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Binding of fusion protein FLSC IgG1 to CCR5 is enhanced by CCR5 antagonist Maraviroc

Olga Latinovic^{a,b,*}, Kate Schneider^{a,b}, Henryk Szmazinski^c, Joseph R. Lakowicz^c, Alonso Heredia^{a,d}, Robert R. Redfield^{a,d}

^aInstitute of Human Virology, University of Maryland, University of Maryland School of Medicine, Baltimore, MD, USA

^bDepartment of Microbiology and Immunology, University of Maryland School of Medicine, Baltimore, MD, USA

^cDepartment of Biochemistry and Molecular Biology, University of Maryland School of Medicine, Baltimore, MD, USA

^dDepartment of Medicine, University of Maryland School of Medicine, Baltimore, MD, USA

Abstract

The CCR5 chemokine receptor is crucial for human immunodeficiency virus type 1 (HIV-1) infection, acting as the principal coreceptor for HIV-1 entry and transmission and is thus an attractive target for antiviral therapy. Studies have suggested that CCR5 surface density and its conformational changes subsequent to virion engagement are rate limiting for entry, and consequently, infection. Not all CCR5 antibodies inhibit HIV-1 infection, suggesting a need for more potent reagents. Here we evaluated full length single chain (FLSC) IgG1, a novel IgG–CD4–gp120_{BAL} fusion protein with several characteristics that make it an attractive candidate for treatment of HIV-1 infections, including bivalency and a potentially increased serum half-life over FLSC, the parental molecule. FLSC IgG1 binds two domains on CCR5, the N-terminus and the second extracellular loop, lowering the levels of available CCR5 viral attachment sites. Furthermore, FLSC IgG1 synergizes with Maraviroc (MVC), the only licensed CCR5 antagonist. In this study, we used both microscopy and functional assays to address the mechanistic aspects of the interactions of FLSC IgG1 and MVC in the context of CCR5 conformational changes and viral infection. We used a novel stochastic optical reconstruction microscopy (STORM), based on high resolution localization of photoswitchable dyes to visualize direct contacts between FLSC IgG1 and CCR5. We compared viral entry inhibition by FLSC IgG1 with that of other CCR5 blockers and showed FLSC IgG1 to be the most potent. We also showed that lower CCR5 surface densities in HIV-1 infected primary cells result in lower FLSC IgG1 EC₅₀ values. In addition, CCR5 binding by FLSC IgG1, but not CCR5 Ab 2D7, was significantly increased when cells were treated with MVC, suggesting MVC allosterically increases exposure of the FLSC IgG1 binding site. These data have implications for future antiviral therapy development.

*Corresponding author at: Institute of Human Virology, University of Maryland School of Medicine, Baltimore, MD, USA.

Keywords

HIV-1; CCR5; CCR5 antagonist; Maraviroc; Fusion protein

1. Introduction

Human immunodeficiency virus type 1 (HIV-1) entry is mediated by the HIV-1 Env glycoproteins gp120 and gp41 (Moore and Doms, 2003). Successful HIV-1 entry requires a series of processes, many dependent on the conformational state of the viral envelope proteins and cellular receptors. Binding of gp120 to CD4 induces conformational changes in both the gp120 and gp41 (Platt et al., 2005, 2014). The Env-CD4 complex then binds to a cellular coreceptor (either CCR5 or CXCR4), and the resulting tri-complex of gp120-CD4-CCR5 induces further conformational changes in Env, leading to viral-cellular fusion and viral entry (Eckert and Kim, 2001).

CCR5 is expressed on many cell types including macrophages, T lymphocytes, and dendritic cells (Lee et al., 1999a). Primary HIV-1 infections, especially those acquired during sexual contact, predominantly involve CCR5-tropic viruses (Shaw and Hunter, 2012) as they appear to be more effectively transmitted than X4-tropic strains (Michael et al., 1997). The importance of CCR5 is reflected by the fact that individuals homozygous for a deletion mutant of the CCR5 gene (Δ32) in the protein coding region are strongly resistant to infection even after repeated exposure to the virus (Burke et al., 2013). These individuals do not have serious health defects (Liu et al., 1996), unlike individuals lacking CD4 or CXCR4 receptors, who have critical immunological issues (Zou et al., 1998). CCR5 is thus a promising target for therapeutic interventions in HIV-1 infection.

As shown in Fig. 1, full length single chain (FLSC) IgG1 is a fusion protein encoded by a synthetic gene that contains an R5 gp120_{BAL} (Fouts et al., 2000) and the binding domain of CD4 linked to the hinge-C_H2-C_H3 portion of human immunoglobulin γ subtype 1 (Vu et al., 2006). FLSC IgG1 binds to the same CCR5 domains as do HIV-1 virions, namely the N-terminus and second extracellular loop (ECL2) (Huang et al., 2007). The N-terminal domain of CCR5 is more important for gp120 binding (Lee et al., 1999b), while the extracellular loops (ECLs) are more important for inducing the conformational changes in the viral envelope that lead to membrane fusion and virus infection (Siciliano et al., 1999). Because FLSC IgG1 binds to both locations on the CCR5 receptor, it is an attractive candidate for antiviral therapy.

In this study we extend our previous characterization of FLSC IgG1 (Latinovic et al., submitted for publication) as a potential synergistic partner to the CCR5 small molecule antagonist Maraviroc (Gorry et al., 2010) (MVC, the only CCR5 antagonist currently approved by the U.S. Food and Drug Administration for treatment of HIV-1 infections). MVC blocks coreceptor activation without masking the binding sites for chemokines or the HIV Env glycoproteins, but apparently causes a conformational shift that reduces the ability of CCR5 to functionally bind virions (Tan et al., 2013). MVC thus differs from natural CCR5 ligands which directly mask HIV-1 binding sites and promote CCR5 cell surface down-regulation (Gong et al., 1998). FLSC IgG1 specifically binds CCR5 without triggering

Ca²⁺ mobilization in peripheral blood mononuclear cells (PBMCs) (Vu et al., 2006) and blocks virus binding and subsequent infection. The protein's bivalency reduces the concentration required for half maximal binding to CCR5 by more than an order of magnitude (Fouts et al., 2000) and also increases FLSC stability and serum half-life, important for *in vivo* efficacy (Ashkenazi and Chamow, 1997).

We previously demonstrated that the antiviral activity of MVC increases at lower CCR5 densities (Latinovic et al., 2011a), and that reduction of CCR5 levels by FLSC-IgG1 treatment lowers the dose of MVC required for antiviral activity (Latinovic et al., submitted for publication). We also showed antiviral synergy of FLSC IgG1 with MVC, with both MVC-sensitive and MVC-resistant viruses, further suggesting its potential as a therapeutic agent against HIV-1 (Latinovic et al., submitted for publication). Here we extend the characterization of FLSC IgG1, demonstrating a more potent antiviral activity than that of other CCR5 antibodies or of FLSC alone. This current study also attempts to better understand the molecular basis of FLSC IgG1 antiviral activity and its high synergy with MVC.

2. Materials and methods

2.1. Cell lines, reagents, and viruses

Virus producer HEK 293T/17 cells were cultured in DMEM supplemented with 10% FBS, 100 µg/ml of penicillin/streptomycin, and 0.5 mg/ml of G418 (Sigma). The HeLa derivatives, JC clones JC10, JC57 and JC53 (Platt et al., 1998), express similar CD4 levels and have CCR5 surface densities of 2000, 9000 and 50,000 mol/cell respectively (JC57 CCR5 densities are with physiologic range). The JC clones and HeLa TZMbls (used in super resolution imaging and as target cells in X-gal virus titer assays) were cultured as above, but without G418. JC clones were a gift from Dr. David Kabat (Oregon Health and Science University, Portland, OR), and HEK 293T/17 and HeLa TZM-bl cells were a gift from Dr. Gregory B. Melikyan (Emory University, GA). Human peripheral blood mononuclear cells (PBMCs) were obtained from healthy donors via the New York Blood Center. PBMCs were isolated by Ficoll-Hypaque density gradient centrifugation and cultured in RPMI medium supplemented with 10% FBS, 100 µg/ml of penicillin/streptomycin, and 100 U/mL IL-2. FLSC IgG1 was expressed from a synthetic gene that links a single chain gp120_{BAL}-CD4 complex containing a codonoptimized R5 gp120_{BAL} sequence and the CD4 binding domain with the hinge-C_{H2}-C_{H3} portion of human immunoglobulin subtype 1 (Vu et al., 2006). PHA used for stimulation of human PBMCs was obtained from Roche. Maraviroc (MVC) and IL-2 (Lahm and Stein, 1985) were obtained from the NIH AIDS Research and Reference Reagent Program, Germantown, MD. FLSC IgG1 protein was a gift from Drs. A.L. DeVico and G. Lewis (IHV, UMB, MD), and FLSC concentrated viral filtrate protein was a gift from Dr. T.R. Fouts (Profectus Biosciences, MD). HIV-1 virus strains Bal and CC1/85 were used for replication competent infection experiments. The Bal used here (Taylor et al., 2008) is an R5 replication competent chimeric virus that contains most of the Bal *env* gene in an HIV-1 IIIB backbone, and is strictly R5 dependent. CC1/85 is an R5 isolate. Bal virus stocks were obtained from the Institute of Human Virology µQuant Core Facility (IHV, UMB, MD). Infectious viral molecular clones derived from CC1/85 were

obtained from Dr. John Moore (Cornell Medical College, NY). Stocks of molecular viral clones were produced by transfection of HEK 293T/17 cells with the infectious plasmids.

2.2. Confocal microscopy

JC53 cells expressing high CCR5 levels were first incubated with a CCR5 N-terminus antibody (purified mouse anti-human CCR5, clone T21/8, BioLegend) or CCR5 ECL2 mAb (mouse monoclonal IgG2b, clone 45531, R&D Systems), or both antibodies in combination, at 100 µg/ml (N-terminus) or 50 µg/ml (ECL2) for 2 h at room temperature. We also tested a multi epitope CCR5 primary antibody, anti-human CCR5 mAb, clone 45523 (Endres et al., 1996) (NIH AIDS Research and Reference Reagent Program, Germantown, MD), which was incubated on the cells in the same manner. Samples were then washed and incubated with a fluorescent FLSC IgG1 Zenon Alexa 488 complex for 1 h in the dark at room temperature. The fluorescent FLSC IgG1 complex was created using a Zenon Alexa 488 human IgG kit (Invitrogen) following manufacturer protocols. Cell-associated fluorescence was visualized using a Zeiss META LSM 510 confocal microscope and inhibition of staining with FLSC IgG1 Zenon Alexa 488 by CCR5 mAbs was analyzed using ZEN Lite 2012 analysis software (Carl Zeiss).

2.3. Super resolution microscopy

HeLa TZMbl cells (4×10^4 cells) used for visualization of CCR5 binding with FLSC IgG1 were plated in phenol red-free medium in 35 mm imaging dishes (MatTek) 1 day prior to imaging. Media was removed before fixation and staining and cells were washed twice with HBSS, then preincubated with BSA containing buffer to remove serum from the culture media. The primary antibody against CCR5, anti-human CCR5 mAb, clone 45523 (Endres et al., 1996) (NIH AIDS Research and Reference Reagent Program, Germantown, MD) was incubated with cells at room temperature for 30 min. The secondary antibody, Alexa 647 goat anti-mouse IgG2b (Invitrogen), was incubated with the cells on ice in the dark for 90 min. Cells were washed and FLSC IgG1 added at 20 µg/ml for 30 min at room temperature. Zenon Alexa 488 (Invitrogen) was added and incubated with cells at room temperature for 7 min. Cells were fixed, washed and kept on ice prior to super-resolution imaging. Images were acquired using Nikon's direct STORM (dSTORM) with a TRIF microscope setup. The system is equipped with the EMCCD technology (Andor iXon Ultra DU987 EMCCD camera), capable of single molecule detection sensitivity. The TIRF angle was adjusted to maximize signal to noise detection for 3D STORM. The TIRF angle was shifted during sample bleaching to angles just below and above the indicated TIRF angle of interest. The sample bleaching is performed to place the target fluorophores in to a ground state and to eliminate non-specific signals from the valid signal of the designated fluorophores. Solid state lasers (488 nm at 80 mW and 647 nm at 125 mW) were used at 100% power at acquisition in order to activate all possible fluorescence molecules and maximize the signal of interest. Images were acquired using a Nikon 100×/1.49 CFI Apo TIRF oil immersion objective for TIRF imaging and processed using Nikon's NIS Elements software.

2.4. β-lactamase virus–cell fusion assays

HIV-1 pseudoviruses containing a β-lactamase (BlaM)–Vpr insert (ref) were produced from HEK 293T/17 cells (3.5×10^6 cells) seeded in a 10 cm dish 24 h prior to transfection. Cells

were cotransfected with JRFL 140T (48 µg), R8 Env (24 µg), pcRev (12 µg) and BlaM-Vpr (12 µg) plasmids using the calcium phosphate method. JRFL 140T plasmid was a gift from Dr. J. Binley (Torrey Pines Institute of Molecular Studies, San Diego, CA), R8DEnv was provided by Dr. C. Aiken (Vanderbilt University Medical Center, Nashville, TN), and pcRev (Malim et al., 1988) and BlaM-Vpr (Tobiume et al., 2003) were both obtained from the NIH AIDS Research and Reference Reagent Program. The cellular medium, containing HIV-1 virion-associated BlaM-Vpr, was collected 48 h post transfection, centrifuged and filtered through a 0.45 µm syringe filter to purify virions from cellular debris and viral clusters. Virus was pelleted onto a 20% sucrose cushion for 1 h at 55,000g (Optima XL-100K Ultracentrifuge; Beckman), then resuspended in PBS. Viral infectivity was determined by X-gal assays (Kimpton and Emerman, 1992) using HeLa TZMbl cells as target cells. Prior to fusion assays, human PBMCs were activated with PHA (5 µg/ml) for 2 days, then with IL-2 (100 U/mL) for 5 days. Before adding virus, 7×10^5 cells/well (PBMCs) or 2×10^4 cells/well (JC clones) were plated in optical bottom 96 well plates and incubated in serum free media with different concentrations of FLSC or FLSC IgG1 for 1 h at 37 °C, then co-centrifuged at 2095g at 4 °C with virus at MOI = 2 (PBMCs) or MOI = 1 (JC clones) for 30 min. The cells were washed with cold PBS to remove unbound virus, then moved to 37 °C for 6 h (PBMCs) or 3 h (JC clones) to initiate fusion. Fusion peptide inhibitor T-20 (a gift from Dr. L.-X. Wang, IHV, UMB, MD) was added prior to virus addition and during the 37 °C fusion process as a positive control. During fusion incubation, cells were kept with the indicated FLSC or FLSC IgG1 concentrations in RPMI supplemented with 10% FBS and 100 U/mL IL-2 (PBMCs) or serum free DMEM (JC clones) during fusion incubation. After fusion, cells were loaded with the fluorescence substrate CCF4-AM (Invitrogen) for 45 min in the dark. Substrate was removed and cells were washed twice and kept in HBSS/10% FBS/2.5 mM Probenecid (Sigma). BlaM activity was developed overnight at room temperature and quantified using an FLx800 fluorescence plate reader and Gen5 software (both from Bio-Tek Instruments). Fused versus unfused viruses were quantified by the ratio of blue (440–480 nm) to green (518–538 nm) emission after exciting the cells at 405–415 nm. All data are given in triplicate, with at least two PBMC donors.

2.5. Singe-cycle HIV-1 entry assay on JC57 cells

Replication-defective HIV-1 luciferase reporter pseudoviruses were produced from HEK 293T/17 cells split at 2×10^6 cells per well in 6-well plates 24 h prior to transfection. Cells were cotransfected with 2.5 µg of pNL4.3-Env-luc3 (a gift from Dr. N. Landau, New York University School of Medicine, New York) and 2.5 µg of pCI-Env-expressing plasmid (MVC-sensitive JRFL HIV-1 R5 Env) using Lipofectamine LTX (Invitrogen). MVC-sensitive plasmid was cloned from MVC-sensitive virus, a gift from Drs. Michael Westby and Hernan Valdez (Pfizer, Sandwich, UK). MVC-sensitive HIV-1 Envs, described originally by Westby et al. (2006), encode Env genes of primary isolate CC1/85 (Pugach et al., 2008) passaged in PBMCs in the absence of MVC. The MVC-sensitive HIV-1 Env contains amino acids 316A, 319A and 323I in V3 (Westby et al., 2006). Pseudoviruses were collected 48 h post-transfection. The virus-containing supernatant was centrifuged and filtered through a 0.45 µm syringe filter to purify virions from cellular debris and viral clusters. The viral titer was determined by p24 ELISA (Perkin-Elmer Life Science, Inc.). One day before infection, JC57 cells were plated in 96 well plates at 1.2×10^4 cells/well. On

the day of the experiment, cells were pretreated for 1 h at 37 °C in serum-free culture media containing varying concentrations of FLSC IgG1 or the indicated CCR5 antibody. 2D7 mAb (purified mouse anti-human CD195, clone 2D7/CCR5) was obtained from BD Biosciences. CD195 mAb (mouse anti-human CD195, clone 2D7/CCR5, Wu et al., 1997), anti-CCR5 N-terminus pAb (Dragic et al., 1996) and anti-human CCR5 mAb, clone 45523 (Endres et al., 1996), were all obtained from the NIH AIDS Research and Reference Reagent Program (Germantown, MD). Cells were then infected with R5 HIV-1 virus (0.41 ng of p24/well) in the presence of polybrene (8 µg/ml, Sigma). Virus and polybrene were removed from cells after 24 h, and medium was replaced. 72 h after infection, the cells were lysed and luciferase activity, measured and expressed as relative luciferase units (RLU) using the Luciferase Assay System (Promega), was determined using a Veritas microplate luminometer with Veritas software (both from Promega). Cells without virus were used to determine the assay background.

2.6. Infection with replication-competent HIV-1

Clones JC10 (2000 CCR5 molecules/cell) and JC57 (9000 CCR5 molecules/cell) were split 1 day prior to the experiment at 5000 cells/well in 96 well plates, then treated with the indicated concentrations of FLSC IgG1 in serum free DMEM for 1 h at 37 °C. R5 Bal HIV-1 replication competent chimeric virus (Taylor et al., 2008) at MOI = 0.1 was added to cells. Cells were shifted to 37 °C for 3 h for infection, then washed and cultured at 37 °C in medium containing the indicated concentrations of FLSC IgG1. The same experiment was also performed using HIV-1 CC1/85 virus. Human PBMCs were activated with IL-2 (100 U/mL) for 7 days, then cultured at 2×10^5 cells/well in 96-well plates. Cells were pretreated in serum-free culture media for 1 h at 37 °C with the indicated concentrations of FLSC IgG1, then infected at MOI = 0.00075 for 3 h at 37 °C (Heredia et al., 2003). After infection, cells were washed and cultured at 37 °C in medium containing IL-2 (100 U/mL) plus the indicated concentrations of FLSC IgG1. Virus production in both cases was measured by p24 ELISA (Perkin–Elmer Life Science, Inc.) on day 4 (cell lines) or 5 (PBMCs) after infection and EC₅₀ values determined using Prism statistic software (Irvine, California).

2.7. CCR5 surface density determinations

Quantitation of CCR5 surface molecules/cell in donor PBMCs was as described (Reynes et al., 2004). Fluorescence was measured using the Quantiquess system (BD Biosciences), which produces a regression line from a series of Quantibrite-PE bead standards (BD Biosciences). The mean number of surface molecules cells labeled with a PE-antibody was determined from the FL-2 value using a linear regression and taking into account the PE antibody ratio for each antibody (1:1 in our reagents).

2.8. Effect of FLSC IgG1 and MVC binding on CCR5 levels

JC57 cells (1×10^6) were pretreated in serum-free medium containing 100 µg/ml FLSC IgG1 alone or 0.5 µM of MVC alone for 1 h at 37 °C, or with 0.5 µM of MVC for 1 h at 37 °C, followed by 100 µg/ml FLSC IgG1 for 1 h at 37 °C. Cells were stained with human CCR5 mAb (clone 45531), and the signal amplified by biotin and PE-conjugated streptavidin, followed by analysis with a FAC-SCalibur flow cytometer and CellQuest software.

2.9. Plasmonic metal-enhanced fluorescence substrate experiments

JC53 cells were used because they express high CCR5 levels, making it easier to detect increases in intensities of labeled CCR5 surface. Cells were pre-treated with 10 μ M MVC (or left untreated) for 1 h at 37 °C, then washed, blocked, and treated with FLSC IgG1 (4 μ g/ 5×10^5 cells) or primary antibody (10 μ g/ 1×10^6 cells) for 30 min at room temperature. Cells were washed again, corresponding secondary antibody was added, and cells were incubated on ice for 90 min. 2D7 primary antibody (purified mouse antihuman CCR5 mAb, clone 2D7/CCR5, BD Biosciences) stained with PE rat anti-mouse IgG2a + b mAb (clone X57, BD Biosciences) secondary antibody. HGS101 primary antibody (Human Genome Sciences, Rockville, MD) was stained with mouse anti-human IgG4 PE (Southern Biotech) secondary antibody. FLSC IgG1 was stained with secondary antibody, Zenon Alexa 555 (Invitrogen). Cells were washed, resuspended in phenol red-free growth medium to minimize autofluorescence, and cell-associated fluorescence was visualized using an Olympus IX confocal microscope integrated with an Alba V fluorescence imaging system (ISS Inc., Urbana, IL). Before imaging, cells were allowed to settle onto the plasmonic substrate to expose the bound fluorescent probes to an enhanced electromagnetic field generated by plasmonic nanostructures within distance of near field interactions (3–50 nm) from the surface. Plasmonic nanostructures contain silver nanoparticles deposited on thin silica layer over the silver mirror on the glass slide. Such multi-layered substrates provide highly enhanced fluorescence from the fluorophores located in proximity to the substrate surface, thus ideal to study the changes in the CCR5 expression on the cell surface (Szmazinski et al., 2012). Cells were kept at 37 °C in 5% CO₂ for at least 2 h before imaging. Images with multiple cells (~15–25 cells/image) within the scanning field of $180 \times 180 \mu\text{m}^2$ were acquired and average cell intensities were measured based on 3–5 images (~45–125 cells). A 473 nm excitation laser was used and PE or Alexa Fluor 555 fluorescence observed through a band pass filter (575/105 nm) (Semrock Inc., Rochester, NY). Images were acquired using a 20 $\times$ NA 0.4 objective (UPlan, Olympus) and FLIM system Alba V.

3. Results

3.1. CCR5 specificity of FLSC IgG1

To establish that FLSC IgG1 specifically binds to CCR5, we performed competition experiments with different CCR5 antibodies. We preincubated JC53 cells (50,000 CCR5 molecules/cell) with antibodies against the CCR5 N-terminus (NT) (purified mouse antihuman CCR5, clone T21/8) and the ECL2 (mouse monoclonal IgG2b, clone 45531), alone or in combination, then probed the cells with FLSC IgG1 conjugated with Zenon Alexa 488. Panel A in Fig. 2 shows a sample treated with FLSC IgG1 Zenon Alexa 488 alone. There is a clear inhibition of FLSC IgG1 binding to cells pretreated with ECL2 [Panel B] or N-terminus [Panel C] antibodies or a combination of both [Panel D], in contrast to that of cells not pre-treated [Panel A]. Panel E presents the quantitation of fluorescence in the same samples. The N-terminus antibody decreased FLSC IgG1 bound to CCR5 fluorescence by more than 20%; ECL2 antibody decreased the CCR5 fluorescence for more than 40% and their combination decreased the CCR5 fluorescence by 50+%. We also tested a multi epitope CCR5 antibody (clone 45523), which resulted in a decrease of fluorescence of

around 50% (data not shown). Cell autofluorescence data was less than 10% [Panel E]. The results are given as average of ten images $\pm$ SD.

3.2. FLSC IgG1 binding to CCR5 visualized via super resolution microscopy

To confirm that FLSC IgG1 binds specifically to CCR5, we used multi-color super resolution microscopy with total internal reflection fluorescence (TIRF), which allows full coreceptor number visualization on the cellular edges up to 200 nm from the cell membrane [Fig. 3]. Using direct stochastic optical reconstruction microscopy (dSTORM) (Rust et al., 2006) to achieve a lateral resolution of 15–20 nm, we visualized and showed binding sites for FLSC IgG1 on the CCR5 coreceptor on the cellular surface. We utilized high-resolution fluorescence microscopy based on localization of photo-switchable fluorophores to visualize binding sites of CCR5 to FLSC IgG1. In each imaging cycle, only a fraction of the fluorophores was turned on at any given time, allowing their positions to be determined with nanometer accuracy (Pereira et al., 2012). We used the fluorophore positions from a series of imaging cycles to reconstruct the final image (Roy et al., 2013). FLSC IgG1 and the CCR5 antibody used in this experiment bind to different sites on CCR5 molecule, and hence do not compete for binding. As a control, we used an antibody to another cell surface antigen, HLA class I, which is ubiquitously expressed on human cells, including HeLa TZMbl, but which does not express in a proximity to CCR5 (data not shown). Super resolution microscopy of CCR5 on the TZMbl cell surface stained with FLSC IgG1 and Zenon Alexa 488 nm is shown as green in Panel A. The CCR5 antibody with Alexa 647 nm goat anti-mouse IgG2B (red) is shown in Panel B. The overlap image is shown in Panel C. The white arrows show the combined binding sites (yellow), consistent with both proteins recognizing CCR5 on the cell surface. Panels D and E show a 3-D close up of direct contacts between CCR5 and FLSC IgG1 on the cell membrane (using Nikon Elements software). For control experiments, cells were stained with FLSC IgG1 alone, with CCR5 Alexa 647 alone, or with only the secondary antibodies and visualized using both laser lines. No fluorescence was evident in any of the control samples (data not shown).

3.3. Comparative antiviral activities of FLSC IgG1 and other CCR5 blockers

We compared the antiviral activities of FLSC IgG1, FLSC alone, and several CCR5 antibodies. We first measured viral entry inhibition by FLSC IgG1 versus FLSC in JC clones expressing two different CCR5 levels (JC53 = 50,000 molecules/cell and JC57 = 9,000 molecules/cell) using the BlaM fusion assay (Cavrois et al., 2002). Both clones express the same CD4 surface density ($\sim 10^5$ molecules/cell). JC cell lines were pretreated in serum-free culture media with 0.01, 0.1, 1, 10, and 100 μ g/ml of FLSC or FLSC IgG1 for 1 h at 37 °C before CCR5-tropic JRFL Env-pseudotyped virions containing Vpr- β -lactamase fusion proteins were added. BlaM activity occurs following insertion of the viral core-associated enzyme into the cytosol and addition of CCF4-AM substrate. Antiviral activity in JC53 cells [Fig. 4A] after treatment with FLSC IgG1 (open circles) and FLSC (solid circles) showed only a moderate decrease, as expected from CCR5 over-expression. The antiviral activity in JC57 cells [Fig. 4B] after treatment with FLSC IgG1 (open circles) and FLSC alone (solid circles) showed a much greater decrease, as JC57 cells express CCR5 at levels similar to those on normal primary CD4⁺ T cells (Hladik et al., 2005). Importantly, FLSC IgG1 treatment of JC57 reduced viral entry to a far greater extent than did FLSC [Fig. 4B]. 50%

inhibition was obtained at 0.1 µg/ml of FLSC IgG1 [Fig. 4B]. As expected, the T-20 positive control (5 µM) strongly inhibited viral entry (data not shown). All data are given as averages of triplicates, normalized to values obtained in the absence of inhibitor and given as a percentage for each cell line separately. Entry of X4-virus was not blocked by FLSC IgG1 or FLSC (not shown).

We performed similar experiments with primary cells. PBMCs were activated with PHA (5 µg/ml) for 2 days, then IL-2 for 5 days, then treated as described for the cell lines. We measured viral entry inhibition with both FLSC and FLSC IgG1 by BlaM fusion assay. FLSC IgG1 was less potent with the PBMCs than in cell lines [Fig. 4C], although there is considerable donor-dependent variability with primary cells and the activation/stimulation procedures used (data not shown). The data are again presented as a percentage of the value with untreated cells. The highest concentration (1000 µg/ml) gave strong (~80%) inhibition with both reagents. A 35% stronger effect, however, was observed with FLSC IgG1 than with FLSC alone at concentrations between 0.01 and 10 µg/ml. Inhibition of HIV-1 entry was similar for cells from both donors, and all experiments were in triplicate and shown as an average ± SD. The positive control, T-20, inhibited up to 90% (not shown). These results indicate that both FLSC and FLSC IgG1 are effective at blocking fusion of R5 enveloped viruses in both cell lines and primary cells, with similar efficacy when CCR5 levels are similar, with FLSC IgG1 being the more potent.

We next tested the antiviral activity of FLSC IgG1 and other CCR5 antibodies using a single-cycle HIV-1 entry assay in JC57 cells. FLSC IgG1 binds at least two domains of CCR5, the NT and ECL2. The CCR5 antibodies used included 2D7 and CD195, which bind to ECL2 (Wu et al., 1997) (open squares and solid triangles), anti-CCR5 NT pAb (Dragic et al., 1996), which binds to the NT (open diamonds), and 45523, a mAb against a multidomain epitope (Endres et al., 1996) (solid diamonds). Inhibition of virus infection by FLSC IgG1 is shown by solid circles and shows greater antiviral potency than any of the antibodies [Fig. 5]. Although 2D7 reached the same level of inhibition as FLSC IgG1, this was only true for the highest concentration (100 µg/ml). The greater potency of FLSC IgG1 is consistent with the interpretation that FLSC IgG1 more closely mimics HIV-1 envelope interactions with CCR5.

3.4. Impact of CCR5 density on the antiviral activity of FLSC IgG1

We next evaluated the influence of CCR5 density on the antiviral activity of FLSC IgG1 using two JC clones expressing similar CCR5 levels to those of normal CD4+ T cells (Platt et al., 1998), JC10 cells, which express 2000 molecules/cell, and JC57 cells, which express 9000 molecules/cell, express similar CD4 levels. Cells were infected with replication competent Bal HIV-1 (MOI = 0.1) in the presence of different concentrations of FLSC IgG1 for 3 h. Four days after infection, culture supernatant p24 was measured by ELISA. Although FLSC IgG1 inhibited viral replication in both JC lines, the EC₅₀ = 0.03 µg/ml with JC10 and EC₅₀ = 0.48 µg/ml with JC57 (Fig. 6A) suggested that variations in CCR5 densities within the range observed in most primary cells affect the antiviral activity of FLSC IgG1. Indeed, the shift in EC₅₀ values was greater than 10-fold when CCR5 levels varied only 5-fold.

We additionally evaluated this apparent CCR5 density effect on FLSC IgG1 inhibition using primary cells from five different PBMC donors. On the day of infection, we measured PBMC CCR5 density by quantitative flow cytometry. Donor cells were infected with Bal HIV-1 replication competent virus (MOI = 0.00075) for 3 h in the presence of various FLSC IgG1 concentrations. Five days later, supernatant p24 was measured by ELISA. For the five donors, CCR5 levels on CD4 T+ cells ranged between 2200 and 5000 molecules/cell. CD4 levels were similar among the donors (23–27,000 molecules/cell). FLSC IgG1 EC₅₀ values varied approximately 80-fold among the different donors. Importantly, they positively correlated with CCR5 densities [Fig. 6B] by Spearman's correlation test using Prism software (Irvine, California); $r = 0.9$, $R^2 = 0.8$. The results in Fig. 6B suggest that the antiviral activity of FLSC IgG1 is strongly affected by CCR5 density variations in PBMCs from different donors as well as with the cell lines. A similar correlation to one in Fig. 6A between CCR5 density and FLSC IgG1 EC₅₀ values was also observed when the same JC clones were infected with HIV-1 CC1/85 [Fig. 6C], another R5 virus, and a similar shift in EC₅₀ values was detected judged by supernatant p24. In another control experiment, infection of JC cells was carried out at various concentrations of the reverse transcriptase inhibitor Efavirenz. EC₅₀ values varied only <3-fold among triplicates and did not correlate with CCR5 density (data not shown).

3.5. Effect of combining FLSC IgG1 and MVC on CCR5 Ab binding

We wanted to test whether FLSC IgG1 binding is increased in the presence of MVC, as we wanted to better understand the basis for their strong cooperative antiviral activity (Latinovic et al., submitted for publication). We measured the binding of FLSC IgG1 alone, MVC alone, and a combination of FLSC IgG1 and MVC [Fig. 7].

We used JC57 cells (expressing a physiologically relevant level of 9000 CCR5 molecules/cell) and flow cytometry to quantify reductions in unbound surface CCR5. The reduction in CCR5 intensity after FLSC IgG1 pretreatment was ~10% on average; after MVC treatment, the reduction was 55%; and after combined treatment ~70%. The isotype control gave a minimal signal of 10% [Fig. 7]. The data are given as averages of triplicates with $\pm$ SD from three independent experiments, normalized to fluorescence values obtained in the absence of any reagent and expressed as a percentage. Together, these data suggests that combined treatment of FLSC IgG1 and MVC decreases levels of free CCR5 beyond what is obtained by either alone. The same trend of inhibition rates was observed in JC53 cell lines expressing 50,000 molecules/cell (data not shown).

3.6. MVC increases binding of FLSC IgG1, but not CCR5 antibodies HGS101 and 2D7, to CCR5

We wondered whether MVC binding might allow increased binding by FLSC IgG1 by altering the conformation of CCR5 allosterically. We previously demonstrated by flow cytometry that the binding of CCR5 antibody HGS101 (Latinovic et al., 2011a) is higher in the presence than in the absence of MVC. In contrast, this was not the case with another CCR5 antibody, 2D7. Both HGS101 and 2D7 bind to ECL2 of CCR5. We used these antibodies and compared their effect with that of MVC on CCR5 binding by FSLC IgG1 in a plasmonic substrate experiment.

In order to measure total CCR5 surface available for FLSC IgG1 binding in the presence of MVC, we employed the plasmonic substrate methodology because of its superior sensitivity, due to an up to 200-fold enhancement of fluorescence compared to standard glass substrate methodology (Szmanski et al., 2012). With this methodology, there is no need to remove unbound detection probe from solution, which allows for real-time detection and localization of cellular events by changes in signal intensities and lifetimes (Szmanski et al., 2013). To evaluate available CCR5 surface upon MVC binding by this technique, we performed fluorescence imaging using PE-labeled CCR5 mAbs 2D7 and HGS101, both of which bind to the ECL2 domain of CCR5. JC53 cells untreated or treated with MVC were incubated with different concentrations of PE labeled CCR5 antibodies, washed and plated in wells on a plasmonic substrate at approximately 20,000 cells/well. Similarly, Alexa Fluor 555-labeled antibody was used for samples incubated with FLSC IgG1. Cells were imaged after 2 h of contact with the plasmonic substrate surface. Adhesion of cells results in exposure of approximately half of the bound PE labeled antibodies on cells to the enhanced electromagnetic field, which causes an amplified PE fluorescence (Szmanski et al., 2012). Amplified fluorescence provides a means for determining differences in brightness for cells treated and untreated with MVC. We measured fluorescence intensities for JC53 cells with or without MVC treatment [Fig. 8]. The mean intensities for cells treated with MVC were clearly higher than those for untreated cells as shown for samples with FLSC IgG1 in Fig. 8A. To facilitate the comparison between samples imaged with different antibodies, the relative changes in mean intensities were calculated [Fig. 8B] and can be interpreted as changes in CCR5 sites available for specific antibody CCR5. CCR5 binding by 2D7 was increased by MVC treatment by approximately 1.13-fold, HGS101 by 1.28-fold and FLSC IgG1 by 1.65-fold. These results clearly suggest that the binding sites for FLSC IgG1 become more exposed when CCR5 is bound to MVC. FLSC IgG1 binds at least two domains of CCR5, the N-terminus and the ECL2, while 2D7 and HGS101 bind only ECL2. This difference in binding sites likely explains the higher MVC-induced fluorescence of CCR5 labeled with FLSC IgG1, as would occur with an allosteric effect of MVC binding.

4. Discussion

CCR5 antagonists are a recently employed class of antiviral agents with a unique mechanism of action that offers additional treatment options for patients. Understanding how CCR5 and its blockers functionally interact among themselves and together with CCR5 is therefore important. Here, we confirmed that a recently developed fusion protein, FLSC IgG1, is a potent CCR5 blocker that is highly synergistic in antiviral activity with MVC, a CCR5 antagonist in current clinical use. We showed that, like FLSC, the parent molecule, FLSC IgG1 binds specifically to CCR5. It appears to bind to at least two distinct CCR5 domains, as judged by its competition with mAbs against both the N-terminal and ECL2 domains. The lack of complete fluorescence knock down upon treatment with both NT and ECL2 Abs suggests that there are alternate CCR5 conformations it recognizes. Other explanations are that it also binds to CD4, and there is an existence of nonspecific binding. The possibility of an additional binding site will be a subject of future investigations. We visualized direct binding sites between CCR5 and FLSC IgG1 in 3D via super resolution

microscopy, giving a more detailed demonstration of their contacts than what has previously been shown [Fig. 3].

We compared the antiviral activity of FLSC IgG1 with that of other CCR5 binding proteins. The IgG1 FLSC chimera was more effective in blocking virus–cell fusion than FLSC alone, both in cell lines and in primary cells [Fig. 4]. The greater efficacy of FLSC IgG1 is likely due to stabilization as a dimer by the IgG1 moiety, conferring bivalency with stronger binding. This makes FLSC IgG1 more attractive than FLSC itself for antiviral treatment. We also compared the antiviral activity of FLSC IgG1 with that of several CCR5 antibodies using single round infection assays. FLSC IgG1 had greater antiviral potency than all but 2D7 antibody, and 2D7 was only equivalent at the highest concentrations of each (100 µg/ml). This is in agreement with other studies showing that FLSC IgG1 is approximately 17-fold more potent than 2D7, based on EC₅₀ values (Vu et al., 2006). The greater potency of FLSC IgG1 is consistent with the idea that it more closely mimics HIV-1 envelope interactions with CCR5 than do the other proteins tested here.

The antiviral activity of FLSC IgG1 is partially an inverse function of CCR5 surface density. In Fig. 6, we showed the effect on EC₅₀ of CCR5 density, using two cell lines differing only in CCR5 expression. As expected, infection of JC10 cells (surface CCR5 = 2000 molecules/cell) was more strongly inhibited than was infection of JC57 cells (surface CCR5 = 9000 molecules/cell), confirming the relation between CCR5 density and inhibition by FLSC IgG1 of entry by two different R5 HIV-1 isolates. More significantly, CCR5 density in primary PBMCs inversely correlated with FLSC IgG1 potency. FLSC IgG1 EC₅₀ values were approximately 80 times higher with donors having 2.2×10^3 CCR5 molecules/cell than with donors with 5×10^3 CCR5 molecules/cell. Thus, donor CCR5 density (number of receptors/CD4+ T cell) impacts the antiviral activity of FLSC IgG1. The antiviral activity of FLSC IgG1 is strongly synergistic with that of the small molecule CCR5 antagonist MVC. To help understand the basis of this strong synergy, we measured residual surface CCR5, using CCR5 Abs, in the presence of MVC and FLSC IgG1 alone and in combination. The greatest reduction was seen with the combination of the two reagents [Fig. 7].

The greater inhibition of infection by FLSC IgG1 of MVC treated PBMCs than of untreated PBMCs suggests that the FLSC IgG1 binding site(s) remain exposed on MVC-bound CCR5. We asked whether MVC treatment might make in fact the FLSC IgG1 binding site(s) on CCR5 more exposed by inducing an allosteric change in CCR5. We measured FLSC IgG1 binding to CCR5 before and after treatment with MVC using a plasmonic substrate methodology [Fig. 8], which has higher sensitivity than does flow cytometry and allows actual visualization. FLSC IgG1, but not 2D7, gave higher mean fluorescence intensities (MFI) with MVC-treated versus untreated CD4+ T cells, suggesting that the FLSC IgG1 binding site, but not that of 2D7, indeed becomes more available when CCR5 is occupied by MVC. As the formation of a fusion pore by R5 HIV-1 likely requires the engagement of several CCR5 molecules (4–6 as shown by Kuhmann et al., 2000), it is likely that this greater proportion of occupied CCR5 molecules would more effectively prevent viral engagement of multiple CCR5 residues. The increase in CCR5 binding suggests that combined MVC/FLSC IgG1 will offer new possibilities for effective antiviral therapy and supports further clinical testing.

The studies described here further our longstanding interest in identifying effective combinatorial antiviral approaches (enhanced potency with reduced side effects and drug costs) targeting CCR5 and in understanding the mechanistic basis of drug synergies. Previously, we and others showed that MVC enhances the antiviral activity of several CCR5 antibodies and strongly synergizes with antibodies that target the ECL2 domain of CCR5 (Ji et al., 2007; Latinovic et al., 2011b). We suggested that this might mean that MVC induces an allosteric change in CCR5 that increases epitope exposure of ECL2 and the N-terminus, as judged by flow cytometry using fluorescent CCR5 antibodies. We have subsequently expanded on these observations using a more potent CCR5 blocker, FLSC IgG1, in combination with MVC. Our original hypothesis was that there is strong *in vivo* synergy between MVC and the fusion protein FLSC IgG1 because of recognition by MVC and FLSC IgG1 of distinct regions of CCR5. FLSC IgG1 binds to the N-terminus of CCR5 and other extracellular sites recognized by HIV-1 Env. In contrast, MVC binds to the transmembrane region of CCR5 (Wood and Armour, 2005), locking it into a conformation that no longer can recognize distal regions of the V3 loop of Env gp120, which is one domain of the gp120 that interacts with CCR5. Using a plasmonic substrate and metal-enhanced fluorescence imaging methodology, we extended our original observations of FLSC IgG1/MVC interactions and demonstrated that MVC conformationally alters CCR5 so that it becomes more exposed and consequently more available for binding with FLSC IgG1, although the altered conformation is not functionally recognized by virus. Together, these data further suggest that MVC-induced CCR5 conformational changes enhance binding by FLSC IgG1, resulting in antiviral synergy, and highlight the potential of their combined use for antiviral therapy.

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Fig. 1. Schematic representation of the FLSC IgG1 fusion construct. To produce FLSC IgG1, the FLSC sequence was fused to the hinge to C_H3 domains of an IgG1 heavy chain lacking the V_H and C_H1 domains (Vu et al., 2006).

Fig. 2.

Competition between FLSC IgG1 and MAbs for CCR5. JC53 cells were stained with FLSC IgG1 alone or in competition with CCR5 mAbs. Panel A shows cells treated with FLSC IgG1 Zenon Alexa 488, Panel B shows cells treated with ECL2 mAb and FLSC IgG1 Zenon Alexa 488, Panel C shows cells treated with the N-terminus antibody and FLSC IgG1 Zenon Alexa 488, and Panel D shows cells after combined and simultaneous treatment with both mAbs and FLSC IgG1 Zenon Alexa 488. Panel E shows the normalized relative staining intensity of CCR5 in samples pretreated or not with antibodies and then with FLSC IgG1 Zenon Alexa 488. AF samples were an unstained autofluorescence control. Bar size is 10 μ m.

Fig. 3.

Contacts of FLSC IgG1 and CCR5. FLSC IgG1 Zenon Alexa 488 (green – Panel A) binding to CCR5 stained with CCR5 mAb, clone 45523 and an Alexa 647 conjugated secondary antibody (red – Panel B), their overlap (yellow – Panel C) and a 3-D close up of direct contacts between CCR5 and FLSC IgG1 on the TZMbl cell membrane (Panels D and E) were imaged by super resolution microscopy (STORM) and total internal reflection fluorescence methodology (TIRF) (Rust et al., 2006).

Fig. 4.

Entry inhibition by FLSC compared with FLSC IgG1. Shown are comparisons of HIV-1 entry inhibition by FLSC and FLSC IgG1 in high CCR5-expressing JC53 cells (Panel A), low CCR5-expressing JC57 cells (Panel B), and primary human PBMCs (Panel C). Results were from BlaM fusion assays.

Fig. 5.

Antiviral activities of FLSC IgG1 and CCR5 antibodies. Antiviral activity was measured by single cycle pseudovirus infection and quantified by luciferase activity. Solid circles represent the antiviral activity of FLSC IgG1; open squares, 2D7; triangles, CD195; open circles, anti-CCR5 NT; and solid diamonds, 45523. Experiments were done twice using JC57 cells and each result done in triplicate. Data are given $\pm$ SD.

Fig. 6.

Effect of CCR5 densities on antiviral activity by FLSC IgG1. Panel A shows the effect of CCR5 density on antiviral activity of FLSC IgG1 in cell lines. JC10 cells (2000 CCR5 molecules/cell) and JC57 cells (9000 CCR5 molecules/cell) were infected with replication competent R5 Bal HIV-1 in the presence of the indicated concentrations of FLSC IgG1. Panel B shows that CCR5 density on primary CD4⁺ T cells correlates inversely with the antiviral activity of FLSC IgG1. Activated PBMCs from different donors were subjected to flow cytometry analysis, and then infected with Bal HIV-1 in the presence of varying concentrations of FLSC IgG1. Panel C shows effect of CCR5 density using a different R5 HIV-1 isolate (CC 1/85) to infect JC10 and JC57 cells. Virus production was measured by p24 ELISA on day 4 or 5 after infection and the data used to determine EC₅₀ values. Data are presented as averages of triplicates $\pm$ SD from two experiments. FLSC IgG1 EC₅₀ values were plotted against CCR5 density values (listed in the legend) on the day of infection. Line fit was done by linear regression where r is Spearman correlation coefficient ($r = 0.90$ – red line). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 7.

CCR5 binding by FLSC IgG1, MVC and FLSCIgG1 plus MVC. Data present the relative fluorescence intensity of residual CCR5 on JC57 cells (9000 CCR5 molecules/cell) stained with CCR5 antibody clone 45531 plus biotin and streptavidin amplification of signal, upon treatment with FLSC IgG1 alone, MVC alone and combined treatment. The isotype control shows minimal fluorescence, as expected. The data are presented as averages of triplicates with $\pm$ SD from three independent experiments. Data are shown as percentages and normalized to CCR5 intensity values obtained in the absence of FLSC IgG1 or MVC.

Fig. 8.

Measurements of bound CCR5 molecules with different anti-CCR5 mAb with or without 10 IM MVC treatment on JC53 cell line using plasmonic substrate methodology. Panel A shows average intensities of cells CCR5 molecules expressed on JC53 cells stained with FLSC IgG1 Alexa 555. The color coded symbols present numbered cells from three different images (three colors). Mean intensities (dashed line) and SD were calculated based on multiple cells within three images and corrected for cell autofluorescence (unstained cells). Number of cells varied within the image from 15 to 20 and were numbered from 1 to 20 within image for MVC(—) and from 25 to 25 for MVC(+). The p value was calculated based on mean, standard deviation and number of cells. Panel B shows normalized mean intensities of JC53 cells untreated or treated with MVC and imaged using different sets of CCR5 primary (2D7, HGS101, and FLSC IgG1) and dye-labeled secondary antibodies. The change in CCR5 expression is proportional to change in intensity. Presented data are of two independent experiments in triplicates, three cell images, with $\pm$ SD. The p values were calculated similarly as described in Panel A, (*) mean $p < 0.0001$.