Selection of a picomolar antibody that targets CXCR2-mediated neutrophil activation and alleviates EAE symptoms

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Receptors and their ligands are important therapeutic targets for about one third of marketed drugs. Here, we describe an epitope-guided approach for selection of antibodies that modulate cellular signaling of targeted receptors. We chose CXC chemokine receptor 2 (CXCR2) in the G-protein coupled receptor superfamily as receptor and a CXCR2 N-terminal peptide for antibody selection. We obtain a highly selective, tight-binding antibody from a 1011-member antibody library using combinatorial enrichment. Structural and Hydrogen-Deuterium-Exchange mass spectrometry analyses demonstrate antibody interaction with an N-terminal region of CXCR2 that is part of the IL-8 epitope. The antibody strongly inhibits IL-8-induced and CXCR2-mediated neutrophil chemotaxis in vitro and alleviates hCXCR2-dependent experimental autoimmune encephalomyelitis symptoms in mice. As inappropriate neutrophil migration accompanies many diseases including inflammatory bowel disease, glomerulonephritis, allergic asthma, chronic obstructive pulmonary disease, and cancer, this antibody has potential for development as a therapeutic agent, akin to anti-TNF antibodies. However, an important difference here is that the antibody targets the chemokine receptor and competes with natural ligand, rather than targeting the ligand itself.

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The G protein coupled-receptor (GPCR) superfamily is one of the largest families in the human genome, consisting of over eight hundred members, with a broad spectrum of distribution in various organs and tissues, including the central nervous, immune, and cardiovascular systems. GPCRs are widely involved in a range of physiological and pathological processes in humans. Almost 40% of approved drugs mediate their effects through GPCRs. Based on evolutionary homology and properties of their physiological ligands, most GPCR proteins can be grouped into one of five main families: Rhodopsin, Adhesion, Secretin, Glutamate and Frizzled/TAS2. Rhodopsin is the largest and most heterogeneous family, and can be further divided into four sub-families, α, β, γ, and δ. These proteins have a similar overall structure: an extracellular N-terminal region, seven transmembrane helices, and a cytoplasmic C-terminal domain. GPCRs sense various extracellular stimuli through interactions with a variety of ligands, such as amino acids, nucleic acids, peptides and proteins, and activate intracellular signaling pathways via ligand-induced conformational changes. In canonical signaling, GPCR signals are transduced through recruitment of intracellular GTP-dependent proteins (G proteins) onto specific cytoplasmic regions of the C-terminus - the so-called G protein-dependent signaling. Then, depending on the particular G protein involved, downstream pathways including cAMP or the PI2 pathway are activated. Moreover, recruitment of other intracellular scaffold proteins, e.g., β-arrestins, has been shown to activate G protein-independent signaling events. Multiplex signaling endows GPCRs with parallel functions in a cell, making it difficult to design relevant assays linking any single target receptor to specific downstream cellular activities. It remains a challenge for the pharmaceutical industry to develop highly effective therapeutics against these important targets. In addition, while antibodies have emerged as an ever increasing source of new therapies, as yet only two therapeutic antibodies to GPCRs have been developed (to CCR4 and GPRPR)32,36, although others have been generated, including to CXC chemokine receptor 1 (CXCR1) and CXCR2, but not advanced to therapy7–10. This paucity is largely due to the difficulty in generating antibodies that bind to functional conformations of these membrane proteins.

Combinatorial antibodies have emerged as a powerful tool in drug discovery11,12. Over 80 antibodies from phage panning have entered clinical studies, and more than 10 have been granted marketing approval13. The combinatorial antibody library approach takes advantage of vast diversity consisting of up to 1014 distinct binding molecules14. Antibodies selected from such libraries have shown diverse mechanisms, and can be neutralizing or function as agonists, antagonists, or inverse agonists and, in some cases, function beyond the scope of native ligands15–17.

CXCR2, a member of the chemokine receptor family, is mainly expressed on neutrophils18. CXCR2 is involved in neutrophil chemotaxis, which normally follows inflammatory stimuli19–23. However, unwanted migration of neutrophils can also be a factor in the pathophysiology of a wide variety of diseases associated with inflammation, including colitis, chronic obstructive pulmonary disease (COPD), asthma, and glomerulonephritis.24–26 For example, a previous study has reported that depletion of CXCR2 protects lungs from cigarette smoke-induced inflammation and injury27. Multiple sclerosis (MS), an autoimmune disease of demyelination in the central nervous system with strong inflammation, was previously thought to be caused mainly by the overreaction of T (T17 & T117) and B cells (autoimmune antibody). Current emerging evidences revealed the important role in involvement of neutrophils and CXCR2 signaling in MS28,29. In addition, CXCR2 has been shown to participate in the progression of different types of cancer, playing a significant role in proliferation, survival, and metastasis of tumor cells, and affecting the whole tumor microenvironment30,31.

Given these clinical findings, inhibition of CXCR2-induced neutrophil migration is an important, but as yet unrealized, strategy to treat neutrophil-related inflammatory diseases and some cancers32–34. Several small molecules targeting CXCR2 have already shown remarkable inhibitory effect in both in vitro and animal studies, but not therapeutic efficacy in the clinical setting32,33. This lack of clinical effectiveness is thought to be due to off-target effects35. In contrast, antibodies have the potential to overcome these clinical difficulties in that they have high specificity, high serum stability, high safety and, because of their size, can target the ligand-binding extracellular domains of receptors. However, because of the difficulty in preparing adequate quantities of stable antigen in native conformation for selection, it has been challenging to identify functional antibodies targeting GPCRs.

Here, using an epitope-guided approach, we selected a combinatorial antibody from a 1014-member library that binds CXCR2 in the same epitope region as IL-8 (also known as CXCL8), but with picomolar potency. The superior antibody affinity compared with IL-8 (picomolar vs. nanomolar) overcomes the competition problem and results in total inhibition of IL-8-induced cellular functions, thus indicating potential for development as a therapeutic modality for treatment of neutrophil-related diseases. The selection strategy might also be generalized to develop antibodies for other GPCR-related diseases.

Results

Selection of combinatorial antibodies that bind human CXCR2. The human CXCR2 (hCXCR2) extracellular N-terminus is essential for binding and signal sensing of its cognate ligands, including IL-8, GRO-α (a.k.a. CXCL1), etc36,37. Site-directed mutagenesis demonstrated that acidic residues, E7, D9, E12 and D13, constituted a putative binding pocket for IL-838. Each of the three extracellular loops of hCXCR2 was implicated in binding of IL-8 in different studies39–41. However, nuclear magnetic resonance and crystallography analyses of the IL-8–hCXCR2 complexes indicated IL-8 binds the N-terminus and loop 2 of the extracellular domain42–47. The N-terminus of hCXCR1 and hCXCR2 share only 25% homology (Fig. 1a), as compared to 100%, 55%, and 92% for loops 1, 2, and 3, respectively, indicating that the extracellular N-terminal peptide of hCXCR2 (48 amino acids) could serve as an ideal antigen in panning and optimization of highly selective and potent combinatorial antibodies. Peptide, pepN48 (see Methods and Fig. 1a) was synthesized (Chinese Peptide, Hangzhou) and used in three rounds of phage panning using a 1011-member single-chain variable fragment (scFv) combinatorial antibody library14. Nine scFv sequences showing high enrichment in the panning were sub-cloned into a pFusE expression vector and their expression and affinity confirmed by ELISA screening of the cellular supernatants with pepN48 (Supplementary Fig. 1a). Further analyses of the supernatants using fluorescence-activated cell sorting (FACS) showed that only one clone, H8 (abN48), recognized the membrane-bound hCXCR2-mCherry on U2OS cells (Supplementary Fig. 1b). The H8 (abN48) scFv antibody was overexpressed, purified to homogeneity, and surface plasmon resonance (SPR) analysis indicated its Kd value of abN48 with pepN48 was 2.6 × 10−9 M (Supplementary Fig. 1c).

Generation of full-length IgG1 combinatorial antibody and affinity maturation. To optimize binding of abN48, the scFv was first converted into a full-length IgG1 antibody, abN48-IgG1

References
Fig. 1 Epitope-guided selection of tight binding antibodies for hCXCR2. 

**a** Schematic illustration of antibodies selected by phage panning based on the IL-8 epitope on the extracellular N-terminus of hCXCR2 (pepN48). Binding affinity measurements of two combinatorial antibodies, abN48-IgG1 and abN48-2-IgG1 by SPR assay on a Biacore T200. Curves in the sensogram plots represent a range of concentrations of antibody solutions: 0.1, 0.5, 1, 2, 5 and 10 nM for abN48-IgG1 and 0.1, 0.2, 0.5, 1, 2, and 5 nM for abN48-2-IgG1. HDX exchange for pepN48 in the presence of abN48-IgG1 (upper panel) or abN48-2-IgG1 (lower panel) as compared to free pepN48. The percentage difference in deuterium uptake values at different labeling time points is represented as heatmap, with light gray indicating no significant changes, red indicating increase, and blue indicating decrease in HDX exchange rates. Crystal structure of pepN9-19 in complex with abN48-2 illustrating that the aromatic side chains of Phe14 and Trp15 from pepN9-19 snugly fit into a cavity formed by the antibody CDR loops. The light and heavy chains of abN48-2 are shown as light and dark gray surfaces, respectively. The CDR loops of the heavy chain and light chain are depicted as red and green cartoons respectively and the surface representation was rendered transparent to show the location of the CDR loops. The backbone of bound pepN9-19 is depicted as a yellow tube with the side chains of critical residues as stick models. Close-up view of the interface between pepN9-19 and abN48-2. Key residues involved in the antibody-antigen interface are shown as stick models and color-coded as following: light gray (light chain residues), dark gray (heavy chain residues), and yellow (pepN9-19 residues). Polar and electrostatic interactions are indicated with black dashed lines. The CDRs residues are labeled following Kabat numbering.
Epitopes of abN48-IgG1 and abN48-2-IgG1 overlap with IL-8 binding site. We then utilized hydrogen-deuterium-exchange mass spectrometry (HDX-MS) to map the epitopes of abN48-IgG1 and abN48-2-IgG1 on hCXCR2. In the presence of abN48-IgG1, the HDX rate of residues 11–18, particularly residues 13–16, on pepN48 decreased considerably (Fig. 1c and Supplementary Fig. 4), indicating their possible location at the interface between abN48-IgG1 and pepN48, in accord with the reported binding of IL-8 to the N-terminus of hCXCR2. The major epitope of abN48-2-IgG1 was also identified as residues 13–16 (Fig. 1c and Supplementary Fig. 4). Apparently, the affinity-matured abN48-2-IgG1 preserved the binding mode between abN48-IgG1 and hCXCR2, albeit with higher affinity. The HDX protection by both antibodies gradually decreased at longer labeling time points and disappeared at 120 s (Fig. 1c). Considering the negligible off-rate of the antibodies using Biacore (Fig. 1b), these results suggest that side-chain rather than main-chain interactions rendered on peptide form the major stabilization interactions between peptide and antibody.

To identify a minimal interacting peptide sequence of pepN48 for crystallography studies, a series of truncates of pepN48 were constructed. Consistent with HDX-MS mapping, a 9–19 amino acid (aa) peptide (pepN9–19) bound both abN48 and abN48-2 comparable to pepN48, whereas pepN48 lacking residues 9–19 (pepN48Δ9–19) was no longer recognized (Supplementary Fig. 5). Co-crystal structures of the Fabs of both antibodies with pepN9–19 were then determined. The abN48-2 Fab/pepN9–19 complex was refined to 2.2 Å resolution (Supplementary Table 2) with two protein complexes (protomers) per asymmetric unit (Supplementary Fig. 6a). Electron density for residues 11–16 of pepN9–19 could be clearly visualized in both protomers (Supplementary Fig. 6b). Phe14 and Trp15 insert their bulky aromatic side chains into a binding site cavity (Fig. 1d). The benzene ring of Phe14 forms face-to-edge π–π stacking with Phe46L and Tyr34L from abN48-2, and the Trp15 indole is anchored by the side chains of Tyr96L and Ser35H (Fig. 1e). Gly95H hydrogen bonds with Phe14 amide nitrogen, while Tyr34L and Trp96L side-chains hydrogen bond with Phe14 and Trp15 carbonyl oxygens (Fig. 1e). The snug fit of Phe14 and Trp15 in the antibody cavity also facilitates hydrogen-bonding from the Ala33H backbone nitrogen to OD2 atom of Asp13, whose OD1 hydrogen bonds with the Trp15 amide nitrogen to further stabilize the peptide conformation (Fig. 1e). The hCXCR2 side chains, Phe11, Glu12, and Lys16, also make intimate interactions with abN48-2 Fab (Fig. 1d). Specifically, the Phe11 benzene ring forms face-to-edge π–π stacking with Tyr96L, Glu12 salt bridges with Arg32L and Arg49H, and Lys16 hydrogen bonds with Asn52H and packs its aliphatic region against Trp50H (Fig. 1e). The Glu12 backbone nitrogen and oxygen also hydrogen bond to the backbone oxygen and nitrogen of Cys97H, further strengthening the antibody–antigen interactions (Fig. 1E).

For further epitope validation, we carried out alanine-scan mutagenesis on residues in the N-terminus of hCXCR2. Consistent with Phe14 and Trp15 contributing more than 50% (395 Å2) of the total (~782 Å2) buried solvent-accessible area (SAA) on pepN9–19 upon abN48–2 binding, their mutation completely abolished the interaction between abN48-IgG1 and U2OS cells expressing hCXCR2-mCherry fusion protein (Supplementary Fig. 7). Asp13 mutation also had a detrimental effect, while Lys16 mutation did not (Supplementary Fig. 7). Thus, Asp13, Phe14, and Trp15 are the major contributors to the epitope of abN48–2-IgG1 on hCXCR2, while Phe11, Glu12, and Lys16 play more auxiliary roles.

As previously proposed, Trp10 from hCXCR1 (equivalent to Trp15 of hCXCR2), is important for membrane anchoring of the CXCR1 N-terminus. Displacement of Trp10 from the membrane appeared to be a pre-requisite for the subsequent IL-8 binding. Residues 7, 9, 12, and 13 of hCXCR2 were implicated in IL-8 binding. Hence, the critical epitope residues of abN48 and abN48-2 identified here, i.e., Asp13–Trp15, overlap with the IL-8 binding site, and, therefore, these antibodies might antagonize hCXCR2 by out-competing the natural IL-8 ligand for binding to the hCXCR2 N-terminus. AbN48-IgG1 and abN48-2-IgG1 differ by two residues in CDR3, i.e., Ser99H to Arg99H and Ser100AH to Arg100AH. Why these arginine substitutions in abN48-2-IgG1 result in a 100-fold increase in binding affinity is not obvious from the x-ray structure and suggests a subtle global stabilization by these residues. Entropy stabilization, thus, could be an important factor in addition to the enthalpy changes in the tight-binding of the optimized antibody, abN48-2-IgG1 with hCXCR2.

Species and sub-type specificity. To determine the species and sub-type specificities of abN48-IgG1 and abN48-2-IgG1, gene constructs containing hCXCR1-mCherry, hCXCR2-mCherry, mouse CXCR2 (mCXCR2-mCherry), rat CXCR2 (rCXCR2-mCherry), rabbit CXCR2 (rCXCR2-mCherry), and macaque CXCR2 (mCXCR2-mCherry) were overexpressed in U2OS cells. Immunofluorescence co-localization of abN48-IgG1 and abN48-2-IgG1 on the surface of U2OS cells overexpressing hCXCR1-mCherry, hCXCR2-mCherry, mCXCR2-mCherry showed both antibodies specifically recognize surface hCXCR2 (Fig. 2a). This specificity was further confirmed by FACS analyses, in which both antibodies bound exclusively to hCXCR2 and closely related macaque monkey CXCR2 (72.9% identity), but not to CXCR2 of mouse (45.8% identity), rat (43.8% identity), rabbit (58.3% identity), or to CXCR1 (Fig. 2b).

Potent inhibition of hCXCR2-mediated β-arrestin signaling and calcium influx. To understand cellular functions of the hCXCR2-specific antibody binders, two independent signaling pathways mediated by CXCR2, namely β-arrestin recruitment...
and cytoplasmic Ca\textsuperscript{2+} influx, were evaluated using Tango and FLIPR methods, respectively. \(\beta\)-arrestin recruitment is a G protein-independent intra-cellular event; whereas Ca\textsuperscript{2+} influx is a result of its release from the endoplasmic reticulum (ER) into the cytoplasm in response to IP3 produced by G\(\alpha\) protein activation. Both events are known to be directly associated with CXCR2 activation.

Using Tango assays, two natural ligands and the two selected antibodies were tested for agonistic effects in \(\beta\)-arrestin recruitment. Only natural ligands, IL-8 and GRO-\(\alpha\), induced \(\beta\)-arrestin recruitment, with apparent 50\% effective concentration (EC\textsubscript{50}) of 4.5 and 5.3 nM, respectively (Fig. 3a), whereas abN48-IgG1 and abN48-2-IgG1 showed no activation up to 100 nM. However, the antibodies showed complete inhibition of natural ligand-induced recruitment of \(\beta\)-arrestin (at EC\textsubscript{90} level), with apparent 50\% inhibitory concentration (IC\textsubscript{50}) of 2.8 and 0.90 nM for IL-8, and 4.7 and 0.37 nM for GRO-\(\alpha\), respectively (Fig. 3b). Both antibodies also showed an inverse agonist effect, in which antibody at 20 nM markedly suppressed the intrinsic \(\beta\)-arrestin recruitment by CXCR2 over-expression (Supplementary Fig. 8).

The natural ligands and antibodies were then tested for their ability to stimulate Ca\textsuperscript{2+} influx. IL-8 and GRO-\(\alpha\) showed dose-dependent activation with EC\textsubscript{50} values of 0.42 and 1.7 nM, respectively (Fig. 3c), whereas only the tighter binding antibody, abN48-2-IgG1, displayed a partial agonist effect at high concentration (10–1000 nM), with apparent EC\textsubscript{90} of 310 nM (Fig. 3c). However, both antibodies displayed dose-dependent inhibition of natural ligand-induced Ca\textsuperscript{2+} influx (at EC\textsubscript{90} level), with IC\textsubscript{50} values of 190 and 44 nM, respectively for IL-8, and 1900 and 110 nM, respectively for GRO-\(\alpha\) (Fig. 3d). The observed enhancement on CXCR2-mediated signaling by abN48-2-IgG1 is consistent with its improved binding to the CXCR2 N-terminus.
Potent induction of endocytosis of hCXCR2. IL-8 also induces endocytosis signaling of CXCR2 at 5–10 nM. To test any antibody effect, internalization of CXCR2 was studied using U2OS cells overexpressing membrane-bound hCXCR2. Immunotoxin complexes of abN48-IgG1 or abN48-2-IgG1 with a cytotoxin AL1-PE38KDEL were used as molecular probes. The immunotoxin complex of abN48-IgG1 or abN48-2-IgG1 showed dosedependent cytotoxicity of U2OS cells overexpressing hCXCR2 (Fig. 3e), whereas U2OS cells without hCXCR2 expression, and an immunotoxin complex of an irrelevant antibody, showed no
detectable cytotoxicity. Both abN48-IgG1 and abN48-2-IgG1 showed extremely potent activation of CXCR2 internalization with estimated EC$_{50}$ of less than 0.1 nM.

**Potent inhibition of IL-8-induced neutrophil chemotaxis.** Neutrophil chemotaxis occurs along the gradient of its corresponding chemokine secreted at a distant acutely damaged or infected site$^{49}$. Since CXCR2 is known to be expressed mainly on neutrophils, and its cognate ligand IL-8 is a key regulator in many pathological processes, the IL-8-CXCR2 axis constitutes an important target for therapeutic intervention$^{50}$. We therefore tested abN48-2-IgG1 in a chemotaxis assay using primary human neutrophils. Viable neutrophils were negatively selected via immuno-magnetic bead separation from a whole blood collection, and their purity was verified by immune-staining of CD15$^+$, CD16$^+$, CD11b$^+$, and CD66b$^+$ (MT-Bio, Shanghai). We examined membrane expression of hCXCR2 on neutrophils using abN48-IgG1 and abN48-2-IgG1 (Fig. 4a). FACs analyses showed that the whole population of neutrophils was captured by our CXCR2-specific antibodies. Next, the effect on IL-8-induced neutrophil chemotaxis was tested in a trans-well migration assay. The abN48-2-IgG1 showed dose-dependent inhibition of neutrophil chemotaxis induced by a maximal IL-8 concentration (10 nM) with 75% inhibition at 1 nM and 120% inhibition at 20 nM (Fig. 4b). For comparison, a small molecule CXCR1/CXCR2 dual inhibitor, MK7123, showed 100% inhibition of neutrophil chemotaxis at 1 μM. The observation of complete blockage of neutrophil migration (both chemokine-dependent and chemokine-independent) by abN48-2-IgG1 may suggest the involvement of CXCR2 in the basal intrinsic neutrophil migration.

**Establishment of hCXCR2 knock-in mice.** Since abN48-2-IgG1 showed an exclusive species specificity (Fig. 2), a homozygous strain of hCXCR2 knock-in mice (hCXCR2 mouse) was first generated by replacement of the cxcr2 CDS in the genome of C57BL/6 mice with that of human via gene targeting in the corresponding embryonic stem cells. Both the genotype and phenotype of hCXCR2 knock-in were validated by sequencing and flow-cytometry. As expected, abN48-2-IgG1 was only able to stain the neutrophils from hCXCR2 knock-in not wild-type mice (Supplementary Fig. 9). Since mice do not secrete IL-8 or CXCL6, mCXCL1, the murine orthologue of human chemokine Gro-α, was tested for its chemokine activity on hCXCR2 mediated calcium signaling. Comparing to human Gro-α (Fig. 3c), mCXCL1 showed a compatible stimulation of calcium influx with a similar EC$_{50}$ value (1.7 nM vs. 5.6 nM, respectively) (Supplementary Fig. 10a). Furthermore, abN48-2-IgG1 completely inhibited the mCXCL1 induced hCXCR2 calcium influx with an almost identical IC$_{50}$ value at EC$_{90}$ concentration of mCXCL1 (Supplementary Fig. 10b).

**Efficacy of AbN4-2-IgG1 and SB225002 in hCXCR2 mediated EAE.** Activation and migration of neutrophils are known to play a key role in the rodent experimental autoimmune encephalomyelitis (EAE) model$^{51-53}$, which mimics the autoimmune component of human multiple sclerosis. Using the above hCXCR2 knock-in mice, we established an EAE model and tested the efficacy of hCXCR2 specific antibody, abN48-2-IgG1. The pharmacokinetics of abN48-2-IgG1 in hCXCR2 mice were first examined (Fig. 5b). The naïve hCXCR2 mice were dosed individually with abN48-2-IgG1 via intraperitoneal (i.p.), subcutaneous (s.c.), or intravenous (i.v.) injections. Time courses of plasma concentration changes of abN48-2-IgG1 were monitored for 72 h. The antibody showed an excellent half-life of t$_{1/2}$ > 11 h and bioavailabilities of 73% and 95% for s.c. and i.p. dosing, respectively (Supplementary Table 3).

The dosing schedule for the establishment of EAE in hCXCR2 knock-in mice and the following antibody treatment is illustrated in Fig. 5a. Two different doses of abN48-2-IgG1 (7.5 mg kg$^{-1}$ and 0.075 mg kg$^{-1}$) were studied via i.p. injection (4 mice per group). Vehicle (PBS) and negative (isotype Fc) controls were administered according to the same dosing schedule as that for the antibody. SB225002, a selective small molecule inhibitor against hCXCR2 (150-folds vs. hCXCR1), was used as a benchmark. SB225002 was i.p. dosed at its maximum efficacious concentration (0.5 mg kg$^{-1}$) over the course of EAE development. Results showed that comparing to negative and vehicle controls, abN48-2-IgG1 alleviated the EAE symptoms of hCXCR2 knock-in mice in a dose-dependent manner (Fig. 5c). At 7.5 mg kg$^{-1}$, abN48-2-IgG1 attenuate about 90% EAE scores comparing to about 60% attenuation observed for the benchmark SB225002. It was also noted in a separate experiment that abN48-2-IgG1 did not have any effect on the EAE symptoms in the wild type mice. Furthermore, the plasma neutrophils at 24 h after the first injection of abN48-2-IgG1 were measured and showed a nearly complete attenuation even comparing to the basal plasma neutrophils of naïve hCXCR2 mice (without myelin oligodendrocyte glycoprotein peptide or pertussis toxin (PTX) challenge) (Fig. 5e).

**Discussion**

Biased signaling is a general feature for GPCRs because of the special structure and function of these receptors in cells. The extracellular domains, including the N-terminus and loops of the seven transmembrane regions, provide a combination of diverse sequences capable of sensing differential stimulation from ligands of various origins. Thus, different ligands that sense the same combination of loops or epitopes on the receptor may be expected to have similar cellular functions. This notion appears to
**Fig. 4 Inhibition of IL-8 induced neutrophil chemotaxis.**

**a** Representative flow-cytometry results showing abN48-IgG1 and abN48-2-IgG1 (FITC-conjugated) specifically bind to human primary neutrophils. Blank: cells without hCXCR2 expression; iso-IgG1-FITC: irrelevant human IgG1 antibody conjugated with FITC. (3 independent biological replicates showed the same results)

**b** Inhibition of abN48-2-IgG1 on IL-8 (10 nM) induced neutrophil chemotaxis was determined by a chemotaxis assay. Blank: media without IL-8 or inhibitor; IL-8: 10 nM IL-8; iso: 10 nM irrelevant human IgG1 antibody; MK: 1 μM small molecule CXCR2 inhibitor MK7123. The values of percentage (%) above the blue and green bars represent the percentage of inhibition of neutrophil chemotaxis induced by IL-8 at various concentrations of abN48-2-IgG1 and 1 μM MK7123, respectively. (n = 3 independent cell samples measured in a single experiment, data presented as dots overlapped with mean (column) ± s.d. (error bars). 3 independent experiments with different samples and antibody concentrations showed consistent results).
be supported in our study of the CXCR2 signaling network with IL-8 and GRO-α. However, in many physiological and pathological situations, structurally different ligands acting on the same receptor are able to preferentially activate only one signaling pathway, which is a phenomenon termed biased agonism or functional selectivity that has been studied extensively, especially for chemokines and chemokine receptors. The combinatorial antibody library technology with its vast numbers of diverse
protein binders can therefore be an invaluable tool in decoding the epitope combinations that are essential for biased signaling of membrane receptors.

Our findings here show that, through epitope-guided combinatorial enrichment, monoclonal antibodies can be identified that can directly compete with IL-8 for binding to its cognate epitope on hCXCR2, and are substantially enhanced in potency and efficacy over previous attempts using proteoliposomes or live cells formats that display full length on hCXCR2 surface, chimeric antigen or biparatopic nanobody format7,8. The optimized anti-

Methods

different receptors and cell types. Our understanding of the signaling networks and interplay among loops and epitopes used by natural GPCR ligands would further complex chronic diseases including MS, rheumatoid arthritis, and abN48-2-IgG1 in the treatment of various autoimmune-based, role. Our current study demonstrates the great potential of binding character of abN48-2-IgG1 appears to play an essential

Combinatorial antibody library panning. A peptide consisting of the first 48 residues of the extracellular N-terminus of hCXCR2 (MEDFNMESDFEDFWK-GEDLSNYSYSTLPPDDAACPESLEIN K) with biotin labeled on its N-terminus was synthesized (Chinese Peptide), and used as the antigen (pepN48) for phase panning. The panning procedure followed a modified

ELISA. Avidin (#21121; Pierce) was diluted in carbonate-bicarbonate buffer pH 9.6 (#C3041; Sigma) to a final concentration of 2 ng μL−1. 96-well ELISA plates (Corning Costar) were coated by incubation with avidin solution (25 μL per well) at 4 °C overnight. Wells were washed once with 150 μL per well PBST buffer (0.05% Tween 20 in PBS pH 7.4). A total of 50 μg of pepN48 in PBS pH 7.4 (2 ng μL−1) was added to each well, and incubated for 30 min at room temperature. Wells were washed 3 times with PBST buffer, and blocked with M-PBST (3% milk in PBST, 130 μL per well) at 37 °C for 5 min. After removal of M-PBST, 25 μL sample solution containing purified antibody or phage supernatant (diluted in M-PBST to proper concentration) were added into each well, and incubated for 1 h at room temperature, followed by 5 washes with PBST. Anti-M13 HRP-conjugated secondary antibody (1:13,000 dilution; #27-9421-01, GE) or anti-human Fc HRP-conjugated secondary antibody (1:13,000 dilution; #A1070, Sigma) was added into wells, incubated for 1 h at room temperature, washed five times with PBST, and followed by incubation with 50 μL per well ARTS solution (#1163430201; Roche) at room temperature for 20 min. Absorbance at 405 nm was measured on a plate reader (EnSpire; PerkinElmer).

For abN48-2-IgG1 animal PK study, pepN48 in PBS was coated on 96-well plates at 0.1 μg per well. HRP-labeled anti-human IgG antibody (1:1000 dilution; Promega, #DNA3B) was used for detection. Other reagents and work flow of ELISA were the same as described above. Sample preparation is described in PK study section in the “Methods”.

Expression and purification of antibodies. DNA sequences encoding the candidate scFv antibodies were cloned into a pUSExpression vector (#pUSE-HIgFc; InvivoGen) for expression of scFv-Fc proteins with the entire Fc domain of human IgG1. For antibodies in the full-length IgG1 format, variable regions of heavy chain and light chain (VH and VL) from the scFv sequence were cloned into plasmids with the complete constant domains of IgG1 heavy chain and light chains (CH1 and CL).

The antibodies were expressed through transfection of the scFv-Fc expression plasmid, or co-transfection of equal molar of heavy chain and light chain for full-length antibody, into HEK293F cells followed by cell culture for 5 days. Antibodies in the medium were purified with a HitTrap Protein A HP column (17- 0403-03; GE Healthcare) by AKTExpress purifier (GE Healthcare). Purified antibodies were concentrated and stored in PBS buffer (pH 7.4) at −80 °C.

SEC-HPLC. SEC-HPLC was carried out to determine the thermostability and homogeneity of purified recombinant antibodies. Briefly, antibody solutions were first concentrated to 20 mg mL−1, and incubated at 42 °C for 5 days. The resulting SEC-HPLC profiles were analyzed on an SEC column (SEC-250) with a running buffer of 0.05% DDM / 0.01% GRS in Tris (pH 8.1). Aggregation and degradation of the combinatorial antibody over prolonged incubation at 42 °C were evaluated and compared by homogeneity of its corresponding elution peak.

Flow-cytometry. The interaction of antibody ligand with surface chemokine receptors was studied using cell lines (U2OS or HEK293T) transiently overexpressing the corresponding chemokine receptor. After 24 h, cells were washed twice with PBS (and detached from dish by re-suspended in a FACS buffer (0.5% BSA in PBS pH 7.4, with 2 mM EDTA) to 5 × 106 cells per mL). 500,000 cells per tube were incubated with 2 μg mL−1 corresponding antibody in a FACS buffer at 4 °C for 15 min. The resulting cells were washed twice with a FACS buffer, and then incubated with 2 μg mL−1 secondary antibody, Alexa Fluor 488 goat anti-human IgG (H + L) (#11909; #A11013, Invitrogen), at 4 °C for 15 min. After two washes with the FACS buffer, cells were re-suspended in PBS and analyzed by CytoFLEX S (Beckman Coulter).
For analysis of neutrophils in the animal study, peripheral blood was isolated from canthus vein of mice. After lysis of red blood cells using RBC lysing solution (#702405; Sigma-Aldrich) and incubation for 15 min at 4 °C, the cells were centrifuged at 1500 × g for 5 min at 4 °C and resuspended in PBS. The cell concentration was calculated by a hemocytometer. 2 × 10^6 cells were seeded to 6-well plates (Falcon; BD Biosciences) in RPMI media containing 10% FBS and 1% Pen-Strep, and were cultured overnight at 37 °C in a humidified atmosphere containing 5% CO2. The next day, AL1-PE38KDEL (provided by Prof. Sachdev Sidhu at Temple University) was added to the cultures at a final concentration of 10 nM. A total of 250 μL of the resulting cell suspension was added to each well on FLIPR. Calcium signals were initiated by 2 μM stock solution of ionomycin (Invitrogen), followed by 10 μM of 1 μM stock solution of Fluo-4 DirectTM (Invitrogen), were added into each well in the assay. Calcium dye-loaded CHO cells, followed by equilibration to room temperature for 30 min. Ca2⁺ influx signals were initiated by 2 μM stock solution of ionomycin (Invitrogen), followed by 10 μM of 1 μM stock solution of Fluo-4 DirectTM (Invitrogen), were added into each well in the assay. calcium dye-loaded CHO cells, followed by equilibration to room temperature for 30 min. Ca2⁺ influx signals were initiated by 2 μM stock solution of ionomycin (Invitrogen), followed by 10 μM of 1 μM stock solution of Fluo-4 DirectTM (Invitrogen), were added into each well in the assay. Calcium in each well was excited at 488 nm and 514 nm, respectively, and emissions at 534 nm and 570 nm were recorded in real time as described above.

**Tango assay**

Internalization of CXCR2. A modified method utilizing an adaptor-toxin fusion protein, AL1-PE38KDEL, which binds specifically to IgG and forms an immunotoxin complex, was used to measure the antibody-stimulated CXCR2 endocytosis[35]. The resulting immunotoxin complex induces cell death when it enters cells. Briefly, U2OS cells overexpressed with hCXCR2 (or without hCXCR2 expression as control) were seeded in 96-well plate and cultured at 37 °C for 24 h. The next day, AL1-PE38KDEL (provided by Prof. Sachdev Sidhu’s lab) was added to antibody solution at 1:1 molar ratio and incubated at room temperature for 1 h to generate the immunotoxin complex. Cell medium was changed to Opti-MEM Reduced Serum Medium (#31985070; Gibco). Then antibody only, or cytotoxin only, or assembled immunotoxin, was mixed with cells at different final concentration of 1.6 × 10^−5, 8.0 × 10^−5, 4.0 × 10^−5, 0.20, 0.10, 0.50, 1.0 μM for 4 h at 37 °C. The co-mixed cells were then re-seeded into a fresh media and cultivated for 3 days at 37 °C for 50% WST-1 (WST-1; Dojindo) was used to determine cell viability by measuring the absorbance at 450 nm on a plate reader (EnSpire: PerkinElmer) according to the manufacturer’s protocol.

**Neutrophil chemotaxis**

Neutrophil migration was detected with 24-well transwell chambers with 8-μm pore size membranes (#3422, Corning Costar). Primary human neutrophils (#PBN-1F, MT-Bio) were suspended in chemotaxis medium without phenol red (RPMI 1640 medium #11835030; Gibco) with 0.5% BSA) to 5 × 10^5 cells per mL. These neutrophil samples were incubated with abN48-IgG1, abN48-2-IgG1, isotype antibody, CXCR1/2 inhibitor Navikax (MK7123, #HY-10198, MedChemExpress) at different concentrations indicated in Fig. 3 in chemotaxis medium for 30 min at 37°C. The chemotactic gradient was created by adding 1 μM stock solution of IL-8 (K03262-25, GenScript) to the lower chamber at concentration of 10 nM. A total of 250 μL of neutrophil suspension was added to each upper chamber and incubated for 60 min at 37°C. After incubation, cells in the lower chamber were counted by hemocytometer (#717810, BRAND).

**Cryolocalization and data collection**

pepN9-19 was synthesized by Sangon Biotech and its purity and identity validated by HPLC and mass spectrometry. The abN48-2 Fab was generated by papain (Sigma-Aldrich, at 1:5 w/w ratio) cleavage of abN48-2-IgG1 (lambda type) at 4 °C overnight in PBS buffer pH 7.4. Following cleavage, abN48-2 Fab was loaded onto a HiTrap lambda HP column (GE Healthcare) and eluted with 0.1 M sodium acetate at pH 3.0. The elution fractions were pooled and immediately applied to a Superdex200 increase 10/300GL gel filtration column (GE Healthcare) equilibrated with buffer containing 20 mM Tris-HCl buffer, pH 7.4.
The mixture was incubated overnight at 4 °C before being further concentrated. The column were then pooled and mixed with pepN9-19 at a molar ratio of 1:3. The system equipped with in-line peptic digestion and desalting. The desalted digests analysis of the peptide centroids was carried out with HD-Examiner v2.4 (Sierra software) or abN48-2 or abN48-2-IgG1 in current LC setting.

The quality of the final models was analyzed with MolProbity. A summary of data collection and refinement statistics is outlined in Supplementary Table 2. Structures have been deposited in the Protein Data Bank under accession codes 6XCN, chain L.

Chimeric offspring were selected for cross-breeding to obtain CXCR2 homozygote mice. Male CXCR2 knockout mice were crossed with CXCR2 homozygote females to obtain F1 heterozygote mice. Male F1 heterozygote mice were mated with CXCR2 homozygote females to obtain F2 homozygote littermates. The F2 homozygote littermates were then mated to obtain F3 homozygote mice.

For the PK study of abN48-2-IgG1, each of the hCXCR2 knock-in mice of 6-8 weeks old with body weight of about 20 g received a single i.v., s.c., or i.p. injection of 6.75 mg kg−1 abN48-2-IgG1 antibody. Four mice were included in each injection group. Blood samples of 100 μL per mouse were collected from the periorbital sinus. Two of the 4 mice in each group were sampled at 15 min, 3 h, 24 h, and 72 h post-injection, while the remaining 2 mice in each group were sampled at 1 h, 12 h, and 48 h post-injection. Serum concentration of abN48-2-IgG1 in each sample was measured by ELISA and quantitated using the above standard curve. PK parameters were estimated by fitting the time courses into a single compartment model using WinNonLin. The parameters calculated included the area under the curve (AUC) of time courses for serum concentrations from 0 to infinity (AUC0–∞), clearance (CL), volume of distribution at steady-state (VSS), and maximum concentration (Cmax), terminal half-life (t1/2), maximum plasma concentration (Cmax), etc.

EAE model studies. The EAE model was induced in both wild type and hCXCR2 knock-in mice using synthetic myelin oligodendrocyte glycoprotein peptide (MOG35–55, MEVGWYRSPFS RVHLYRGK; GenScript). Briefly, mice were s.c. injected with an emulsion of 100 μg MOG35–55 peptide mixed with complete Freund’s adjuvant (IFA, 5% vac-IFA, InvivoGen) containing 400 μg Mycobacterium tuberculosis H37Ra (231411, Difco). A total of 400 ng pertussis toxin (180, List Biological) were i.p. injected on the same day of MOG35–55 immunization and a second time at 48 h later.

To evaluate the efficacy of abN48-2-IgG1, 7.5 mg kg−1 or 0.075 mg kg−1 of abN48-2-IgG1 was i.p. injected q.4d. from the day of PTX injection for a total of 3 injections. Vehicle (PBS) and negative (Fc) controls were administrated according to the same schedule as that for the antibody. Negative (Fc) controls were administrated with the dose of 7.5 mg kg−1. SB252002, a small molecule allosteric inhibitor of hCXCR2 was employed as a benchmark. SB225002 was shown as a CXCR2-selective inhibitor, whose binding affinity to CXCR1 was 150-folds lower than that to CXCR2. SB225002 is widely used in in vivo studies of CXCR2 related neutrophil trafficking responsive to inflammation, especially some in the EAE model.25,29,35 In the current experiment, 0.5 mg kg−1 SB252002 was i.p. administered once every day throughout the EAE experiment (from Day 1 to Day23). Each cohort group contained 4 mice. Clinical scores were recorded according to a 5-point scoring system (0, no clinical symptoms; 0.5, Tip of tail is limp; 1, Limp of the whole tail; 2, Limp tail and weakness of hind leg; 3, Limp tail and complete paralysis of hind legs; 4, Complete hind leg and partial front leg paralysis; 5, Dead. Mouse is euthanized due to severe paralysis.)

To analyze the effect of abN48-2-IgG1 on peripheral neutrophils, blood samples were collected 24 h after the first injection of the antibody and subjected to FACS analysis (see “Flow-cytometry” above). All animal use and experiments were approved by the Institutional Animal Care and Use Committee at ShanghaiTech University.

Statistical methods. All statistical tests were performed with Graphpad Prism 7 software. Values of measurements were expressed as mean with error bar of standard deviation (s.d.) in figures. Numbers to get the mean (n value) were chosen according to assay features and indicated in each figure legend. For statistical comparison, Dunnett’s multiple comparisons test of one-way ANOVA with Geisser-Greenhouse correction was employed. Consideration of significance by p values and exact p values were specified in legends.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. Structural data have been deposited in the Protein Data Bank under accession codes 6KVA (abN48-2-IgG1) and 6KVF (abN48-2-IgG1-19). Source data are provided with this paper. All other data that support the findings of this manuscript are available from the corresponding authors upon reasonable request. Source data are provided with this paper.

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**Author contributions**

X.J.S., N.W., J.C.X., I.A.W., Y.W., G.Y., and R.A.L. designed the study, and interpreted the results. G.Y. and R.A.L. conceived and supervised this project, wrote the paper with X.J.S. and B.Y I.A.W reviewed and revised the paper. X.J.S., N.W., Y.W., X.F.Y., and J.W. performed the antibody affinity maturation by yeast display and full-length antibody construction, expression and purification, candidates binding validation, specificity check by immunocytochemistry and flow-cytometry, antibody kinetics and affinity measurement, Tango assay, calcium flux assay, internalization assay, and human neutrophil chemotaxis assay. J.C.X., L.Y., and R.Y. performed epitope mapping, HDX-MS, X-ray data collection, crystal structure determination, and analysis of antibody-targets complex. X.F.Y. and J.W. performed the combinatorial antibody library screen by phage display. Y.W., T.W., X.D., L.D., and X.J.S contributed the EAE in vivo study. T.W. and Y.L. helped with experiments and provided technical support. L.L.L made and maintained all the gene overexpression stable cell lines. S.C.H. and Z.W.Z. provided the antibody adaptor fused cytotoxin AL1-PE38KDEL and helped immunotoxin internalization assay.

**Competing interests**

The authors declare no competing interests.

**Additional information**

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