Full Length Single Chain Fc Protein (FLSC IgG1) as a Potent Antiviral Therapy Candidate: Implications for *In Vivo* Studies

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Abstract

We have previously shown that FLSC, a chimeric protein containing HIV-1_{BAL} gp120 and the D1 and D2 domains of human CD4, blocks the binding and entry of HIV-1 into target cells by occluding CCR5, the major HIV-1 coreceptor. In an effort to improve the antiviral potential of FLSC, we fused it with the hinge-C_H2-C_H3 region of human IgG1. The IgG moiety should increase both the affinity and stability *in vivo* of FLSC, due to the resultant bivalency and an extended serum half-life, thereby increasing its antiviral potency. We previously showed that (FLSC) IgG1 indeed had greater antiviral activity against T cell infections. Here we extend these results to macrophages, for which (FLSC) IgG1 has a more potent antiviral activity than FLSC alone, due in part to its higher binding affinity for CCR5. We also test both compounds in a relevant humanized mouse model and show that, as anticipated, the IgG1 moiety confers a greatly extended half-life. These data, taken together with previous results, suggest potential clinical utility for (FLSC) IgG1 and support further developmental work toward eventual clinical trials.

Introduction

ANTIRETROVIRAL DRUGS THAT TARGET viral enzymes have been extensively employed to treat HIV-1 infection and in general have been highly successful. However, there are drawbacks, including toxicity and side effects as well as the eventual emergence of resistant mutant strains that threaten prophylactic and/or therapeutic efficacy. There remains an urgent need to develop approaches that focus upon alternative targets, particularly those that offer a high threshold of resistance. The next generation of anti-HIV drugs is now focused on agents with prolonged half-lives, especially in the area of preexposure prophylaxis, as well as new agents that block HIV-1 entry into potential target cells.

For target cell entry, HIV-1 requires two cellular proteins, the primary viral receptor, CD4, and either of two coreceptors, CCR5 and CXCR4.^{1–4} CCR5, a G protein-coupled receptor (GPCR), is the predominant HIV-1 coreceptor during horizontal transmission and in the early stages of infection.^{2,5–7} CCR5 is an especially attractive antiviral target

because it is relatively dispensable for normal immune function and human health.^{8–10} People homozygous for a 32-base pair deletion (Δ 32) within CCR5 express a nonfunctional truncated receptor that causes resistance to HIV-1 infection.^{11,12} Entry inhibitors are, in general, attractive since they act at the earliest step of viral replication and immobilize HIV-1 within the extracellular environment, increasing potential exposure to the immune system. Development of CCR5 antagonists, including multiple small allosteric and noncompetitive molecules^{13–20} and several effective CCR5 antibodies^{18,19,21–23} against the ECL2 domain and the N-terminus region of CCR5, has significantly improved the range of choices of antiviral therapies.^{24–26} Our group has identified (FLSC) IgG1, a fusion protein that binds specifically to CCR5,^{27–30} as a more potent CCR5 blocker than commonly used CCR5 antibodies,^{27–30} with faster kinetics in preventing virus binding with T cells.

Pharmaceutical companies have developed multiple small molecule CCR5 blockers that are highly antiviral. Unlike natural β -chemokines, these CCR5 antagonists neither elicit

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CCR5 signal transduction nor cause its internalization. We have previously shown strong synergistic antiviral activity *in vitro*³⁰ using (FLSC) IgG1^{27,28} and a small molecule CCR5 antagonist, maraviroc (MVC).^{13,14,19} This synergy would allow enhanced antiviral efficacy with decreased dosages, costs, and potential side effects of entry inhibitors. A combinatorial approach similar to those used against viral enzymes could maximize the potential of targeting CCR5 and reduce the possibility of the emergence of resistant variants. Although combinatorial antiretroviral therapies against reverse transcriptase and protease have been effectively implemented, no combinatorial approach against the envelope–CCR5 interaction has yet been developed. Our exploratory *in vivo* studies have the potential to provide proof of principle for the development of such a novel approach.

(FLSC) IgG1 is an attractive partner to MVC. Their synergy is due (at least in part) to distinct CCR5 binding sites and mechanisms of activity. (FLSC) IgG1 mimics the CD4-induced CCR5 binding site of HIV-1 gp120_{BAL}, which binds two extracellular sites (N-terminus and ECL2) distinct from the transmembrane binding site of MVC. We previously reported that MVC enhances the activity of (FLSC) IgG1 by allosterically altering the CCR5 conformation, making it more available for binding to the fusion protein.³⁰ In addition, the hinge–CH₂–CH₃ IgG1 region potentially confers several important advantages. First, the IgG1 moiety causes protein dimerization. The resultant bivalency reduces the concentration required for half maximal binding to CCR5 by more than an order of magnitude over FLSC lacking the IgG domain. Second, the IgG domain should also increase protein stability and serum half-life. It should be noted that (FLSC) IgG1 does not induce calcium mobilization or chemotaxis subsequent to CCR5 binding.²⁸ Based upon these observations and our *in vitro* studies showing that MVC is highly synergistic with (FLSC) IgG1,²⁹ we believe that an MVC-(FLSC) IgG1 combination *in vivo* will have high therapeutic efficacy and the potential to be developed for future clinical use.

We extend here our previous work on the greater binding activity and potency of the IgG1 form over the FLSC parental molecule in order to support future *in vivo* studies using a combinatorial approach with (FLSC) IgG1 and MVC. Here we compare the effect on binding of HIV-1 to CCR5 using different CCR5 blockers and we measure the binding affinities of FLSC and (FLSC) IgG1. We demonstrate that the higher antiviral efficacy of the IgG1 version of FLSC in T cells^{29,30} extends to macrophages, another CCR5-rich natural target cell. We also show a 10-fold longer serum half-life of (FLSC) IgG1 compared to FLSC alone, a characteristic that will be important for the development of treatment and prevention strategies.^{31–34}

Materials and Methods

Cell lines and inhibitors

Virus producer HEK 293T/17 cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 100 μ g/ml of penicillin/streptomycin, and 0.5 mg/ml of G418 (Sigma). The HeLa derivative, JC clone JC53 (a gift from Dr. D. Kabat, OHSU, OR),³⁵ expresses CD4 and has a CCR5 surface density of 50,000 mol/cell (measured via flow cytometry). JC53 cells³⁵ and HeLa TZMbls (target cells in the X-gal virus titer assay) were cultured in DMEM supple-

mented with 10% FBS and 100 μ g/ml of penicillin/streptomycin. Human peripheral blood mononuclear cells (PBMCs) were obtained from healthy donors via the New York Blood Center. PBMCs were isolated from blood by Ficoll-Hypaque density gradient centrifugation and cultured in RPMI medium supplemented with 10% FBS and 100 μ g/ml of penicillin/streptomycin. Monocyte-derived macrophages (MDMs) were also isolated from PBMC donors (described later). The (FLSC) IgG1 protein and HIV-1 17b mAb were gifts from Drs. A.L. DeVico and G. Lewis (IHV, UMB, MD). (FLSC) IgG1 was expressed from a synthetic gene that links a single chain gp120_{BAL}–CD4 complex containing a codon optimized R5 gp120_{BAL} sequence with the hinge–C_H2–C_H3 portion of human immunoglobulin subtype 1.²⁷ FLSC itself was a gift from Dr. T. Fouts (Profectus Biosciences, MD). MVC and interleukin (IL)-2³⁶ were obtained from the NIH AIDS Research and Reference Reagent Program (Germantown, MD). PHA used for stimulation of PBMCs was from Roche. Fusion peptide T-20, used as a control in the fusion assay, was a gift from Dr. L.-X. Wang (IHV, UMB, MD). HEK 293T/17 cells, HIV Gag GFP plasmid,³⁷ and TZMbl cells were gifts from Dr. G.B. Melikyan (Emory University, Atlanta, GA).

Confocal microscopy: fluorescent HIV virus binding experiment

JC53 cells used in these experiments express high CCR5 levels due to stable transfection of the parental HI-J clone.³⁵ A fluorescent HIV GFP pseudovirus³⁷ was produced from HEK 293T/17 cells seeded at 3.5×10^6 cells/ml in a 10-cm dish 24 h before transfection. Cells were then transfected with JRFL140T (48 μ g), HIV Gag GFP (7.2 μ g), R8 Δ Env (24 μ g), and pcRev (12 μ g) using calcium phosphate. The cellular medium containing HIV GFP pseudoviruses was collected 48 h posttransfection, centrifuged, and filtered through a 0.45- μ m syringe filter to purify virions from cellular debris and viral clusters. Virus was centrifuged onto a 20% sucrose cushion for 1 h at $55,000 \times g$ (Optima XL-100K Ultracentrifuge; Beckman), then resuspended in phosphate-buffered saline (PBS). JC53 cells (0.5×10^6) were incubated in eight-well imaging chambers (Nunc) with various CCR5 blockers. The CCR5 N-terminus antibody was purified mouse antihuman CCR5, clone T21/8 (BioLegend). The two CCR5 ECL2 antibodies were purified mouse antihuman CD195, clone 2D7 (BD Biosciences), and mouse antihuman CCR5, clone #45531 (R&D Systems). FLSC and (FLSC) IgG1 were also used. Cells were incubated with CCR5 blockers at room temperature for 90 min, washed twice with HBSS, centrifuged with HIV Gag GFP³⁷ at $2095 \times g$ at 4°C for 30 min to allow virus binding to the cell membrane, then fixed with 2% PFA prior to imaging. Cells were analyzed by confocal microscopy on the same day. To analyze the inhibitory effect of CCR5 blockers on virus binding to the cell surface, cell-associated fluorescence was visualized using a Zeiss META LSM 510 confocal microscope and ZEN 2012 lite imaging software (Carl Zeiss, Germany).

Biacore analyses: binding affinities of FLSC alone and (FLSC) IgG1

17b mAb was captured on a CM5 sensor chip by amine-coupling to approximately 125 RU. For kinetic measurements of FLSC and (FLSC) IgG1 binding to immobilized antibody,

sensorgrams were obtained by passing various concentrations of FLSC/(FLSC) IgG1 (0.39–12.5 nM). A buffer injection served as a negative control. Upon completion of each association and dissociation cycle, surfaces were regenerated with 0.1 M glycine, pH 3.0. The data were double referenced by subtraction of the blank surface (no ligand) and a blank injection (no analyte). Binding curves were globally fit to a 1:1 binding model and the binding kinetics [association rates (k_a), dissociation rates (k_d), and affinity constants (K_D)] were calculated with the BIAevaluation software.

β -Lactamase assay for direct virus-cell fusion

Human PBMCs obtained from healthy donors via the New York Blood Center were isolated by Ficoll-Hypaque density gradient centrifugation, and monocytes were immediately isolated using the Monocyte Isolation Kit II (Miltenyi Biotec) and magnetic column separation. Monocytes were then cultured in 10-cm dishes as 10 million monocytes/dish in RPMI containing 20% FBS, 2% penicillin/streptomycin, and 25 ng/ml MCSF (GenScript). Monocytes were allowed to adhere and grow in the presence of MCSF for 3–5 days, and then washed and used for experiments. StemPro Accutase (Invitrogen) was used to resuspend the attached macrophages from dishes.

HIV-1 pseudoviruses containing the BlaM-Vpr insert were produced from HEK 293T/17 cells seeded at 3.5×10^6 cells/ml in a 10-cm dish 24 h before transfection. Cells were then transfected with JRFL140T (48 μ g), R8 Δ Env (24 μ g), pcRev (12 μ g), and BlaM-Vpr (12 μ g) using calcium phosphate. The cellular medium containing HIV-1 BlaM-Vpr virions was collected 48 h posttransfection, centrifuged, and filtered through a 0.45- μ m syringe filter to purify virions from cellular debris and viral clusters. Virus was concentrated on a 20% sucrose cushion for 1 h at $55,000 \times g$ (Optima XL-100K Ultracentrifuge; Beckman), then resuspended in PBS. Viral infectivity was determined to be 1.0×10^7 IU/ml by X-gal assays³⁸ using TZMbl cells as targets. The pCAGGS plasmid encoding JRFL (Binley *et al.*, 2003) was a gift from Dr. J. Binley (Torrey Pines Institute of Molecular Studies, San Diego, CA), the HIV-based packaging vector pR8 Δ Env lacking the “Env” gene was provided by Dr. C. Aiken (Vanderbilt University Medical Center, Nashville, TN), and pcRev³⁹ and BlaM-Vpr⁴⁰ were both obtained from the National Institutes of Health AIDS Research and Reference Reagent Program.

Before adding virus, macrophages (3×10^5 cells/treatment) were incubated at 37°C in serum-free media with various concentrations of FLSC alone or (FLSC) IgG1 for 1 h, then virus was added at MOI=2, and cells continued incubating at 37°C for an additional 2 h. Fusion peptide inhibitor T-20 was added prior to virus addition and during the 37°C fusion process as a positive control. After 2 h of incubation with virus, cells were loaded with the fluorescence substrate CCF4-AM (Invitrogen) for 1 h in the dark. Then the substrate was removed and cells were kept in HBSS/10% FBS/2.5 mM Probenecid (Sigma). BlaM activity was developed overnight at room temp ($\sim 18^\circ\text{C}$) 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, one experiment per human donor, two donors total.

Humanized NSG mouse model

For two *in vivo* experiments, we used NSG mice engrafted with human PBMCs originally established by Nakata *et al.*³¹ They developed human PBMC-transplanted nonobese diabetic-SCID, interleukin 2 receptor γ -chain-knock-out (NOG) mice, which are susceptible to massive and systemic HIV-1 infection with the R5 human immunodeficiency virus type 1 isolate JR-FL (HIV-1_{JR-FL}). Cells were prescreened and identified from several donors to select samples expressing the highest levels of CCR5. PBMCs were cultured with IL-2 for 7 days prior to engraftment. Stimulated PBMCs (10^7) were injected intraperitoneally (ip) in each mouse. After 3 weeks of the engraftment, mice were bled retroorbitally and human CD4/CD8 cell levels were checked to monitor engraftment levels.

Those mice with 70% +human cells were treated as follows: 10 mice were injected ip with FLSC alone and 10 mice were injected with IP with (FLSC) IgG1. Mice were bled 0, 3, 5, 7, 12, 24, and 48 h postinjection, and the half-life in serum for both proteins was monitored on the same day by quantitative ELISA. In a second *in vivo* experiment to further validate the model and measure CD4⁺ T cells, CCR5⁺ cells, and CCR5 expression, mice were engrafted for 3 weeks with a prescreened CCR5-expressing donor. Three weeks post-engraftment, mice were infected with HIV-1_{BAL} or inoculated with PBS. All mice were bled retroorbitally on days 0, 7, 12, 18, 24, and 28 postinfection, and flow cytometry evaluation was done on the same day. *In vivo* experiments were approved by the IACUC, protocol number 0213014.

Flow cytometric analyses of CD4⁺ T cells, CCR5⁺ cells, and CCR5 expression

Mouse whole blood (30 μ l) was mixed with 30 μ l of RPMI medium containing 20% FBS and 2% penicillin/streptomycin and incubated at room temperature for 10 min. Fluorescent antibodies were then added to blood and the samples were vortexed and incubated at room temperature in the dark for 20 min. The fluorescent antibody mixture for each sample contained 3.5 μ l mouse antihuman CD3 APC (BD Biosciences, clone UCHT1), 1.5 μ l mouse antihuman CD4 FITC (BD Biosciences, clone RPA-T4), and 20 μ l mouse antihuman CD195 (BD Biosciences, clone 2D7/CCR5). After incubation with antibodies, 500 μ l of 1 $\times$ BD lysis solution (BD Biosciences) was added to each sample and the samples were vortexed and incubated at room temperature for 4 min. Following that, 150 μ l of Beckman Coulter Fixative solution (Beckman Coulter) was added to each sample, and the samples were again vortexed. Then 1 ml of wash buffer (1% FBS in PBS) was added to the samples, which were again vortexed, and an additional 2 ml of washing buffer was added. Samples were centrifuged at 2,500 rpm for 5 min. Supernatant was discarded, and samples washed again in 2 ml wash buffer. Samples were resuspended in 150 μ l 1% paraformaldehyde (Electron Microscopy Sciences) and analyzed using a BD FACS Calibur flow cytometer (BD Biosciences).

Results

Inhibition of virion binding by CCR5 blocking agents

We tested the ability of different CCR5 blocking agents to inhibit the binding of fluorescent HIV-1 pseudovirions to

pretreated JC53 HeLa clones. Virus binding was performed by spinoculation as previously described.^{37,41} Cell-associated fluorescence due to bound virus was visualized and quantified using standard confocal microscopy (Zeiss META 510) (Fig. 1). The target cells were pretreated for 1 h with the indicated CCR5 blockers. Figure 1A shows HIV Gag GFP bound to JC53 cells after centrifugation with virus at 4°C, with no pretreatment. The other panels show the effects of pretreatment by CCR5 blockers. Figure 1B shows the results from pretreatment with 100 μ g/ml of the N-terminus antibody. Figure 1C shows the results from pretreatment with 50 μ g/ml 45531 (anti ECL2) antibody. Figure 1D shows the results from pretreatment with 50 μ g/ml 2D7 Ab (anti ECL2). Figure 1E shows the results from pretreatment with 50 μ g/ml FLSC. Figure 1F shows the results from treatment with 50 μ g/ml (FLSC) IgG1 Ab. Figure 1G shows the normalized relative staining intensity of CCR5 in these experiments. Virion binding showed only a 10% decrease after pretreatment with N-terminus antibody in comparison to nontreated cells, a 24% decrease after pretreatment with 45531 and a 50% decrease when pretreated with 2D7. When cells were pretreated with FLSC or (FLSC) IgG1, binding was decreased 60% and 80%, respectively. Thus, the strongest inhibition of virion binding at comparable levels of protein was obtained with (FLSC) IgG1. A similar experiment performed with PBMC-derived macrophages showed the same trend among CCR5 blockers (data not shown).

FLSC and (FLSC) IgG1 binding affinities

To determine whether its greater inhibitory effect is due to the increased affinity of (FLSC) IgG1 for CCR5, we compared binding affinities of FLSC and (FLSC) IgG1 to Ab 17b. 17b specifically binds to the CCR5 binding site and is therefore a CCR5 proxy. (FLSC) IgG1 bound to 17b with a 10-fold higher affinity than FLSC, as judged by surface plasmon resonance (SPR) analysis (Fig. 2A–C). These data strongly suggest that the greater blocking potency of (FLSC) IgG1 is at least in part the result of an increased affinity for CCR5. In addition, however, (FLSC) IgG1 is dimeric, with two FLSC arms, is directly involved in CCR5 engagement, and contains an Fc portion. Its greater size could possibly interfere with the accessibility of CCR5 to viral epitopes by greater steric hindrance.

(FLSC) IgG1 inhibition of HIV-1 entry into macrophages is greater than that of FLSC

We previously demonstrated that (FLSC) IgG1 had greater antiviral activity than did FLSC in T cells and PBMCs.^{29,30} To further establish the potential utility of (FLSC) IgG1, we performed similar experiments in macrophages, another important target cell in HIV-1 infection. Monocytes were isolated from human PBMCs and allowed to differentiate into macrophages as described in Materials and Methods. Macrophages were pretreated in serum-free media with 0, 0.01, 0.1, 1, 10, 100, and 1,000 μ g/ml of either FLSC or (FLSC) IgG1 for 1 h at 37°C. CCR5-tropic JRFL Env-pseudotyped virions containing a Vpr-BlaM fusion protein were added. BlaM activity occurs following release of the viral core-associated enzyme into the cytosol and the addition of the CCF4-AM fluorescent substrate. As shown in Fig. 3, open symbols represent the antiviral activity of FLSC and solid

FIG. 1. Effect of CCR5 blockers on virion binding. JC53 cells were preincubated with or without different CCR5 blockers, and then centrifuged with HIV Gag GFP pseudovirus at 4°C. (A–F) HIV Gag GFP bound to JC53 cells after centrifugation at 4°C: (A) with cells not pretreated; (B) cells pretreated with the N-terminus Ab; (C) cells pretreated with 45531 Ab; (D) cells pretreated with 2D7 Ab; (E) cells pretreated with FLSC; and (F) cells pretreated with (FLSC) IgG1 Ab. (G) The normalized relative staining intensity of CCR5 in the samples shown in A–F. AF represents unstained cells as an autofluorescence control. Bar size is 10 μ m.

FIG. 2. FLSC and (FLSC) IgG1 binding to gp120 Ab 17b. Binding was measured by surface plasmon resonance using a Biacore machine. (A, B) Binding curves were generated using different concentrations of FLSC or (FLSC) IgG1 (0.39–12.5 nM, 2-fold dilutions) injected over mAb 17b captured on CM5 sensor chips. The data were double referenced by subtraction of the blank surface (no antibody) and a blank injection (no analyte). Binding curves were globally fit to a 1:1 binding model. All experiments were repeated with similar results. (C) Binding kinetics of 17b mAb to monomeric FLSC and (FLSC) IgG1. Antibody association rates (k_a), dissociation rates (k_d), and affinity constants (K_D) were calculated with the BIAevaluation software.

symbols represent the antiviral activity of (FLSC) IgG1. Both proteins show strong dose-dependent antiviral activity. However, (FLSC) IgG1 inhibited HIV-1 entry more potently; EC₅₀ values for FLSC and (FLSC) IgG1 were 10 μg/ml and 0.3 μg/ml, respectively, in general agreement with our previous data in T cells and PBMCs.³⁰

Extended in vivo half-life of (FLSC) IgG1 in NSG mice

Future studies will require a convenient and reliable animal model that mimics the natural course of infection in humans. The immunodeficient NSG mouse transplantation/engraftment model is the best model currently available. The specific NOG.Cg-PrkdcscidIL-2rg-/- strain is better suited to our purposes than other SCID/NOG strains, as they lack NK, T, and B cells and allow development of these lineages, as well as macrophage lineage cells, of human origin.^{31,40} This strain is superior to other SCID mice at accommodating human

FIG. 3. Comparative antiviral activity of FLSC and (FLSC) IgG1 on human macrophages. The entry of virus into macrophages in the presence of the indicated input of blockers was measured by BlaM fusion assays. The experiment was done with two different donors, each in triplicate. Results are averaged and given as ± SD.

PBMCs,⁴² requiring approximately a 10- to 100-fold lower dose of human cells, and does not develop thymomas, which occur in ~30% of classic NSG mice by the age of 6 months.³¹

It was important to confirm that these mice were infectable under our conditions. They were injected intraperitoneally (ip) with 20,000 units of 50% tissue culture infective dose (TCID_{50%}) of HIV-1, a viral titer that gave the highest productive infection among the three titers tested (10,000, 20,000, and 30,000 TCID_{50%}) (not shown). Both groups of mice, noninfected and infected, were injected daily with PBS for the duration of the experiment (28 days) to mimic future experiments with drug treatments. We also included a control group of HIV-1-infected mice that were not injected with PBS in order to monitor needle and dehydration influences on infected mice, and the overall effect was similar to that of HIV-1-infected mice injected with PBS. CD4⁺ T and CCR5⁺ cells and CCR5 surface densities were measured on the same days as the other two groups of mice (data not shown). Each experiment was performed twice. Figure 4 presents results from two representative experiments. By standard quantitative RT-PCR,^{32,43} there was clear evidence of productive infection. HIV-1 RNA was not detectable in noninfected mice ($n=5$). With HIV-1-infected mice ($n=20$), mean serum HIV-1 RNA varied between thousands and hundreds of thousands of copies/ml on most days postinfection (data not shown).

We then quantified CD4⁺ T cells, CCR5⁺ cells, and CCR5 expression levels to further evaluate the validity of the model. Figure 4 shows data from days 0, 7, 12, 18, 24, and 28 in both noninfected and infected mice, obtained by flow cytometry.^{32,43} Both CD4⁺ (Fig. 4A) and CCR5⁺ cells from within the CD4⁺ T cell population (Fig. 4B) showed a constant number in uninfected mice during the course of the experiment. There was a slight decrease in CCR5 expression in Fig. 4C, which may be due to small changes in lymphocyte metabolism. Infected mice, presented in Fig. 4A-1 and 4B-1, showed a steep decline in both CD4⁺ T cells and CCR5⁺ cells,⁴⁴ as anticipated, as well as a somewhat greater decrease

FIG. 4. CD4⁺ T and CCR5⁺ cell survival and CCR5 expression in noninfected and HIV-infected NSG humanized mice. CD4⁺ T and CCR5⁺ cells (within the CD4⁺ T cell population) and CCR5 expression/cell were measured as described in Materials and Methods. (A, B, C) The values obtained with HIV-negative mice; (A-1, B-1, C-1) data obtained with the HIV-positive mice. Numbers in the legends present individual, tagged mice.

in CCR5 expression (Fig. 4C-1), starting at day 7 postinfection. Similar differences between HIV-1-positive and HIV-1-negative mice were observed with CD4/CD8 ratios (data not shown). These data rule out the possibility that the decrease in CD4⁺ T and CCR5⁺ cells in infected mice is due to an engraftment failure, but is rather due to productive HIV-1 infection. We monitored weight and hair loss at each of the bleeding dates and confirmed the relative stability of both (within 10–20% variation), in agreement with Lockridge *et al.*⁴⁵ and Pitcher *et al.*⁴⁶ until the end of the experiment (data not shown). Taken together, these data show the utility of this NSG humanized mouse model for our purposes.

Confident that these NSG mice will be suitable for future studies, we next compared the serum half-life of FLSC and (FLSC) IgG1 *in vivo*. We injected both proteins into two groups of mice and measured the residual protein in serum from blood taken at 0, 3, 5, 7, 12, 24, and 48 h postinjection. Figure 5 presents the FLSC levels (ng/ml) at the indicated times after protein injection. Triangles represent (FLSC) IgG1 and solid circles represent FLSC. Both black curves correspond to FLSC and (FLSC) IgG1, respectively, obtained by performing nonlinear fits using Prism software. These gave a half-life of 30 h for (FLSC) IgG1 and 3 h for FLSC. These data suggest that, as anticipated, the IgG1 moiety confers a far greater stability *in vivo* to FLSC.

Discussion

The current rate of 2.5 million new HIV-1 cases per year indicates an ongoing need for effective prevention, treatment, and cure. Recently clinical indications for antiviral therapy have been expanded to include preexposure prophylaxis (PreP).⁴⁷ This has added to the interest in developing long

FIG. 5. Circulating half-lives: FLSC alone versus (FLSC) IgG1 in humanized NSG mice. Curved lines are mathematical nonlinear fits calculated based on Prism software.

acting antiretroviral agents that enhance compliance, especially in the setting of preexposure prophylaxis.^{48–50} Antibody-based products have a long history of clinical utility for preventing viral infections and might represent improvements over existing approaches,^{51–53} especially since by their nature they would be administered by a directly observed modality of therapy.

In previous work, we identified a CCR5 binding protein, (FLSC) IgG1, which is more potent than commonly used CCR5 Abs^{27–30} in blocking HIV-1 entry. FLSC mimics complexes of gp120 and CD4 in which the CCR5 binding site is relatively exposed. (FLSC) IgG1 is derived from FLSC by fusion with the hinge-C_H2-C_H3 region of human IgG1. (FLSC) IgG1, like the parental FLSC, efficiently binds to CCR5, inhibiting HIV-1 infection *in vitro*.^{27–30} The IgG1 moiety provides several important theoretical advantages, due to protein dimerization. The resultant bivalency should reduce the concentration required for half maximal binding to CCR5, and we show this to be the case by more than an order of magnitude, at least partially explaining why it blocks viral entry more effectively than the monomeric FLSC. It should also increase protein stability and serum half-life,^{54,55} and we show that its half-life in mouse serum is indeed greatly lengthened. We also confirm that after pretreatment with several CCR5 mAbs, FLSC, or (FLSC) IgG1, the greatest inhibitory effect is seen in T cells with (FLSC) IgG1, and we extend this observation to include macrophages, another HIV-1 important target cell.

(FLSC) IgG1 thus represents a strong antiretroviral agent candidate with an ideal drug profile for use, particularly in the setting of preexposure prophylaxis, which can be hampered by patient noncompliance. These results support the development of (FLSC) IgG1 *in vivo* in a model system as a potential antiviral agent. We have previously shown that the antiviral efficacy of (FLSC) IgG1 is synergistically increased when used in combination with the only clinically approved CCR5 antagonist, MVC (Pfizer, 2007), *in vitro*.^{30,56,57} It therefore seems important to proceed with studies using the same combination [MVC/(FLSC) IgG1] to test the inhibition of HIV-1 infection *in vivo*. We plan to pursue this using a well-established humanized SCID/NOG mouse model³¹ as proof of principle for potential further clinical studies.

To confirm the validity of the humanized NSG mouse model, we determined the optimum infectious dose and measured CD4⁺ T and CCR5⁺ cell levels and CCR5 expression per cell in noninfected and infected mice. We observed steady levels of both CD4⁺ T and CCR5⁺ cells for up to day 28 postengraftment of donor cells in noninfected mice. In infected mice, in contrast, both CD4⁺ T and CCR5⁺ cell levels decreased significantly by 7 days after infection (3 weeks after cell engraftment). Therefore, in our future studies, we will use this mouse model to evaluate the efficacy of (FLSC) IgG1 *in vivo*, both alone and in combination with MVC. We note that as only T cells are engrafted in SCID/NOG mice, we do not expect Fc cell receptors to interact with the Fc portion of the (FLSC) IgG1, since the T cells lack such receptors.

We recognize that a potential drawback of (FLSC) IgG1 in a clinical setting could result from an induction of antibodies against CD4-induced sites on gp120 (CD4i Abs) in infected people, which would diminish its potency and its pharmacodynamic advantages. In principle, this could be overcome by an increase in dosage. However, few of the epitopes are

cross-reactive⁵⁸; thus, most would not recognize (FLSC) IgG1. The few potential cross-reactive epitopes can be ablated by additional mutations introduced into the gp120 or the CD4 moieties to further abrogate binding of such antibodies.⁵⁶ Alternatively, use of a SIVgp120–CD4 complex, which also binds CCR5,⁵⁶ would avoid cross-reactivity with antibodies to the HIV gp120 coreceptor binding site. Recognizing these limitations, we believe that the potential advantages of a combination of anti-CCR5 therapy that includes a stabilizing IgG moiety, as proposed here for (FLSC) IgG1, hold potential, especially as related to the expanded clinical indications for preexposure prophylaxis.

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Author Disclosure Statement

No competing financial interests exist.

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