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CCR5 tyrosine sulfation heterogeneity generates cell surface receptor subpopulations with different ligand binding properties

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ABSTRACT

Background: Chemokine receptor tyrosine sulfation plays a key role in the binding of chemokines. It has been suggested that receptor sulfation is heterogeneous, but no experimental evidence has been provided so far. The potent anti-HIV chemokine analog 5P12-RANTES has been proposed to owe its inhibitory activity to a capacity to bind a larger pool of cell surface CCR5 receptors than native chemokines such as CCL5, but the molecular details underlying this phenomenon have not been elucidated.
Methods: We investigated the CCR5 sulfation heterogeneity and the sensitivity of CCR5 ligands to receptor sulfation by performing ELISA assays on synthetic N-terminal sulfopeptides and by performing binding assays on CCR5-expressing cells under conditions that modulate CCR5 sulfation levels.
Results: Two commonly used anti-CCR5 monoclonal antibodies with epitopes in the sulfated N-terminal domain of CCR5 show contrasting binding profiles on CCR5 sulfopeptides, incomplete competition with each other for cell surface CCR5, and opposing sensitivities to cellular treatments that affect CCR5 sulfation levels. 5P12-RANTES is less sensitive than native CCL5 to conditions that affect cellular CCR5 sulfation.
Conclusions: CCR5 sulfation is heterogeneous and this affects the binding properties of both native chemokines and antibodies. Enhanced capacity to bind to CCR5 is a component of the inhibitory mechanism of 5P12-RANTES.
General significance: We provide the first experimental evidence for sulfation heterogeneity of chemokine receptors and its impact on ligand binding, a phenomenon that is important both for the understanding of chemokine cell biology and for the development of drugs that target chemokine receptors.

1. Introduction

The chemokine receptor CCR5 is a G protein-coupled receptor expressed on a subset of leukocytes whose main physiological role is in the recruitment of effector cells to sites of inflammation [1–3]. In addition to being implicated in the pathology of cancer [4–6], neuroinflammation [7] and several inflammatory diseases [8,9], CCR5 is the principal HIV co-receptor [10–12] and a validated target for HIV prevention and therapy [13,14]. The native chemokines of CCR5, CCL3, CCL4 and CCL5, show modest anti-HIV potency [15], which has been explained in terms of a combination of their capacity to sterically block the receptor and to induce its internalization [16,17]. Certain chemokine analogs with modified N-terminal regions have been shown to exhibit strikingly higher anti-HIV potency, due in some cases to enhanced receptor internalization [18,19] and in others, including 5P12-RANTES, to improved steric blockade of CCR5 [19]. Structural studies indicate that the increased anti-HIV potency of 5P12-RANTES is due to an improved molecular fit between its modified N-terminal region and the transmembrane domain of CCR5 [20].

A number of studies using anti-CCR5 monoclonal antibodies (mAbs) and chemokine ligands have provided evidence that CCR5 exists in different conformational states at the cell surface [21–27]. Several explanations have been put forward for the existence of these cell surface subpopulations, including local differences in plasma membrane lipid composition [22,28], as well as the oligomerization [29] and the G protein-coupling state of CCR5 [26,30]. The increased anti-HIV potency of 5P12-RANTES has been explained in terms of its capacity, unlike native CCL5 from which it was derived, to bind with equally high affinity to different CCR5 subpopulations [26].

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Tyrosine sulfation is a post-translational modification catalyzed by the Golgi-resident enzymes tyrosylprotein sulfotransferases 1 and 2 (TPST-1 and TPST-2) that occurs on a subset of secreted and transmembrane proteins [31], including chemokine receptors [32]. CCR5 contains four tyrosine residues in its extracellular N-terminal domain (Tyr^3, Tyr^{10}, Tyr^{14} and Tyr^{15}) that can be sulfated [33], and sulfation at these positions has been shown to play a key role in the binding of both native chemokines [33,34] and HIV envelope glycoprotein [33]. It has been proposed that tyrosine sulfation of chemokine receptors is heterogeneous at the cell surface [32,35], but this phenomenon has not been investigated experimentally.

In this study we identified, among a panel of well-characterized anti-CCR5 mAbs that bind to epitopes in the extracellular N-terminal region of CCR5, a pair of mAbs that are sensitive to the sulfation state of the receptor. We used these mAbs as probes to investigate sulfation heterogeneity of cell surface CCR5, and made use of fluorescently labelled chemokine analogs to test the hypothesis that CCR5 variants with different levels of sulfation give rise to cell surface subpopulations that differ in their binding affinities for native CCL5 but are bound at uniformly high affinity by 5P12-RANTES.

2. Materials and methods

2.1. Reagents

2.1.1. Chemokines

Human CCL5 and 5P12-RANTES used in this study were prepared by chemical synthesis as previously described [18,19]. Fluorescent chemokines were generated as previously described [36], except that the fluorochrome used for derivatization was 5-(and-6)-carboxytetramethylrhodamine rather than Cy5.

2.1.2. Antibodies

The following anti-CCR5 monoclonal antibodies were used: mouse mAb 3A9 (NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH), phycoerythrin (PE)-labelled mAb 3A9 (BD Biosciences), rat mAb HEK/1/85a fluorescent derivatives (Bio-Rad), mouse mAb 2D7, unlabeled and PE-labelled (BD Biosciences), mouse mAb T21/8 (BioLegend), mouse mAb R22/7 (Novus Biologicals), and mouse mAb MC-5 (kindly provided by Dr. Nathalie Signoret, University of York, UK).

2.1.3. Sulfopeptides

Human CCR5 N-terminal peptides were synthesized using a standard Fmoc solid phase peptide synthesis strategy on a MultiPep RSi synthesizer (Intavis AG, Germany). Sulfotyrosine incorporation was performed as recommended by the manufacturer of the sulfotyrosine building block [37]. Essentially, sulfotyrosine residues protected by neopentenyl ester (nP) were used during synthesis, where they remained protected during trifluouroacetic acid cleavage and purification by reverse-phase HPLC and were then deprotected in sodium azide prior to a second reverse-phase HPLC purification using pH 6.5 ammonium acetate running buffers. Purified peptides were lyophilized and analyzed for purity and integrity by reverse-phase HPLC and MALDI mass spectrometry, respectively.

2.2. Cells and constructs

2.2.1. HEK-CCR5 and CHO-CCR5

Clonal cell lines (human embryonic kidney 293 (HEK) and Chinese Hamster Ovary (CHO)) stably expressing human CCR5 were obtained by lentiviral transduction [18] followed by clonal selection by fluorescence-activated cell sorting. Cells were maintained in DMEM (for HEK-CCR5) or RPMI (for CHO-CCR5) supplemented with 10% FBS and 1% Penicillin/Streptavidin (ThermoFisher Scientific).

2.2.2. CHO-CCR5 FFFF

The human CCR5 phenylalanine mutant (FFFF) was generated by Gibson Assembly® (New England Biolabs) (forward primer 5′- GGCTG CAGGTCGACTCTAGAGGATCCAGAACCATGGATTTTCAAGTGTCAAGT CCAATCTTTGACATCAATTTTTTTACATCGGAGCCC–3′, reverse primer 5′-ATAAGCTTGATATCGAATTCtcaCAAGCCCcccACAGATATTTCC-3′) and cloned into the FUGW lentiviral vector (Addgene #14883). CHO cells were stably transfected with FUGW-CCR5 FFFF as previously described [18]. Positive clones were selected by fluorescence-activated cell sorting using anti-CCR5 PE-labelled mAb 2D7 antibody. Cells were maintained in RPMI supplemented with 10% FBS and 1% Penicillin/Streptavidin (ThermoFisher Scientific).

2.2.3. HEK-TPST-2-T2A-mPlum

Human TPST-2 and mPlum genes were obtained from Addgene (catalog #66618 and #54629 respectively). The self-cleaving peptide T2A sequence (EGRGSLLTCGDVEENPGP) was inserted between TPST-2 and mPlum genes by PCR assembly and the assembled open reading frame was cloned into the pCDNA3.1(−) expression vector. HEK-CCR5 and HEK-WT were grown to 70–80% confluence in 10-cm dishes and transiently transfected with 7.5 μg of pCDNA3.1(−)TPST-2-T2A-mPlum using jetPRIME® transfection reagent, according to the manufacturer's protocol. Culture medium was changed 5 h post-transfection cell, and cells were detached for assay 96 h later.

2.2.4. Primary human T cell blasts

Peripheral blood mononuclear cells were isolated from buffy coat preparations obtained from anonymized human donors (HUG Blood Transfusion Center (BTC), Geneva, Switzerland) and cultured in RPMI 1640 medium supplemented with 20 mM HEPES, 20% FBS, 100 units/mL penicillin, 0.1 mg/mL streptomycin and 2 mM L-glutamine. T cell blasts were established by 3-day culture in medium supplemented with 1.5% phytohemagglutinin, M form (Gibco®) followed by a further 15 days' culture in medium supplemented with IL-2 (20 ng/mL; PeproTech). Competition binding experiments were performed between day 10 and day 15 of culture.

2.3. ELISA assay

Wells of Nunc™ MaxiSorp™ ELISA plates were coated overnight at 4 °C with streptavidin (5 μg/mL) in PBS, then washed three times with washing buffer (25 mM Tris, 150 mM NaCl, 0.1% BSA, 0.05% Tween-20, pH 7.2) and blocked with blocking buffer (3% BSA in PBS) for 1 h at RT. Wells were then incubated with biotinylated peptide (0.5 μg/mL) for 1 h at RT and washed three times with washing buffer prior to incubation with different concentrations of primary antibody, 1 h at RT. After washing three times with washing buffer, wells were revealed by incubation with a horseradish peroxidase-conjugated goat anti-rat (Sigma-Aldrich) or goat anti-mouse (Sigma-Aldrich) secondary antibody for 1 h at RT, washing three times with washing buffer, and addition of 50 μL of 3,3′,5,5′-Tetramethylbenzidine substrate. Signal development was stopped using 50 μL of 2 N H_2SO_4. Absorbance at 450 nm was measured on a POLARstar Omega (BMG Labtech).

2.4. Flow cytometry

Cells were washed with PBS, detached by trypsin and incubated with ligands (chemokines or mAbs) diluted in FACS buffer (PBS, 1% BSA, 0.05% NaN_3). Following incubation, ligand binding was measured by flow cytometry on a Cytoflex instrument (Beckman Coulter) with 10^4 events collected. Binding signals on CCR5-expressing cells is subtracted of the binding signal on the parental cell line (background signal).

2.4.1. Saturation binding experiments

Serial dilutions of rhodamine-labelled chemokine (100 μM stock solution) or labelled antibody (PE-labelled 3A9 or Alexa647-labelled HEK/

1/85a) in FACS buffer were performed in order to obtain the target concentrations. 10^5 cells were incubated with rhodamine-labelled chemokine or labelled antibody at different concentrations for 2 h at 4 °C.

2.4.2. Competition binding assays

Labelled reagents (tracers) were used at concentrations estimated to provide binding levels lower than 50% of B_{max}, based on saturation binding experiments (Fig. 7A–B and Supplementary Fig. 1). Rhodamine-labelled 5P12-RANTES was used at 10 nM (CHO cells) and 5 nM (HEK cells) and phycoerythrin-conjugated mAb 3A9 and Alexa647-conjugated mAb HEK/1/85a were used at 1:40 of their initial concentration. Unlabeled chemokines and antibodies were serially diluted in FACS buffer to obtain target concentrations. 10^5 cells were incubated with rhodamine-labelled 5P12-RANTES or labelled-antibody in presence of the unlabelled chemokine or antibody competitor respectively at a specific concentration, for 2 h at 4 °C. Binding signals were determined as the percentage control binding signal (100 x [binding signal at data point - background signal] / [binding signal in absence of unlabelled mAb - background signal), where 100% corresponds to the binding of tracer alone. Competition binding experiments carried out at ambient temperature and for longer time periods (4 h, 8 h) yielded qualitatively similar results (Supplementary Fig. 2).

For primary human T cells, cells were resuspended in ice-cold RPMI 1640 supplemented with 20 mM HEPES, 2 mM L-glutamine containing 2% BSA and 25 μg/mL human immunoglobulin G. Cells were then centrifuged, resuspended in PBS, 1% BSA and incubated with rhodamine-labelled 5P12-RANTES or labelled-antibody in the presence of the unlabelled chemokine or antibody competitor respectively for 2 h at 4 °C.

2.4.3. Binding assay on CCR5 FFFF

Parental CHO cells or clones stably expressing either native CCR5 or CCR5 FFFF were incubated with either fluorochrome-labelled mAbs at saturating concentrations or rhodamine-labelled chemokines at 300 nM prior to analysis.

2.4.4. Sodium chlorate treatment

Parental CHO cells and CHO-CCR5 cells growing in 75 cm^2 flasks were incubated in sulfate-free media (DMEM/Nutrient Mixture F-12 Ham from Sigma-Aldrich) supplemented with 10% FBS and Penicillin/Streptavidin (ThermoFisher Scientific), with or without sodium chlorate (100 mM, Sigma-Aldrich). 16 h later, cells were washed with PBS and detached with 5 mM EDTA in PBS prior to analysis.

2.4.5. Cells overexpressing TPST-2

HEK-CCR5 and HEK-WT cells either transiently transfected with the TPST-2-T2A-mPlum expression vector or mock-transfected (jetPRIME® reagent only), were detached 96 h post-transfection with trypsin prior to analysis. Binding signals for TPST-2-T2A-mPlum expressing cells were obtained by gating on mPlum-positive cells (see Supplementary Fig. 3) and collecting ligand binding data for 10^4 events.

2.5. Western blot

2.5.1. CCR5 expression

CHO-CCR5 WT, CHO-CCR5 FFFF or Parental CHO cells at 80–90% confluency were scraped and the resulting suspensions were centrifuged 15 min at 900 rpm in a benchtop centrifuge. The pellets were resuspended in lysis buffer (1% NP40, 10 mM Tris HCl pH 7.5, 2 mM EDTA, 150 mM NaCl, supplemented with phosphatase inhibitor (cOmplete™ ULTRA Tablets from Roche) and protease inhibitor (PhosSTOP™ from Roche)). Samples were incubated on ice for 40 min, then centrifuged 10 min (4 °C) at 14′000 rpm in a benchtop centrifuge. The supernatants were harvested, submitted to SDS-PAGE 4–12% and transferred onto PVDF membranes (BioRad, USA). Membranes were incubated overnight at 4 °C with primary antibody rabbit anti-CCR5 ab63123 (Abcam) (1:1000) in PBS, 3% BSA and 0.1% Tween (PBS-BSA-T). Membranes were then incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:80,000) (DakoCytomation, Glostrup, Denmark) for 1 h at room temperature and detected with Pierce SuperSignal West Pico Chemiluminescent Substrate (ThermoFisher Scientific). After extensive washing and blocking 1 h at 37 °C in PBS-BSA-T, membranes were re-blotted using primary antibody mouse anti-transferrin (1:500) (Invitrogen) and secondary antibody goat anti-mouse HRP (1:3000) (DakoCytomation, Glostrup, Denmark).

2.5.2. TPST2 expression

CHO-CCR5 and HEK-CCR5 cells were cultivated to semi-confluency (approximately 8–10 million cells) in 10 cm Petri dishes. Dishes were placed on ice, washed 2 times in cold PBS (1 ×) and incubated on ice with 1 mL per plate of lysis buffer (Tris 50 mM, 1% NP40 supplemented with Halt™ Protease inhibitor 1× (Thermo Scientific)). Lysis supernatant was centrifuged for 10 min at 14000 rpm in a bench top centrifuge and lysates were dosed for protein content using Pierce™ BCA Protein Assay Kit (Thermo Scientific). Samples were mixed with NuPAGE LDS Sample Buffer (Invitrogen) and DTT (final concentration of 0.1 M)), heated to 100 °C for 3 min and loaded on a 15-lane Nu-PAGE™ 4–12% Bis-Tris gel (Invitrogen) prior to electrophoresis in MES SDS running buffer (Novex®) for 35 min at 200 V. Samples were then transferred on to nitrocellulose membranes using iBlot® 2NC Regular stacks (Invitrogen). Membranes were blocked at room temperature (1 h) in PBS (1 ×) 4% nonfat dried milk, then were incubated at room temperature (1 h) with primary antibodies (Rabbit anti-TPST2 monoclonal antibody (ab157191 Abcam) diluted 1:500 and mouse anti-beta cytoplasmic actin [38] diluted 1:200) in PBS (1 ×) - 0.1% Tween- 4% nonfat dried milk (PBS-T 4% nonfat dried milk). Membranes were washed once in PBS-T 4% nonfat dried milk (10 min) and twice in PBS-T (15 min), then incubated for at room temperature (1 h) with the secondary antibodies (goat anti-rabbit HRP (P0448 Dako) 1:2000 and goat anti-mouse HRP (170–6516 Bio-Rad) 1:10000) in PBS-T 4% nonfat dried milk. After one washing step in PBS-T 4% nonfat dried milk (10 min) and two washing steps in PBS-T (15 min), membranes were developed using WesternBright™ Quantum western blotting detection kit (1:1).

2.6. Real time quantitative PCR

Total RNA from CHO-CCR5 and HEK-CCR5 cells was extracted using a Monarch® Total RNA Miniprep Kit (New England Biolabs) and RNA quality was verified using an RNA 6000 Nano Kit (Agilent). cDNA was synthesized from 0.5 μg of total RNA using a mix of random hexamers and oligo d(T) primers and PrimerScript reverse transcriptase enzyme (Takara) according to the manufacturer's instructions. SYBR green assays were designed using Primer Express v2.0 software (Applied Biosystems) with default parameters. Custom primers were obtained from Thermo Fisher and PCR was performed on an SDS 7900 HT instrument (Applied Biosystems). Each reaction was performed in three replicates on 384-well plate. Ct values were obtained using SDS 2.2 software (Applied Biosystems).

2.7. Data analysis and statistics

For statistical analysis GraphPad Prism software was used. For saturation and competition binding assays, curves were fitted using non-linear regression analysis (one site, total and non-specific binding). For binding assays at a single concentration, repeated measure one-way ANOVA with Dunnett's multiple comparisons tests were used for experiments involving three mAbs, and two-tailed unpaired *t*-tests for experiments comparing CCL5 and 5P12-RANTES.

3. Results

3.1. Anti-CCR5 mAbs recognize different CCR5 N-terminal sulfopeptides

We first assessed the capacity of a panel of anti-CCR5 mAbs to distinguish between fully sulfated and unsulfated human CCR5 N-terminal peptides (residues 1–19) (Fig. 1). As expected, mAb 2D7,

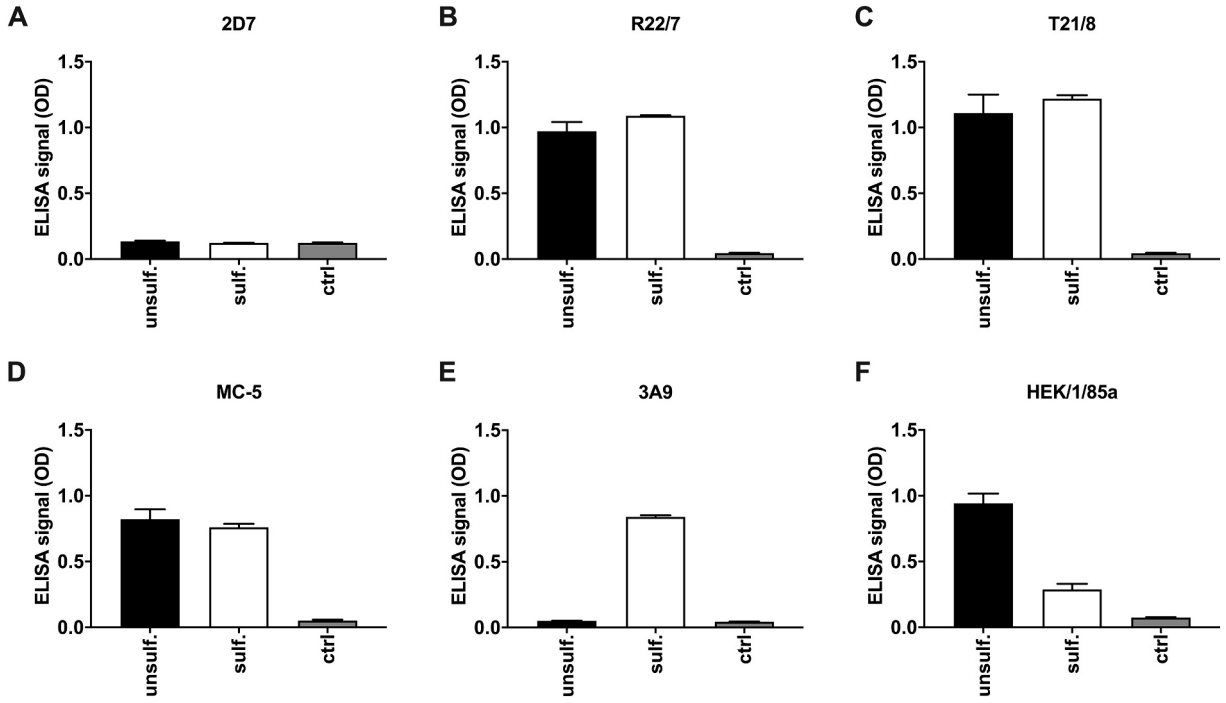

Fig. 1. Anti-CCR5 mAbs show distinct binding preferences for sulfated or unsulfated CCR5 N-terminal.

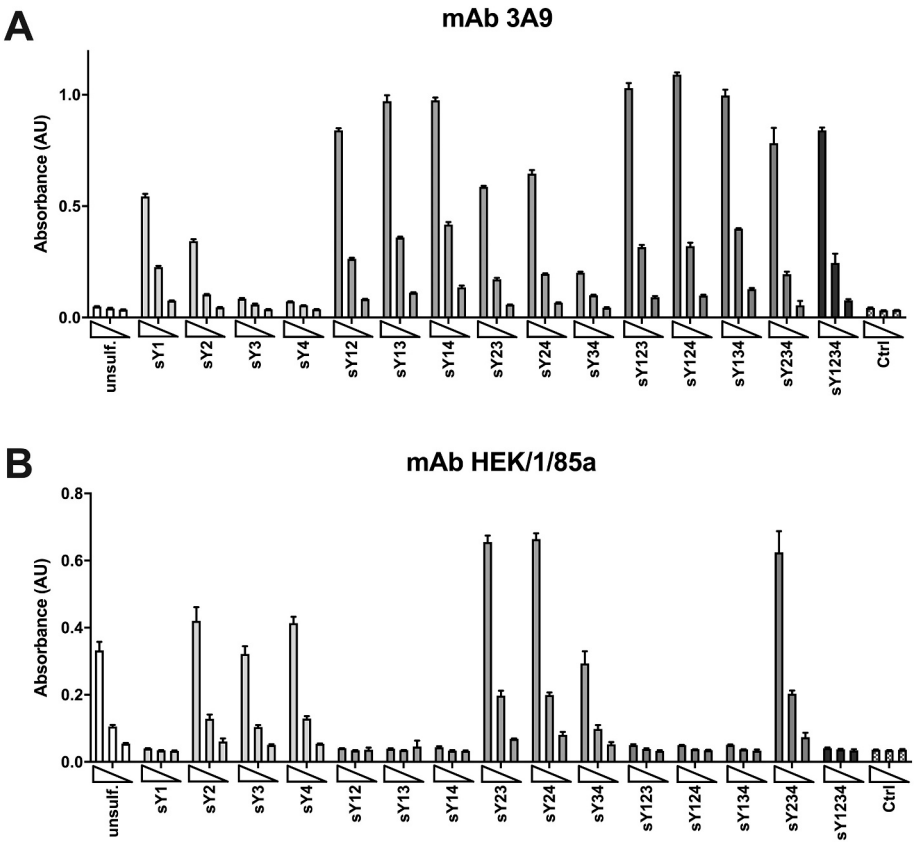

Fig. 2. Binding patterns of mAbs 3A9 and HEK/1/85 on a panel of CCR5 N-terminal sulfopeptides. Chemically synthesized C-terminally biotinylated peptides corresponding to CCR5 residues 1–19 with the indicated sulfation patterns (unsulf.: unsulfated peptide, sY#: sulfated tyrosine at position #, where position 1 corresponds to Tyr^{3}, position 2 to Tyr^{10}, position 3 to Tyr^{14} and position 4 to Tyr^{15}), as well as an unrelated control peptide (Ctrl), were immobilized on streptavidin-coated multiwell plates, and tested by ELISA using the indicated anti-CCR5 mAbs at three different dilutions (2 μg/mL, 0.4 μg/mL, 0.08 μg/mL) Data represent mean absorbance signal of triplicates ± SD and are representative of three independent experiments.

which recognizes a conformation-dependent epitope mainly located in the second extracellular loop of CCR5 [38], was unable to bind to either of the CCR5 N-terminal peptides (Fig. 1A). Several mAbs known to have epitopes in the CCR5 N-terminal region, including mAbs R22/7 [39], T21/8 [40] and MC-5 [25], gave binding signals that were equally high for both peptides (Fig. 1B–D). Interestingly, however, we identified two mAbs that are sensitive to the sulfation state of the peptide, with mAb 3A9 only binding to the sulfated peptide (Fig. 1E), and mAb HEK/1/85a showing a clear preference for the unsulfated peptide (Fig. 1F).

3.1.1. peptides

Chemically synthesized C-terminally biotinylated peptides corresponding to residues 1–19 of CCR5 with (sulf.) or without (unsulf.) sulfation at Tyr^{3}, Tyr^{10}, Tyr^{14} and Tyr^{15}, as well as an unrelated control

peptide (ctrl), were immobilized on streptavidin-coated multiwell plates, and tested by ELISA using the indicated anti-CCR5 mAbs (2 μg/mL). Data represent mean absorbance signal of triplicates and are representative of two independent experiments. Error bars represent SD.

3.2. Binding studies using a set of CCR5 sulfopeptides provide evidence that partially sulfated CCR5 variants exist at the cell surface

To further characterize differences in CCR5 N-terminal sulfation specificity for mAbs 3A9 and HEK/1/85a, we next compared the capacity of these two mAbs to bind to a set of CCR5 sulfopeptides covering all sulfation permutations at each of the four tyrosine residues (in the figure: sY# indicates sulfated tyrosine at position #, where position 1 corresponds to Tyr^3, position 2 to Tyr^{10}, position 3 to Tyr^{14} and position 4 to Tyr^{15}) (Fig. 2, Supplementary Table 1).

This experiment revealed that while certain CCR5 N-terminal sulfation permutations are exclusive for one mAb or the other (e.g. fully sulfated for mAb 3A9, fully unsulfated for mAb HEK/1/85a), others are recognized by both. Analysis of the CCR5 sulfopeptide binding profile of mAb 3A9 shows that it absolutely requires sulfation at Tyr^3 or Tyr^{10} and is increased when Tyr^{14} and Tyr^{15} are also sulfated. In contrast, binding of mAb HEK/1/85a to the CCR5 N-terminal peptide does not require any tyrosine sulfation and indeed does not tolerate sulfation at Tyr^3, but is augmented by sulfation at any of the other positions.

3.3. Removal of tyrosine sulfation sites differentially affects binding of anti-CCR5 mAbs

We next investigated binding of mAb 3A9 and mAb HEK/1/85a on intact cell surface CCR5 carrying previously described mutations that abrogate tyrosine sulfation [33]. In the CCR5-FFFF variant, Tyr^3, Tyr^{10}, Tyr^{14} and Tyr^{15} are substituted with phenylalanine so that the aromatic side-chains present in tyrosines are retained but no sulfation is possible. We used flow cytometry to compare binding levels of mAbs 3A9 and HEK/1/85a on CHO cells expressing native CCR5 with those on CHO cells expressing CCR5-FFFF. To control for differences in CCR5 expression levels between the two cell lines we used mAb 2D7, whose epitope is outside of the N-terminal domain of CCR5 and hence would not be expected to be sensitive to changes in this region, as a reference antibody (Fig. 3).

While all three mAbs show relatively lower levels of binding on CCR5-FFFF (Fig. 3A), presumably due to the comparatively lower expression level of the CCR5-FFFF in the cell line used (Supplementary Fig. 4), the reduction in mAb 3A9 binding is proportionally greater (Fig. 3A, right panel). Analysis of relative mAb binding (CCR5-FFFF: native CCR5) normalized to that of mAb 2D7 across a pooled series of experimental replicates (Fig. 3B) revealed no change in normalized relative binding of HEK/1/85a. In contrast, normalized relative binding of mAb 3A9 showed a clear decrease (2.8-fold). These results are consistent with CCR5 sulfation playing a key role in the binding of mAb 3A9, not only to synthetic sulfopeptides but also in the context of intact cell surface CCR5.

3.4. mAbs 3A9 and HEK/1/85a show incomplete competition for CCR5 binding at the cell surface

We next tested the two mAbs in competition binding experiments on cell surface CCR5. Having established sub-maximal binding concentrations for each fluorescently labelled mAb by flow cytometry (Supplementary Fig. 1), we performed flow cytometry-based competition binding assays on a CHO-CCR5 cell line using both mAb 3A9 and mAb HEK/1/85a as tracers (Fig. 4). As expected, in homologous competition assays the unlabeled version of each mAb was able to fully displace the labelled version of itself. In heterologous binding assays, while unlabeled mAb HEK/1/85a was able to achieve almost full displacement of labelled mAb 3A9 (8% of control binding signal remaining at highest concentration of competitor, Fig. 4A), competition between unlabeled mAb 3A9 and labelled mAb HEK/1/85a was clearly incomplete, approaching a plateau corresponding to approximately 45% of the control binding signal at the highest concentration tested (Fig. 4B).

Since mAbs 3A9 and HEK/1/85a show clear differences in their capacities to recognize sulfation variants in the context of both CCR5 N-terminal peptides (Figs. 1 and 2) and cell surface CCR5 (Fig. 3), the incomplete competition between the two antibodies may be a reflection of cell surface CCR5 sulfation heterogeneity. Indeed, the capacity of the two mAbs to compete with each other changed when competition binding experiments were carried out on the non-sulfated CCR5 FFFF variant (Fig. 5). We observed that binding levels of labelled mAb 3A9 were reduced to close to background levels even in the absence of competitor (Fig. 5C). Binding levels of labelled mAb Hek/1/85a remained detectable, but the capacity of unlabeled mAb 3A9 to compete for binding was completely abrogated (Fig. 5D).

3.5. Modulation of total cellular tyrosine sulfation differentially affects binding of anti-CCR5 mAbs

We next compared the capacities of the two mAbs to bind to WT CCR5 under conditions expected to raise or lower cellular sulfation levels. In order to investigate lower sulfation levels, binding studies were performed on CHO-CCR5 cells that had been incubated with sodium chlorate (100 mM) for 16 h, a treatment previously shown to inhibit protein sulfation via blockade of ATP-sulfurylase [33,41], the first enzyme in the biosynthesis of the sulfate donor PAPS (3′-phosphoadenosine-5′-phosphosulfate) [42] (Fig. 6). Not unexpectedly, Sodium chlorate treatment led to a degree of cellular toxicity, but more than 75% of cells nonetheless remained viable after 16 h treatment (Supplementary Fig. 5). Sodium chlorate treatment reduced the binding level of the reference mAb 2D7 (Fig. 6A, central panel), indicating that it reduces levels of CCR5 at cell surface, a possible consequence of lowered protein biosynthesis and trafficking stemming from inhibition of cellular sulfotransferase activity [43]. Binding of mAb HEK/1/85a was also reduced, but to a lesser extent than that of mAb 2D7 (Fig. 6A, left panel), and binding of mAb 3A9 clearly showed the most pronounced reduction (Fig. 5A, right panel). Analysis of pooled experiments with binding signals normalized to those of mAb 2D7 (Fig. 6B) revealed a modest increase in normalized relative binding of mAb HEK/1/85a under sodium chlorate treatment (1.2-fold) in contrast to a strong reduction in normalized relative binding of mAb 3A9 (4.9-fold).

To raise sulfation levels, we generated HEK-CCR5 cells overexpressing the TPST-2 enzyme by transfecting the parent cell line with a bicistronic expression vector in which the TPST-2 gene is fused to the mPlum gene via a self-cleaving T2A peptide [44], so that TPST-2 and mPlum are expressed as separate proteins and levels of mPlum fluorescence can be used in flow cytometry experiments to distinguish cells overexpressing TPST-2 from those expressing endogenous levels (Supplementary Fig. 3). Four days after transfection, binding of anti-CCR5 mAbs on HEK-CCR5 cells was analyzed by flow cytometry, comparing mAb binding levels of mPlum-positive versus those of mPlum-negative cell populations (Fig. 7). While TPST-2 overexpression did not affect binding of mAb 2D7 (Fig. 7A, central panel), it had opposing effects on the binding of the other two mAbs, with a small reduction in the mAb HEK/1/85a binding signal (Fig. 7A, left panel) and a more substantial increase in the mAb 3A9 binding signal (Fig. 7A, right panel). Analysis of pooled experiments normalizing mAb binding to that of mAb 2D7 (Fig. 7B) revealed a modest decrease (1.2-fold) in normalized relative binding of mAb HEK/1/85a, in contrast to a more substantial increase (1.6-fold) in relative binding of mAb 3A9.

3.6. Native CCL5 has a much lower total binding level for cell surface CCR5 than the chemokine analog 5P12-RANTES

Since CCR5 sulfation has been shown to play an important role in the binding of native chemokines [33,34,45], and in the light of our anti-CCR5 mAb-based evidence of CCR5 sulfation heterogeneity, we next set out to investigate whether the previous observations that cell

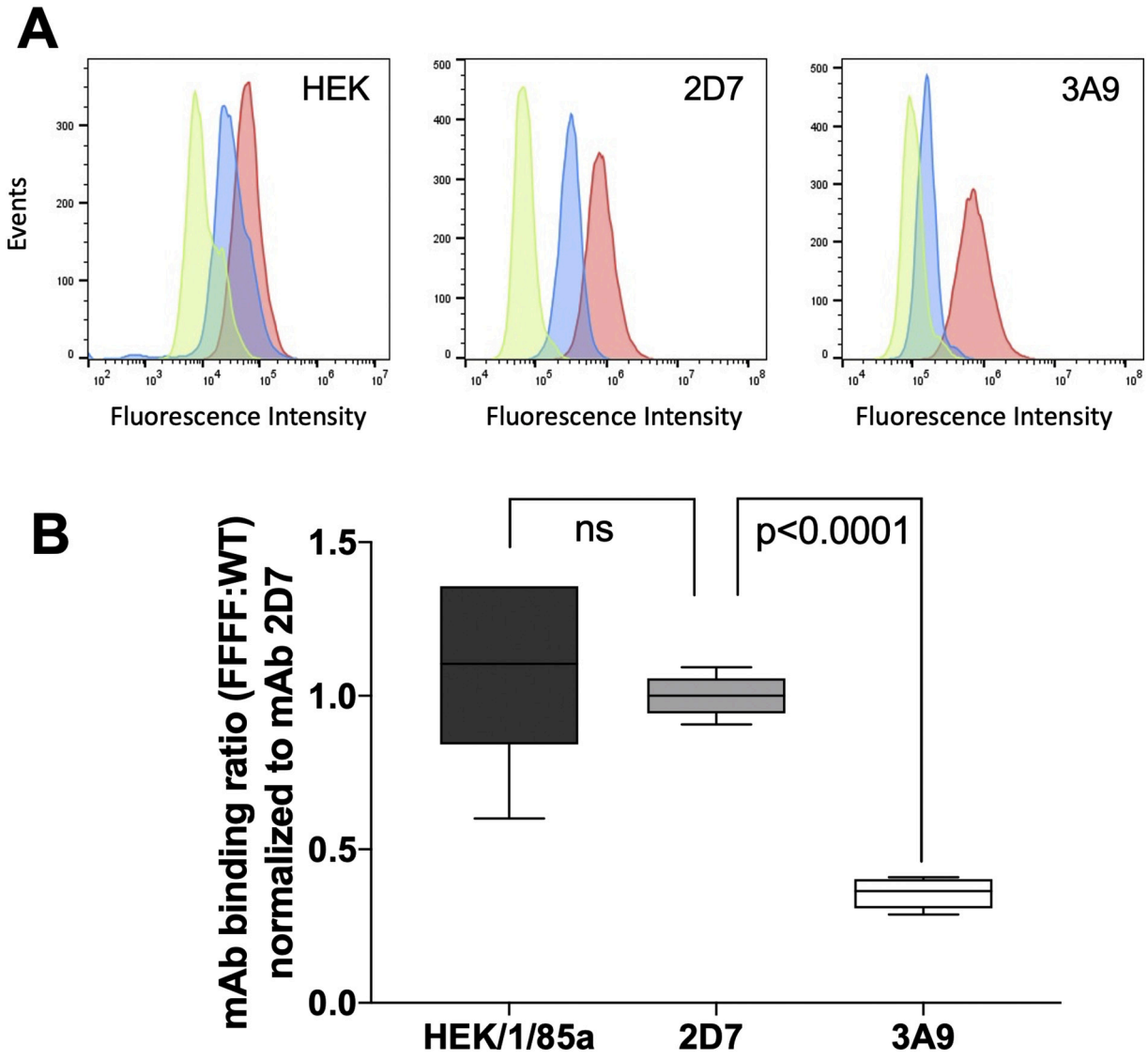

Fig. 3. Mutation of CCR5 tyrosine residues Y3, Y10, Y14 and Y15 into phenylalanine disproportionally affects binding of mAb 3A9. A. Parental CHO cells (CHO WT, green) and cell lines expressing native CCR5 (red) or CCR5-FFFF cells (blue) analyzed by flow cytometry using saturating concentrations of the indicated fluorochrome-labelled anti-CCR5 mAbs (Supplementary Fig. 1). Representative histograms are shown. B. Normalized relative binding signals across a series of the independent experiments. Data are represented as box-and-whiskers plots (median and quartiles, with bars representing 95% confidence intervals). *P*-values were calculated using a one-way ANOVA test.

surface CCR5 consists of subpopulations with high and low binding affinities for native chemokines [26,27], and that certain potent anti-HIV chemokine analogs can engage these subpopulations with equally high affinity [26], relate to the existence of subpopulations of cell surface CCR5 with different sulfation patterns.

First, we synthesized derivatives of CCL5 and 5P12-RANTES site-specifically labelled with rhodamine at C-terminus, a region known not to participate in receptor interaction [46], and used them to perform flow cytometry-based saturation and competition binding experiments on CCR5-expressing cell lines (Fig. 8).

Fitted B_{max} levels determined in the saturation binding experiments (Fig. 8A–B) indicate that 5P12-RANTES is able to access a substantially larger number of receptor binding sites than CCL5, with fitted B_{max} values of 5.2×10^4 RFU (95% confidence interval 4.9×10^4–5.7×10^4) in CHO-CCR5 and 9.7×10^4 RFU in HEK-CCR5 (9.0×10^4–1.0×10^5) for 5P12-RANTES versus 2.0×10^4 RFU (1.2×10^4–7.4×10^4) in CHO-CCR5 and 2.0×10^4 RFU in HEK-CCR5 (1.4×10^4–3.1×10^4) for CCL5 across the concentration range used. In competition binding assays using rhodamine-labelled 5P12-RANTES as tracer (Fig. 8C–D), unlabeled 5P12-RANTES fully displaced itself in both cell lines with fitted IC_{50} values of 4.9 nM (95% confidence interval 4.4–5.4) on CHO-CCR5 cells and 3.9 nM (3.0–5.0) on HEK-CCR5 cells. In contrast, CCL5 only displaced a small fraction of tracer, with 75% of signal remaining on CHO-CCR5 cells and 90% of signal remaining on HEK-CCR5 cells at the highest concentration used. The differences in displacement of rhodamine-labelled 5P12-RANTES by CCL5 between CHO-CCR5 and HEK-CCR5 cells are consistent with the differences in relative binding levels of the two chemokines in the saturation binding experiments performed on the two cell lines (Fig. 8A–B). These results provide further evidence for the existence of a sizeable CCR5 cell surface subpopulation which, at nanomolar concentrations, is recognized by 5P12-RANTES but not by CCL5.

3.7. Modulation of CCR5 sulfation disproportionally affects binding of CCL5 compared to 5P12-RANTES

We next compared the binding capacity of rhodamine-labelled chemokines (300 nM) on CHO cells expressing native CCR5 or the CCR5-FFFF mutant. Binding of both 5P12-RANTES and CCL5 is reduced

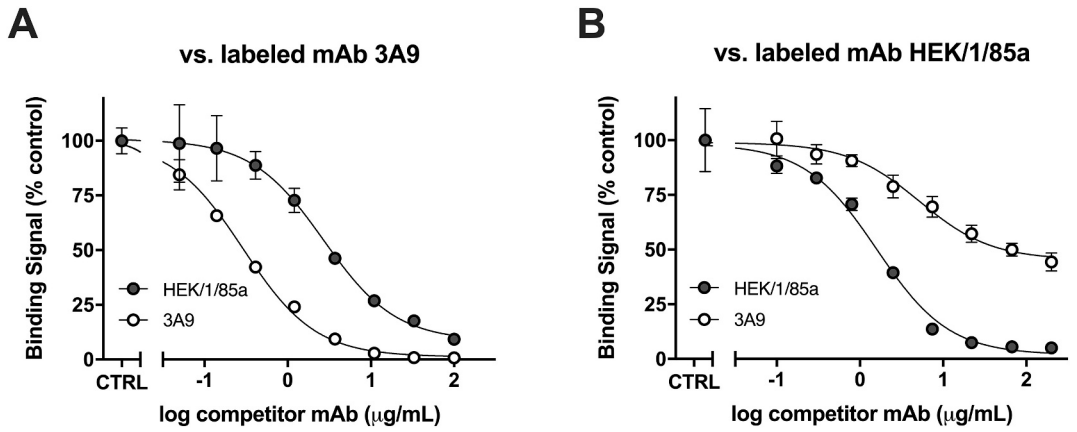

Fig. 4. Incomplete competition between mAbs 3A9 and HEK/1/85a for cell surface CCR5. CHO-CCR5 cells were incubated with unlabeled mAbs at the indicated concentrations in the presence of fixed concentrations of fluorescently labelled mAb 3A9 (A) or mAb HEK/1/85a (B) prior to analysis of fluorescent mAb binding by flow cytometry. Signals are expressed in percentage, having sample with labelled-mAbs alone as 100%. Data points represent mean signals ± SD (n = 2) and are representative of three independent experiments.

on the mutant CCR5 compared to the wild-type counterpart (Fig. 9), a phenomenon likely to be at least partly due to differences in CCR5 expression levels (Fig. 3A and Supplementary Fig. 4). Notably however, mean relative binding (CCL5: 5P12-RANTES) decreases 1.8-fold on cells expressing the CCR5-FFFF mutant across a series of pooled experimental replicates (Fig. 9B). This observation is consistent with previous studies showing that the CCR5-FFFF mutant affects binding of native chemokines [33,34], but indicates that the binding of 5P12-RANTES is less sensitive to the absence of sulfation on CCR5.

We next investigated the effect of treating CHO-CCR5 cells with sodium chlorate (100 mM, 16 h), in order to reduce overall sulfation levels, on binding levels of rhodamine-labelled chemokines (300 nM) by flow cytometry. While treatment modestly reduced binding of 5P12-RANTES (Fig. 10A, left), we noted a much more substantial decrease in the binding of CCL5 (Fig. 10A, right). Results from pooled experiments showed a 2.3-fold decrease in the binding level of CCL5 relative to that

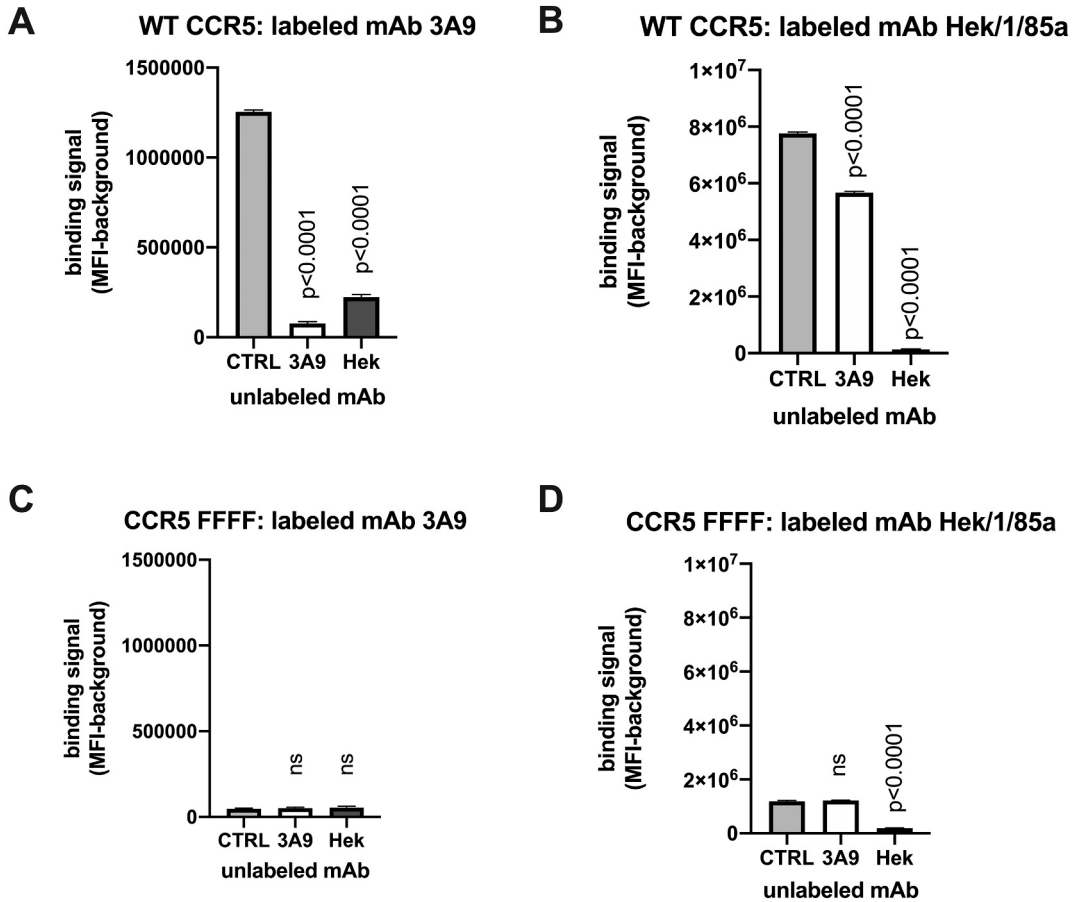

Fig. 5. Elimination of CCR5 sulfation abrogates the capacity of mAb 3A9 to compete with mAb HEK/1/85a for cell surface CCR5. CHO-CCR5 cells (A-B) or CHO-CCR5 FFFF cells (C-D) were incubated with unlabeled mAbs at (11 μg/mL) in the presence of fluorescently labelled mAb 3A9 (A, C) or mAb HEK/1/85a (B, D) prior to analysis of fluorescent mAb binding by flow cytometry. Signals are expressed as mean binding signal [median fluorescence intensity (MFI) minus background (no fluorescent antibody)] ± SD (n = 3) and are representative of two independent experiments. P-values were calculated using a one-way ANOVA test.

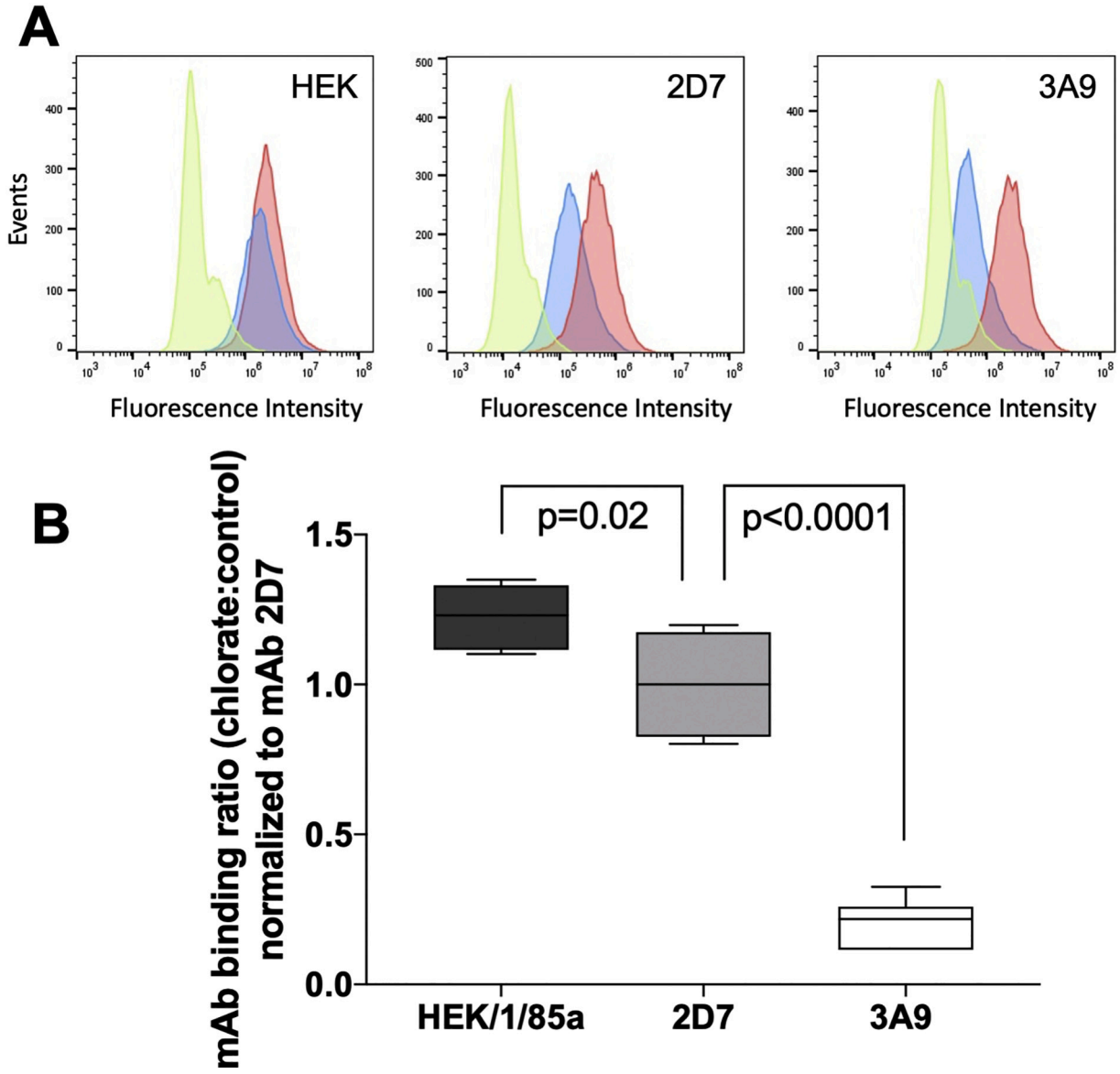

Fig. 6. Sodium chlorate treatment disproportionally affects binding of 3A9. A. Parental CHO cells (CHO WT, green) and CHO-CCR5 cells were incubated for 16 h at 37 °C with either sulfate-free medium (red) or sulfate-free medium supplemented with sodium chlorate (100 mM) (blue), prior to analysis by flow cytometry using the indicated fluorescent mAbs. Representative histograms are shown. B. Normalized relative binding signals across a series of three independent experiments. Data are represented as box-and-whiskers plots (median and quartiles, with bars representing 95% confidence intervals). *P*-values were calculated using a one-way ANOVA test.

of 5P12-RANTES under these conditions (Fig. 10B). This observation provides further evidence that 5P12-RANTES is less sensitive to reduced receptor sulfation than native CCL5 for binding to CCR5.

Finally, we observed the effect of TPST-2 overexpression on the binding of rhodamine-labelled chemokines (300 nM) to cell surface CCR5 in flow cytometry experiments. While the level of 5P12-RANTES binding was modestly reduced on cells overexpressing TPST-2 (Fig. 11A, left panel), CCL5 binding was substantially increased (Fig. 11A, right panel). Analysis of pooled experiments revealed that TPST-2 overexpression results in an 9.7-fold increase in the binding level of CCL5 relative to that of 5P12-RANTES (Fig. 11B). These observations provide further evidence that binding of CCL5 is more sensitive to changes in CCR5 sulfation than that of 5P12-RANTES.

3.8. Using 5P12-RANTES as a tracer in competition binding assays reveals differences in ligand properties that are not observable using native chemokines as tracers

It was previously noted that certain CCL5 analogs with lower anti-HIV potency than 5P12-RANTES exhibited indistinguishable binding properties in competition assays using radiolabeled native CCL4 as tracer [19]. To address this apparent contradiction, we investigated whether one such lower-potency analog, 2P3-RANTES [19], would show distinguishable binding properties in competition assays using labelled 5P12-RANTES rather than a native chemokine as tracer. Using rhodamine labelled 5P12-RANTES, we performed flow cytometry-based competition binding assays with unlabelled 5P12-RANTES and 2P3-RANTES, as well as with the native CCR5 chemokines CCL3 and CCL4 (Fig. 12). We determined an IC_{50} value of 8.2 nM (95% confidence interval 6.9–9.8) for 5P12-RANTES in homologous competition. As seen previously with CCL5 (Fig. 8), the native chemokines CCL3 and CCL4 were capable of displacing only a small fraction of tracer, with 63% and 62% of control signal remaining, respectively, at the highest concentrations used. These results are consistent with previous evidence indicating that not only CCL5 but also the native chemokines CCL3 and CCL4 bind only a fraction of cell surface CCR5 [26], and that like CCL5, CCL3 and CCL4 require CCR5 sulfation for high affinity binding [33]. 2P3-RANTES

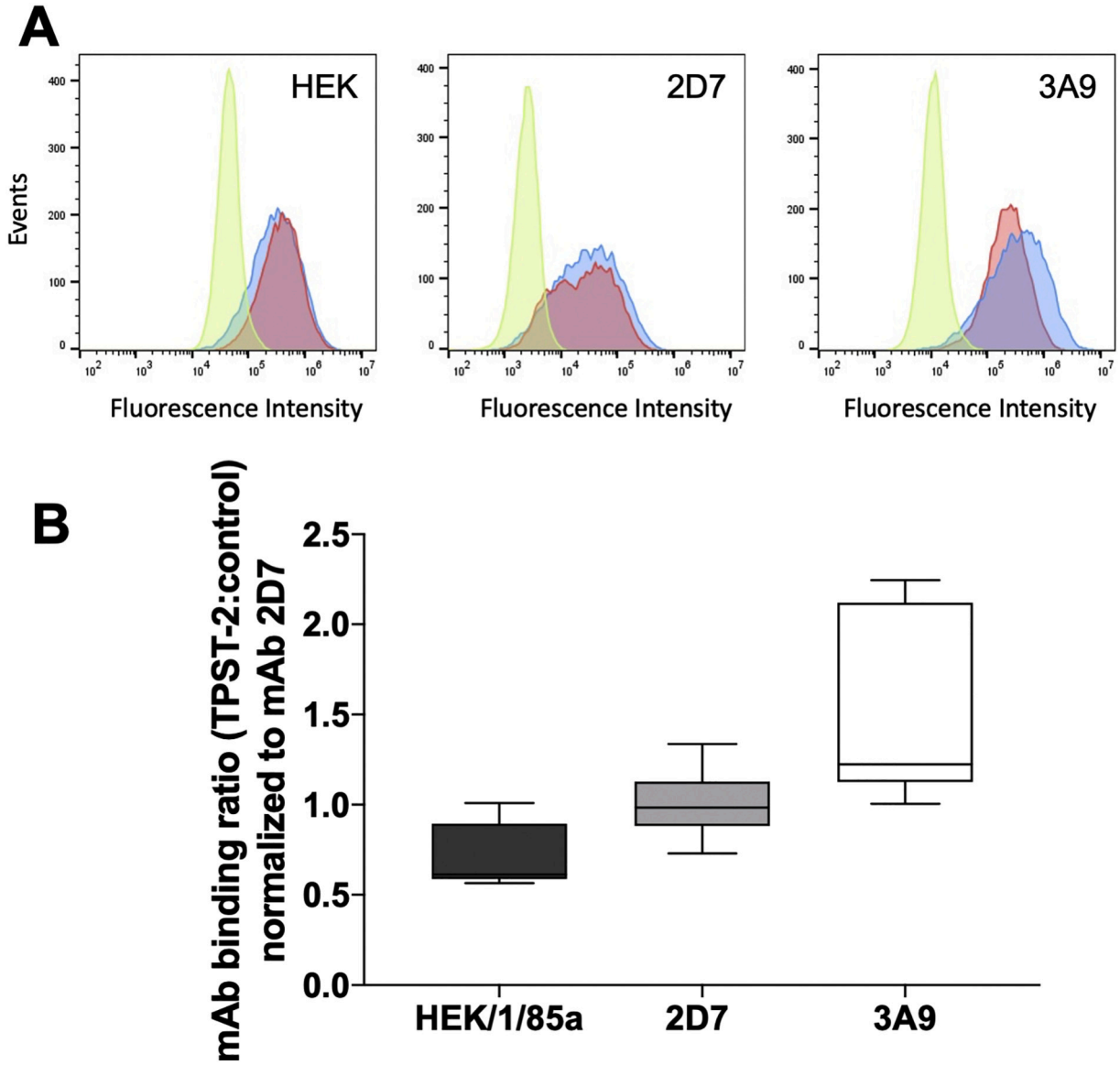

Fig. 7. TPST2 overexpression disproportionally affects antibody binding. A. Parental HEK cells (green) or HEK-CCR5 cells transfected with a TPST2-T2A-mPlum expression vector and defined as TPST2-negative (red) or TPST2-positive (blue) 4 days-post transfection (see Supplementary Fig. 3) were analyzed by flow cytometry with the indicated fluorescent mAbs. Representative histograms are shown. B. Normalized relative binding signals across a series of three independent experiments. Data are represented as box-and-whiskers plots (median and quartiles, with bars representing 95% confidence intervals). *P*-values were calculated using a one-way ANOVA test.

exhibited an improved binding profile with respect to the native chemokines, with only 20% of control tracer signal remaining at the highest concentration used, and a fitted IC_{50} value of 23 nM (95% confidence interval 18–30). By using a CCR5 sulfation-insensitive ligand such as 5P12-RANTES, rather than a CCR5 sulfation-sensitive native ligand it is possible to reconcile the anti-HIV potency of 2P3-RANTES, which is improved with respect to native chemokines but lower than that of 5P12-RANTES [19], with differences in CCR5 binding properties.

Finally, we performed flow cytometry-based competition binding assays on primary human T cells using rhodamine-labelled 5P12-RANTES (5 nM) with unlabelled native CCL5 and 5P12-RANTES (300 nM) (Fig. 13). Consistent with the results obtained on both HEK-CCR5 and CHO-CCR5 cell lines, we observed that while CCL5 is a poor competitor (tracer binding level reduced to 89% (SD = 3.6, n = 6) of control levels, 5P12-RANTES is much stronger, reducing binding levels to 50% (SD = 4.9, n = 6) under these conditions (Fig. 13).

4. Discussion

Previous studies using antibodies [22,23], chemokines [26,27] and HIV glycoproteins [29] have provided evidence that CCR5 exists in distinct cell surface subpopulations that exhibit different ligand binding properties. Here we provide further evidence in support of the existence of these subpopulations, but in contrast to earlier studies, which implicated either local membrane lipid composition [22], receptor oligomerization state [29,47] or receptor G-protein coupling state [26,30] in their formation, our results demonstrate that heterogeneity at the level of cell surface CCR5 sulfation plays an important role in defining the binding properties of both native chemokines and certain anti-CCR5 mAbs. Our results are consistent with earlier observations that CCR5 sulfation affects binding of native chemokines [33,34,45]. They also provide the first experimental results in support of the suggestion that chemokine receptor sulfation is heterogeneous [32,35].

We identified anti-CCR5 mAbs that show clear differences in binding levels depending on the sulfation state of both chemically

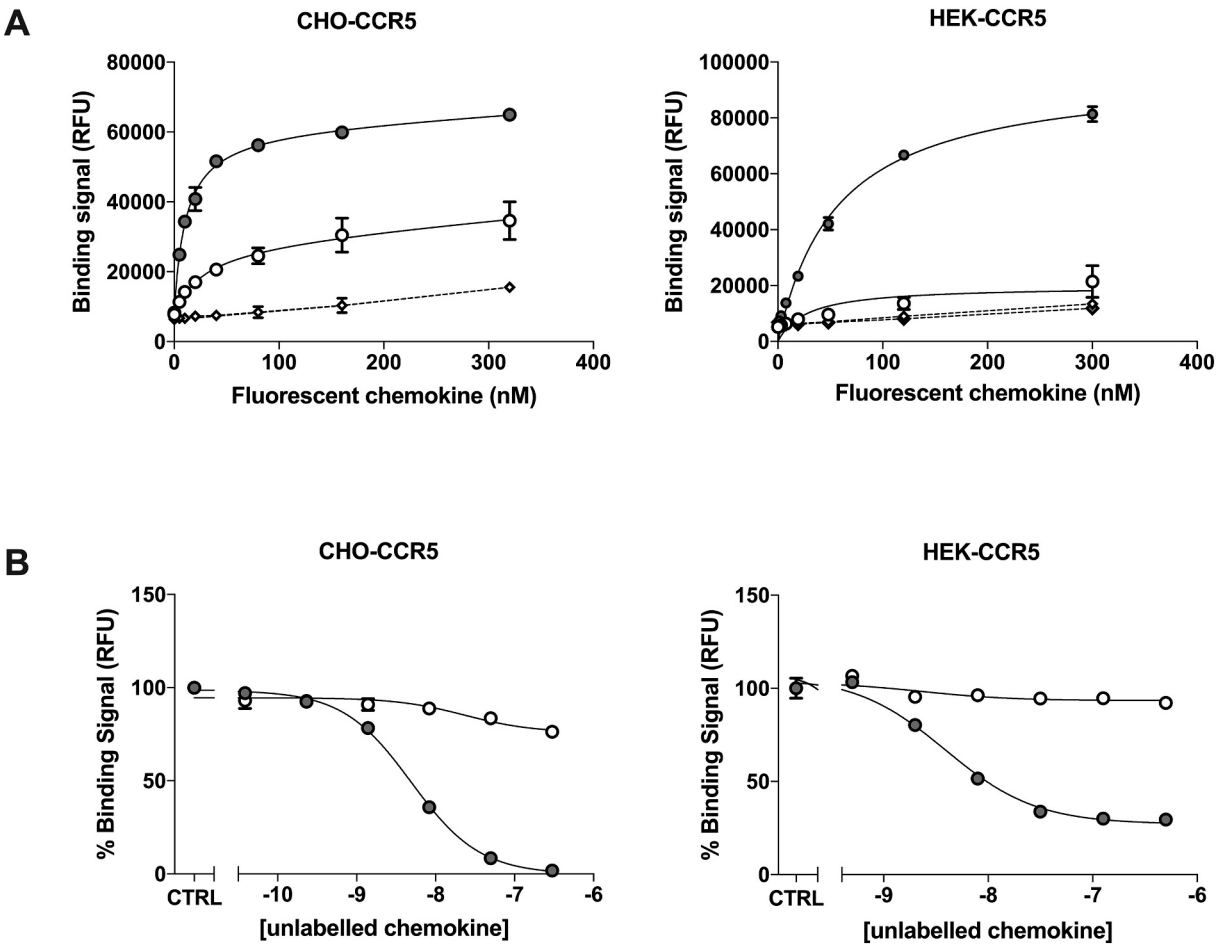

Fig. 8. The potent chemokine analog 5P12-RANTES binds to a larger population of cell surface CCR5 than the native chemokine CCL5. CHO-CCR5 (A) and HEK-CCR5 cells (B) were incubated with rhodamine-labelled 5P12-RANTES (filled circles) or rhodamine-labelled CCL5 (open circles) at the indicated concentrations prior to analysis of fluorescent chemokine binding levels by flow cytometry. Background binding levels for both rhodamine-labelled 5P12-RANTES (filled diamonds, dotted line) and rhodamine-labelled CCL5 (open diamonds, dotted line), obtained on the parental CHO and HEK cell lines, are also indicated. CHO-CCR5 (C) and HEK-CCR5 cells (D) were incubated with 5P12-RANTES (filled circles) or CCL5 (open circles) in the presence of rhodamine-labelled 5P12-RANTES (10 nM for CHO-CCR5, 5 nM for HEK-CCR5). Data represent mean tracer binding signal ± SD of duplicate measurements and are representative of three independent experiments.

synthesized CCR5 N-terminal peptides (Figs. 1, 2) and intact cell surface CCR5 (Figs. 3, 6, 7). These antibodies show incomplete cross-competition with each other for cell surface CCR5 (Fig. 4), despite recognizing adjacent / overlapping epitopes in the extracellular N-terminal domain of the receptor [39] (Fig. 2). The most probable explanation for this observation is that cell surface CCR5 comprises receptor subpopulations with different levels of sulfation, an idea that is supported by further experiments showing that the relative binding level of these antibodies can be modulated by cellular treatments expected to either decrease (Fig. 6) or increase (Fig. 7) CCR5 sulfation.

Detailed analysis of the binding patterns of the two anti-CCR5 mAbs used in this study (Fig. 2) allow us to put forward hypotheses concerning the relative abundance of different sulfoforms of CCR5 at the surface of the cells used in this study. The capacity of mAb HEK/1/85a to almost completely displace labelled mAb 3A9 (Fig. 3A) suggests that sulfovariants that are not permissive for mAb HEK/1/85a binding (i.e. those incorporating sulfation at Tyr^3) are present at low levels at the cell surface. Similarly, the inability of mAb 3A9 to achieve more than partial displacement of mAb HEK/1/85a (Fig. 3B) suggests that CCR5 low-sulfation variants that are not permissive for mAb 3A9 (i.e. those lacking sulfation at both Tyr^3 and Tyr^{10}) are relatively abundant at the cell surface. Our experiments in which sulfation levels were decreased (Fig. 6) or increased (Fig. 7) could equally be interpreted in terms of lowering or augmenting levels of CCR5 sulfation variants that are required for binding of mAb 3A9 (i.e. Tyr^3 and Tyr^{10}) and which are not permissive for binding of mAb HEK/1/85a (i.e. Tyr^3). Indeed the capacity of mAb 3A9 to compete with mAb HEK/1/85a for cell surface CCR5 is eliminated on the sulfation-abrogated CCR5 FFFF variant (Fig. 5).

More robust mass spectrometry-based methods for determining sites of receptor sulfation, analogous to those used to probe cytosolic phosphorylation patterns on GPCRs [48–50], will be required to test these hypotheses, however. Nonetheless, our results already sound a note of caution for experimenters working with anti-receptor mAbs, for which the general starting assumption is that they recognize the entire cellular pool of receptors. It is plausible that other sulfated GPCRs, including most if not all chemokine receptors [32], are also subject to sulfation heterogeneity, and that among other sulfated GPCRs, certain anti-receptor mAbs might only recognize a subset of cellular receptors.

In this study we confirmed the previous observation [26] that native chemokines only bind to a limited subset of cellular CCR5 compared to that accessible to the potent anti-HIV chemokine 5P12-RANTES (Fig. 8). Because we were able to use fluorescently labelled chemokines, our experiments provided direct measurements of chemokine and chemokine analog binding rather than the indirect measurements obtained using radiolabeled HIV envelope as a tracer in the previous study [26]. This is potentially important because binding of HIV envelope is itself sensitive to CCR5 sulfation [33]. Our results suggest that the differences in binding capacity between native chemokines and 5P12-RANTES are due at least in part to heterogeneity at the level of cell surface CCR5 sulfation, since the binding of 5P12-RANTES, unlike that of native CCL5 shows little

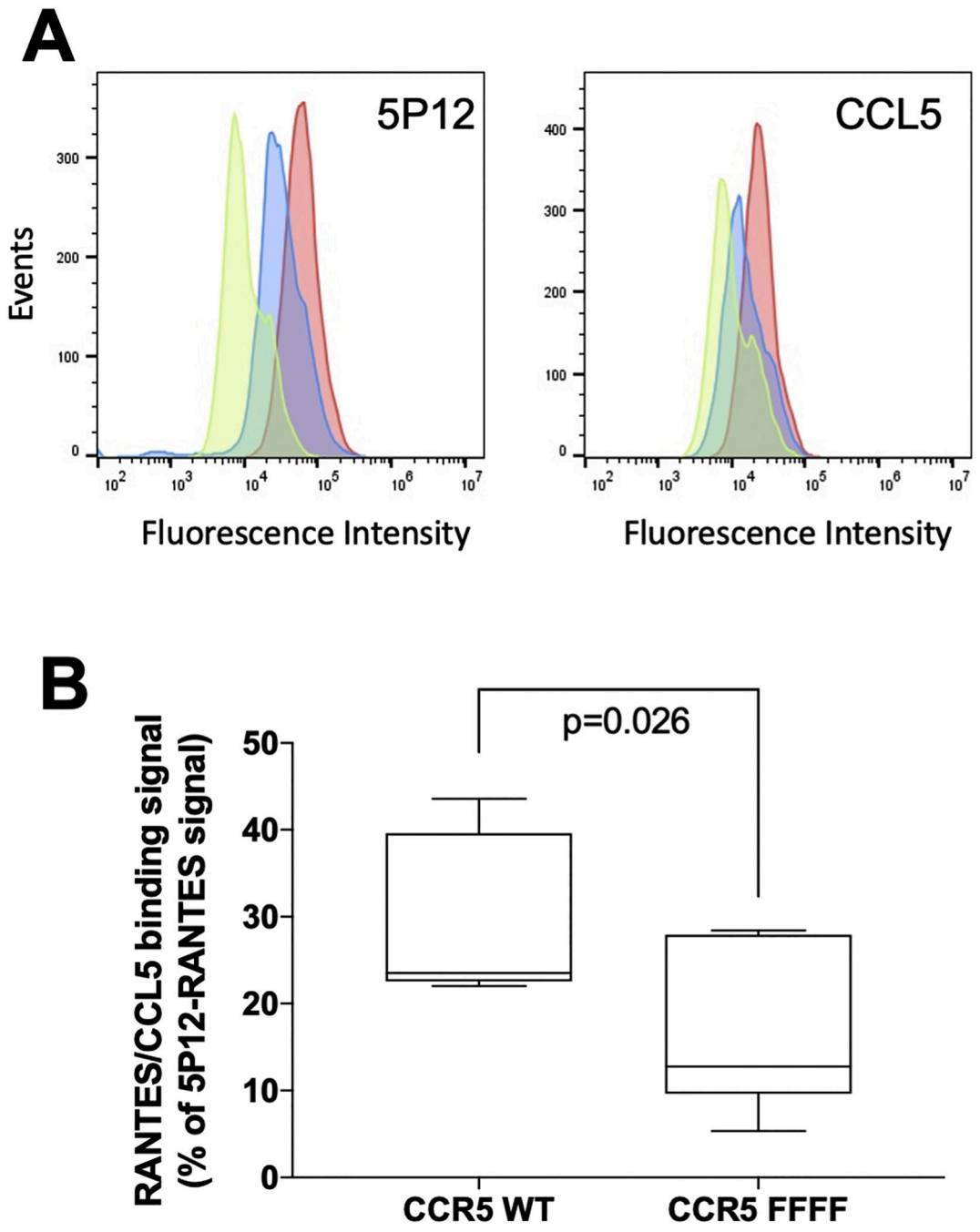

Fig. 9. Mutation of CCR5 tyrosine residues Y3, Y10, Y14 and Y15 into phenylalanine disproportionally affects binding of CCL5 compared to 5P12-RANTES. A. Parental CHO cells (green) as well as CHO cells expressing native CCR5 (red) or CCR5-FFFF (blue) were incubated with rhodamine-labelled 5P12-RANTES or rhodamine-labelled CCL5 (300 nM) and then analyzed for fluorescent chemokine binding levels by flow cytometry. Representative histograms are shown. B. Relative binding signals across a series of three independent experiments. Data are represented as box-and-whiskers plots (median and quartiles, with bars representing 95% confidence intervals). The *P*-value was calculated using an unpaired *t*-test.

sensitivity to CCR5 mutations that abrogate sulfation (Fig. 9) and to treatment that reduces (Fig. 10) or increases (Fig. 11) cellular tyrosine sulfation. A likely explanation for the capacity of 5P12-RANTES to overcome the dependence on CCR5 sulfation shown by native CCL5 is provided by a structural study of a chemokine analog closely related to 5P12-RANTES in complex with CCR5, in which it was proposed that while both native CCL5 and the 5P12-RANTES use common structures in the core region of the chemokine to engage the sulfated extracellular domain of the receptor, the modified N-terminal domain of 5P12-RANTES makes a substantially better molecular fit with the transmembrane domain [20]. The increased binding contribution provided by the enhanced CCR5 transmembrane domain interaction of 5P12-RANTES might thus enable the analog to overcome the diminished binding interaction with the unsulfated CCR5 extracellular domain.

While qualitatively similar results for chemokine binding were obtained on the CHO-CCR5 and HEK-CCR5 cell lines, we noted that CCL5 binding relative to that of 5P12-RANTES was lower on HEK-CCR5 cells than on CHO-CCR5, both in saturation binding and competition binding (Fig. 8). We interpret this to be a consequence of HEK-CCR5 cells harboring a lower proportion of sulfated CCR5 at the cell surface population compared to CHO-CCR5 cells. A possible explanation of this phenomenon is that HEK cells express lower endogenous levels of TPST

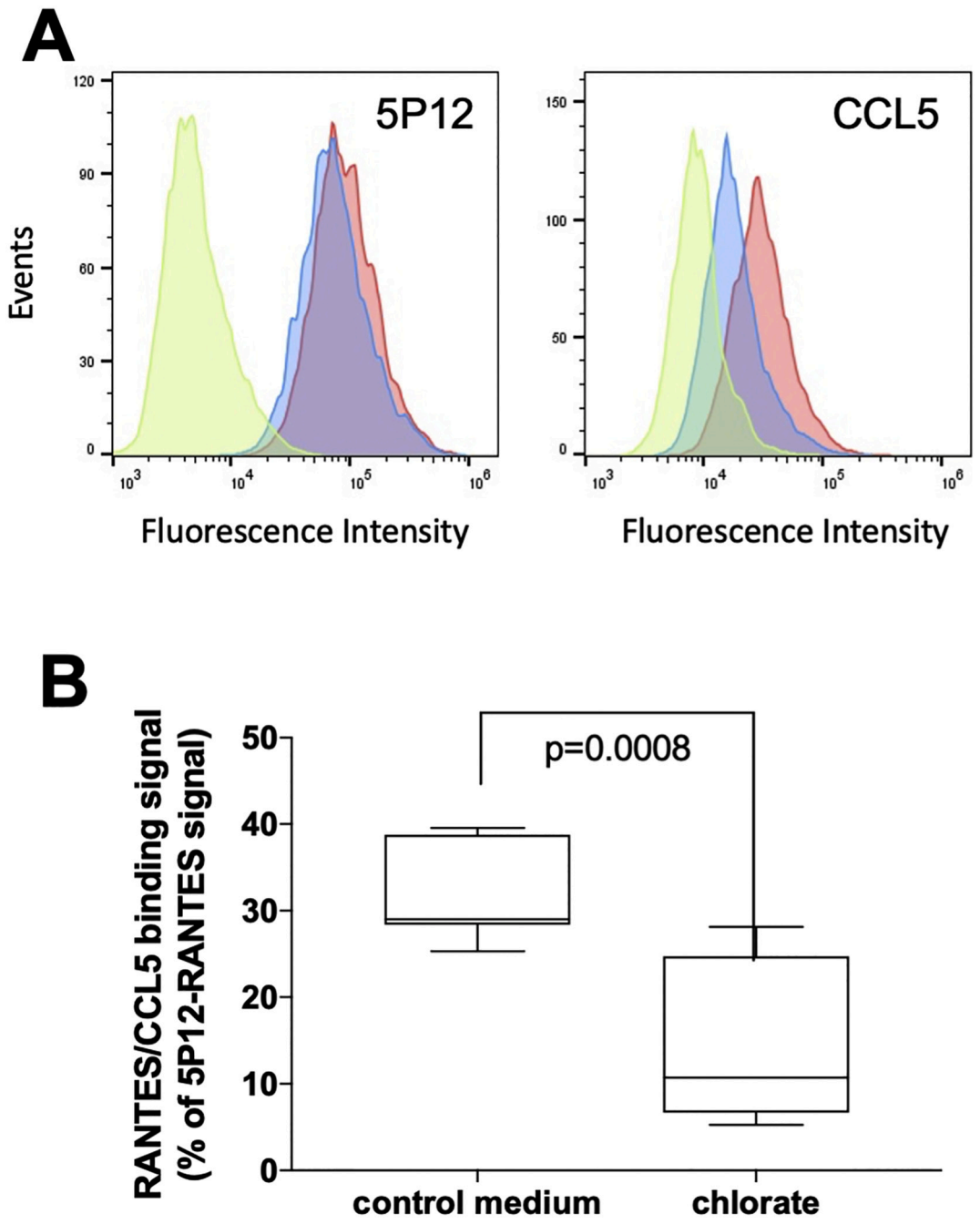

Fig. 10. Sodium chlorate treatment disproportionally affects binding of CCL5 compared to 5P12-RANTES. A. Parental CHO cells (CHO WT, green) and CHO-CCR5 cells were incubated for 16 h at 37 °C with either sulfate-free medium (red) or sulfate-free medium supplemented with sodium chlorate (100 mM) (blue), prior to incubation with rhodamine-labelled 5P12-RANTES or rhodamine-labelled CCL5 (300 nM) and analysis of fluorescent chemokine binding by flow cytometry. Representative histograms are shown. B. Relative binding signals across a series of three independent experiments. Data are represented as box-and-whiskers plots (median and quartiles, with bars representing 95% confidence intervals). The *P*-value was calculated using an unpaired *t*-test.

than CHO cells. Indeed, although the two cell lines were comparable at the level of RNA expression levels of both TPST1 and TPST2 (Supplementary Fig. 6A), endogenous TPST2 protein was only detectable by Western blot in CHO-CCR5 cell lysates, being presumably below the limit of detection in HEK-CCR5 cells (Supplementary Fig. 6B).

Understanding of how CCR5 heterogeneity affects the binding of chemokines and chemokine analogs enabled us to reconcile the apparent contradiction between binding affinities and anti-HIV potencies of chemokine analogs related to 5P12-RANTES. 2P3-RANTES, an earlier lead analog from which 5P12-RANTES was derived, showed more than 25-fold difference in potency compared to 5P12-RANTES in an anti-HIV assay, but with equivalent apparent binding affinity in a CCR5 competition binding assay using radiolabeled CCL4 as a tracer [19]. Our results (Fig. 12) support previously published work [26] showing that CCL3 and CCL4, like CCL5 (Fig. 8), only engage a small proportion of the total pool of CCR5. It is only by using 'super-binding' ligands such as 5P12-RANTES rather than native chemokines as tracers, that differences in binding profiles between native chemokines, partially optimized chemokine analogs such as 2P3-RANTES, and fully optimized highly potent analogs such as 5P12-RANTES become apparent. Since anti-HIV potency relates to blocking CCR5 binding sites for HIV envelope glycoprotein, it is noteworthy that like native chemokine ligands, CCR5-tropic HIV envelopes require CCR5 sulfation for optimal coreceptor activity [33,51]. Hence the increased anti-HIV potency of

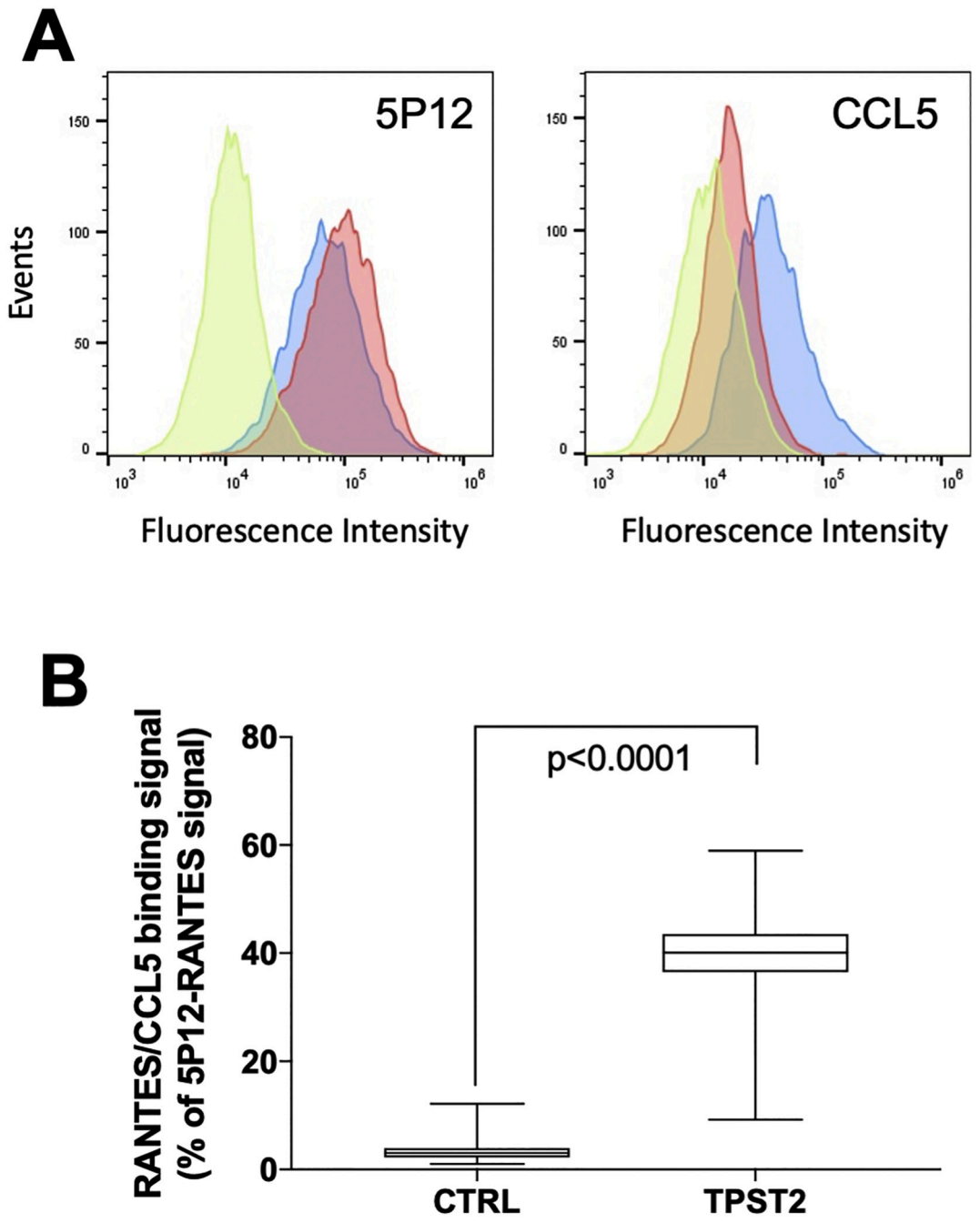

Fig. 11. TPST2 overexpression disproportionally affects binding of CCL5 compared to 5P12-RANTES. A. Parental HEK cells (green) or HEK-CCR5 cells transfected with a TPST2-T2A-mPlum expression vector and defined as TPST2-negative (red) or TPST2-positive (blue), (see Supplementary Fig. 3) were incubated 4 days-post transfection with either 5P12-RANTES or CCL5 (300 nM) prior to analysis of fluorescent chemokine binding by flow cytometry. Representative histograms are shown. B. Relative binding signals across a series of two independent experiments. Data are represented as box-and-whiskers plots (median and quartiles, with bars representing 95% confidence intervals). The P-value was calculated using an unpaired t-test.

optimized chemokine analogs is likely to be due to an increased capacity to compete with HIV envelope for sulfated CCR5 rather than their capacity to bind to unsulfated receptors. Finally, although the majority of the experiments presented in this study were carried out on cell lines engineered to heterogeneously express CCR5, our preliminary competition binding studies on primary human T cells (Fig. 13) indicate that the phenomenon of sulfation heterogeneity is likely to occur on physiological cells that endogenously express the receptor.

Further studies of the interaction of antibody and chemokine ligands with chemokine receptors will help to better elucidate the extent to which sulfation heterogeneity exists across the chemokine receptor family, and the role it plays in receptor cell biology and pharmacology. Such information will be valuable in gaining a better understanding of the inhibitory mechanisms of existing chemokine receptor inhibitors, and in informing strategies for the discovery of new medicines targeting chemokine receptors and other sulfated GPCRs.

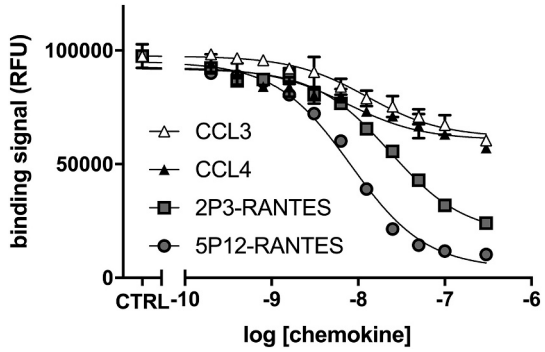

Fig. 12. Competition binding assays using fluorescent 5P12-RANTES as a tracer reveal previously unobserved differences in CCR5 ligand binding properties. CHO-CCR5 cells were incubated with the indicated chemokines and chemokine analogs in the presence of rhodamine-labelled 5P12-RANTES (10 nM). Fluorescent chemokine binding levels were then determined by flow cytometry, with signals expressed in percentage, Data represent mean tracer binding signal ± SD of duplicate measurements and are representative of two independent experiments.

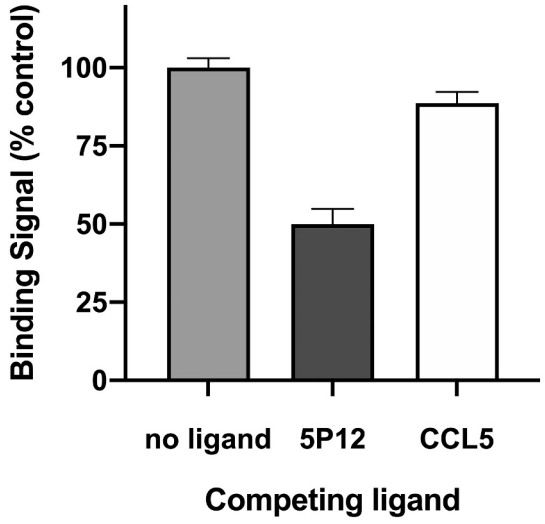

Fig. 13. Evidence for CCR5 heterogeneity on primary human T cells. PHA / IL2-activated human T cells were incubated with the indicated competing ligands in the presence of rhodamine-labelled 5P12-RANTES (5 nM). Data represent mean tracer binding signal ± SD, n = 6 and are representative of two independent experiments.

Declaration of Competing Interest

O. H. is the inventor of the chemokine analog 5P12-RANTES described in this study and a shareholder of Orion Biotechnology Switzerland, to whom the rights to 5P12-RANTES have been assigned.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbagen.2020.129753.

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