), ECL2 (RTTYYSSNVSPACEDMGNNSTANWR) and ECL3 (DTLMRTQVIQETCERRNHIDR), using overlapping linear and single looped 16-mers (Cys-X_16-Cys format, overlapping double looped 16-mers in a Cys-X_16-Cys-X_16-Cys format and overlapping looped 7 mers of the combined N-terminus, ECL1, ECL2, and ECL3 constructs in a X_7-Cys-X_7-Cys-X_7-Cys format. Conformational loops were created by connecting the cysteine residues with a CLIPS scaffold.48 Additional mapping was performed using a 455 linear and CLIPS peptide array consisting of an alanine scan of peptides with the highest binding levels from the 3604 peptide array and a dedicated N-terminus/ECL3 library.

Epitope mapping of monoclonal antibodies was performed according to published protocols47,48 with binding of each antibody tested in a PEPSSCAN-based ELISA. Briefly, peptide arrays were incubated with antibody (1–10 μg/ml) diluted in PBS blocking solution containing 4% horse serum, 5% ovalbumin and 1% Tween. After washing, peptides were incubated with a 1000-fold dilution of antibody peroxidase conjugate for one hour at 25 °C. The antibody was removed by washing and signal developed by addition of peroxidase substrate 2,2'-azino-di-3-ethylbenzthiazoline sulfonate and 2 μl of 3% H_2O_2. The extent of color development was quantified with a charge coupled device camera and an image processing system.

Disclosure of Potential Conflicts of Interest

Epitope mapping was performed by Pepscan Presto B.V on a fee for service basis. Additional data interpretation and input into manuscript preparation was provided by Joris Benschop and Jerry Slootstra without additional cost.

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