CCR5: A RECEPTOR AT THE CENTER STAGE IN INFECTION

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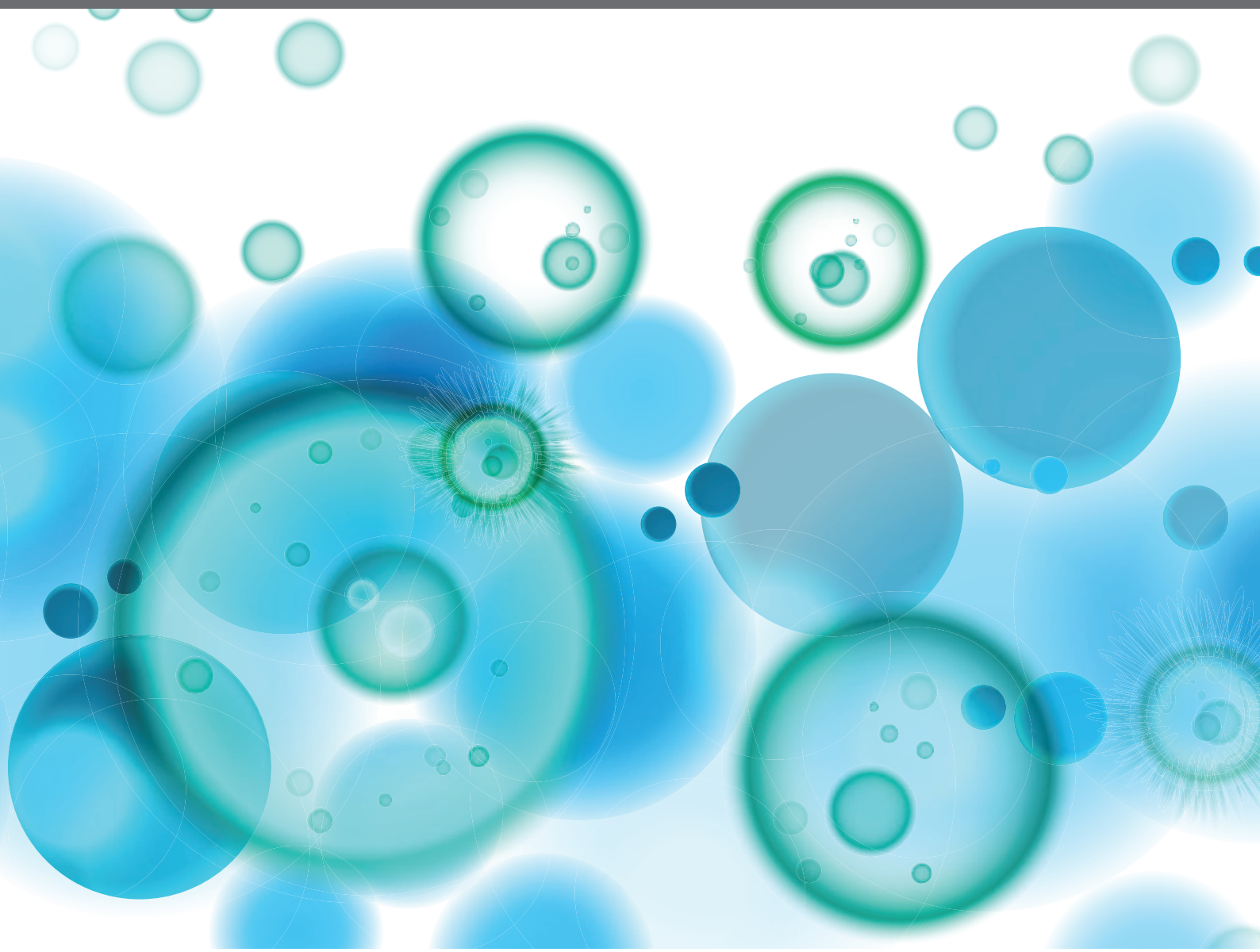

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CCR5: A RECEPTOR AT THE CENTER STAGE IN INFECTION

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Editorial: CCR5: A receptor at the center stage in infection

Joel Henrique Ellwanger[1], Massimiliano Secchi[2], Julio Aliberti[3] and Luca Vangelista[4]*

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Editorial on the Research Topic
CCR5: A receptor at the center stage in infection

CCR5: As receptor that has shaped science

The human C-C chemokine receptor type 5 (CCR5) is mostly expressed on the surface of leukocytes, playing a pivotal role in inflammatory responses and other immune functions (Figure 1) (1, 2). In 1996, CCR5 was reported as the HIV-1 co-receptor (3), and the 32-nucleotide deletion in the CCR5 gene (CCR5Δ32) was reported as a resistance factor to HIV-1 infection (4, 5). These discoveries massively advanced HIV-1 research, bringing insights into resistance mechanisms against HIV-1 and leading to the development of new anti-viral therapies. The clinical use of the CCR5 antagonist maraviroc for HIV-1 treatment was approved in 2007, and cases of sustained remission of HIV-1 infection following stem-cell transplantation using CCR5Δ32 homozygous cells were reported in the following years (e.g., the Berlin Patient in 2009 and the London Patient in 2019) (2, 6, 7).

CCR5 was initially studied in different populations in the context of HIV-1 infection. More recently, it has become clear that CCR5 influences various health and pathological conditions, including infectious diseases other than HIV-1 infection. For example, CCR5 and its agonists participate in the immune responses to Zika virus (8), severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (9), *Schistosoma* spp. (10), among other pathogens (2, 10), and the CCR5 Δ32 variant is a critical risk factor for symptomatic West Nile virus infection (11, 12). Moreover, human pathogens, such as *Toxoplasma gondii* produce CCR5-binding molecules that can affect immune response to infection as well as block R5 tropic HIV-1 entry in CCR5-expressing cells (13–15).

Therapies involving CCR5 blockade have advanced substantially, with the potential to be applied in infectious and non-infectious diseases (1, 16, 17). CCR5 also drives several debates, including those on gene-editing technologies (18) and chemokine system

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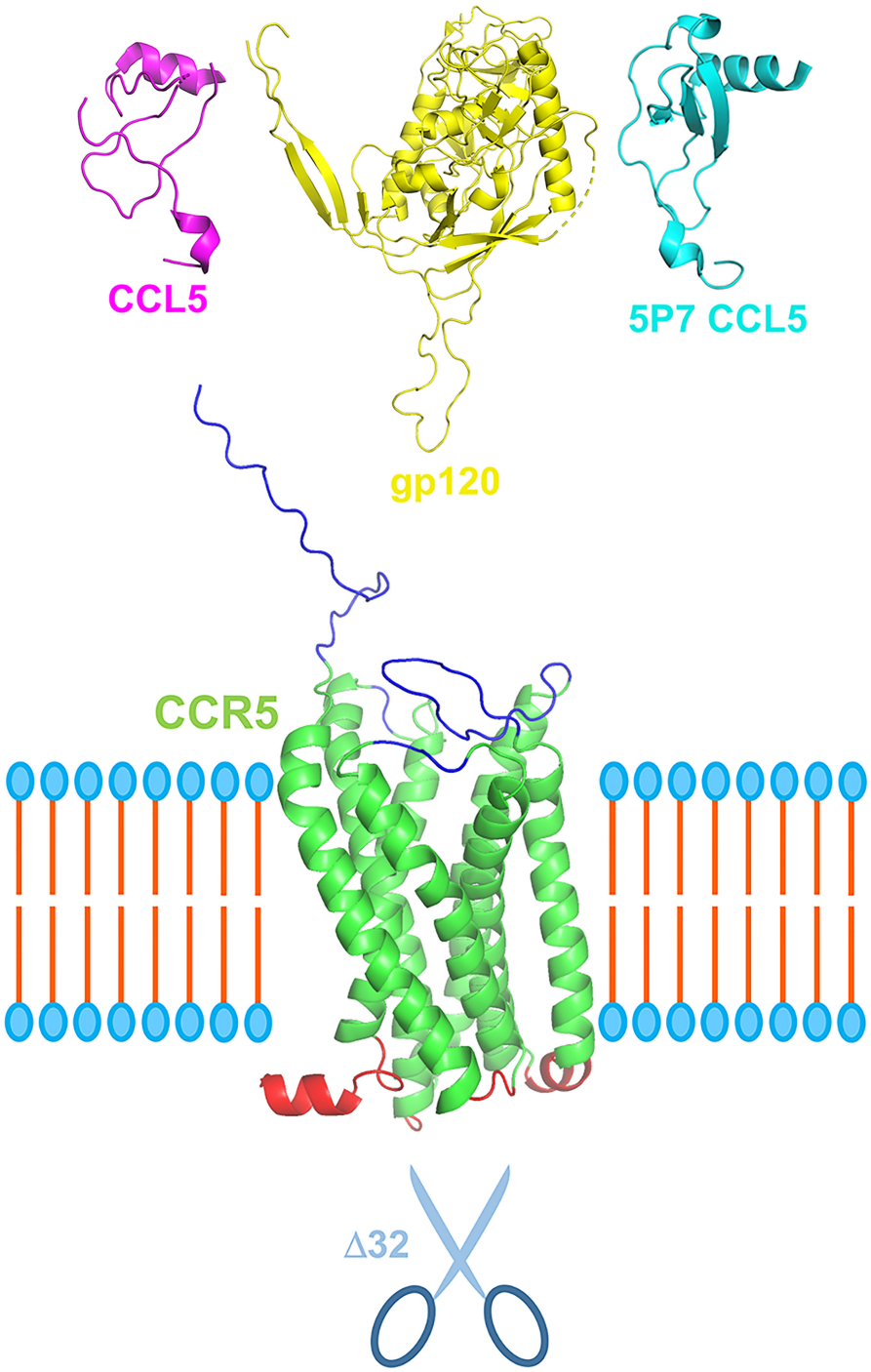

FIGURE 1
Schematic representation of CCR5 in its central role in pathophysiology. CCR5 (green), depicted here with extracellular portions in blue and intracellular in red, can be engaged by its natural ligands, e.g., CCL5 (purple), but also by HIV-1 gp120 (yellow) *via* its V3 loop. CCR5 antagonists, e.g., 5P7 CCL5 (light blue), can block the receptor in its inactive form preventing receptor internalization and engagement by HIV-1 gp120. More in general, CCR5 antagonists such as chemokine derivatives, small chemical compound (e.g., maraviroc) and monoclonal antibodies (e.g., leronlimab) could provide a therapeutic landscape for the array of infectious and inflammatory diseases in which CCR5 plays a central role. Scissors represent the CCR5 Δ32 deletion variant that confers resistance to HIV-1 infection and proved relevant for other CCR5-centered diseases. Three dimensional ribbon representation were generated using PyMOL: CCR5 and gp120 from PDB entry 6MEO, CCL5 from 7F1R, and 5P7 CCL5 from 5UIW. The relative sizes of the different proteins are not to scale. The cell membrane bilayer is schematized.

redundancy and robustness (19). CCR5 is exemplary of how a single molecule may shape different research fields, and many advances related to CCR5 continue to be made, as highlighted by articles published on this Research Topic.

New contributions to CCR5 research

This Research Topic brings together important contributions to the understanding of CCR5 biology and participation of this receptor in numerous aspects of infectious diseases. Using machine learning methods and data from human samples, Patterson et al. explored the immune spectrum of SARS-CoV-2 infection, including the impact of CCR5 and its ligands on COVID-19. Analyzing different patient profiles, the study showed that severe COVID-19 cases are characterized by excessive inflammation and dysregulated T cell activity. Patterson et al. also characterized the immune profile of post-acute sequelae of COVID-19 (PASC) patients. In brief, this study reports important data to the understanding of the participation of CCR5 and other immune molecules in the COVID-19 spectrum, including the intriguing PASC cases, and describes tolls to predict COVID-19-related immune outcomes.

Exploring basic aspects of CCR5-HIV-1 interactions, the article by Picton et al. evidenced the genetic predisposition to lower CCR5 expression in individuals who naturally control HIV-1, based on data from black South African individuals. This is a relevant and updated contribution to the understanding of differential progression of HIV-1 infection, especially by focusing on a sub-Saharan population. The debate about the CCR5-HIV-1 interactions was also advanced and updated by Jasinska et al., who addressed the CCR5 as a co-receptor for HIV-1 and simian immunodeficiency viruses in an interesting review. This is a great reference for those seeking accurate and relevant information concerning CCR5 in evolutionary and host-pathogen interaction perspectives. In a complementary way, Mohamed et al. reviewed the efficacy of CCR5-based HIV-1 therapies, also describing important information concerning CCR5 biology. Several mechanisms to control HIV-1 infection progression are discussed in the article, including the use of small-molecule inhibitors, anti-CCR5 antibodies, disruption of CCR5 expression, and *CCR5*-editing strategies.

Considering the next generation of HIV-1 therapies, the work by Karuppusamy et al. described important data on the use of *CCR5*-edited $CD34^+CD90^+$ hematopoietic stem cells as a graft source for HIV-1 gene therapy. This is an exciting and detailed study with numerous *in vitro* and *in vivo* (animal) experiments. The potentialities of *CCR5*-editing were also evaluated by Scheller et al., who showed in a proof-of-concept study that targeting cells using CRISPR-Cas9 mediated HDR (homology directed repair) enables the selection of mutant cells that are CCR5 deficient and highly resistant to HIV-1 infection. Together, results from Karuppusamy et al. and Scheller et al. move forward the research on HIV-1 gene therapies. Amerzhanova and Vangelista explored structural details of the occupancy of CCR5 orthosteric site by several antagonist ligands, focusing on the 3D modeling analysis of CCL5 mutants, and discussing their likely contributions to HIV-1 therapy as well as the entire spectrum of diseases where this receptor is central. Classic and novel strategies to block G protein-coupled receptors (GPCRs) have been discussed and related to CCR5 blockade. Finally, the study by Chang et al. also brings an important contribution to HIV-1 treatment investigation based on CCR5 blockade. With data obtained from humans and non-human primates, the authors evidenced increased peripheral blood $CCR5^+CD4^+$ T cells following treatment with leronlimab, a promising anti-CCR5 antibody. Chang et al. also bring contributions concerning the monitoring of the use of anti-CCR5 therapeutic antibodies and the impacts of CCR5 blockade on the immune function.

Kulmann-Leal et al. reviewed the impacts of CCR5Δ32 on the Brazilian population, discussing how the colonization of Brazil shaped the CCR5Δ32 distribution in different regions of the country. The article showed that CCR5Δ32 affects cancer, inflammatory conditions, and infectious diseases heterogeneously in Brazilians, with particular influences on each disease. The influences of CCR5 on influenza virus infection were reviewed by Ferrero et al.; with an informative and didactic figure, the authors addressed specifically the dual role of CCR5 during influenza-related immune responses, bringing insights into treatment opportunities. Taken together, these two reviews show how the CCR5 impact on different diseases is relatively complex and cannot be generalized. In this context, Bauss et al. used the CCR5 as a study model to combat biological reductionism. Using a set of bioinformatics tools and student participation, the article evidenced the biological complexity of CCR5, highlighting its involvement in numerous biological contexts, beyond HIV-1 infection. This article brings contributions to a deep understanding of CCR5 functions in the human body, and demonstrates how CCR5 can be used as a tool for addressing biological and social debates.

Conclusion

This Research Topic highlights studies addressing different aspects of CCR5 in infection, contributing to the understanding of CCR5 participation in immune responses (in health and disease contexts), and reporting updated information and new data on therapeutic potentials of CCR5 modulation and gene-

editing. This Research Topic will be useful to readers from multiple fields, and confirm that CCR5 continues to be under the spotlight of research involving immunology and infectious diseases.

Author contributions

JE wrote the first draft of the manuscript. LV prepared the figure. LV, MS and JA edited and complemented the text. LV supervised the work. All authors revised and approved the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Immune-Based Prediction of COVID-19 Severity and Chronicity Decoded Using Machine Learning

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Expression of CCR5 and its cognate ligands have been implicated in COVID-19 pathogenesis, consequently therapeutics directed against CCR5 are being investigated. Here, we explored the role of CCR5 and its ligands across the immunologic spectrum of COVID-19. We used a bioinformatics approach to predict and model the immunologic phases of COVID so that effective treatment strategies can be devised and monitored. We investigated 224 individuals including healthy controls and patients spanning the COVID-19 disease continuum. We assessed the plasma and isolated peripheral blood mononuclear cells (PBMCs) from 29 healthy controls, 26 Mild-Moderate COVID-19 individuals, 48 Severe COVID-19 individuals, and 121 individuals with post-acute sequelae of COVID-19 (PASC) symptoms. Immune subset profiling and a 14-plex cytokine panel were run on all patients from each group. B-cells were significantly elevated compared to healthy control individuals ($P<0.001$) as was the CD14+, CD16+, CCR5+ monocytic subset ($P<0.001$). CD4 and CD8 positive T-cells expressing PD-1 as well as T-regulatory cells were significantly lower than healthy controls ($P<0.001$ and $P=0.01$ respectively). CCL5/RANTES, IL-2, IL-4, CCL3, IL-6, IL-10, IFN-γ, and VEGF were all significantly elevated compared to healthy controls (all $P<0.001$). Conversely GM-CSF and CCL4 were in significantly lower levels than healthy controls ($P=0.01$). Data were further analyzed and the classes were balanced using SMOTE. With a balanced working dataset, we constructed 3 random forest classifiers: a multi-class predictor, a Severe disease group binary classifier and a PASC binary classifier. Models were also analyzed for feature importance to identify relevant cytokines to generate a disease score. Multi-class models generated a score specific for the PASC patients and defined as S1 = (IFN-γ + IL-2)/CCL4-MIP-1β. Second, a score for the Severe COVID-19 patients was defined as S2 = (IL-6+sCD40L/1000 + VEGF/10 + 10*IL-10)/(IL-2 + IL-8). Severe COVID-19 patients are characterized by excessive inflammation and dysregulated T cell activation, recruitment, and counteracting activities. While PASC patients are characterized by a profile able to induce the activation of effector T cells with pro-inflammatory properties and the capacity of generating an effective immune response to eliminate the virus but without the proper recruitment signals to attract activated T cells.

Keywords: COVID-19, PASC, cytokines, chemokines, CCR5

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INTRODUCTION

Post-acute sequelae of COVID-19 (PASC) is a group of previously infected individuals who experience a multitude of symptoms from several weeks to months after recovering from their acute illness and presumably months after viral clearance. The prevalence of PASC ranges from 10% to 30% of all individuals infected with SARS-CoV-2 (1). These symptoms include joint pain, muscle aches, fatigue, "brain fog" and others. These symptoms can commonly resemble rheumatic diseases such as rheumatoid arthritis, autoimmune disorders, and others such as fibromyalgia and chronic fatigue syndrome (2). Many of these common disorders are caused by inflammation, hyper- and/or auto-immunity and some such as chronic fatigue are associated with viral persistence after an acute infection with pathogens such as Epstein Barr virus (EBV) and Human Cytomegalovirus (CMV) (3). Previous studies demonstrated that elevations of CCL5/RANTES, IL-6 and to a lesser extent TNF-α were elevated in acute COVID-19 (4). Although patients improved using CCR5 antagonists, the levels of these cytokines decreased but not to normal levels suggesting persistent cytokinemia following discharge from hospitals. In addition, studies including those from our laboratory, have suggested that PASC may be caused by persistent SARS-CoV-2 itself (5). Here, we sought to identify possible immunologic signatures of COVID-19 severity and to determine whether PASC might represent a distinct immunologic condition compared to Mild to Moderate (MM) or Severe COVID-19. Further, we addressed the question whether the immunologic profile represents an immune response indicative of prolonged or chronic antigenic exposure. Using machine learning, we identified algorithms that allowed for accurate determination of PASC and Severe COVID immunotypes. Finally, we present a quantitative immunologic score that could be used to stratify patients to therapy and/or non-subjectively measure response to therapy.

MATERIALS AND METHODS

Patients

Following informed consent, whole blood was collected in a 10 mL EDTA tube and a 10 mL plasma preparation tube (PPT). A total of 224 individuals were enrolled in the study consisting of 29 healthy control individuals (negative for both SARS-CoV-2 RNA and SARS-CoV-2 IgM/IgG serology), 26 Mild-Moderate COVID-19 patients, 48 Severe COVID-19 patients and 121 chronic COVID (PASC) individuals (enrolled through the Chronic COVID Treatment Center following informed consent, Protocol CCTC 20-001). PASCs symptoms are listed in **Figure 1**. Study subjects were stratified according to the following criteria.

Mild

1. Fever, cough, sore throat, malaise, headache, myalgia, nausea, diarrhea, loss of taste and smell
2. No sign of pneumonia on chest imaging (CXR or CT Chest)
3. No shortness of breath or dyspnea

Moderate:

1. Radiological findings of pneumonia fever and respiratory symptoms
2. Saturation of oxygen (SpO2) ≥ 94% on room air at sea level

Severe:

1. Saturation of oxygen (SpO2) < 94% on room air at sea level
2. Arterial partial pressure of oxygen (PaO2)/fraction of inspired oxygen (FiO2) < 300mmHG
3. Lung infiltrate > 50% within 24 to 48 hours
4. Heart Rate ≥ 125 bpm
5. Respiratory rate ≥ 30 breaths per minute

PASC

1. Extending beyond 12 weeks from the initial onset of first symptoms.

High Parameter Immune Profiling/Flow Cytometry

Peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood using Lymphoprep density gradient (STEMCELL Technologies, Vancouver, Canada). Aliquots (6) of 5 x 10^5 cells were frozen in media that contained 90% fetal bovine serum (HyClone, Logan, UT) and 10% dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO) and stored at -70°C. Cells (5 x 10^5) were stained and analyzed as previously described (4) using a 14-color antibody cocktail with the volumes indicated (**Supplementary Table 1**). Samples were analyzed on a Beckman Coulter CytoFlex LX 6-laser flow cytometer using Kaluza Analysis Software (Beckman-Coulter, Miami, FL). All statistical analysis was performed using the Mann-Whitney test and a P value ≤0.05 was considered statistically significant.

Multiplex Cytokine Quantification

Fresh plasma was used for cytokine quantification using a customized 14-plex bead based flow cytometric assay (IncellKINE, IncellDx, Inc) on a CytoFlex flow cytometer as previously described using the following analytes: TNF-α, IL-4, IL-13, IL-2, GM-CSF, sCD40L, CCL5 (RANTES), CCL3 (MIP-1α), IL-6, IL-10, IFN-γ, VEGF, IL-8, and CCL4 (MIP-1β) (4). For each patient sample, 25 µL of plasma was used in each well of a 96-well plate. Samples were analyzed on a Beckman Coulter

Abbreviations: IL, interleukin; RANTES, regulation on activation, healthy control T-expressed and secreted; CCR, chemokine receptor; IFN, interferon; TNF, tumor necrosis factor; MIP, macrophage inflammatory protein; GM-CSF, granulocyte-macrophage colony-stimulating factor; VEGF, vascular endothelial growth factor; HIV, human immunodeficiency virus; HCV, hepatitis C virus.

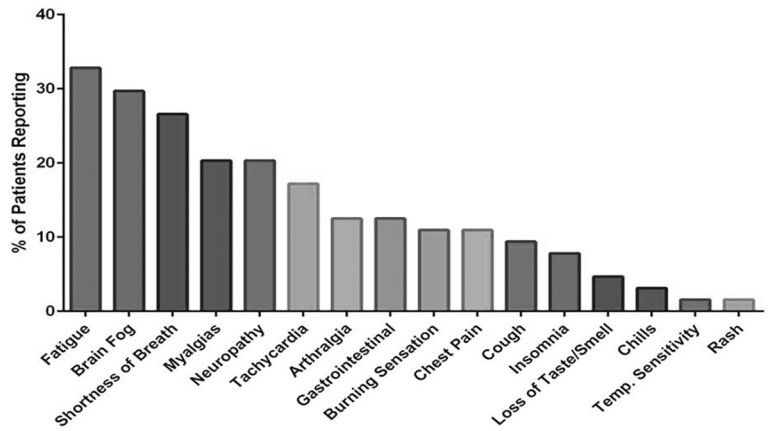

FIGURE 1 | Symptoms reported by PASC patients enrolled in the study.

CytoFlex LX 3-laser flow cytometer using Kaluza Analysis Software (Beckman-Coulter, Miami, FL). All statistical analysis was performed using the Mann-Whitney test and a P value ≤0.05 was considered statistically significant.

Data Processing

Although we have previously defined healthy, Mild, Moderate, Severe, and PASC patients, for downstream analysis we have divided the patients into 4 classes: Healthy control (healthy patients), Mild-Moderate (including the Mild and Moderate patients), Severe, and PASC. Data was imported and processed using Python 3.8.3, using the *pandas* library (version 1.1.0) (7). and the numeric python module, *numpy* version 1.18.5 (8). Our data consisted of 224 instances representing 4 classes (healthy control, Mild-Moderate, Severe and PASC). The dataset consisted of 16 columns, of which 14 represented the different cytokine/chemokine analytes, one for the patient identifier and one column for the label, or the class to which the patient belonged (healthy control, Mild-Moderate, Severe or PASC).

We identified imbalanced class labels in our dataset, and thus decided to proceed to balance the dataset. In order to adequately do data balancing, it was necessary to separate the data into training, validation and test sets. We used the 60/20/20 schema, with a 20% validation partition to assess model overfitting after training, and 20% of data for class label prediction. Data partitions needed to be implemented in order to ensure that generated samples would be present in the training set only. It is necessary to avoid generated samples in either the validation or test set because their presence in either can lead to overfitting and spurious results.

Data Balancing With Synthetic Oversampling of the Minority Class

The 4 classes in our dataset were composed of different numbers of instances. If the variation between the number of classes is large enough, it can lead to a phenomenon identified as class imbalance. The potential existence of class imbalance in our dataset was further supported by the fact that 50% of the dataset, or 121 individuals, were PASCs, while only 26 and 29 were mild-moderate and healthy controls, respectively, and the remaining 48 corresponded to the Severe class. Class imbalance leads to differences in the ratios between classes, for example we identified a 2.5 ratio between PASC and Severe, and a 4-fold ratio between PASC and both Mild-Moderate and Control. These differences in ratios can lead to biased predictions, which are often reflected as poor model performance metrics and generalizations (6, 9, 10). In order to avoid this potential pitfall, balancing methods have been proposed, which include random under-sampling and oversampling methods. However, it has been reported that random under-sampling can lead to information loss (11), whereas basic/randomized oversampling can lead to model overfitting.

Chawla et al. (11) proposed a solution in synthetic oversampling of the minority class. This method, known as SMOTE, uses interpolation between minority class instances to generate new data points to balance the dataset. SMOTE has been used in imbalance, including those of biological context, in conjunction with machine learning models (12). We pipelined SMOTE from the python library imbalanced-learn (13) to balance the training set, which was used in random forest classifier construction.

Random Forest Classifier

The random forest (RF) classifier is an ensemble method that groups multiple decision trees. Random Forests can be used for both classification and regression problems, as developed in 2001 (14). This method has been used to analyze biological datasets and in biological context knowledge discovery (11, 15, 16). Random forest classifiers possess the advantage of incorporating the option of assessing feature importance, which can be of great importance when undertaking downstream analysis, like assessing the biological significance of a feature, understanding its relevance in a given biological

process like immune response, or its potential role as a biomarker (17).

The ability to both be a predictor and identify relevant features makes random forests embedded selection methods. We used the Python's machine learning library, scikit learn, version 0.24.1 to construct the random forest classifier (18). Additionally, in order to adjust model hyperparameters (number of features, tree depth and number of trees) we used an exhaustive grid search with 10-fold cross validation (CV). It is important to note that variable importance was only implemented to identify significant features and not for dimensionality reduction.

Defining Precision, Recall and F1 Score for Model Performance

To estimate the random forest classifier performance, we selected three different metrics: precision (equation 1) which is a measure of the percentage of the results that are relevant, recall which measures the total relevant results that are correctly classified by the predictor (equation 2), and the F1 score (equation 3), which is the harmonic mean between these two measures and ranges from 0 to 1. If the F1 score is close to 1, the better the model performs. The F1 score for both false positives (FP) and false negatives (FN) as well as for true positives (TP).

$$Precision = \frac{TruePositive}{TruePositive + FalsePositive} \quad (1)$$

$$Recall = \frac{TruePositive}{TruePositive + FalseNegative} \quad (2)$$

$$F1 = \frac{2 * Precision * Recall}{Precision + Recall} = \frac{TP}{TP + \frac{1}{2}(FP + FN)} \quad (3)$$

RESULTS

Immune Profiling

To determine if immunologic abnormalities remain in PASCs, we performed high parameter immune cell quantification and characterization in a subset of individuals with preserved PBMCs (**Table 1A**). We quantified B-cells, T-cells, and monocytes including subsets and including CD4/CD8 activation and T-cell exhaustion. All T-cells determinations were initially gated on CD3 expression and all monocyte subsets were initially gated on CD45 (**Supplementary Figure 1**). Unlike acute COVID-19 (4), the CD4 and CD8 T-cell populations in PASC were within healthy control limits and there was no evidence of T-cell exhaustion. In fact, CD4 and CD8 positive T-cells expressing PD-1 were significantly lower than normal controls (P<0.001 and P=0.01 respectively). Further, there was a significant decrease in total T regulatory cells compared to healthy control individuals (P<0.001) possibly exacerbating the hyper-immunity in PASC. B-cells were significantly elevated compared to healthy control individuals (P<0.001) as was the CD14+, CD16+, CCR5+ monocytic subset (P<0.001) (**Table 1A**). Interestingly, these two immune cell populations have been shown to be chronically infected by different viruses. B-cells are infected by Epstein-Barr and the CD14+, CD16+, CCR5+ monocytic subset by HIV-1 and by HCV (19).

To further characterize the immune response in PASCs, we performed a quantitative, multiplex cytokine/chemokine panel on 29 healthy control individuals to establish the healthy control range of the assay. We then analyzed Mild-Moderate, Severe, and PASCs plasma samples and compared the cytokine/chemokine profiles (**Table 1B**). CCL5/RANTES, IL-2, IL-4, CCL3, IL-6, IL-10, IFN-γ, and VEGF were all significantly elevated compared to healthy controls (all P<0.001). Conversely GM-CSF and CCL4 were in significantly lower levels than healthy controls P=0.005.

Construction of a Multi-Class Random Forest Predictor for the Discrimination of the Analytical Groups in the Dataset

We proposed to differentiate the analytical groups (or diseases groups) of the dataset by constructing a multi-class random forest classifier. During the exploratory data analysis phase, we identified that the current dataset presented the characteristic of being imbalanced, with an overrepresentation of the PASC class. This dataset can also be considered medium-sized due to the number of instances. To address these potential pitfalls, and to avoid model overfitting, we implemented a balancing technique

TABLE 1A | T-, B-cell, and monocyte immunophenotyping.

Average	CD3 +%	CD4%	CD8 +%	CD4 +PD1%	CD4 +LAG3%	CD4 +CTLA4%	CD4 +FoxP3%	CD8 +PD1%	CD8 +LAG3%	CD8 CTLA4%	CD8+ FoxP3%	CD19%	CD14 +CD16- %	CD16 +CD14 +%	CD16 +CD14- %
Healthy Controls	64.40	53.80	33.83	35.62	0.94	1.51	6.21	43.75	4.35	1.38	0.67	6.04	42.79	9.00	32.67
Lower CI	54.39	43.21	27.20	28.36	0.49	0.75	4.54	33.50	2.71	0.74	0.37	5.04	34.41	4.60	25.49
Upper CI	74.50	64.57	40.46	42.89	1.39	2.26	7.87	54.01	5.99	2.03	0.97	7.04	51.16	13.41	39.86
PASC	48.98	56.18	35.36	17.78	0.72	4.06	2.58	31.99	0.71	3.11	1.01	13.14	19.01	29.3	33.86
Lower CI	44.78	52.44	32.56	15.73	0.36	2.32	2.01	29.46	0.55	2.04	0.80	11.72	15.65	25.65	30.28
Upper CI	53.18	59.92	38.70	19.83	1.08	5.80	3.15	35.52	0.87	4.18	1.22	14.56	22.37	32.95	37.44

TABLE 1B | Cytokine and other soluble factors quantification.

Average (pg/ml)	TFN-α	IL-4	IL-13	IL-2	GM-CSF	sCD40L	CCL5 (RANTE S)	CCL3 (MIP-1α)	IL-6	IL-10	IFN-γ	VEGF	IL-8	CCL4 (MIP-1β)
Healthy Controls	9.09	4.18	3.94	6.17	51.27	7192.39	10781.84	22.82	2.21	0.67	1.94	9.32	16.87	76.84
Lower CI	7.37	2.17	1.79	5.53	25.72	5148.85	9764.99	13.05	1.65	0.42	0.63	6.36	13.03	61.00
Upper CI	10.81	6.18	6.09	6.82	76.82	9235.92	11798.68	32.60	2.77	0.92	3.26	12.28	20.72	92.67
PASC	7.72	17.03	4.21	16.16	12.46	18302.41	12505.06	97.81	20.47	12.23	86.60	41.03	35.98	35.10
Mild-Mod	6.82	2.33	2.40	5.90	56.13	10673.72	11627.70	18.75	8.74	0.63	1.15	17.39	17.37	94.40
Severe	5.39	2.39	2.26	5.43	20.31	12306.39	11581.47	16.54	144.15	3.10	2.06	25.52	10.87	64.84

as described above. The implementation of SMOTE is thus useful to counter overfitting and to generate new samples from interpolation for the underrepresented or minority classes. By using SMOTE to balance the minority classes to 100% of the PASC class, it resulted in each class having 76 instances in the training set. This represented a 4-fold increase in the healthy control and the Mild-Moderate classes, and a 2.5-fold increase for the Severe class.

The balanced dataset was used to construct the multi-class RF predictor, which was fine-tuned using the grid-search and cross validation approach. This implementation of grid search and 10-fold CV was used as a fine-tuning approach for this and all subsequently constructed classifiers. The multi-class model was then analyzed for overfitting with the validation set (**Table 2**). During this analysis, we noticed a slight decrease in the model's predictive performance when discriminating between the healthy control and Mild-Moderate class, however the overall performance in the validation set was high, as seen by the recall (sensitivity) and the f1 score. However, these differences were heavily accentuated in the performance metrics of the test set (**Table 2**). This can be further appreciated in the confusion matrix for the multi-class classifier (**Figure 2**), which demonstrates that in the test split, both the Severe and PASC classes were properly identified but the healthy control and Mild-Moderate classes incurred in multiple misclassifications. Furthermore, when analyzing the feature importance (cytokines) of the dataset, we noticed the differences between variables are of small magnitude, only amplified by the scale of the axis (**Figure 2**), but apart perhaps the difference between IFN-ɣ and CCL5 (RANTES), differences might not be that obvious. Because of these findings, we decided to proceed with the construction of the binary RF classifiers focused on Severe and PASC classes.

TABLE 2 | Random forest classifier predictor performances on the validation and test partitions.

Model	Accuracy	Precision	Recall	F1
Multi-class-Val	0.97	0.97	0.92	0.93
PASC-Val	1.00	1.00	1.00	1.00
Severe-Val	0.94	0.95	0.94	0.94
Multi-class-Test	0.8	0.62	0.65	0.63
PASC-Test	0.96	0.95	0.96	0.95
Severe-Test	0.95	0.97	0.93	0.94

The partition is indicated next to the model, either as Val for validation or Test for the test partition. The presented performance metrics were calculated using the classification report and the confusion matrix form sci-kit learn (18).

Construction of a Binary PASC Random Forest Classifier Allows Identification of Relevant Features for the Development of a Heuristic Score for PASC Patient Identification

After constructing the multi-class predictor, we proceeded with the development of a binary classifier furthering our understanding of the PASC disease group. The PASC class was comprised of long-term disease carriers, and thus the random forest classifier was tasked with separating the long-term carriers from those instances that did not belong to this class, and to identify the cytokines or features that were relevant for the discrimination of the disease groups. To achieve this, we separated the data into two major groups, one that consisted of all the classes (healthy control, Mild-Moderate and Severe) representing non-long term disease carrier groups, and a second with the PASCs. This new dataset was split into 60/20/20 (training/validation/test) and the training set was balanced using SMOTE. The trained classifier was fine-tuned to determine the best hyperparameter combination (tree-depth, feature number, number of trees) using and exhaustive grid search. We then used the model on the validation set in order to detect model overfitting, and did not identify indications of model overfitting (**Table 2**). The model was implemented on the test set, to predict the classes for the instances in this partition. When analyzing the confusion matrix (**Figure 3**), the model's predictive capabilities seemed very high, with only 2 instances being misclassified, this is further supported by the predictors metrics (**Table 2**), where the F1 score, the balance between precision and recall was 0.95. Additionally, when looking at the variable importance analysis (**Figure 3**), we identified that the top 5 most relevant cytokines were (in order): IFN-ɣ, IL-2, IL-4, IL-10 and GM-CSF. Other relevant identified cytokines include: IL-8, CCL4 (MIP-1β) and CCL3 (MIP-1α).

The resulting features identified from the variable importance analysis were fundamental for the subsequent development for a novel heuristic that was constructed using feature engineering. Through the use of the score derived from this heuristic, we aimed to simplify our model and gain biological insight about the PASC phenotype. We obtained a "PASC Score" defined as S1 = (IFN-γ + IL-2)/CCL4-MIP-1β (**Figure 4**). Setting an optimized threshold of S1 = 0.5 as a tradeoff of sensitivity and specificity, it was possible to classify the majority of PASCs as such (118/121 with S1 > 0.5) for a sensitivity of 97.5%. No healthy control or MILD-Moderate cases were classified as PASCs (specificity of 100% for healthy control and MILD-

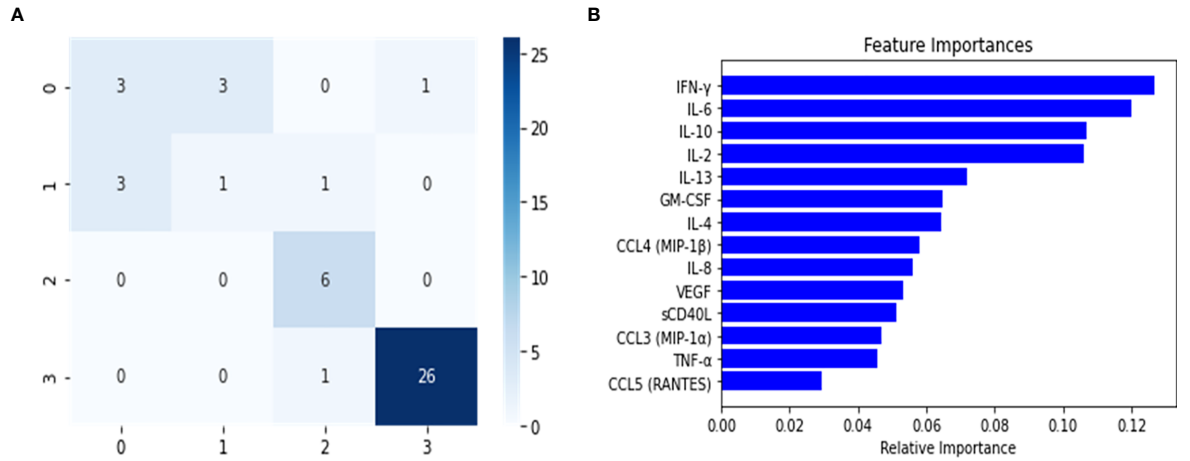

FIGURE 2 | Confusion Matrix **(A)** and Feature importance **(B)** for multi-class classifier using Random Forest predictor. The confusion matrix was calculated from the predictions of the random forest classifier on the test set. The classes were assigned in the following manner: (0) healthy controls, (1) Mild-Moderate, (2) Severe and (3) PASC.

Moderates). In contrast, 7/48 Severe cases were classified as PASCs (S1>0.5) for a specificity of 85%, suggesting that these 'misclassified' Severe cases could indeed become PASCs.

Construction of a Binary Random Forest Classifier and Variable Importance Enables the Feature Engineering of a Score for Severe Patient Identification

The random forest classifier for discriminating between Severe and non-Severe individuals was constructed by grouping the balanced healthy control and Mild-Moderate classes into a single group that was labeled as non-Severe. In this dataset, the PASC class was excluded based on the scope of potentially identifying the cytokines that separate the Severe disease group from those that are in a non-Severe state. These non-Severe individuals however, do not belong to a long-term carrier group. In addition, the results from the disease score generated using the important variables allowed us to discriminate the PASCs.

The model was constructed and fine-tuned using the same approach implemented in the multi-class and binary models. The model with the best parameters was then selected to identify model overfitting in the validation set. We were not able to determine any evidence of overfitting, and proceeded to use this model to undertake predictions in the test set. As the confusion matrix for this Severe binary classifier indicates (**Figure 5**), it was possible to discriminate between what we defined as Severe and

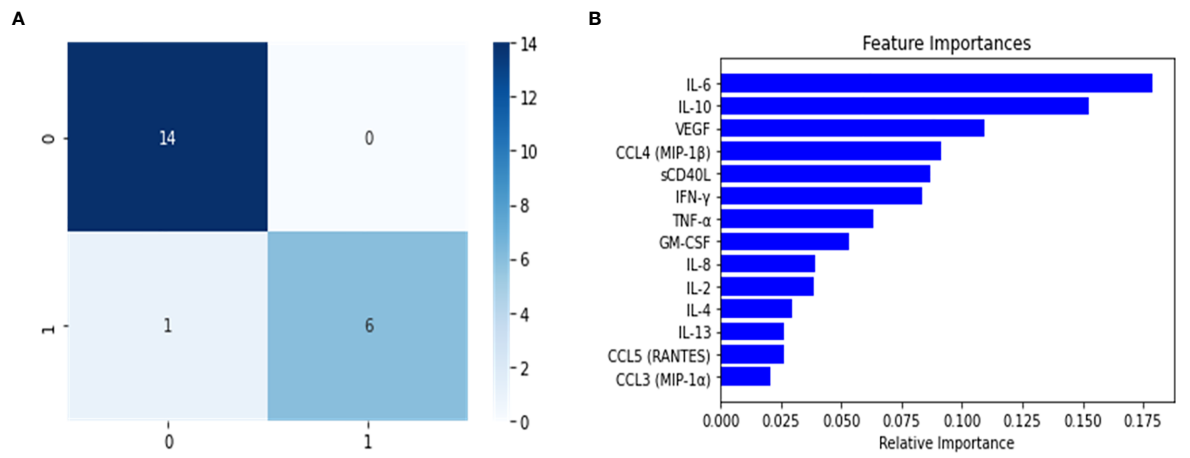

FIGURE 3 | Confusion Matrix **(A)** and feature importance **(B)** for the PASC binary random forest classifier to enable the feature engineering of a score for the identification of PASC patients. The positive class (1) are PASCs while the negative (0) class are the non-PASCs (healthy control, Mild-Moderate-Severe).

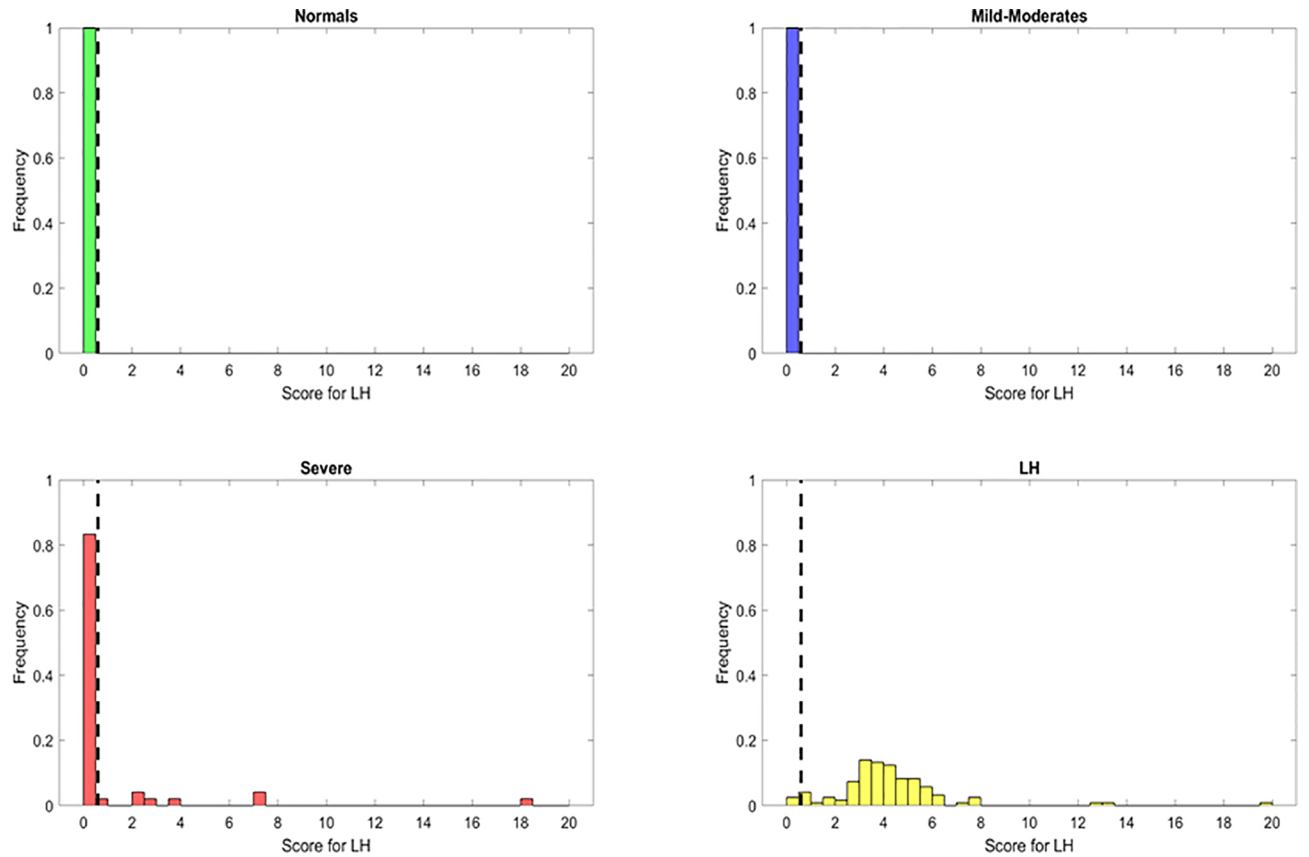

FIGURE 4 | Discrimination ability of the long hauler score for the identification of PASC patients (S1) with reduced or most important features identified using Random Forest classifier. The dots represent the data points, where yellow are PASCs, red-Severe, dark blue-Mild-Moderate and green-healthy control.

non-Severe instances. The number of incorrectly classified instances was 1 non-Severe misclassified as Severe (**Figure 5**). The model performed very well, as indicated by its metrics in the test set (**Table 2**). Both precision and recall were high (0.97 and 0.93, respectively, with an F1 score of 0.94). Additionally, as we will report, this model also identified important features (cytokines) that were relevant to discriminate between the disease groups. This information would be useful to develop a heuristic score for the Severe disease group. We also undertook variable importance analysis (**Figure 5**) where we identified as the most relevant features: IL-6, IL-10, VEGF, with IFN-γ, CCL4-MIP-1β and sCD40L being informative to a lesser degree.

Using these important features we developed a score to identify patients. Based on the same principle, but using the relevant features from the Severe random forest binary classifier, we engineered a score for the identification of Severe cases. This new score, identified as S2, was calculated as follows: S2 = (IL6+sCD40L/1000+VEGF/10+10*IL10)/(IL2+IL8). Setting an optimized threshold of S2 = 1.5 as a tradeoff between sensitivity and specificity, it was possible to apply the heuristic to classify the majority of Severe as such (46/48 with S2 > 1.5) for a sensitivity of 95.8%. Only 2/29 healthy control and 5/26 MILD-Moderate cases were classified as Severe (specificity of 93% for healthy control and 81% for Mild-Moderates which may be disease status misclassification) (**Figure 6**). However, using this score alone, the original PASCs cannot be separated as most of them will be classified as Severe.

A Combined Heuristic Enables an Optimal Classification of PASCs and Severe Cases of COVID-19

In order to integrate the PASC and Severe identification, we aimed to develop a combined heuristic using both scores and the optimized thresholds defined above. This heuristic identifies the PASC cases first using the 'PASC score' and then identifies the Severe cases from the remaining data points. The graphical representation in **Figure 7** shows a very good separation of the PASC and Severe cases from the healthy control and Mild-Moderates. All PASCs (121) were classified either as PASCs (118) or Severe (3) indicating a sensitivity of 100% to identify pathology. On the other hand, only 1 Severe case was classified as Mild-Moderate, indicating that most Severe cases were classified either as Severe (n=40) or PASC (n=7) indicating a sensitivity of 97.9% to detect pathology. In addition, the presence of those 7 'mis-classified' Severe cases as PASCs suggests that some Severe cases are underway to become PASCs.

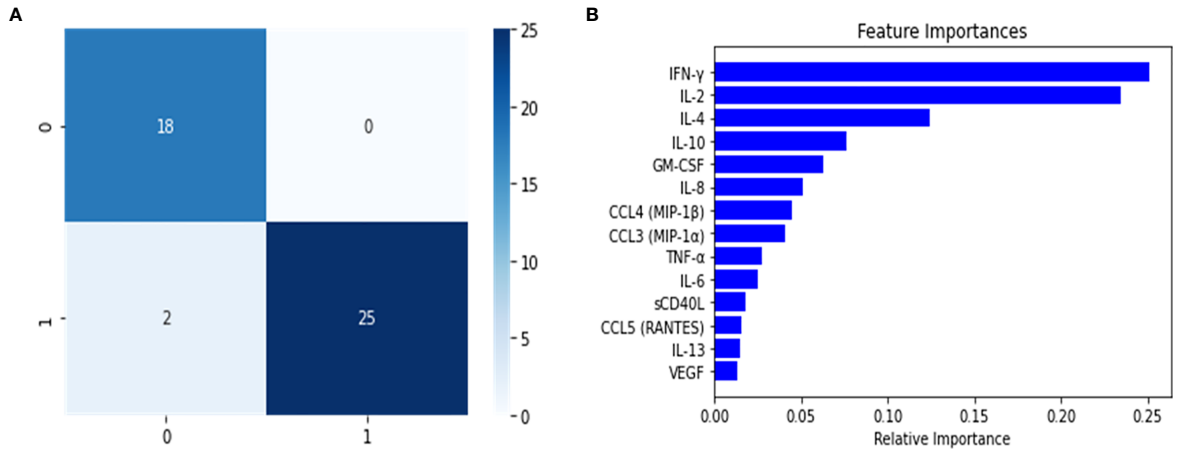

FIGURE 5 | Confusion matrix **(A)** and variable importance **(B)** for the Severe binary classifier constructed using the random forest classifier. The results shown in the confusion matrix were calculated for test split, where 0 represents the grouped Mild-Moderate and healthy control instances, and 1 are the Severe cases. For B, the most significant variables were to calculate a disease group score for Severe patients.

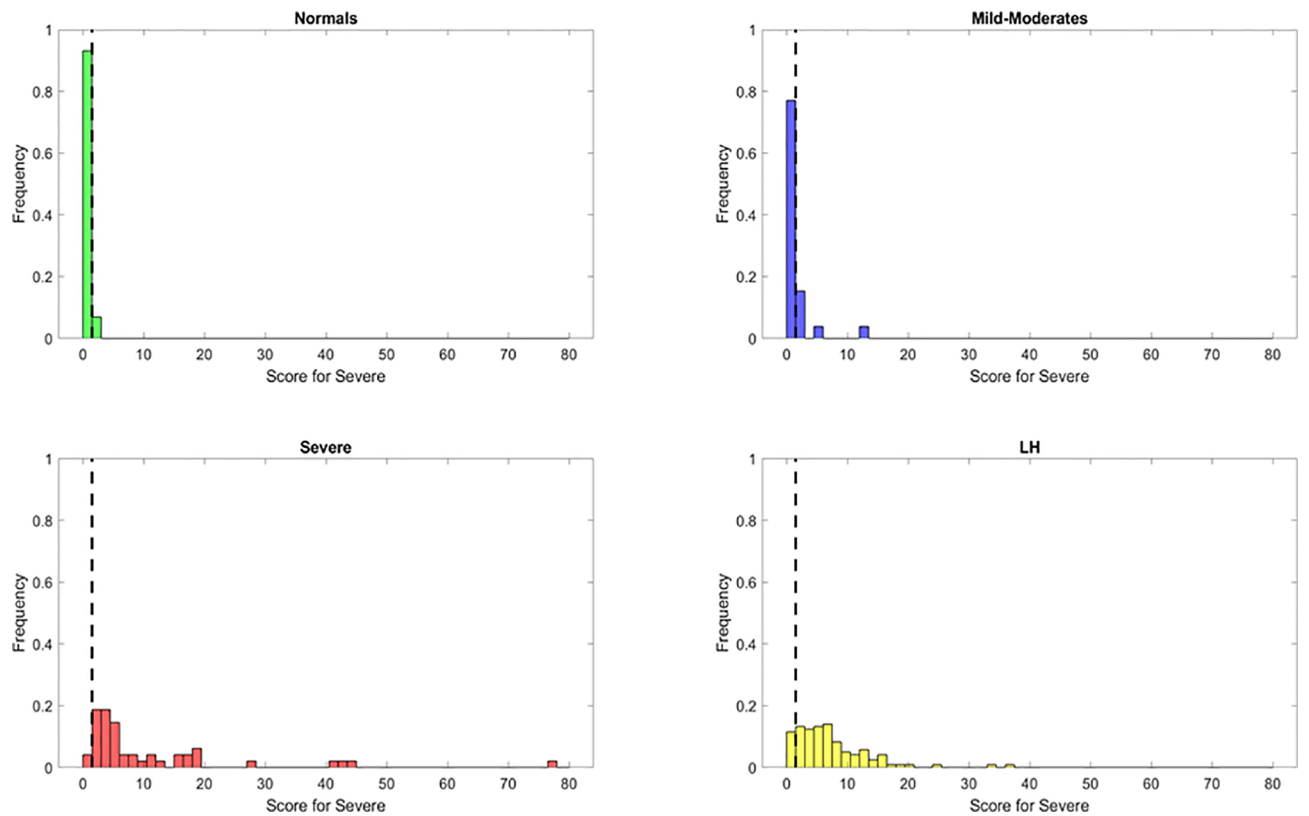

FIGURE 6 | Discrimination ability of the Severe score for the identification of Severe patients (S1) with reduced or most important features identified using Random Forest classifier. The dots represent the data points, where yellow are PASCs, red-Severe, dark blue-Mild-Moderate and green-healthy control.

Finally, we simplified our prediction model by feature engineering of two classification scores based on the top informative features. First, a "PASC Score" was defined as S1 = (IFN-γ + IL-2)/CCL4-MIP-1β. Second, "Severe Score" was defined as S2 = (IL6+sCD40L/1000+VEGF/10+10*IL10)/(IL2+IL8). Using a combined heuristic to first classify the PASCs (S1>0.4) and second the Severe COVID-19 patients (S2>0), we obtained a sensitivity of 97% for PASCs with a 100% specificity

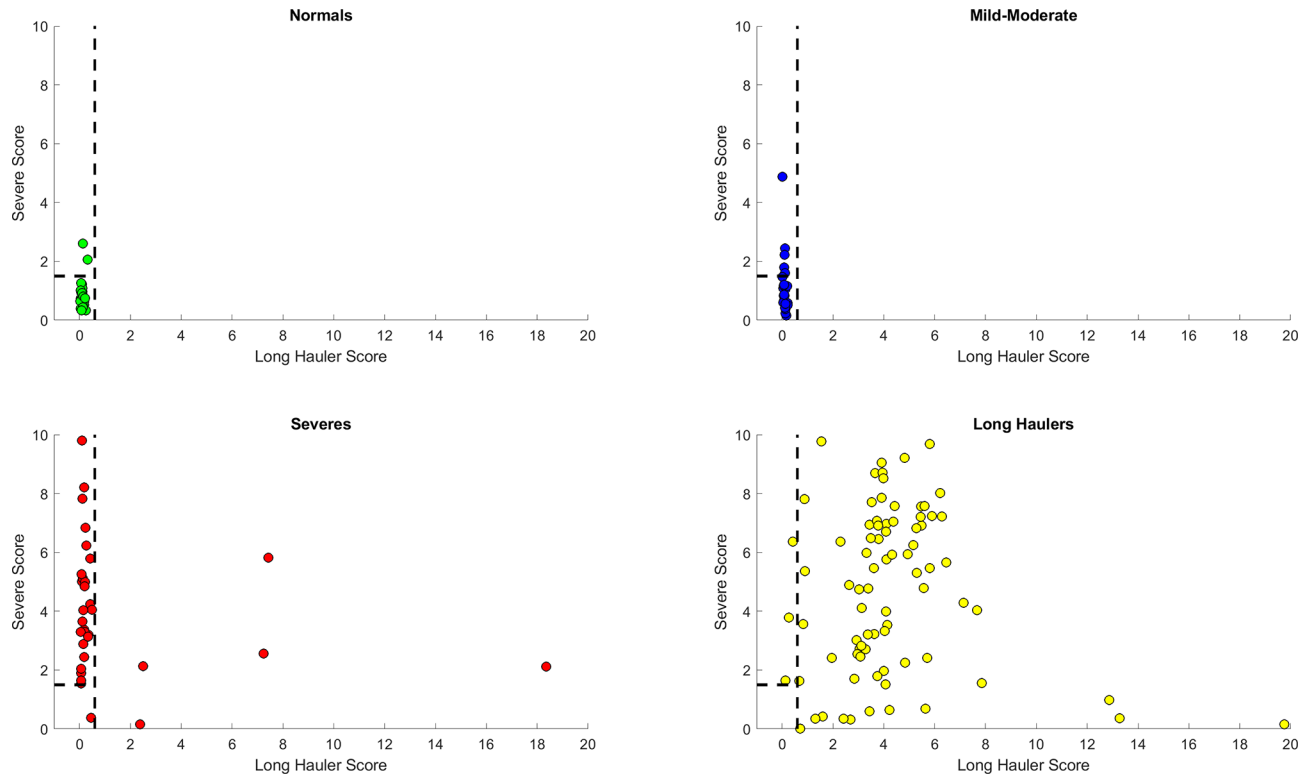

FIGURE 7 | Discrimination ability of the heuristic with both Long Hauler (S1) and Severe (S2) scores. The PASC patients are first identified with an S1>1.5. From those remaining patients, the Severe cases are identified with an S2> 1.5. The dots represent the data points, where yellow are PASCs, red-Severe, dark blue-Mild-Moderate and green-normal.

and a sensitivity of 88% for Severe patients with a specificity of 96% (**Figure 7**).

DISCUSSION

Individuals infected with SARS-CoV-2 exhibit distinct severity patterns which have been associated with different immune activation profiles. Interestingly, in some cases longer times are required to experience full recovery, representing a particular pathological type recently described as long-COVID or PASC.

The scientific evidence generated during the last months strongly supports that the different outcomes on COVID-19 patients are determined by the immune mechanisms activated in response to the viral infection (20).

The immune response to SARS-CoV-2 induces a release of different molecules with inflammatory properties such as cytokines including interleukins and chemokines. This event, known as cytokine storm (20), is an immunopathological feature of COVID-19 and it has been associated with the severity of the disease. The increase in blood concentrations of different cytokines such as interleukins and chemokines such as IL-6, IL-8, IL-10, TNF-α, IL-1β, IL-2, IP-10, MCP-1, CCL3, CCL4, and CCL5 has been described for COVID-19 patients (4). Some of these molecules have been proposed as biomarkers to monitor the clinical evolution and to determine treatment selection for COVID-19 patients (21–23). Nevertheless, it is important to consider that some of these molecules function in a context dependent manner, therefore the clinical relevance of analyzing single cytokine changes is limited.

One of the most important challenges during the pandemics is to avoid the saturation of the health systems, therefore the determination of predictive biomarkers that allow a better stratification of the patients is paramount. Even though cytokines such as IL-6 and IL-8 have been proposed as indicators of the disease severity, and in some studies they were strong and independent predictors of patient survival (24), their predictive value when analyzed alone is debatable (24). The generation of scores considering blood levels of cytokines such as interleukins and chemokines with different immunological functions incorporates the importance of the context-dependent function of these molecules.

In order to predict Severe cases, a score was generated considering blood concentrations of inflammation-associated factors such as IL-10, IL-6, IL-2, and IL-8, as well as sCD40L and VEGF which are associated with vascular homeostasis (25, 26). In this classification, Severe cases are characterized by high IL-6 and IL-10 levels, both cytokines previously attributed to increase the immunopathogenesis of COVID-19 and predictive value in Severe cases (22, 23). In different backgrounds, IL-6 has

been associated with oxidative stress, inflammation, endothelial dysfunction, and thrombogenesis (25–28) which are characteristic features of Severe COVID-19 cases caused by excessive myeloid cell activation (29). Consistently, increased IL-10 levels interfere with appropriate T-cell responses, inducing T-cell exhaustion and regulatory T cell polarization leading to an evasion of the antiviral immune response (30). Furthermore, besides its anti-inflammatory function on T cells, in some backgrounds IL-10 induces STAT1 activation and a pro-inflammatory response in type I IFN-primed myeloid cells (30, 31). Therefore, elevated levels of IL-6 and IL-10 promote myeloid cell activation, oxidative stress, endothelial damage, which might affect an adequate antiviral T cell activation (26–30).

Furthermore, Severe cases show high levels of sCD40L and VEGF, which are associated with vasculitis and vascular remodeling. The cytokine storm observed in SARS-CoV-2 infection is accompanied by hemostatic alterations and thrombosis. sCD40L is a platelet activation marker, which has been associated with increase severity in COVID-19 patients (32–34). Moreover, sCD40L levels are higher in male patients compared with females and it is the sex-associated differences in the severity of the disease (33). Another vascular alteration associated to SARS-CoV-2 infection is endothelial hyperactivation. According to the proposed severity score, VEGF levels were significantly elevated in hospitalized COVID-19 patients when compared to Mild-Moderate cases. Additionally, to strengthen the classification presented here, the score differentiates the Severe cases by the denominator of IL-2 and IL-8, which are cytokines related to proper T cell activation (IL-2) and recruitment (IL-8) (35, 36).

According to the score generated for distinguishing PASC, these patients are characterized by an increased IFN-γ and IL-2 and a reduced CCL4 production. In the context of a viral infection, the combination of IFN-γ and IL-2 would induce the activation of effector T cells with pro-inflammatory properties and the capacity of generating an effective immune response to eliminate the virus. However, PASC are characterized by longer periods of time with clinical signs and symptoms such as fatigue and lung damage. This suggests that the inflammatory context created by these cytokines that leads to T cell activation is not enough to generate an adequate anti-viral response without the proper recruitment signals to attract activated T cells. CCL4 signals through the receptor CCR5 to attract T cells to the site of inflammation and depending on the immune context, this molecule recruits differently activated T cells (37, 38). Moreover, it was recently shown, by single cell analysis, down regulation of CCL4 expression in peripheral myeloid cell compartments in patients with Mild and Severe COVID-19 (39). In PASC, IFN-γ and IL-2 would create an immune context favoring the Th1 polarization, but the low levels of CCL4 affect the recruitment of these cells thus impairing the antiviral response should SARS-CoV-2 RNA or protein persist. The effect of increased IFN-γ and IL-2 on T cell activation is evident in the reduction of the frequency of exhausted (CD4+PD1+/CD8+PD1+) and total regulatory T cells (FoxP3+) compared to healthy donors. Therefore, proper T cell activation (high IFN-γ+IL-2) but ineffective T cell recruitment (low CCL4) are characteristic features of the failed anti-viral response observed in the PASC group supporting virus persistence.

The significant increase of B cells in the PASC group is associated with high IL-2 levels promoting B cell proliferation and differentiation (40). Interestingly, increased IFN-γ affects B-cell homing to lymph nodes (41), reduces total IgG production, and inhibits pre-activated B cells (42). This could be associated with virus persistence in the PASC group as supported by the low CCL4 levels observed in these patients, since CCL4 has been proposed as a biomarker for B cell receptor pathway activation (43).

Additionally, increased IFN-γ promotes myeloid cell activation which is observed in the augmented frequency of inflammatory CD14+, CD16+, CCR5+ monocytes in the PASC group compared to healthy donors, supporting lymphopenia and virus persistence in these patients. This is in line with recent findings describing increased gene expression in response to IFN-γ in Mild and Severe COVID-19 patients in peripheral myeloid cells (39) and the dysregulation in the balance of monocyte populations by the expansion of the monocyte subsets described in COVID-19 patients (39). Finally, we propose that long-lasting pulmonary damage observed in PASC, is caused by a combination of factors including 1) virus persistence influenced by the PASC immune profile as characterized by high IFN-γ and IL-2 levels. This in turn induces Th1 polarization which is ineffective with low CCL4-induced T cell recruitment, leading to an inflammatory myeloid cell activation; and 2) the immunopathological pulmonary effects of this PASC immune profile. Regarding the immunopathological effects of the PASC immune profile, it has been shown using murine models that high IFN-γ levels could affect the kinetics of the resolution of inflammation-induced lung injury as well as thrombus resolution (44–46), which could be related to long-lasting symptoms of PASC associated to pulmonary coagulopathy and immune-mediated tissue damage.

Interestingly, COVID-19 individuals (including PASC, Mild, Severe) show high levels of CCL5, a chemokine that like CCL4 signals through CCR5. Indeed, the disruption of the CCL5-CCR5 pathway restores immune balance in critical COVID-19 patients (4). In the specific case of PASC, despite the statistically significant elevation of CCL5 compared to healthy controls, a reduction in the CCL4-mediated recruitment of activated T cells is proposed. This could be related to different factors:

(1) Reduction of total recruitment signals in PASC with low CCL4 concentrations.
(2) Different functional responses of CCL4 and CCL5 to polymorphic variants of the CCR5 gene. Distinct functional features have been reported in CCR5 variants regarding binding avidity, receptor internalization, Ca++ influx and chemotactic activity (47). Even though, clear mechanistic differences between CCL4 and CCL5 interaction with CCR5 are missing, even considering the knowledge gained on CCR5 polymorphisms in HIV/AIDS context (48).
(3) Signaling through alternative receptors for CCL5. Besides CCR5, CCL5 can signal through the receptors CCR1 and CCR3 (49) whereas CCL4 effects are restricted to CCR5. It has been shown that CCL4 can bind to CCR1 but is not able to induce the intracellular pathway necessary for activating the chemoattractant

stimulus (49). Therefore, CCL4 has been proposed as an antagonist of CCR1 (50), however further analysis of this needs to be performed. Interestingly, CCR1 is expressed on blood myeloid cells such as monocytes and neutrophils, and it is upregulated on COVID-19 patients (51). Additionally, high levels of IFN-γ (a feature of PASC) have been associated with an increase in CCR1 expression on human neutrophils (52). Therefore, in PASC, high levels of CCL5 (combined with low levels of potential CCR1-antagonist CCL4) leads to a higher recruitment of myeloid cells expressing CCR1.

CONCLUSION

In conclusion, we developed a bioinformatics pipeline that analyzed cytokines of the immunological landscape of COVID-19 using machine learning methods to discriminate between PASC and Severe individuals from other classes. The implementation of random forest classifiers allowed for the identification of the critical cytokines for this discrimination, which in turn was used to calculate highly sensitive heuristics for PASC and Severe individuals. These models, which can be incorporated into clinical laboratory information systems, enabled a highly accurate, immune-based classification of severe COVID-19 infection and PASC. This workflow could greatly aid the triage, treatment, and prognosis of those affected. An interesting caveat affecting the specificity of the PASC classification was that 7 Severe COVID-19 patients classified as PASC that, while affecting the specificity of PASC classification, may represent a subset of acute COVID-19 patients destined to become affected by PASC.

These data also indicate that with an effective classification of severe and PASC individuals based on cytokine profiles, precision therapies guided by the machine learning output may result in lower severity and PASC scores and possibly in more favorable clinical outcomes. CCR5 antagonism has already been demonstrated to reduce IL-6, and VEGF (4, 53), numerators in the severity score, and to reduce IFN-γ, a numerator in the PASC score (54).

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

Informed consent was obtained from all participants. Samples were considered exempt for the purposes of this study and results were not used to manage patients. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

RY organized the clinical study and actively recruited patients. BP, AP, HR, and EL performed experiments and analyzed the data. JG-C, RM-R, and JM performed the bioinformatics. BP, JM, JG-C, RM-R wrote the draft of the manuscript. All authors contributed to the article and approved the submitted version.

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The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.700782/full#supplementary-material

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CCR5 Receptor Occupancy Analysis Reveals Increased Peripheral Blood CCR5+CD4+ T Cells Following Treatment With the Anti-CCR5 Antibody Leronlimab

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CCR5 plays a central role in infectious disease, host defense, and cancer progression, thereby making it an ideal target for therapeutic development. Notably, CCR5 is the major HIV entry co-receptor, where its surface density correlates with HIV plasma viremia. The level of CCR5 receptor occupancy (RO) achieved by a CCR5-targeting therapeutic is therefore a critical predictor of its efficacy. However, current methods to measure CCR5 RO lack sensitivity, resulting in high background and overcalculation. Here, we report on two independent, flow cytometric methods of calculating CCR5 RO using the anti-CCR5 antibody, Leronlimab. We show that both methods led to comparable CCR5 RO values, with low background on untreated CCR5+CD4+ T cells and sensitive measurements of occupancy on both blood and tissue-resident CD4+ T cells that correlated longitudinally with plasma concentrations in Leronlimab-treated macaques. Using these assays, we found that Leronlimab stabilized cell surface CCR5, leading to an increase in the levels of circulating and tissue-resident CCR5+CD4+ T cells *in vivo* in Leronlimab-treated macaques. Weekly Leronlimab treatment in a chronically SIV-infected macaque led to increased CCR5+CD4+ T cells levels and fully suppressed plasma viremia, both concomitant with full CCR5 RO on peripheral blood CD4+ T cells, demonstrating that CCR5+CD4+ T cells were protected from viral replication by Leronlimab binding. Finally, we extended these results to Leronlimab-treated humans and found that weekly 700 mg Leronlimab led to complete CCR5 RO on peripheral blood CD4+ T cells and a statistically significant increase in CCR5+CD4+ T cells in peripheral blood. Collectively, these results establish two RO calculation methods for longitudinal monitoring of anti-CCR5 therapeutic antibody blockade efficacy in both macaques and humans, demonstrate

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that CCR5+CD4+ T cell levels temporarily increase with Leronlimab treatment, and facilitate future detailed investigations into the immunological impacts of CCR5 inhibition in multiple pathophysiological processes.

Keywords: CCR5, CD4, HIV, receptor occupancy (RO), flow cytometry, antibody

INTRODUCTION

C-C chemokine receptor type 5 (CCR5) is a G protein-coupled receptor involved in cell signaling and migration. CCR5 is primarily expressed in lymphocytes, macrophages, dendritic cells, and natural killer cells, but can also be found on the epithelium, endothelium, vascular smooth muscle, and fibroblasts from multiple organs, as well as neurons, astrocytes, and microglia in the central nervous system (CNS) (1, 2). Chemokines CCL3 (MIP-1α), CCL4 (MIP-1β), and CCL5 (RANTES) are the primary CCR5 ligands and are expressed in sites of inflammation to recruit CCR5+ immune cells; thus, the number of CCR5+ cells often correlate with the severity of inflammation (1, 3, 4).

While CCR5 is known for its role in facilitating human immunodeficiency virus (HIV) infection of CD4+ T cells, it has a wide range of roles in normal and pathophysiological processes. In addition to HIV, CCR5 is a critical host receptor for Dengue virus (5) and Staphylococcus aureus (6) infection. Furthermore, high expression of CCR5 is associated with cancer progression and tumorigenesis (7–9), development of insulin resistance *via* adipose tissue macrophage recruitment (10), and suppression of cortical plasticity, learning, and memory (11–13). Moreover, individuals homozygous for the naturally occurring CCR5 mutation, CCR5Δ32, lack cell surface expression of CCR5 receptors, which has protective effects against HIV infection (14, 15), asthma (16, 17), severe SARS-CoV-2 symptoms (18), and development of rheumatoid arthritis (19), and is associated with improved hepatitis B virus infection recovery rates (20) and lower incidence of cardiovascular disease (21, 22). However, CCR5 is critical for viral clearance after infection by West Nile (23), Japanese encephalitis (24), and influenza A viruses (25, 26) because of its role in trafficking immune cells to sites of infection. Exemplifying the complexity of CCR5, the lack of CCR5 receptors protected against parasitic *Toxoplasma gondii* infection (27) while the presence of CCR5 was essential for disease control after infection (28, 29). Because of the myriad roles played by CCR5, the ability to target CCR5 with therapeutic agents will have a diverse range of applications.

Widely used HIV-specific therapeutics, such as antiretroviral (ARV) drugs and HIV-specific broadly neutralizing antibodies (bNAbs), exert their antiviral effects by directly targeting viral proteins, and thus their efficacy is correlated to plasma concentration (30–32). However, for immunomodulatory and antiviral agents that target host receptors, their efficacy relies on the level of receptor occupancy (RO) achieved. Indeed, in HIV infection the density of CCR5 on CD4+ T cells correlated closely to both susceptibility to HIV infection *in vitro* and plasma viral loads in HIV-infected individuals (33–35). Additionally, the paucity of CCR5+ CD4+ T cells present in natural hosts of simian immunodeficiency virus (SIV) during infancy protects against mother-to-offspring transmission during breastfeeding by viremic mothers (36). Thus, the level of CCR5 occupied by a CCR5-targeting drug is a critical predictor of its therapeutic efficacy.

Currently, Maraviroc is the only FDA-approved CCR5 antagonist. Maraviroc inhibits CCR5 internalization following ligand binding, and thus Maraviroc CCR5 RO is indirectly measured by a MIP-1β internalization assay where CCR5 RO is defined by the percentage of cell surface CCR5 that is not down-regulated following treatment with MIP-1β (37). This indirect method of measuring CCR5 RO results in background RO of approximately 25% (38), with reports of 120% CCR5 RO in peripheral blood CD4+ T cells from both Maraviroc-treated and -untreated rhesus macaques (39). These issues of extremely high background and overcalculation of CCR5 RO are major limitations of the MIP-1β internalization assay, especially when CCR5 RO is a critical parameter in studying CCR5-blocking agents.

Directly measuring CCR5 RO with monoclonal antibodies also presents challenges as CCR5 expression is a dynamic process that must be controlled for. Indeed, the frequency of CCR5+ cells change longitudinally in response to inflammatory and homeostatic stimuli and can be impacted by the CCR5-targeting reagent itself (39–43), leading to inaccuracies in methods that use baseline CCR5 values to calculate CCR5 RO (44). Not accounting for the ability of CCR5 expression to change over time in the CCR5 RO calculation for the anti-CCR5 antibody HGS004 resulted in baseline pre-treatment CCR5 RO values of 20% in HIV-1 infected participants (45). Thus, no robust and highly sensitive method for the calculation of CCR5 RO currently exists.

Here, we report on two sensitive methods to measure CCR5 RO by the anti-CCR5 antibody Leronlimab (PRO-140; Vyrologix). We demonstrate the sensitivity of this method to longitudinally quantify CCR5 RO on blood and tissue CD4+ T cells from Leronlimab-treated macaques and describe increased levels of CCR5+CD4+ T cells in the blood of both Leronlimab-treated macaques and humans. Finally, we translate the macaque CCR5 RO method to Leronlimab-treated, HIV-naïve human participants, demonstrating the direct use for monitoring CCR5 RO by Leronlimab in human clinical trials.

MATERIALS AND METHODS

Study Approval and Design (Non-Human Primates)

All animal work occurred at the Oregon National Primate Research Center (ONPRC), a Category I facility that is fully

credited by the American Association for Accreditation of Laboratory Animal Care (AAALAC), with approved Assurance (#A3304-01) for the use and care of animals on file with the NIH Office of Laboratory Animal Welfare. Animal experimental care plans, protocols, procedures, and administered reagents were approved by ONPRC Institutional Animal Care and Use Committee (IACUC). The ONPRC IACUC adhers to the national guidelines established in the Animal Welfare Act (7 U.S.C. Sections 2131–2159) and the Guide for the Care and Use of Laboratory Animals (8th Edition) as mandated by the U.S. Public Health Service Policy.

Macaques (*Macaca mulatta and Macaca fascicularis*) used in this study were housed at the Oregon National Primate Research Center (ONPRC) in Animal Biosafety level (ABSL)-2+ rooms with autonomously controlled temperature, humidity, and lighting. At assignment, macaques were free of Cercopithecine herpesvirus 1, D-type simian retrovirus, simian T-lymphotropic virus type 1, and Mycobacterium tuberculosis. Macaque specialists designed and oversaw daily wellness and dietary enrichment plans. Ketamine HCl (Ketathesia™, Henry Schein Animal Health) with or without Dexmedetomidine (Dexmedesed™, Dechra, Overland Park, KS) was used to sedate macaques for procedures, including subcutaneous (SC) injections of Leronlimab, venipuncture, tissue biopsy, and viral challenge, and they were performed by certified veterinarians or trained animal technicians with veterinarian oversight.

Adult rhesus macaques (n=12) were used in this study, with six macaques in the single 10 mg/kg SC Leronlimab group and six macaques in the single 50 mg/kg SC Leronlimab group. All but one animal in the 10 mg/kg group were female. Baseline biopsies and whole blood were collected before SC Leronlimab injections. Blood was collected at eight, 24, 48, and 72 hours post Leronlimab injection, and then weekly afterwards. Biopsies were collected at one and four weeks after Leronlimab injection and processed as described below. This was a non-terminal study and macaques were returned to the ONPRC colony after study conclusion.

One adult female chronically SIVmac239-infected Mauritian cynomolgus macaque received weekly doses of 50 mg/kg Leronlimab subcutaneously for 11 weeks.

Study Approval and Design (Human)

De-identified peripheral blood samples were obtained from participants in a phase 2, randomized, double blind, placebo-controlled study to evaluate for the efficacy and safety of Leronlimab treatment in human participants experiencing prolonged SARS-CoV-2 symptoms (termed Long-Haulers). Participants were randomized to receive weekly 700 mg SC Leronlimab or placebo. Participants of all sexes, over 18 years of age, and with prior confirmed positive SARS-CoV-2 RT-PCR test were eligible. Participants experienced at least two clinical symptoms consistent with a SARS-CoV-2 infection for more than 12 weeks. The trial took placed at the Arthritis & Rheumatic Disease Specialties in Aventura, Florida and Center for Advanced Research & Education (CARE) in Gainesville, Georgia under the ClinicalTrials.gov identifier (NCT number): NCT04678830. All participants provided written informed consent prior to inclusion in study.

Tissue Processing

Whole blood, lymph node, and bone marrow samples were processed as previously described (40). Briefly, peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by density gradient centrifugation using Ficoll-Paque PLUS (Cytiva) and centrifuged at 1,860 x g for 30 minutes. Plasma was saved for viral nucleic acid and Leronlimab concentration detection. Buffy coat containing PBMCs was collected and washed with R10 (RPMI 1640 containing 10% fetal bovine serum (FBS)) before use. Lymph nodes were collected in R10, diced with a scalpel, and forced through a 70 μm cell strainer to a single cell suspension in R10. Bone marrow aspirates were collected in R10 and pelleted by centrifugation at 830 x g for 4 minutes. Cell pellets were resuspended by shaking in PBS containing 2 mM EDTA, and then centrifuged at 830 x g for 4 minutes. Cell pellets were then resuspended in 70% isotonic Percoll (GE Healthcare, Buckinghamshire, UK) and layered under 37% isotonic Percoll. Layers were centrifuged at 500 x g for 20 minutes. Mononuclear cells in the interface were collected into R10.

CCR5 RO Assay

Equation 1 Flow Staining

Equation 1 measured for Leronlimab-occupied CCR5 by using anti-IgG4 FITC in comparison to total CCR5 measured by *ex vivo* incubation with a saturating concentration of Leronlimab (5 μg/mL). Three staining tubes were used, with one tube serving as the fluorescence minus one (FMO) control (**Supplemental Table 1**). A minimum of 50 μL of whole blood or 3 x 10^5 mononuclear cells were used per staining tube. Cells were washed twice with 1mL of PBS by centrifuging at 830 x g for 4 minutes, then supernatants were aspirated to leave ~100 μL, and finally cell pellets were resuspended by vortex. Next, 5 μg/mL of parental, unconjugated Leronlimab was added to tube 3 according to **Supplemental Table 1** and incubated for 30 minutes at room temperature in the dark. Cells were washed once with PBS, and then anti-IgG4 FITC was added to tubes 2 and 3 and incubated for 30 minutes at room temperature in the dark. Afterwards, cells were washed once with PBS + 10% FBS and once with PBS. Anti-CCR5 APC was added to tubes 2 and 3, while surface antibodies (CD3, CD4, CD8, CD45, CD95, and CD28) and amine-reactive dye (cell viability) were added to all tubes. A description of antibodies used can be found in **Supplemental Table 2**. Cells were incubated for 30 minutes at room temperature in the dark. For whole blood samples, 1 mL of 1X FACS Lysing solution (BD Biosciences) was added to each tube to lyse red blood cells for 8 minutes, and then immediately centrifuged and washed three times with PBS + 10% FBS. Cells were stored at 4°C prior to running on BD™ LSR II flow cytometer (BD Biosciences). For mononuclear cells, cells were washed twice with PBS and then fixed by adding 100 μL of 2% paraformaldehyde (PFA) and incubating for at least 10 minutes before running on BD™ LSR II flow cytometer (BD Biosciences). Flow analysis was done using FlowJo 10.4, where cells were progressively gated on singlets (FSC-H vs. FSC-A), live, CD45+, CD3+, CD4+/CD8-, and CCR5+ cell populations, as described in **Supplemental Figure 1**. Staining tube 1 served as the FMO control to assist with gating on desired cell populations, tube 2 stained for the frequency of Leronlimab-occupied CCR5+CD4+ T cells, and tube 3 was saturated with Leronlimab *ex vivo* to measure

for the total frequency of CCR5+CD4+ T cells. The equation to calculate CCR5 RO using equation 1 is as follows:

$$\%\,RO = \frac{\%\,IgG4\,(tube\,2)}{\%\,IgG4\,(tube\,3)} \times 100\,\%$$

Equation 2 Flow Staining

Equation 2 measured for unoccupied CCR5 by using Leronlimab that was conjugated to Pacific Blue (termed Leronlimab-PB). Three staining tubes were required, with two tubes serving as the FMO controls (**Supplemental Table 1**). Similar to equation 1, a minimum of 50 µL whole blood or 3 x 10^5 mononuclear cells were used per staining tube. Cells were washed two times with PBS and anti-IgG4 FITC was added to tube C following **Supplemental Table 1** and incubated for 30 minutes at room temperature in the dark. Because anti-IgG4 FITC could interact with downstream Leronlimab-PB, leading to false positive staining of anti-IgG4 FITC, cells were washed once with PBS + 10% FBS and at least three times with PBS. Afterward, anti-CCR5 APC was added to tubes B and C, Leronlimab-PB was added to tube C, surface antibodies (CD3, CD4, CD8, CD45, CD16, and CD14) and amine-reactive dye were added to all tubes (**Supplemental Table 2**). Cells were incubated for 30 minutes at room temperature in the dark. Afterward, whole blood or mononuclear cells were lysed with 1X FACS Lysing solution or fixed with 2% PFA, respectively, as described in the staining for equation 1. Samples were collected on a BD™ LSR II flow cytometer (BD Biosciences) and analyzed with FlowJo 10.4, where cells were progressively gated on CD45+, singlets (FSC-H vs. FSC-A), live, CD3+, CD4+/CD8-, and CCR5+ cell populations (**Supplemental Figure 1**). Here, staining tube A served as a FMO control to assist with gating on desired cell populations, and tube B served as a FMO control to assist with gating on IgG4+ and Leronlimab-PB+ cell populations. Tube C stained for cells with unoccupied CCR5 (Leronlimab-PB) and occupied CCR5 (anti-IgG4 FITC), with the total frequency of CCR5+CD4+ T cells represented by the sum of IgG4+ and Leronlimab-PB+ cell frequencies. The equation to calculate CCR5 RO using equation 2 is as follows:

$$\%RO = \frac{\%IgG4\,(tube\,C)}{\%IgG4\,(tube\,C) + \%Leronlimab\text{–}PB\,(tube\,C)} \times 100\%$$

Lastly, the percentage of CCR5+CD4+ T cells was determined with tube B because it was free from *ex vivo* incubation of unconjugated Leronlimab or conjugated Leronlimab-PB, which, described later in the Results session, was found to stabilize and increase CCR5 expression on CD4+ T cells.

Combined Equations 1 and 2 Flow Staining

In the clinically-adapted CCR5 RO assay, changes were made to 1) fluorophore conjugates to adjust for the higher-wattage lasers used in FACSymphony A5 (BD Biosciences), a machine that allowed for easier detection of rare events, 2) the FACSymphony A5's lack of a 651nm (green) laser and expanded 488nm (blue) laser, 3) antibody clones to improve species reactivity to macaque and human samples, and 4) combine both CCR5 RO equations into one staining panel to minimize the number of cells required for staining. Here, four staining tubes were required, with two tubes serving as FMO controls (**Supplemental Table 1**). At least 50 µL of whole blood or 3 x 10^5 PBMC samples were placed into each staining tube and samples were washed two times with PBS. Next, 5 µg/mL of the parental, unconjugated Leronlimab was added to tube H4 (**Supplemental Table 1**) and incubated for 30 minutes at 2-8°C in the dark. Afterward, tubes H1 and H2 were washed once with 3 mL cold PBS while tubes H3 and H4 were washed with 1 mL cold PBS with 10% mouse serum (Equitech) in order to minimize nonspecific binding of anti-human IgG4 FITC. Cells in tubes H3 and H4 were then incubated with 100 µL of cold mouse serum and incubated for 60 minutes at 2-8°C followed by another wash of 1 mL cold PBS with 10% mouse serum. Anti-IgG4 FITC and 100 µL of cold mouse serum were then added to tubes H3 and H4 and incubated for 30 minutes at 2-8°C in the dark. Next, cells were washed once with DPBS and 0.1% BSA and then at least three times with PBS. Anti-CCR5 APC was added to tubes H2, H3, and H4, Leronlimab-PB was added to tube H3, surface antibodies (CD3, CD4, CD8a, CD45, CD16, CD14, CD28, and CD95), and amine-reactive dye (cell viability) were added to all tubes (**Supplemental Table 3**). Cells were incubated for 30 minutes at room temperature in the dark. Finally, whole blood or PBMC samples were lysed with 1X FACS Lysing solution, incubated for 10 minutes in the dark, and then immediately washed once with DPBS and 0.1% BSA. Cells were then permeabilized with 0.5 mL 1.5X FACS Lysing solution and 0.05% Tween-20, incubated for 10 minutes in the dark, and then washed twice with DPBS and 0.1% BSA. Samples were run using the FACSymphony A5 (BD Biosciences) and analyzed with FlowJo 10.4. Cells were progressively gated on CD45+, singlets (FSC-H vs. FSC-A), live, CD3+, CD4+/CD8-, and CCR5+ cell populations (**Supplemental Figure 1**). Here, tube H1 and H2 served as FMO controls to assist with gating on cell populations, tube H3 stained for the frequency of Leronlimab-occupied CCR5 with anti-IgG4 FITC and Leronlimab-unoccupied CCR5 with Leronlimab-PB, and tube H4 was saturated with Leronlimab *ex vivo* to measure for the total frequency of CCR5+CD4+ T cells. The equations to calculate CCR5 RO are as follow:

$$\%RO = \frac{\%IgG4\,(tube\,H3)}{\%IgG4\,(tube\,H4)} \times 100\% \qquad \text{Equation 1}$$

$$\%\,RO = \frac{\%\,IgG4\,(tube\,H3)}{\%\,IgG4\,(tube\,H3) + \%\,Leronlimab\text{–}PB\,(tube\,H3)} \times 100\,\%$$

Equation 2

In Vitro CCR5 Ligand Binding and Staining

PBMCs were incubated with or without 5 µg/mL of Leronlimab for 30 minutes at 37°C. Next, cells were incubated at 37°C for an additional 30 minutes with no additional ligand or 50 nM of one of the following CCR5 ligands: MIP-1α, MIP-1β, or RANTES. Afterwards, cells were stained with CCR5 (clone 3A9), CD3, CD4, and CD8 cell surface receptors and amine-reactive dye. Samples were collected using the BD™ LSR II flow cytometer

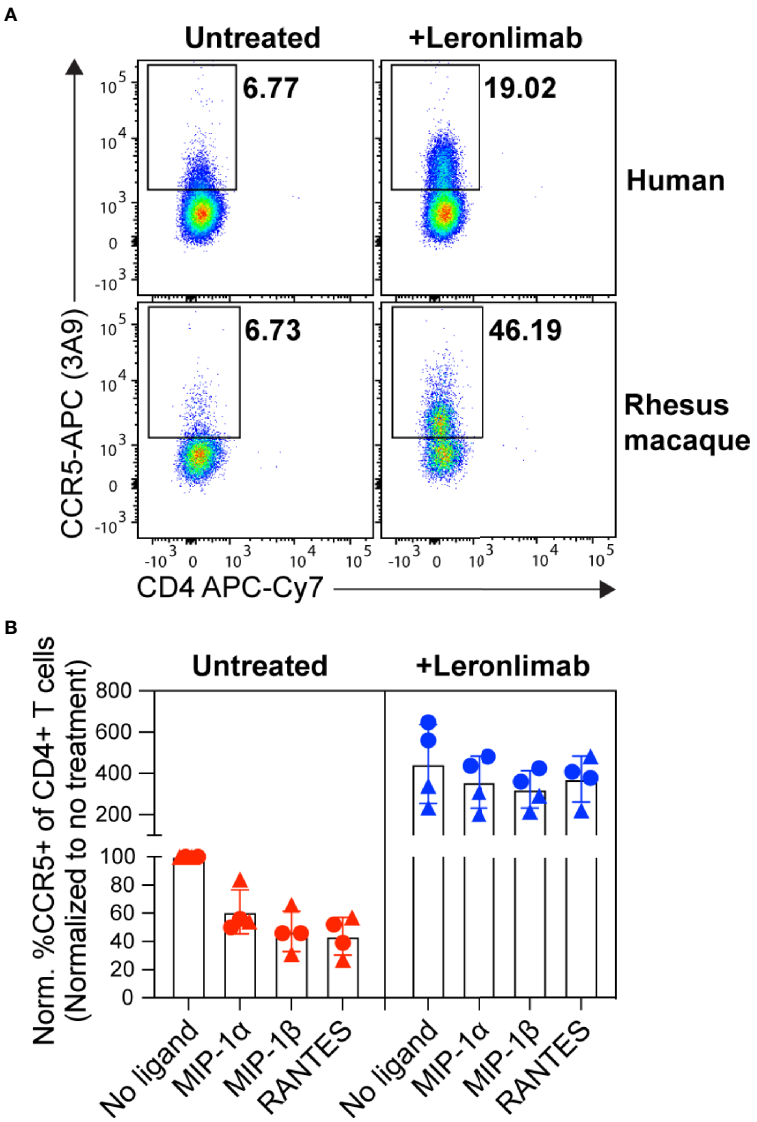

FIGURE 1 | Leronlimab increases cell surface CCR5 expression that is resistant to internalization. PBMC from humans (n=2; triangles) and rhesus macaques (n=2; circles) were incubated without (left, red) or with (right, blue) 5 μg/mL Leronlimab for 30 minutes at 37°C, then with either no treatment or 50nM of one the following CCR5 ligands: MIP-1α, MIP-1β, or RANTES, for an additional 30 minutes. After incubation, cells were stained for CCR5 (clone 3A9) and CD3, CD4, and CD8 surface markers. Cells were gated within live, CD3+, CD4+/CD8-, singlet populations. **(A)** Representative flow plots. **(B)** Graphs show normalized frequencies of CCR5+ of CD4+ T cells to frequency observed with untreated cells (no Leronlimab, no CCR5 ligands) from the respective donor.

and analyzed with FlowJo 10.4 by gating on live, CD3+, CD4+/CD8-, singlet, and CCR5+ cell populations. Data was presented as normalized %CCR5+ for each treatment condition that was normalized to frequency observed in Leronlimab-untreated, ligand-free cells from each respective donor.

Measurement of Leronlimab Concentration in Plasma

Enzyme-linked immunosorbent assay (ELISA) was used to detect for Leronlimab levels in plasma samples, as previously described (40). Briefly, plates were coated with 1.5 μg/mL PA22 (CytoDyn, Vancouver, WA) in carbonate-bicarbonate buffer (ThermoFisher) overnight at 4°C. Plates were washed three times with PBS-T (PBS with 0.1% Tween-20) and blocked for at least two hours in room temperature with Blocking Buffer (PBS with 0.4% Tween-20 and 10% bovine serum albumin (Fisher Scientific). Standard curve generated with serial dilutions of Leronlimab and samples were plated onto blocked plates and incubated for 30 minutes at room temperature. Plates were washed three times with PBS containing 0.5 M NaCl, and then incubated with 20,000-fold diluted mouse anti-human IgG4 pFc'-horseradish peroxidase (Southern Biotech) in Blocking Buffer for 30 minutes at room temperature. Finally, plates were washed three times with PBS-T and 3,3',5,5'-

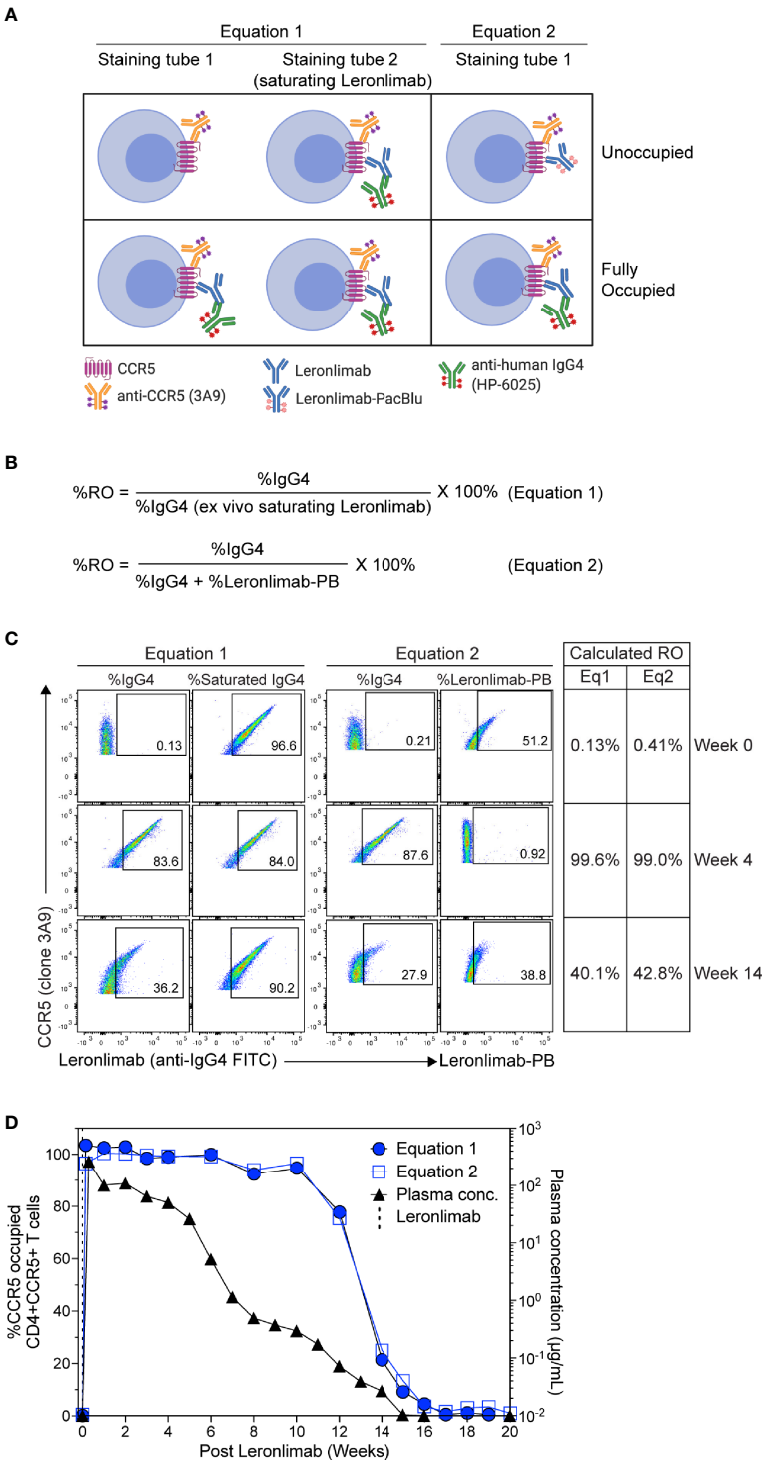

FIGURE 2 | CCR5 receptor occupancy assay overview. **(A)** Flow cytometry diagram showing the interactions between anti-CCR5, Leronlimab, Leronlimab-PacBlu (PB), and anti-human IgG4 for the two equations using a CCR5 unoccupied (top) and fully occupied (bottom) scenario. **(B)** Equations for calculating CCR5 RO. **(C)** Representative flow cytometry plots displaying the different components needed to calculate for the two equations using a rhesus macaque that received a single 50 mg/kg SC Leronlimab injection. Equation 1 used %IgG4+ events within CD45+, singlet, live, CD3+, CD4+/CD8-, and CCR5+ events. Equation 2 used %IgG4+ and Leronlimab-PB+ events within CD45+, singlet, live, CD3+, CD4+/CD8-, and CCR5+ events. Table on the right shows the calculated CCR5 RO calculated by the two equations at study weeks 0, 4, and 14 post single Leronlimab injection. **(D)** Left Y-axis is for CCR5 RO by Leronlimab on peripheral blood CD4+CCR5+ T cells calculated by equation 1 (solid blue circle) and equation 2 (open blue square). Right Y-axis is for the longitudinal plasma concentration (solid black triangle) in blood samples from the treated macaque.

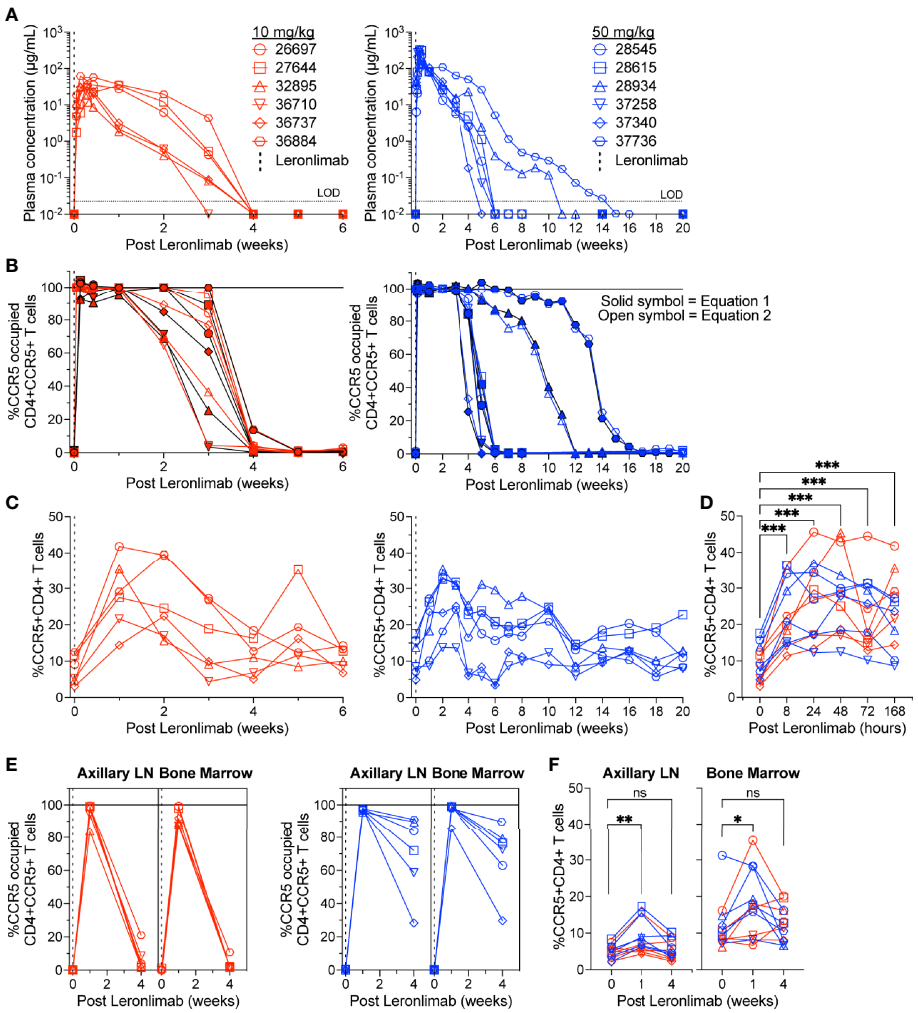

FIGURE 3 | CCR5+CD4+ T cell frequencies increase with CCR5 RO. Leronlimab-naïve, SIV-naïve, rhesus macaques received a single 10 mg/kg (n=6; red; left) or 50 mg/kg (n=6; blue; right) SC Leronlimab. **(A)** Longitudinal Leronlimab concentration in plasma. **(B)** Longitudinal CCR5 RO by Leronlimab on peripheral blood CD4+CCR5+ T cells. **(C, D)** Peripheral blood CCR5+CD4+ T cell frequency for **(C)** longitudinal weekly timepoints, separated by treatment group and **(D)** hourly timepoints for both treatment groups within the first week post Leronlimab. **(E, F)** Axillary lymph node and bone marrow **(E)** CCR5 RO by Leronlimab and **(F)** % CCR5 on tissue-resident CD4+ T cells. **(B, E)** Solid symbol represents CCR5 RO calculated from equation 1 while open symbol represents CCR5 RO calculated from equation 2. P-values in panels **(D, F)** generated by Wilcoxon signed-ranked test; ns, not significant, *p < 0.05; **p < 0.01; ***p = 0.0005.

Tetramethylbenzidine (TMB) substrate (Southern Biotech) was added to develop the plates for two minutes, after which, 1 N H2SO4 was added to stop the reaction. Developed plates were read on Synergy HTX Multi-Mode Microplate Reader (BioTek) using the Gen5 v3.10 software to read two absorbance wavelengths: 650 nm for the developing reaction and 450 nm for the developed reaction. Plasma concentration for each sample (µg/mL) was determined using the generated standard curve, with an assay limit of detection of 0.0226 µg/mL.

Viral Nucleic Acid Detection

SIV nucleic acid detection assays were performed by members of the ONPRC Molecular Virology Core. Detection of SIV nucleic acids was performed as previously described (40, 46, 47). Briefly, viral nucleic acids from 300 µL of plasma were extracted using LEV Viral Nucleic Acid Kit and the Maxwell 16 instrument (Promega, Madison, WI) following manufacturer's protocols. RT-qPCR reaction was performed to quantify SIV viral RNA in the plasma. The reaction used the total extracted RNA, 900 nM of SGAG21 forward primer (GTCTGCGTCATPTGGTG CATTC), 900 nM of SGAG22 reverse primer (CACTAGKTG TCTCTGCACTATPTGTTTTG), and 250 nM of pSGAG23 probe (5′-6-carboxyfluorescein [FAM]-CTTCPTCAGT KTGTTTCACTTTCTCTTCTGCG-black hole quencher [BHQ1]-3′) for a final reaction volume of 30 µL. Standard curve was created by using *in vitro* transcribed SIVgag RNA that was serially diluted in 5 ng/µL yeast tRNA (Sigma R5636). Applied Biosystems QuantStudio 6 Flex instrument (Life

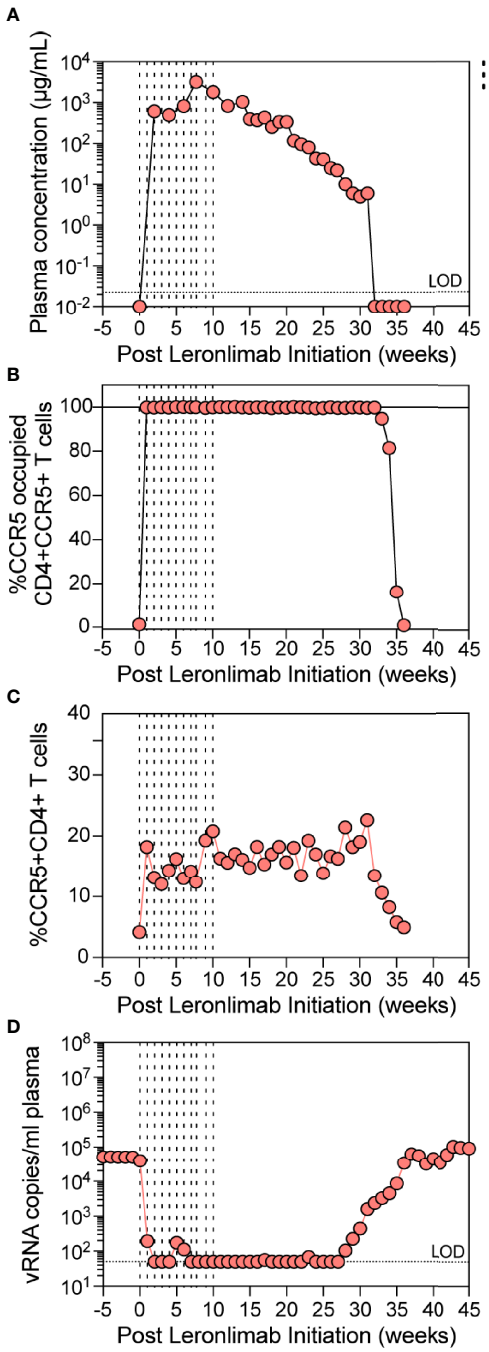

FIGURE 4 | Leronlimab treatment of an SIVmac239-infected macaque. A cynomolgus macaque (n=1; 36484) was chronically infected with SIVmac239 prior to receiving weekly 50 mg/kg SC Leronlimab. **(A)** Longitudinal Leronlimab concentration in plasma. **(B)** Longitudinal CCR5 RO by Leronlimab on peripheral CD4+CCR5+ T cells. **(C)** Longitudinal peripheral blood CCR5+CD4+ T cell frequency. **(D)** Longitudinal SIV RNA copies per mL plasma. Vertical dashed lines represent each SC Leronlimab injection.

Technologies) was used to run the RT-qPCR reactions at the following setting: 50°C for 5 min; 95°C for 20 s; [95°C for 3 s, 60°C for 30 s] × 45 cycles. The limit of quantification for this assay is 50 copies/mL.

Statistical Analysis

Wilcoxon signed-rank test was used to analyze %CCR5+CD4+ T cells between baseline and Leronlimab treatment timepoints in macaques. Two-way repeated measures analysis of variance (ANOVA) with Sidak's correction for multiple comparisons was used to compare the %CCR5+CD4+ T cells between Leronlimab-treated and placebo human participants. Analysis was performed using GraphPad Prism Version 8.31 (332).

RESULTS

Development of the Leronlimab CCR5 Receptor Occupancy Assay

Rhesus macaques are vitally important pre-clinical models used to validate potential prophylactic and therapeutic modalities as they share similar immune systems with humans. Indeed, CCR5 sequence, structure, and function are highly conserved between the two species (48). Previously, we demonstrated that Leronlimab specifically binds the same CCR5 epitope on the surface of human and rhesus macaque leukocytes (40). Therefore, to facilitate both pre-clinical macaque and clinical human studies, we analyzed the effect of Leronlimab on CCR5 expression on CD4+ T cells from both species.

First, we examined the impact of treatment with a saturating concentration of 5 µg/mL Leronlimab on surface CCR5 levels of primary human and macaque CD4+ T cells *in vitro*. Similar to Maraviroc, Leronlimab stabilized cell surface CCR5 and directly increased the frequency of CCR5+CD4+ T cells from humans and rhesus macaques (**Figure 1A**). Next, we explored if Leronlimab-occupied CCR5 was resistant to internalization following treatment with CCR5 ligands, a phenomenon that forms the basis for the MIP-1β internalization Maraviroc CCR5 RO assay (37, 38). In samples without Leronlimab, we observed CCR5 internalization in response to MIP-1α, MIP-1β, and RANTES (**Figure 1B**). Following treatment with Leronlimab, we found increased frequencies of CCR5+CD4+ T cells where CCR5 was resistant to internalization following treatment with all three CCR5 ligands, indicating that Leronlimab both stabilized surface CCR5 expression and prevented its internalization. Thus, it is critical to account for this Leronlimab-induced increase in surface CCR5 levels for CCR5 RO measurements.

To measure CCR5 RO, we designed Leronlimab CCR5 RO assays based on methods previously established for RO measurements of anti-PD-1 antibodies in clinical trials (49, 50). The Leronlimab CCR5 RO assays consist of the three following critical components: 1) the CCR5-specific antibody clone 3A9 that does not compete with nor sterically hinder Leronlimab binding of CCR5 to track overall CCR5 expression, 2) the anti-human IgG4 antibody clone HP6025, which binds to the humanized IgG4 Fc of Leronlimab and 3) Pacific Blue-labeled Leronlimab (termed Leronlimab-PB), which binds to

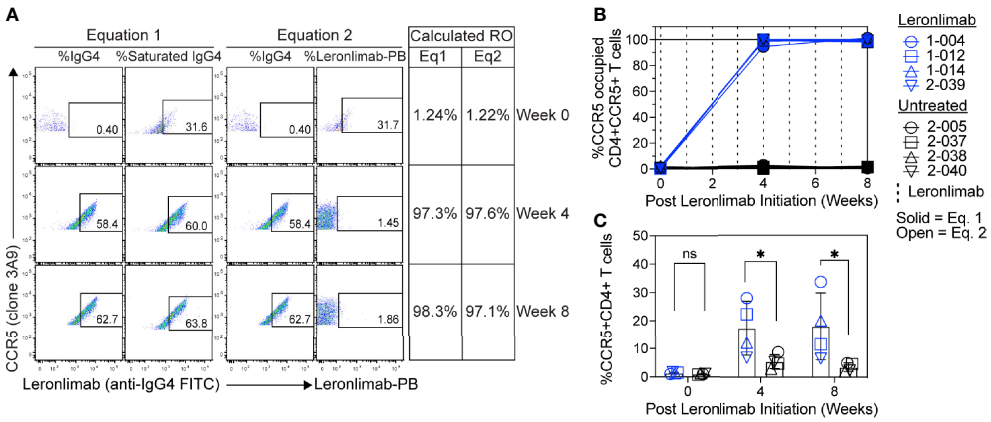

FIGURE 5 | CCR5 RO assay on human samples. Blood samples were collected from human participants in ClinicalTrials.gov Identifier: NCT04678830 at study weeks 0, 4, and 8 post Leronlimab initiation. Leronlimab-treated (n=4; blue) participants received weekly 700 mg SC Leronlimab, while controls were untreated (n=4; black). **(A)** Representative flow cytometry plots showing the different components required to calculate CCR5 RO for equation 1 and 2. Table on the right shows the calculated RO at study weeks 0, 4, and 8. **(B)** Longitudinal CCR5 RO by Leronlimab on peripheral blood CD4+ T cells calculated with equation 1 (solid blue) and equation 2 (open blue). **(C)** Longitudinal CCR5+CD4+ frequency in peripheral blood. Vertical dashed line represents each SC Leronlimab injection. P-values in panel **(C)** calculated by two-way repeated measures ANOVA with Sidak's correction for multiple comparisons; ns, not significant; *$p < 0.05$.

free CCR5 receptors not occupied by Leronlimab (**Figure 2A**). To measure the percentage of CCR5 RO on the surface of CD4+ T cells, two different methods were used as described in detail in the Materials and Methods section. CCR5 RO equation 1 measures Leronlimab-bound receptor by using the anti-human IgG4 antibody to measure Leronlimab-occupied CCR5 receptor directly (**Figure 2A**). CCR5 RO equation 2 measures unoccupied CCR5 receptors by using Pacific Blue-conjugated Leronlimab. In both methods, CCR5 staining with the 3A9 antibody is used to account for weekly variations in CCR5 expression prior to calculation of CCR5 RO (**Supplemental Figure 1**). CCR5 RO for Equation 1 is defined by the percentage of cells CCR5+ (measured by clone 3A9) and Leronlimab+ (measured by anti-human IgG4) divided by the percentage of cells CCR5+ and Leronlimab+ following incubation with 5 μg/mL, a saturating concentration of unlabeled Leronlimab (**Figures 2B, C**).

$$\%\,RO = \frac{\%\,IgG4}{\%\,IgG4\ (ex\ vivo\ saturating\ Leronlimab)} \times 100\,\% \quad \text{(Equation 1)}$$

CCR5 RO for Equation 2 is defined as the percentage of cells CCR5+ (measured by clone 3A9) and Leronlimab+ (measured by anti-human IgG4) divided by the percentage of cells CCR5+ and Leronlimab+ (measured by the sum of anti-human IgG4 and Leronlimab-PB) cells following incubation with 5 μg/mL Leronlimab-PB.

$$\%\,RO = \frac{\%\,IgG4}{\%\,IgG4 + Leronlimab\text{–}PB} \times 100\,\% \quad \text{(Equation 2)}$$

Next, we tested the Leronlimab CCR5 RO assay using longitudinal peripheral blood samples from a rhesus macaque that received a single 50 mg/kg subcutaneous (SC) dose of Leronlimab. Representative flow cytometric plots and calculated CCR5 RO values using the two equations at three different study weeks are shown to demonstrate how the RO values are calculated (**Figure 2C**). We observed no CCR5 RO on peripheral blood CD4+ T cells immediately prior to Leronlimab dosing, followed by 100% CCR5 RO within eight hours following the SC injection (**Figure 2D**). CCR5 RO was then maintained at approximately 100% until the Leronlimab plasma concentration fell below 5 μg/mL at approximately six weeks post injection. Following this, CCR5 RO continually decreased to baseline level following complete washout of Leronlimab from plasma. Importantly, both RO methods yielded similar CCR5 RO measurements throughout the study and correlated with the Leronlimab plasma concentration. These results demonstrate the sensitivity and reproducibility of the CCR5 RO assay for monitoring Leronlimab RO *ex vivo*.

TABLE 1 | Calculated CCR5 RO by Leronlimab on peripheral blood CD4+ T cells from Leronlimab-treated or untreated humans and macaques.

Leronlimab condition	Species	Mean % CCR5 RO (SD, N)	
		Equation 1	Equation 2
Untreated	Human	1.02% (0.62, 8)	0.65% (0.52, 8)
	Macaque	0.38% (0.41, 12)	0.08% (1.06, 12)
Treated	Human	98.39% (2.47,4)	99.73% (0.57, 4)
	Macaque	100.83% (4.33, 12)	99.84% (0.59, 12)

Leronlimab Treatment Increases CCR5+CD4+ T Cell Counts in Healthy Rhesus Macaques

To further verify that CCR5 RO calculated by equation 1 and equation 2 were robust methods to longitudinally track Leronlimab CCR5 RO over time in blood and tissue, as well as to monitor the impact of CCR5 RO on CCR5+CD4+ T cell levels, we treated 12 rhesus macaques with a single 10 mg/kg or 50 mg/kg

SC Leronlimab injection (n=6 per group). As expected, Leronlimab plasma levels peaked at lower levels and washed out more rapidly in macaques who received 10 mg/kg versus 50 mg/kg (**Figure 3A**). In both groups, however, Leronlimab treatment yielded full CCR5 RO on peripheral blood CD4+ T cells by eight hours post injection, and maintained >90% CCR5 RO for an average of 12.8 days and 32.6 days for the 10 mg/kg and 50 mg/kg groups, respectively (**Figure 3B**).

CCR5 antagonists impact expression of cell surface CCR5 and thus modulate the levels CCR5+CD4+ T cells circulating *in vivo*. Maraviroc treatment increased CCR5 expression on peripheral blood T cells in both humans and macaques, but the effect on CCR5 expression on tissue-resident T cells is unclear (42, 43, 51). In contrast, longitudinal treatment of macaques with the anti-CCR5 antibody HGS101 did not increase CCR5 expression, but rather decreased levels of CCR5+CD4+ T cells in both peripheral blood and lymph nodes (41). Therefore, we next sought to assess the impact of Leronlimab on CCR5+CD4+ T cells levels in both peripheral blood and within tissues. Following administration of Leronlimab at either dose we observed an increase in the frequencies of CCR5+CD4+ T cells circulating within peripheral blood that tracked with CCR5 RO (**Figure 3C**). In line with the rapid stabilization of CCR5 *in vitro* shown in **Figure 1**, statistically significant increases in peripheral blood CCR5+CD4+ T cell frequencies were found as early as eight hours post injection and maintained at every timepoint examined throughout the first week post injection (**Figure 3D**). Next, we examined the impact of Leronlimab on CCR5+CD4+ T cells from lymph node and bone marrow biopsies. Similar to peripheral blood results, we found no CCR5 RO on lymph node and bone marrow CD4+ T cells prior to Leronlimab injection, followed by high levels of CCR5 RO on CD4+ T cells from these tissues at one week post injection (**Figure 3E**). Reflective of plasma concentration and peripheral blood CCR5 RO results, levels of CCR5 RO by Leronlimab on tissue CD4+ T cells were very low at four weeks post injection in the 10 mg/kg treated group, while significant, but variable CCR5 RO levels remained on tissue CD4+ T cells from the 50 mg/kg treated group (**Figure 3E**). In line with these CCR5 RO values, we observed a statistically significant increase in frequencies of tissue CCR5+CD4+ T cells at one week post Leronlimab injection, concomitant with high levels of CCR5 RO on CD4+ T cells in those tissues (**Figure 3F**). Upon loss of CCR5 RO at week four post Leronlimab injection, the levels of CCR5+CD4+ T cells decreased and were no longer statistically different from pre-injection levels. These observations demonstrate that Leronlimab treatment increases CCR5+CD4+ T cell frequencies in both the peripheral blood and within lymphoid tissues, and that this phenomenon depends upon the degree of Leronlimab occupancy of CCR5.

Leronlimab Treatment Suppresses SIVmac239 Replication Despite Increasing CCR5+CD4+ T Cell Levels

The observed increase in CCR5+CD4+ T cells in Leronlimab-treated macaques raised the possibility that Leronlimab could inadvertently exacerbate ongoing SIV infection by providing additional susceptible targets for viral replication. To study the impact of Leronlimab on CD4+ T cell dynamics and viral replication in an ongoing SIV infection, we treated a chronically SIVmac239-infected Mauritian cynomolgus macaque with weekly 50 mg/kg SC Leronlimab injections for 11 weeks.

Similar to a single 50 mg/kg injection, complete CCR5 RO on peripheral blood CD4+ T cells was observed one week after the first dose and subsequently maintained for weeks after the final dose. However, compared to a single 50 mg/kg injection, repeated weekly 50 mg/kg injections for 11 weeks resulted in a longer duration of plasma Leronlimab, where complete Leronlimab plasma wash out and loss of CCR5 RO occurred at study weeks 32 and 36 post first dose, respectively (**Figures 4A, B**). As expected, based on our results above in SIV-naïve macaques, the frequency of CCR5+CD4+ T cells circulating in peripheral blood tracked with CCR5 RO, where it immediately increased after the first injection, began to decline with Leronlimab plasma washout, and returned to baseline level with loss of CCR5 RO (**Figure 4C**). Importantly, the increased frequencies of CCR5+CD4+ T cell targets did not exacerbate SIV replication. Instead, Leronlimab potently and completely suppressed SIV replication for approximately 20 weeks, during the time period where both full CCR5 RO and increased CCR5 +CD4+ T cells were present in the blood (**Figure 4D**). As the Leronlimab plasma concentration declined and CCR5 RO was lost on CD4+ T cells, viral rebound occurred. SIVmac239 plasma viremia ultimately returned to pre-Leronlimab levels after complete loss of CCR5 RO. Therefore, the Leronlimab-induced increase in CCR5+CD4+ T cell targets did not exacerbate ongoing SIV replication; rather, the binding of Leronlimab to the CCR5 co-receptor used for viral entry protected these cells from infection and greatly diminished ongoing SIV replication, resulting in minimal plasma viremia during the period of complete CCR5 RO.

Measurement of CCR5 RO in Leronlimab-Treated, HIV-Free Human Participants

Currently, Leronlimab is undergoing testing in clinical trials for both HIV and HIV-unrelated indications. With the successful demonstration of the CCR5 RO assay in non-human primate models, we next sought to extend the pre-clinical CCR5 RO technique for clinical applications. While the majority of the antibodies utilized in the macaque-specific assay are human-specific antibodies that cross react with the macaque orthologue, we adapted and refined the antibody clones utilized for optimal staining on human cells (**Supplemental Table 2**). To determine the performance of the clinical-grade CCR5 RO assay, we obtained blinded clinical samples from eight participants enrolled in a phase 2, two-arm, randomized, double blind, placebo-controlled study on the effects of Leronlimab treatment in long COVID-19 (ClinicalTrials.gov Identifier: NCT04678830). Enrolled participants were randomized to receive either weekly 700 mg SC Leronlimab injections or placebo throughout an eight-week study period, with a total of three clinical visits at weeks 0, 4,

and 8 after the first Leronlimab initiation. As in our pre-clinical study models, the two equations calculated comparable CCR5 RO percentages from all three clinical visits in Leronlimab-treated human participants (**Figure 5A**). Furthermore, the CCR5 RO assay correctly identified individuals treated with Leronlimab versus placebo following unblinding, as treated individuals presented at baseline with no CCR5 RO, but then achieved full CCR5 RO on peripheral blood CD4+ T cells at the two follow-up clinical visits, while placebo-treated participants never presented with CCR5 RO (**Figure 5B**). In line with the preclinical macaque results, we observed increased frequencies of circulating CCR5+CD4+ T cells following Leronlimab dosing. After Leronlimab initiation, frequencies of CCR5+CD4+ T cells circulating in the peripheral blood in Leronlimab-treated participants were statistically higher than in untreated participants (**Figure 5C**). Thus, the longitudinal CD4+ T cell CCR5 RO in Leronlimab-treated participants and lack of measurable CCR5 RO in Leronlimab-untreated participants demonstrates the robustness and sensitivity of the clinical CCR5 RO assay. Indeed, in both macaques and humans, CCR5 RO in the absence or presence of saturating plasma concentrations of Leronlimab was approximately 1% and 99%, respectively (**Table 1**). Cumulatively, these results establish precise methods to measure CCR5 RO in a pre-clinical nonhuman primate species and in human participants in clinical trials, and demonstrate that Leronlimab CCR5 RO induces increased frequencies of CCR5+CD4+ T cells.

DISCUSSION

Here, we created and validated two different methods of calculating CCR5 RO by the anti-CCR5 antibody Leronlimab. The methods generated comparable longitudinally CCR5 RO percentages in rhesus macaques that received a single 10 mg/kg or 50 mg/kg SC Leronlimab injection. Additionally, both methods were highly sensitive, with baseline values of 1% and fully saturated values of 99% when tested in human and non-human primates. These results are in contrast to the commonly used MIP-1β internalization assay utilized for Maraviroc, which is associated with background levels of approximately 25% in human samples (38) and yields values in excess of 100% in maraviroc-treated and -untreated rhesus macaques (39). Higher RO percentages calculated by the MIP-1β internalization assay may be due to fluctuating CCR5 frequencies or incomplete CCR5 internalization upon MIP-1β binding. In contrast, our methods did not depend on receptor internalization and all mathematical components used were gated on CCR5+ cells, compensating for any fluctuation in CCR5 frequency and allowing for precise calculation of RO. Finally, the pre-clinical Leronlimab CCR5 RO assay was extended into human participants, demonstrating the ability to longitudinally and robustly monitor CCR5 RO.

Similar to maraviroc, we found that Leronlimab stabilized surface CCR5 molecules and prevented its internalization following ligand binding. Indeed, this shared feature of both drugs likely explains their shared ability to increase frequencies of CCR5+CD4+ T cells in both humans and macaques. Because CCR5+CD4+ T cells are target cells for HIV/SIV infection, increasing the frequencies of susceptible cells could raise concerns of increased HIV/SIV replication. However, we found that weekly Leronlimab treatment in a chronically SIVmac239-infected macaque fully suppressed plasma viremia for over 20 weeks despite a rise in the CCR5+CD4+ T cell frequency immediately after the first Leronlimab injection. Both suppression of viral replication and increased CCR5+CD4+ T cell levels were temporally associated with full CCR5 RO on peripheral blood CD4+ T cells, underscoring the need to measure CCR5 RO in studies utilizing CCR5-blocking agents.

Because CCR5 is involved in multiple pathophysiologic processes, Leronlimab is being explored in clinical trials for both HIV and non-HIV indications. In HIV-positive participants, Leronlimab suppressed plasma viremia after a single 5 or 10 mg/kg SC Leronlimab injection, while Leronlimab monotherapy contributed to the maintenance of viral suppression for over six years (52–55). Moreover, the only two cases of HIV cure occurred after hematopoietic stem cell transplantation (HSCT) with donor cells homozygous for CCR5Δ32 (56, 57), while similar studies using donor cells that were wild-type CCR5 or heterozygous CCR5Δ32 led to eventual viral rebound (58). For this reason, there is intense focus on genetically disrupting *ccr5* to abolish cell surface CCR5 expression in HIV-positive individuals (59, 60). However, CCR5 has many protective roles, making it difficult to predict the long-term biological effects or consequences of permanently disrupting *ccr5* expression in humans. A logical approach to reproduce the phenotypic protection seen in homozygous CCR5Δ32 individuals is to instead use CCR5-blocking pharmacologic agents such as Leronlimab. Moreover, the therapeutic use of Leronlimab extends beyond HIV treatment due to its diverse roles. CCR5 is expressed in over 95% of triple-negative breast cancers (61) and influences breast cancer progression (9). In a murine model, Leronlimab prevented and reduced breast cancer metastasis suggesting a role for Leronlimab in the treatment of neoplasia (62). As CCR5 is central in inflammatory immune responses, it is currently being studied as a therapeutic for severe and critical SARS-CoV-2 infections (44, 63–65) and graft-versus-host disease (GVHD), where Leronlimab treatment reduced xeno-GVHD after HSCT of human cells to mice (66). Finally, Leronlimab is currently in phase 1 and 2 clinical studies to treat metastatic colorectal cancer, nonalcoholic Steatohepatitis, and long COVID after SARS-CoV-2 infection, demonstrating the diverse applicability of this safe and effective CCR5-targeting agent.

The appeal of monoclonal antibody-based therapeutic agents is growing due to their longer half-life and promising safety profile, and this is reflected in the dramatic rise in FDA approvals and commercial use of antibody treatments in recent years (67, 68). The CCR5 RO assays described here will be an important study measurement for any CCR5 antibody-based agent, and can be modified for any antibody-based agents that have a clearly defined cell surface protein target. In the case of the anti-CCR5 antibody Leronlimab, the ability to accurately measure CCR5 RO

will advance both pre-clinical and clinical studies, furthering our understanding of the immunological impacts of CCR5 for multiple pathophysiologic processes.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The protocol was approved by the institutional review board at Amarex Clinical Research, Arthritis and Rheumatic Disease Specialties, and Center for Advanced Research and Education (CARE). ClinicalTrials.gov identifier (NCT number), NCT04678830. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by Oregon National Primate Research Center (ONPRC) Institutional Animal Care and Use Committee (IACUC).

AUTHOR CONTRIBUTIONS

JBS conceptualized the studies and experiments. XC and HW managed macaque studies, assisted by JR, and JH. CR ran the clinical trial, while CH, AS, DM, KB, and SH performed analysis. MF, RB, and JS managed the animal care and procedures. XC, HW, GW, MT, CH, JR, JH, CW, CB, CP, AS, DM, KB, SK, NP, CR, SH, and JBS investigated. XC and JBS designed and validated CCR5 RO assays in macaques, while JBS and SH adapted assay for human clinical use. XC and JBS drafted the manuscript, and all authors contributed to editing. JBS, SH, SK, and NP acquired funding. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.794638/full#supplementary-material

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CCR5 and Biological Complexity: The Need for Data Integration and Educational Materials to Address Genetic/Biological Reductionism at the Interface of Ethical, Legal, and Social Implications

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In the age of genomics, public understanding of complex scientific knowledge is critical. To combat reductionistic views, it is necessary to generate and organize educational material and data that keep pace with advances in genomics. The view that CCR5 is solely the receptor for HIV gave rise to demand to remove the gene in patients to create host HIV resistance, underestimating the broader roles and complex genetic inheritance of CCR5. A program aimed at providing research projects to undergraduates, known as CODE, has been expanded to build educational material for genes such as *CCR5* in a rapid approach, exposing students and trainees to large bioinformatics databases and previous experiments for broader data to challenge commitment to biological reductionism. Our students organize expression databases, query environmental responses, assess genetic factors, generate protein models/dynamics, and profile evolutionary insights into a protein such as CCR5. The knowledgebase generated in the initiative opens the door for public educational information and tools (molecular videos, 3D printed models, and handouts), classroom materials, and strategy for future genetic ideas that can be distributed in formal, semiformal, and informal educational environments. This work highlights that many factors are missing from the reductionist view of CCR5, including the role of missense variants or

expression of CCR5 with neurological phenotypes and the role of CCR5 and the delta32 variant in complex critical care patients with sepsis. When connected to genomic stories in the news, these tools offer critically needed Ethical, Legal, and Social Implication (ELSI) education to combat biological reductionism.

Keywords: CCR5, viral infections, expression analysis, evolutionary profiling, molecular dynamic simulations, microglia, educational material generation

INTRODUCTION

Genetics and genomics are complex. Nearly every scientist is trained to integrate the scientific method into research design, formulating a hypothesis and testing it. However, this method of probing scientific insights was formulated in an age with limited data and resources in a simplified, often reductionistic, biological understanding. As the amount of data generated now often overcomes what a mind can comprehend, hypothesis-driven research becomes more and more challenging, especially when clinical, real-world decision-making occurs. Focused, hypothesis-driven research in genomics can often result in overly simplified views of genes that result in reductionism when not balanced with a full view of the biological complexity. To combat these reductionistic views in genomics, it is critical to look more broadly, often non-hypothesis driven and based on the larger data analysis. It is the responsibility of the genetics community to build tools that combat misunderstanding and reductionism (1), particularly when Ethical, Legal and Social Implications (ELSI) are involved. The big data community often is embraced to move beyond gene to single-function insights to broaden our view of how genetics contributes to biology.

Throughout our educational pipeline, genomic literacy has been a growing weakness, even in well-educated individuals, potentially elevating genetic essentialism (2, 3). We cannot utilize only scientific publications to combat genomic reductionism, as these are not accessible to most individuals. Studies in high school standards (4), undergraduate education (5), medical school training (6), specialized medical fields such as nursing (7, 8), practicing physicians (9), and general public education (10, 11) all suggest weaknesses in our genomics education pipelines. Many of our genomics classes, textbooks, and resources still focus on reductionistic genetics of Punnett squares and monogenic inheritance, missing the complexity of genomics (12). In a randomized control trial, it has been shown that students with more genomic literacy prevents essentialist views of genetics (13). The Public Understanding and Attitudes towards Genetics and Genomics (PUGGS) instrument applied to first-year university students suggested that the challenges of genetic reductionism also include social factors of age and religion (14). However, a more recent assessment of the PUGGS suggests the need for reform and further applications to educational assessments (15). Through a mixed-methods approach, others have shown the need to implement culturally and linguistically diverse backgrounds into our genomics education (16), which could be accomplished with more visual aids and interactive forums. The increasing education on gene regulation, epigenetics, and gene-by-environment regulation is critically needed at earlier levels to counter reductionistic views (17). Implementing more mechanistic reasoning abilities into genomic literacy is also critical (18). Here we lay out a strategy to engage students in complex research on genes, integrating large data resources into educational tools that others can be used to broaden genomics perspectives.

The fundamental insight of CCR5 as a receptor for HIV to infect cells, and that a human variant known as delta32 (hg38 3_46373452_TACAGTCAGTATCAATTCTGGAAGA ATTTCCAG_T, rs333/rs775750898, CCR5 p.Ser185IlefsTer32) in the protein corresponds to HIV resistance (19, 20), gave rise to the potential to target *CCR5* for HIV treatment and prophylaxis (21–23). What started as a potential to edit blood cells to give host HIV resistance (24, 25) created ambiguity and opportunities for scientists to perform the gene editing in human germline experiments. Yet, these germline experiments leave out many of the risks of the experiments ranging from off-target activity (26) to the role *CCR5* plays in normal cell, tissue, and organ biology (27), which can be compensated by the complex multivariant inheritance of delta32. While the potential for CRISPR editing of *CCR5* to create HIV resistance is intriguing (28), a more complex understanding of *CCR5* biology is critical. HudsonAlpha Institute for Biotechnology and Michigan State University formulated the Characterizing Our DNA Exceptions (CODE) program to advance knowledge of genetic variation and provide insights into genetics through a research program for undergraduate and graduate students in performing gene-centric data surveillance and integration into knowledge. This program created an opportunity for students within our CODE program to build CCR5 tools for educational use to broaden understanding of the biology of CCR5. This work describes the tools and resources integrated for a richer, more complex view of CCR5, with tools and resources accessible outside of our traditional publication system that does not often reach those needing enhanced genomic literacy.

The C-C Chemokine Receptor Type 5 (CCR5) is a G-protein coupled receptor (GPCR) prominently known for its role as the co-receptor (with CD4 as the primary receptor) in HIV infection. However, this receptor has many roles outside of the infectious disease realm. CCR5 is predominantly located on the cell membrane of macrophages, T-cells, Hofbauer cells, and Kupffer cells with minor expression on epithelial cells, type 2 alveolar cells, fibroblasts, and B-cells (29). When comparing *CCR5* expression among T cell subpopulations, it was found to be specific to TH1 T-cells (30) and CD8+ T-cells (31) as opposed

to the TH2 subpopulation involved in allergy and parasitic responses, which were more specific for CCR3 (32). Ellwanger et al. have laid out many of the pros and cons of CCR5 removal, including a detailed literature review of the many experiments performed for CCR5 biology outside of HIV (33). In 2009, it was well laid out that the recent emergence of the HIV infection could not be a sole explanation for the emergence of delta32, instead suggesting a push-pull aspect of immune activation, where inhibition of immune overactivation due to infection or autoimmunity could be evolutionarily advantageous, but with consequences to immune system components (34). This is further defended by data in CCR5 knockout models, which suggest advantageous roles in decreased immune activation (35–37) while having neurological complications (38, 39) and, in some cases, blunted immune response to pathogens (40, 41). As CCR5 has also been extensively linked to autoimmunity and autoimmune liver diseases, targeting it with therapeutics has been suggested (42–44).

CCR5 is a receptor for several CC-chemokines, including CCL3 (MIP-1-alpha), CCL4 (MIP-1-beta), and CCL5 (RANTES), which induce intracellular signal amplification *via* activation of the AKT and NF-KB pathways (45, 46). Both CCL3 and CCL4 are predominantly produced and secreted by T-cells, Hofbauer cells, macrophages, and Kupffer cells, while CCL5 has higher expression and secretion by T-cells (29). When bound to CCR5, CCL3 plays a significant role in T-cell chemotaxis and transmigration with similar activities in macrophages and other immune cells (47, 48). CCL4 is a potent chemotactic factor for neutrophils (49), with knock-out studies demonstrating decreased neutrophil chemotaxis to sites of inflammation (50). CCL5 plays a role in the cellular migration of T-cells, NK cells, macrophages, eosinophils, and basophils (51). CCL5 production has also been shown to reduce HIV entry into host cells (52). Homology within the C-C Chemokine Receptor family may compensate for some of the CCR5 biology, but the extent to which these mechanisms can compensate for the broad phenotypes of CCR5 ligand activation within individuals carrying the delta32 or other CCR5 variants is not well understood. Therefore, we have integrated our CCR5 knowledgebase with that of the larger C-C Chemokine Receptor family and broader phenotypic knowledge, using publicly available data, to expand our understanding of CCR5, which is critical in establishing a broader biological context for understanding the consequences of genetic manipulation. This example demonstrates how public data needs to be better integrated before setting out on high-risk clinical experiments.

METHODS

Amino Acid Knowledgebase and Human Genomic Variants

The human CCR5 protein sequence (UniProt P51681) was assessed on NCBI BLAST (53) against the *Homo sapiens* UniProtKB/Swiss-Prot database, and the top 100 hits were extracted for the canonical UniProt isoform. These 100 sequences were aligned using ClustalW (54), alignment available at https://doi.org/10.6084/m9.figshare.16619983, and a phylogenetic tree (https://doi.org/10.6084/m9.figshare.16619950) was constructed using MEGA (55) with 500 bootstrap calculations. Amino acids of the alignment were exported into Excel, where the conservation to all 100 GPCR sequences was calculated for each amino acid of human CCR5. The conservation was also calculated for the top 16 BLAST hits with an E-value less than 1E-50 (CCR5, CCR2, CCR1, CCR4, CCR3, CCR8, CCRL2, CCR9, CX3CR1, CCR6, XCR1, CCR7, CXCR4, ACKR2, ACKR4, CXCR6). Vertebrate orthologs of *CCR5* were extracted from NCBI ortholog as RefSeq transcripts, which were parsed for open reading frames using TransDecoder-v5.5 (56) and aligned using ClustalW codon (alignment available at https://doi.org/10.6084/m9.figshare.16619986). The translated amino acid sequences were assessed for percent conservation relative to the human CCR5 sequence or were assessed for functional conservation based on hydrophobic (A, V, I, L, M, F, Y, W), aromatic (F, Y, W, H), polar basic (R, H, K), polar acidic (D, E), or Ser/Thr (S, T) amino acids. Codon selection and linear motif analysis of the open reading frame alignment were calculated as previously described (57, 58). Knowledge for human CCR5 topology, modifications, mutagenesis, and natural variants were extracted from the UniProt database (59) on 6/15/2021.

The human CCR5 (UniProt P51681) protein was modeled using homology modeling in YASARA (60), which merged PDB files 5UIW, 5T1A, 5LWE, and 4RWS. The single merged structure was energy minimized with a pH-based pka setting of 7.4 within a phosphatidyl-ethanolamine (PEA) lipid membrane and 0.997g/mL water equilibrated across the membrane using YASARA md_runmembrane macro. Molecular dynamic simulations (mds) were run for the membrane-embedded CCR5 with 14,206 explicit water molecules, 48 Cl, and 33 Na giving a compiled 67,402 atoms for 300 nanoseconds (ns) using the AMBER14 force field (61), and atomic positions collection every 100 picoseconds for analysis. The analysis was performed using YASARA macros md_analyze and md_analyzeres (yasara.org/macros.htm), using a correlation cutoff for each amino acid of >0.9 in dynamic cross-correlation matrix (DCCM) calculations. All the mds trajectory and analysis files can be found at https://doi.org/10.6084/m9.figshare.15134979, allowing for a full reanalysis as needed.

All CCR5 missense and loss-of-function (LoF) variants were extracted from gnomADv2.1 nonTOPmed (62), COSMIC (63), Bravo for TOPmed variants (64), and ClinVar (65) on 11/29/2018. CCR5 missense variants were extracted from Geno2MP (66) on 6/15/2021. All missense and LoF variants were compiled, and each unique change was assessed with PolyPhen2 (67), Provean (68), SIFT (69), and Align-GVGD (70), where the variant was scored 1 for damaging equivalent predictions of each tool. A variant impact score was calculated by adding the functional prediction scores with our codon selection score (max of 2) and multiplying that by the functional conservation score, our linear motif conservation score, and the total allele observations for the variant from gnomAD, TOPmed, ClinVar,

COSMIC, and Geno2MP. The top five highest impact scores had the Geno2MP phenotypes extracted on 6/15/2021.

Public Dataset Generation

The 3D model of CCR5 was recorded for molecular videos using python scripted movement within the YASARA molecular modeling tools (60). The video files were uploaded into FigShare and YouTube, with links provided in the results section. The 3D coordinates were saved as a PDB file and loaded into PyMol (https://pymol.org/2/) to generate colored files for 3D printing, saving the files in VRML format and submitted to FigShare or Shapeways. Delta32 variant allele frequency was extracted from gnomADv2.1 (62). The CCR5 website was built using WordPress.

RNA Expression Analysis

The genome browser images and all GWAS variants near *CCR5* were extracted from the UCSC genome browser (71) on 9/6/2021. *CCR5* eQTLs were extracted from GTEx version 8 (72) on 9/6/2021. Open Targets Genetics (73) was used for the understanding of GWAS and pheWAS associations. Samples from our previous RNAseq work and details of methods used can be found in the three publications for MODS, RSV, or COVID-19 (74–76). All PAXgene tube blood RNAseq datasets within the NCBI SRA were downloaded with the SRA toolkit (https://trace.ncbi.nlm.nih.gov/Traces/sra/sra.cgi?view=software) and processed for abundance using Salmon_0.14.1 (77) and the Gencode38 transcriptome (78). Microglia datasets were extracted from BioProjects PRJNA649597, PRJNA662330, PRJNA665286, PRJNA667596, PRJNA688478, PRJNA689841, PRJNA387182, PRJNA483247 and the blood RNAseq datasets from BioProjects PRJEB14743, PRJEB20731, PRJEB23048, PRJEB27958, PRJEB27965, PRJEB33892, PRJEB36928, PRJEB41073, PRJEB44660, PRJNA201433, PRJNA230906, PRJNA232593, PRJNA251404, PRJNA277352, PRJNA305001, PRJNA315611, PRJNA327986, PRJNA329148, PRJNA352062, PRJNA354367, PRJNA357628, PRJNA358580, PRJNA369684, PRJNA378794, PRJNA380820, PRJNA384259, PRJNA390289, PRJNA397222, PRJNA398240, PRJNA401870, PRJNA427575, PRJNA437114, PRJNA454694, PRJNA476781, PRJNA493832, PRJNA494155, PRJNA504827, PRJNA511891, PRJNA526259, PRJNA526839, PRJNA533086, PRJNA552286, PRJNA562305, PRJNA588242, PRJNA591657, PRJNA600846, PRJNA601661, PRJNA607120, PRJNA630674, PRJNA632871, PRJNA634938, PRJNA638653, PRJNA639278, PRJNA647880, PRJNA664368, PRJNA679264, PRJNA679331, PRJNA680771, PRJNA683803, PRJNA686397, PRJNA702558, PRJNA728070 in addition to our groups studies on MODS, RSV, and COVID-19 (74–76). All Gencode38 mapped reads for these samples can be found at https://doi.org/10.6084/m9.figshare.16658449.v1. To calculate CCR5 delta32 read frequency we created a fasta file containing all isoforms of CCR5 and several additional paralog isoforms (https://doi.org/10.6084/m9.figshare.16649830.v1) that was indexed and assessed using Salmon, where the percent of reads containing delta32 were compared to the reads without the variant to calculate abundances for the variant from RNAseq datasets.

RESULTS

CCR5 Evolutionary Insights

CCR5 is a member of the GPCR superfamily. A BLAST analysis of the human CCR5 against other human protein sequences revealed the top 100 hits have E-values less than 5.23E-13, and percent identify greater than 22%. Phylogenetic reconstruction of these 100 GPCR human proteins shows that CCR5 clusters next to CCR2 and near CCR1, CCR3, CCRL2, CCR4, CCR8, CX3CR1, XCR1, and ACKR2 (**Figure 1A**). Using these 100 GPCR sequences, the percent of amino acids the same as CCR5 was calculated for each of the human CCR5 amino acids, where 12 amino acids (3.4%) are conserved >90%. In addition, the top 16 BLAST hits were also assessed for conservation with CCR5, where 26 amino acids (7.4%) are conserved >90%. A total of 98 vertebrate orthologs of *CCR5* were assessed for codon selection, linear motifs, amino acid conservation, and functional amino acid conservation. The alligator CCR5 represents the most divergent sequence within CCR5 orthologs with 54% conservation of amino acids with human. A total of 186 amino acids (52.8%) are conserved >90% in CCR5 orthologs. These conserved amino acids at the GPCR, top 16, and CCR5 ortholog levels mapped onto a model of the CCR5 structure reveal a broad GPCR conservation in the core, 16 most similar conservation in several clusters, and broad CCR5 conservation of the transmembrane, intracellular, and extracellular residues (**Figure 1B**). The fact that other chemokine receptors show a lack of conservation at the ligand-binding interface challenges the notion that they could potentially compensate for CCR5 loss, in agreement with Ellwanger et al. (79).

CCR5 Amino Acid Knowledgebase

The conservation data from above was compiled with molecular dynamic simulation (mds) data, UniProt insights, and known genomic variants for each of the human CCR5 amino acids to make a CCR5 amino acid knowledgebase (https://doi.org/10.6084/m9.figshare.16619974). The mds were generated by embedding the CCR5 protein of amino acids 6-352 into a PEA membrane, equilibrating water on the intra and extracellular portions, and simulating the protein movement for 300 nanoseconds. The mds tools use physics approximations of atomic movement at the femtosecond time scale, allowing users to determine the chemical environment around each amino acid of the modeled structure, providing information on the stability of movement, secondary structure, and how each amino acid correlates with every other amino acid. By recording the trajectory of amino acid movement using root-mean-squared deviation (RMSD) of the carbon alpha position, we know that the protein reaches an equilibrium of movement around ten nanoseconds of simulation, allowing for us to capture hundreds of nanoseconds of stabilized movement. The seven transmembrane helices of the GPCR structure all have a stable, low movement as reflected by a root-mean-square fluctuation (RMSF) below 4Å. The N- and C-termini both have high levels of RMSF, >10Å, reflective of decreased stability of the structure. A total of 15.0% of the amino acids are predicted in the initial

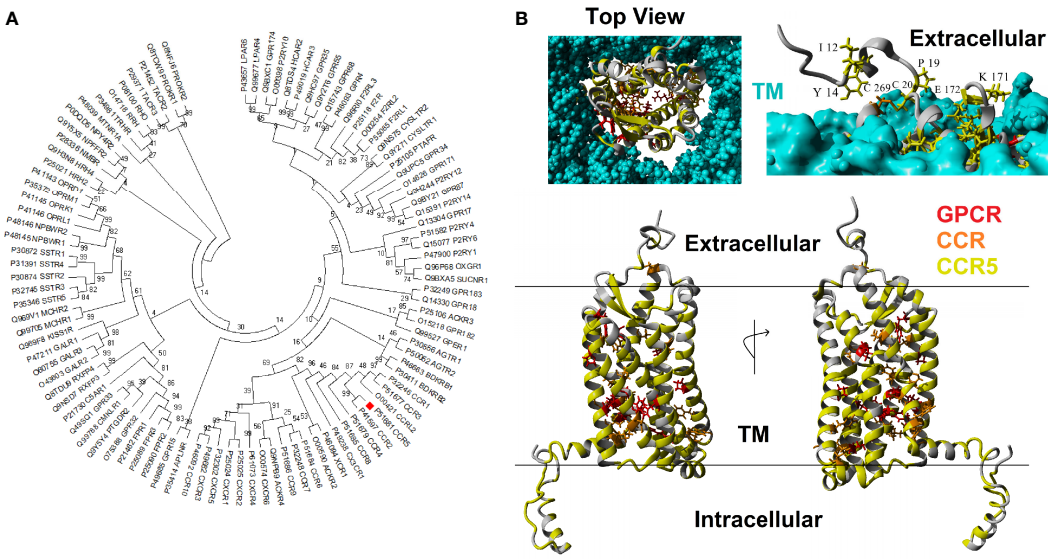

FIGURE 1 | Human CCR5 paralogs. **(A)** Phylogenetic tree of the top 100 human paralogs for CCR5 protein. Values at each node represent the percent of trees that cluster out of 500 bootstrap analyses. CCR5 is marked with a red box.**(B)** Model of human CCR5 with conservation colored. The transmembrane is colored cyan, amino acids conserved >90% in 100 GPCRs in red, conserved >90% in 16 most related CCR5 paralogs in orange, and conserved >90% in CCR5 vertebrate orthologs in yellow. The top left shows the view of CCR5 from extracellular space looking into the GPCR. The top right shows the side view with the transmembrane to visualize exposed and conserved residues, which are labeled. The bottom shows a 180° rotation of side view of CCR5.

structure to have coiled structure, 76.9% helical, 2.9% beta-sheet, and 5.2% as turns. A total of 70% of the amino acids have one or more amino acids that correlate with their movement greater than 0.9 based on dynamics cross-correlation matrix calculations. A total of eight amino acids have 10 or more amino acids in correlation >0.9 (10 = 54,55,69,149; 11 = 52,66,67; 13 = 70). These calculation values were included in the supplemental file's main amino acid knowledgebase matrix (https://doi.org/10.6084/m9.figshare.16619974).

Genomic missense variant extraction from gnomAD (population variants useful for allele frequency insights), TOPmed (population variants), ClinVar (disease-associated variants), COSMIC (somatic cancer variants), and Geno2MP (disease-associated variants with correlated phenotype) identified 403 unique variants for CCR5. Based on gnomAD allele frequencies, the average variant was found in 0.0079% of the population, with only a single missense variant (L55Q) found in more than 1% of individuals. Of the 403 variants, they fell on amino acids with an average of 89.6% conservation in CCR5 orthologs with 39% of variants with a conservation >99%. A total of 27% of the variants were predicted probably damaging by PolyPhen2, 54% deleterious by Provean, 60% damaging by SIFT, and 12% as class C55/C65 by Align-GVGD. Only 9% of these variants were predicted bad outcomes in all four tools. To prioritize variant assessments, we used a combined variant impact calculation with an average score of 89,761. The top ten variants were L55Q, R223Q, A73V, V131F, S63C, T288A, L121R, G106R, V46M, R60S. Finally, we added into the amino acid knowledgebase the UniProt extracted data for topology (extracellular, transmembrane helices, or intracellular), posttranslational modification (PTMs: sulfotyrosine, O-linked GalNAc, disulfide bonds, S-palmitoyl, phosphorylation), and known experimental mutagenesis/natural variant insights on ligand binding and protein expression/size. The number of variants and the top impact score for variants were brought into the compiled amino acid matrix with all other datasets, allowing for multidimensional data insights for each variant, for example, the top ten variants and the PTMs (**Table 1**).

Launch of Public Tools for CCR5 Education

Our CODE students and faculty integrated our amino acid knowledgebase into additional tools and resources for the education of CCR5 protein structural insights, gene sequences throughout many species, and human variants in large databases (**Figure 2**). Many of the tools developed focus on the CCR5 delta32 variant (Ser185IlefsTer32). From the 3D models, we have generated a video of CCR5 and delta32 that brings to light the extreme mutation that results in 0% protein function from the allele. A long ~2 min video is available as a MPG file on FigShare (https://doi.org/10.6084/m9.figshare.16628905) and a video file on YouTube (https://youtu.be/74w2N51tSOg). The video shows the model of CCR5 embedded into a lipid membrane, rotating around all axes with the location of critical conserved amino acids and the delta32 variant. A shortened 14-second video of only delta32 is available as a MPG file (https://doi.org/10.6084/m9.figshare.16628956). A one-minute video of the movement of residues from the mds trajectory is available as an MPG file (https://doi.org/10.6084/m9.figshare.16628854) and a YouTube link (https://youtu.be/WaoPfQXA8Pg). To facilitate a hands-on

TABLE 1 | Top functional amino acids of CCR5 from amino acid knowledgebase.

AA	Codon	AA	Inclusion	CCR5 Conserved (%)	GPCR Conserved	Top 16 GPCR (E<1E-50)	Secondary Structure	RMSF (Å)	mds DCCM >0.9
3	TAT	Y	PTM	82.65	2.02	0.00	–	–	–
6	TCA	S	PTM	78.57	14.14	13.33	C	12.161	2
7	AGT	S	PTM	80.61	27.27	6.67	C	9.246	2
10	TAT	Y	PTM	80.61	4.04	13.33	T	4.615	1
14	TAT	Y	PTM	98.98	6.06	33.33	T	3.63	0
15	TAT	Y	PTM	19.39	4.04	20.00	T	4.047	1
16	ACA	T	PTM	27.55	3.03	6.67	C	2.959	1
17	TCG	S	PTM	69.39	7.07	33.33	C	3.384	0
20	TGC	C	PTM	98.97	11.11	53.33	C	1.325	0
46	GTG	V	Top 10	97.96	19.19	40.00	H	1.786	4
55	CTG	L	Top 10	100.00	23.23	60.00	H	1.738	10
60	AGG	R	Top 10	100.00	25.25	33.33	C	2.658	8
63	AGC	S	Top 10	94.90	21.21	46.67	C	1.85	8
73	GCC	A	Top 10	97.96	82.83	80.00	H	1.229	6
101	TGT	C	PTM	100.00	100.00	100.00	H	1.202	7
106	GGG	G	Top 10	91.75	13.13	46.67	H	1.277	4
121	CTC	L	Top 10	100.00	13.13	40.00	H	1.262	5
131	GTC	V	Top 10	100.00	52.53	80.00	T	2.255	0
178	TGC	C	PTM	100.00	10.10	53.33	E	1.055	1
223	CGG	R	Top 10	89.80	20.20	40.00	C	3.459	0
269	TGC	C	PTM	98.97	47.47	93.33	H	1.181	2
288	ACG	T	Top 10	88.78	9.09	26.67	H	1.222	1
321	TGC	C	PTM	89.69	2.02	13.33	C	3.514	0
323	TGC	C	PTM	26.80	0.00	0.00	C	5.452	4
324	TGT	C	PTM	97.94	2.02	13.33	C	6.36	5
336	AGC	S	PTM	83.67	13.13	40.00	C	9.765	4
337	TCA	S	PTM	100.00	22.22	46.67	C	9.244	3
342	TCC	S	PTM	100.00	27.27	40.00	T	7.834	1
349	TCT	S	PTM	98.98	13.13	26.67	C	4.292	1

Sites were included based on known posttranslational modifications (PTMs) or for being a top 10 scoring variant.

interface with CCR5, we have created a 3D printing model of CCR5 and delta32 available as a VRML file (https://doi.org/10.6084/m9.figshare.16628962) and as large (https://www.shapeways.com/product/8XEK5N2EF/ccr5-large?optionId=147670860&li=shops), or small (https://www.shapeways.com/product/VNX7TE26B/ccr5-protein-model?optionId=127830241&li=shops) sized print that can be ordered from Shapeways for delivery. The small model provides a low-cost option, which our group has made into jewelry or keychains for distribution at genomic educational events. The large print works well for classrooms for students to hold and explore.

To make access to these files easier, we have created a web resource page (https://prokoplab.com/ccr5-and-hiv/). From this site, anyone can obtain the video of CCR5, sequence data, insights on delta32, and a handout used in classrooms. In addition, visitors can order 3D printed models of CCR5 with the delta32 location marked in red. The two-sided handout (https://doi.org/10.6084/m9.figshare.16628815) walks students through the biological role of CCR5, how many sites on the protein are conserved throughout evolution, and details of delta32. This handout pairs well with the large and small 3D prints of the protein. All material is provided to the public for free except the 3D printed models, offered at production cost with no markup. The generation of this material by students and faculty from the CODE program represents an exciting new potential framework in the genomic era that can be expanded to many additional variants and proteins moving forward.

CCR5 Expression Insights

Additional insights about *CCR5* are available through public datasets of expression and noncoding variants, broadening the insights and knowledge to challenge genomic reductionism. One effective way to combat genetic reductionism is to demonstrate that the expression of CCR5 is not only subject to variation in the gene but is also highly dependent on the molecular context, such as genes proximate to CCR5, epigenetic factors, as well as the type of cell in which expression occurs. The *CCR5* gene is located on chromosome 3 from bases 46,370,854 to 46,376,206 (based on hg38 annotation). Near *CCR5* are multiple cytokine receptor genes and many known genomic associations from genome-wide association studies (GWAS), including the strongest locus for severe COVID-19 (**Figure 3A**). This COVID-19 locus (80) has its highest association signal over the *SLC6A20* gene and does not overlap the *CCR5* gene body (**Figure 3B**). Located around 57 kilobases near *CCR5* are associations for multiple immune system-connected phenotypes notable for lymphocytes, monocytes, and macrophages (**Table 2**). Of the variants in this region, several contribute to observed changes in *CCR5* expression based on expression quantitative trait loci (eQTL), with the strongest associations seen in whole blood and lung, which contain large portions of monocytes and macrophages (**Table 3**). It should also be noted that multiple eQTLs were observed in brain tissue. The top SNP for blood *CCR5* expression influence, rs76258812, is also an eQTL for the other paralogs of cytokine receptors near *CCR5*, including *CCR1*, *CCR3*, and *CCR2*

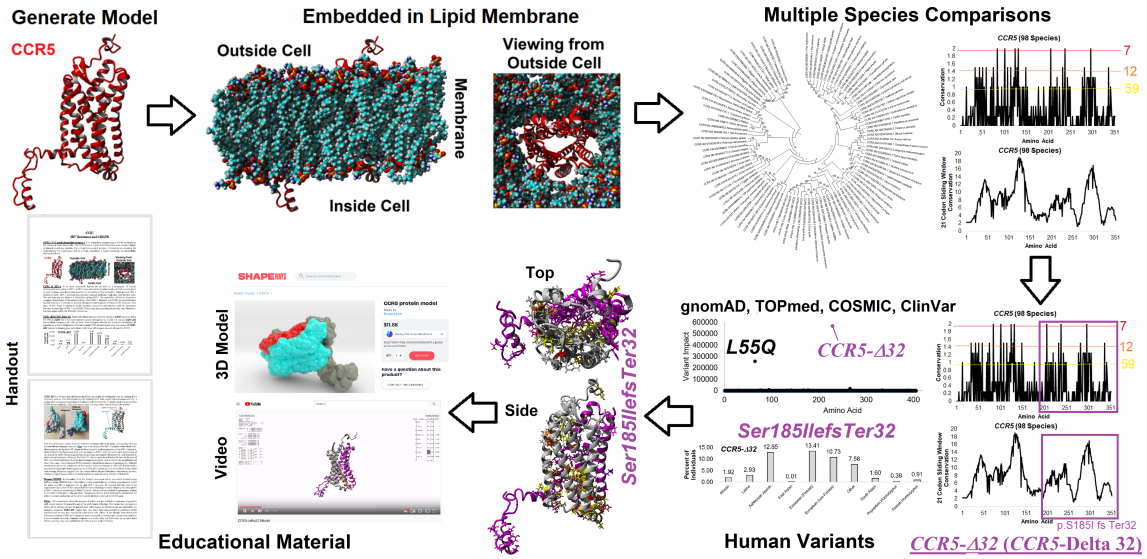

FIGURE 2 | Amino acid knowledgebase of CCR5 used for educational insights. CODE students generated protein models, which were embedded into a lipid membrane and run for molecular dynamics simulations. These values were combined with multiple species analysis of CCR5 evolution and genomic variant extractions from gnomAD, TOPmed, COSMIC, and ClinVar. This amino acid knowledgebase was then used to assess the delta 32 variant and generate various educational handouts, videos, and 3D printed models.

(https://genetics.opentargets.org/variant/3_46318831_T_C), making it difficult to determine if the monocyte associated GWAS are from *CCR5* or these other genes. This variant is also found throughout multiple subpopulations with the highest known allele frequency in East Asian ancestry. Contrary, the top two variants for the brain *CCR5* expression, rs9862021, and rs140177427, are rarer. rs9862021 is found highest in 10% of African ancestry and has no trait associations. rs140177427 is found highest in 10% of Finnish ancestry and is associated with various lymphocyte and macrophage phenotypes (https://genetics.opentargets.org/variant/3_47234712_G_A).

Expression across broad tissues of the human protein atlas (HPA) (29) and GTEx (72) show higher levels in immune tissues such as spleen, tonsils, appendix, and lymph nodes and tissues associated with immune cells like blood, lung, and intestine. Further dissection of cell types within HPA shows high *CCR5* expression in T-cell levels in blood and macrophages in peripheral tissues. Therefore, we utilized a large single-cell RNAseq repository, PanglaoDB (81), to identify further cell types of importance for *CCR5* expression, identifying a large number of microglia and macrophage identified cell experiments (**Figure 4A**). The macrophage and monocyte annotations come from broad tissues, including the liver, vessels, lung, and heart (**Figure 4B**). The microglia annotations come from whole-brain isolates and specific brain regions (**Figure 4C**). It should be noted that all of the microglia insights come from isolates of mouse brains, where there are no human brain single-cell RNAseq datasets integrated into PanglaoDB. In the mouse, the knock-out of *CCR5* is associated with multiple neurological phenotypes, including microglial alterations and abnormal spatial learning (**Table 4**).

CCR5 Role in Brain Development

To further dissect the brain and microglia insights into human biology, we integrated several additional datasets. The human brain microarray of the Allen Brain atlas suggests 16 genes that correlate >0.7 in expression with *CCR5* in 500 human brain samples. Most are also highly expressed in the mouse single-cell datasets of PanglaoDB for microglia (**Figure 5A**). Most of these genes code for proteins that are known to interact and enrich synapse pruning and microglia phenotypes (**Figure 5B**). To further show the expression of *CCR5* in isolated microglia, we pulled all human RNAseq paired-end datasets from the NCBI SRA mentioning "microglia." We annotated them relative to the Gencode38 transcriptome using a quasi-based alignment strategy (**Figure 5C**). All Gencode38 mapping data for each sample can be found at https://doi.org/10.6084/m9.figshare.16649842. The HMC3 cell line (PRJNA649597) that is supposed to mimic microglia cells does not express any *CCR5*. Contrary iPS (induced pluripotent stem cell) derived microglia (PRJNA662330, PRJNA665286, PRJNA688478, PRJNA483247) and purified primary microglia (PRJNA387182) all show expression of *CCR5*.

Broadening to the more extensive integrated STRING network for CCR5 shows interaction with many chemokines while identifying multiple genes connected to the central nervous system and microglia biology relative to HIV (**Figure 6**) (89, 90). Using our amino acid knowledgebase, we ranked the top five variants (L55Q, R223Q, A73V, V131F, S63C) followed by extracting human phenotypes associated with these changes in the Geno2MP database. A total of 273 affected individuals have a phenotype with one of these top five variants. Of these, 73 affected individuals (27%) are indicated as "Abnormality of the

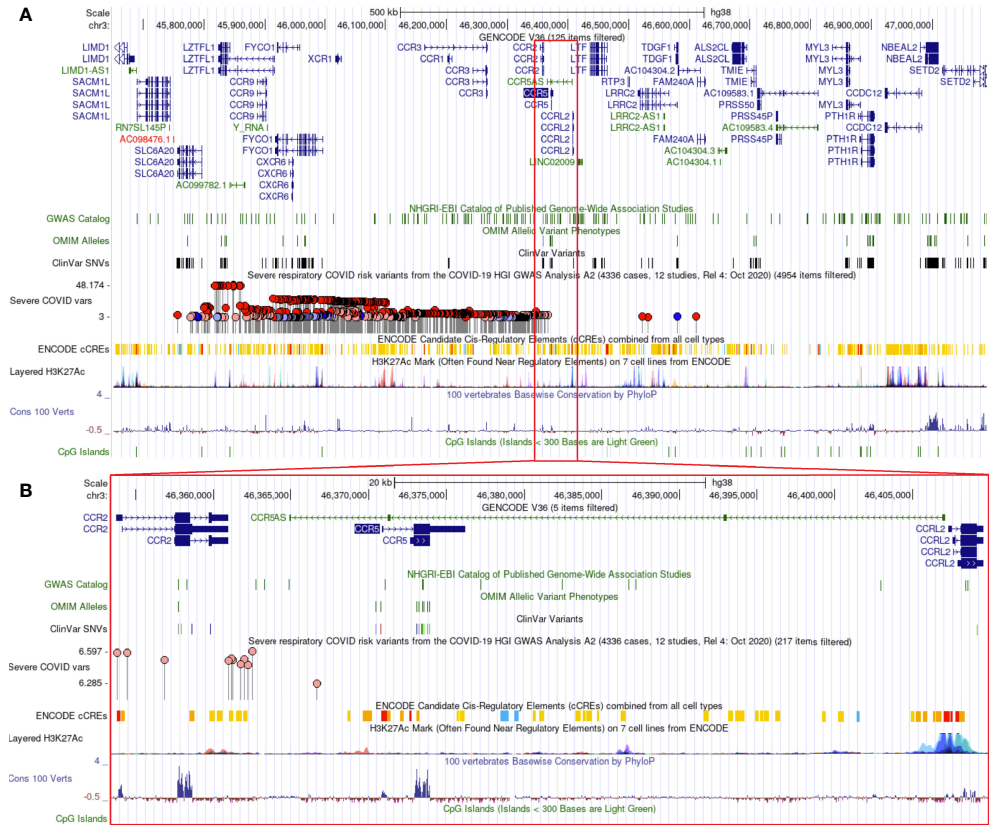

FIGURE 3 | Genomic architecture around CCR5. The genome browser view (hg38) of around one million bases near *CCR5* **(A)** or zoomed into around 40,000 bases **(B)**. Tracks shown include the Gencode transcripts, known variants from GWAS/OMIM/ClinVar, the high-risk COVID-19 loci (higher dots are the strongest signal), various gene regulation insights (ENCODE cCTEs, H3K27Ac, CpG Islands), and evolutionary conservation (Cons 100 Verts).

nervous system" in the broad term (**Table 5**). This is the top-ranked phenotype with "Abnormality of the cardiovascular system" at 21% and "Abnormality of the musculature" at 11%. The only neurological phenotype associated with homozygous CCR5 variants was L55Q associated with "Abnormality of brain morphology" with seven additional heterozygous individuals with the variant and individuals with R223Q, A73V, V131F, and S63C also having this annotation. Individuals with all five variants are also identified with "Intellectual disability". Several variants are associated with "Epileptic encephalopathy". The L55Q is found in ~1.5% of the population, and therefore association to phenotypes could be random; however, the 21 patients with neurological phenotypes with R223Q (AF=0.005) and 18 with the other three (A73V, S63C, V131F, AF average of 0.0006) suggest an enrichment from random probability. The Geno2MP database contains data for 19,344 individuals with phenotypes, where 458 have one of these five CCR5 variants. Based on allele frequencies, this number is expected to be 384, with an enrichment of 1.2. The V131F has an enrichment of 8.2 in observations relative to expect allele frequency, A73V of 4.3, and S63C of 2.2, suggesting these variants are hyper observed within Geno2MP. Moreover, with the enrichment of neurological phenotypes for individuals with these variants, it seems likely that there is an association with rare variants in CCR5 for neurological phenotypes.

CCR5 in Human Blood Samples

The phenotypes seen in the Geno2MP data suggested a broader analysis of CCR5 in disease pathologies. With the robust expression of *CCR5* in blood and our history in studying the blood of immune challenged individuals, we selected to process *CCR5* biology for blood-based RNAseq of 7,280 samples from 62 different BioProjects and three of our studies where we have patient-to-transcriptome insights (74–76) (**Figure 7**). All these samples were blood collected into RNA PAXgene tubes to standardize sample collection. Expression of *CCR5* is highly variable across BioProjects (**Figure 7A**), suggesting that the TPM data is influenced by the RNA isolation or sequencing technique (polyA vs. total RNAseq). Therefore, we normalized the TPM data with a BioProject Z-score, identifying several samples with elevated CCR5 expression (**Figure 7B**). The highest Z-score was observed in SRR5225514 (12.4), a 17-year-old (yo) female control sample, yet very little is deposited into the SRA about this individual. This can also be said for SRR3236097 (8.8, 17 yo male control), SRR12291502 (8.3, male sepsis case), SRR3236039 (8.0, 17 yo male TB patient), SRR5902058 (7.4,

TABLE 2 | Traits associated with variants within the *CCR5* gene region (chr3:46,353,419-46,409,888, hg38) of **Figure 3B**.

Study ID	Trait	P-value	Beta	Publication
GCST004433	Macrophage inflammatory protein 1b levels	7.57E-115	0.4985	PMID:27989323
GCST90002340	Monocyte count	1.11E-75	0.035585	PMID:32888493
NEALE2_30190_raw	Monocyte percentage	4.35951E-50	0.0963857	UKB Neale v2
GCST004609	Monocyte percentage of white cells	1.051E-33	0.04394357	PMID:27863252
NEALE2_6149_1	Mouth ulcers | mouth/teeth dental problems	4.03372E-32	0.093964676	UKB Neale v2
GCST004608	Granulocyte percentage of myeloid white cells	2.358E-25	-0.03789086	PMID:27863252
NEALE2_30130_raw	Monocyte count	1.90975E-22	0.00516017	UKB Neale v2
GCST004625	Monocyte count	5.757E-22	0.03506334	PMID:27863252
GCST004438	Monocyte chemoattractant protein-1 levels	1.05E-19	0.2902	PMID:27989323
GCST90002292	Basophil count	4.85E-17	-0.018406	PMID:32888493
GCST90002316	Lymphocyte counts	4.44E-14	0.021045	PMID:32888493
NEALE2_30300_raw	High light scatter reticulocyte count	7.77011E-14	0.000252296	UKB Neale v2
NEALE2_30180_raw	Lymphocyte percentage	2.13306E-11	0.159232	UKB Neale v2
NEALE2_30120_raw	Lymphocyte count	2.24757E-10	0.0233973	UKB Neale v2
NEALE2_30290_raw	High light scatter reticulocyte percentage	3.2664E-10	0.00704487	UKB Neale v2
GCST003045	Ulcerative colitis [EA]	1.3229E-08	0.0757873	PMID:26192919
NEALE2_6149_100	None of the above | mouth/teeth dental problems	2.22533E-08	-0.027486511	UKB Neale v2
NEALE2_30150	Eosinophill count	2.81799E-08	0.00995217	UKB Neale v2
NEALE2_30260_raw	Mean reticulocyte volume	7.16666E-08	-0.137991	UKB Neale v2
NEALE2_30250_raw	Reticulocyte count	8.74646E-08	0.000690186	UKB Neale v2

TABLE 3 | Top eQTL for CCR5 expression.

Tissue	SNPs	Lowest P-Value	NES	rsID
Whole Blood	206	1.1E-09	-0.24	rs76258812
Lung	123	3.7E-06	-0.14	rs9110
Brain - Caudate (basal ganglia)	10	1.3E-05	1.1	rs9862021
Brain - Cortex	2	1.9E-05	-1.6	rs140177427
Skin - Sun Exposed (Lower leg)	3	1.9E-05	0.17	rs1388604
Esophagus - Mucosa	47	2.1E-05	-0.17	rs202207288
Esophagus - Muscularis	26	2.4E-05	0.16	rs2133660
Colon - Sigmoid	114	2.7E-05	-0.39	rs6765904
Skin - Not Sun Exposed (Suprapubic)	51	2.8E-05	-0.18	rs9872946
Nerve - Tibial	4	1.0E-04	-0.71	rs80257961

male malaria vaccinated individual), SRR13224554 (6.1, 41 yo HIV-infected patient), and SRR3235984 (5.9, 14 yo female TB patient).

Therefore, we focused analysis on three cohorts our team collected blood PAXgene tube RNAseq in hospitalized patients with MODS, RSV, or COVID-19 in an age range from weeks of life to elderly. The adult hospitalized COVID-19 cohort shows highest *CCR5* expression in the male Hispanic control patient 21 age 50-59 (**Figure 7C**), who had the highest interferon response, was noted to have a unique transcriptome, was the furthest outlier of the control samples, had markers of multiple organ damage, and generally seemed to be a highly divergent sample from the cohort (74). The second highest was a 50-59 yo European ancestry male who had a lethal case of COVID-19 marked by a SAPSII score of 61, suggesting multiple organ complications, had a robust interferon response, elevated cytokine expression profile, the highest cell markers of peripheral monocytes, and a weak clonal expansion of the immune repertoire. The third outlier of the study was a 40-49 yo European ancestry male with a SAPSII score of 30, a high activation of mitotic cell cycle control genes, an elevation of interleukin-7 and histone genes, and detectable reads in the blood related to *Paraburkholderia* and *Streptomyces tsukubensis*. None of the samples were identified as outliers of *CCR5* expression on the low end, but samples with *CCR5* expression <0.8 standard deviations were all noted in COVID-19 hospitalized patients and not controls. The depth of sequencing for this COVID-19 study allowed for various gene panels, immune repertoire, and foreign RNA mapping for all samples, which were correlated to the *CCR5* values. The CIBERSORTx absolute values for CD8 T-cells were highly correlated (R^2 = 0.8) to *CCR5* expression (**Figure 7D**).

Further evaluation of our pediatric MODS and infant RSV cohorts (**Figure 7C**) reveals diseased samples as outliers of *CCR5* expression. Sample 24, female 16 yo with European ancestry, within the MODS study had two separate measurements with high *CCR5* and an *Epstein Barr* virus (EBV) infection that required ECMO and was also identified as having the highest levels of CIBERSORTx annotated CD8 T-cells (75). Sample 18, a male who required ventilation, had the highest levels of *CCR5* within the MODS cohort and was noted to have elevated genes for interferon response, had a clinical *Serratia marcescens* infection, and was coronavirus positive. The lowest *CCR5* levels in the MODS cohort were observed in sepsis patients, one of which (patient 27) was lethal. The RSV study's top three outliers for CCR5 expression were all hospitalized RSV cases

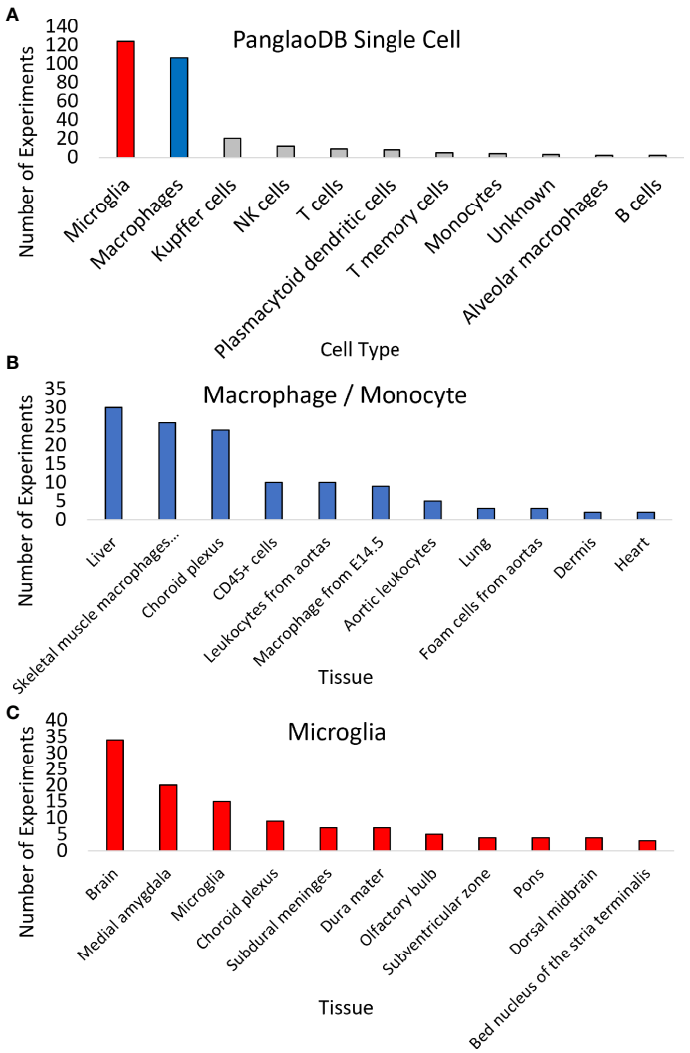

FIGURE 4 | Expression of CCR5 in 1,063 mouse single-cell datasets. **(A)** PanglaoDB analysis of experiments that CCR5 was detected in various cells based on single-cell analysis. **(B, C)** The number of times sample types showed CCR5 expression for macrophages/monocytes **(B)** or microglia **(C)**.

(patient 1, 4, 19) (76). Patient 1 had high levels of *Agromyces aureus* and *Caulobacter vibrioides* like reads in the blood in addition to clinically confirmed RSV, while also having multiple levels of elevated RNA associated with lung hyperinflammation. Patient 4 had elevated reads belonging to type-1 Alveolar cell and an elevation of viral defense response genes relative to the cohort. All three samples with a z-score below -1 were from hospitalized RSV samples. These three cohorts suggest that *CCR5* levels, either high or low, often are found in sick patients and rarely seen in healthy controls within our cohorts.

To probe whether RNAseq samples contained the *CCR5* delta32 variant, we developed a novel Salmon-based indexing file containing the wild-type *CCR5* transcripts, the delta32 transcripts, and several of the top human paralog transcripts. Mapping samples with delta32 reads over our 179 in-house RNAseq samples reveals 75.4% of the samples to be homozygous wild type (<0.01% delta32 reads), 21.2% heterozygous, and 3.4% homozygous for delta32 (**Figure 7E**). The heterozygous samples were highly variable for the % of reads with delta32 and included the MODS sample 18, with the highest overall *CCR5* z-score of the three studies. Surprisingly, all three samples of the ECMO patient 24 showed homozygous *CCR5* delta32, where an additional two-year follow-up of the patient also showed homozygous *CCR5* (data not shown as the z-score cannot be calculated as it was a single sample RNAseq). This patient was the focus of our 2020 MODS paper (75), where we discovered the 16 yo patient to have Hemophagocytic lymphohistiocytosis (HLH) likely driven by a dominant-negative splicing variant in RNASEH2B that is activated by the EBV suppression of nonsense-mediated decay. As this patient is an N=1 case, it is interesting to note the severe, nearly lethal phenotype of this patient and to be *CCR5* delta 32 homozygous,

TABLE 4 | Mouse knock-out phenotypes.

Phenotype	Publication	Neurological
abnormal astrocyte physiology	(82)	Yes
abnormal glial cell physiology	(82)	Yes
abnormal long term spatial reference memory	(82)	Yes
abnormal nervous system physiology	(83)	Yes
abnormal spatial learning	(82)	Yes
decreased microglial cell activation	(82)	Yes
abnormal CD4-positive, alpha beta T cell morphology	(84)	
abnormal CD8-positive, alpha beta T cell morphology	(84)	
abnormal cytokine level	(84)	
abnormal hepatocyte physiology	(85)	
abnormal Ito cell morphology	(86)	
abnormal Kupffer cell morphology	(86)	
abnormal locomotor behavior	(84)	
abnormal NK T cell physiology	(85)	
decreased NK cell number	(87)	
decreased susceptibility to induced colitis	(84)	
decreased susceptibility to Retroviridae infection	(83)	
impaired macrophage chemotaxis	(88)	
increased NK T cell number	(84)	
increased susceptibility to fungal infection	(88)	
liver failure	(85)	

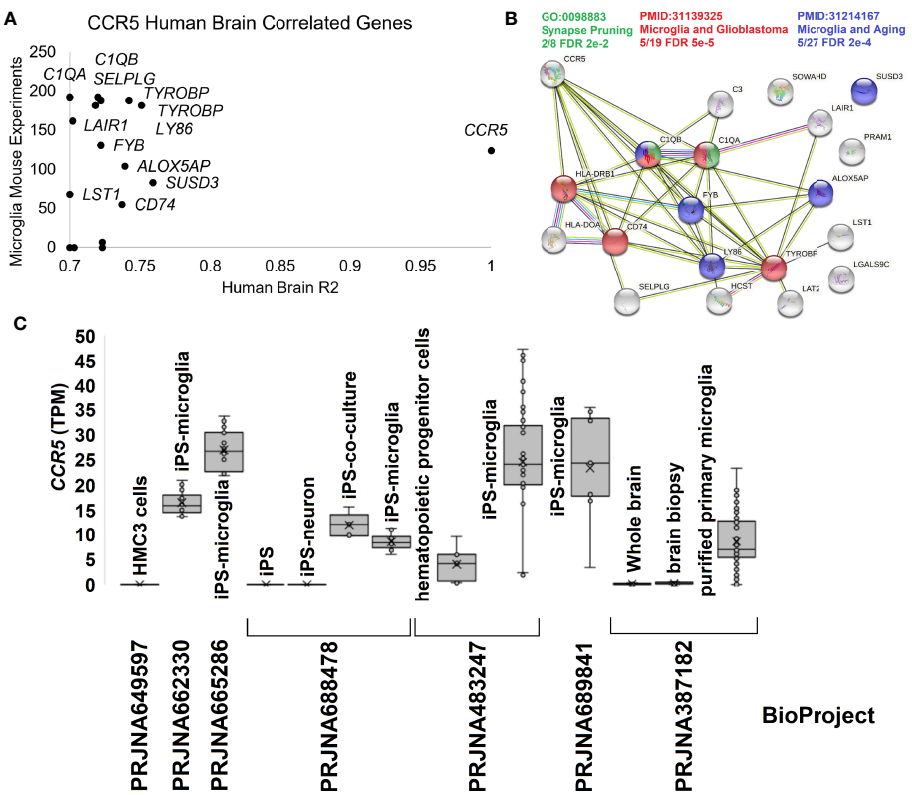

FIGURE 5 | Human brain CCR5 and microglia. **(A)** The correlation of genes to CCR5 from the Allen Brain Atlas Human Brain microarray data for 500 samples relative to their expression in mouse microglia single-cell experiments. The x-axis shows the genes R^2 from microarray relative to CCR5 expression, while the y-axis shows the number of experiments the gene is detected in mouse single-cell datasets for microglia. **(B)** STRING protein network for genes in panel **(A)** showing the enrichment of synapse pruning (green), microglia/glioblastoma (red), and microglia/aging (blue) genes. **(C)** Expression of *CCR5* in transcripts per million (TPM) from seven BioProjects of human microglia cells or related experiments.

TABLE 5 | Neurological phenotypes for the top human CCR5 missense variants from Geno2MP.

Variant	Het	Hom	Phenotype
L55Q	7	1	Abnormality of brain morphology
L55Q	4	0	Epileptic encephalopathy
L55Q	4	0	Abnormality of nervous system physiology
L55Q	3	0	Neurodevelopmental abnormality
R223Q	3	0	Intellectual disability
L55Q	2	0	Microcephaly
L55Q	2	0	Dystonia
L55Q	2	0	Fatigable weakness
R223Q	2	0	Agenesis of corpus callosum
R223Q	2	0	Abnormality of brain morphology
R223Q	2	0	Cerebral cortical atrophy
R223Q	2	0	Abnormality of nervous system morphology
A73V	2	0	Epileptic encephalopathy
S63C	2	0	Intellectual disability
L55Q	1	0	Autism, Intellectual disability
L55Q	1	0	Spastic paraplegia
L55Q	1	0	Abnormality of hindbrain morphology
L55Q	1	0	Seizures
L55Q	1	0	Abnormality of nervous system morphology
L55Q	1	0	Intellectual disability
L55Q	1	0	Global developmental delay, Autism
L55Q	1	0	Global developmental delay
L55Q	1	0	Abnormality of the nervous system
R223Q	1	0	Behavioral abnormality
R223Q	1	0	Seizures
R223Q	1	0	Neurodegeneration
R223Q	1	0	Abnormality of hindbrain morphology
R223Q	1	0	Seizures
R223Q	1	0	Epileptic encephalopathy
R223Q	1	0	Abnormality of nervous system physiology
R223Q	1	0	Intellectual disability
R223Q	1	0	Intellectual disability
R223Q	1	0	Global developmental delay
A73V	1	0	Intellectual disability
A73V	1	0	Agenesis of corpus callosum
A73V	1	0	Abnormality of hindbrain morphology
A73V	1	0	Abnormality of brain morphology
A73V	1	0	Intellectual disability
V131F	1	0	Abnormality of hindbrain morphology
V131F	1	0	Abnormality of brain morphology
V131F	1	0	Fatigable weakness
V131F	1	0	Abnormality of nervous system physiology
S63C	1	0	Spastic paraplegia
S63C	1	0	Microcephaly
S63C	1	0	Abnormality of brain morphology
S63C	1	0	Seizures
S63C	1	0	Abnormality of movement

The Het is the number of heterozygous individuals with the variant and the annotated phenotype and Hom are homozygous individuals with phenotype.

suggesting that *CCR5* complete inhibition does not remove HLH risks as others have proposed for COVID-19 (91). It is also interesting that as the EBV infection cleared in the patient, the *CCR5* levels, even when delta32 homozygous, decreased in sample 3. The other three samples where we observed *CCR5* homozygous delta32 were in severe outcomes, including patient 15 who had lethal COVID-19, the MODS patient 20 who had Alveolar rhabdomyosarcoma and human betaherpesvirus 7, and the MODS patient 27 who required ECMO and passed away. Our cohort of 98 unique individuals from these three studies had only 13 lethal cases, of which two were delta32 positive (15%). This trend demands further evaluation of delta32 status in sepsis and critical care patients.

DISCUSSION

Creating modification in genomic backgrounds that are not commonly present with the variant yields unknown risks that need to be assessed before moving to human editing, particularly *in utero*. The delta32 variant is unique to subpopulations of humans, and there could be unknown variants co-evolved in these populations that modulate the deleterious effects of the delta32 variant. As *CCR5* is found within a region of the genome containing multiple cytokine receptor paralogs and has a large and complex linkage disequilibrium block and eQTLs often overlap the different paralogs, genetic variants can be in linkage disequilibrium to compensate for deleterious outcomes.

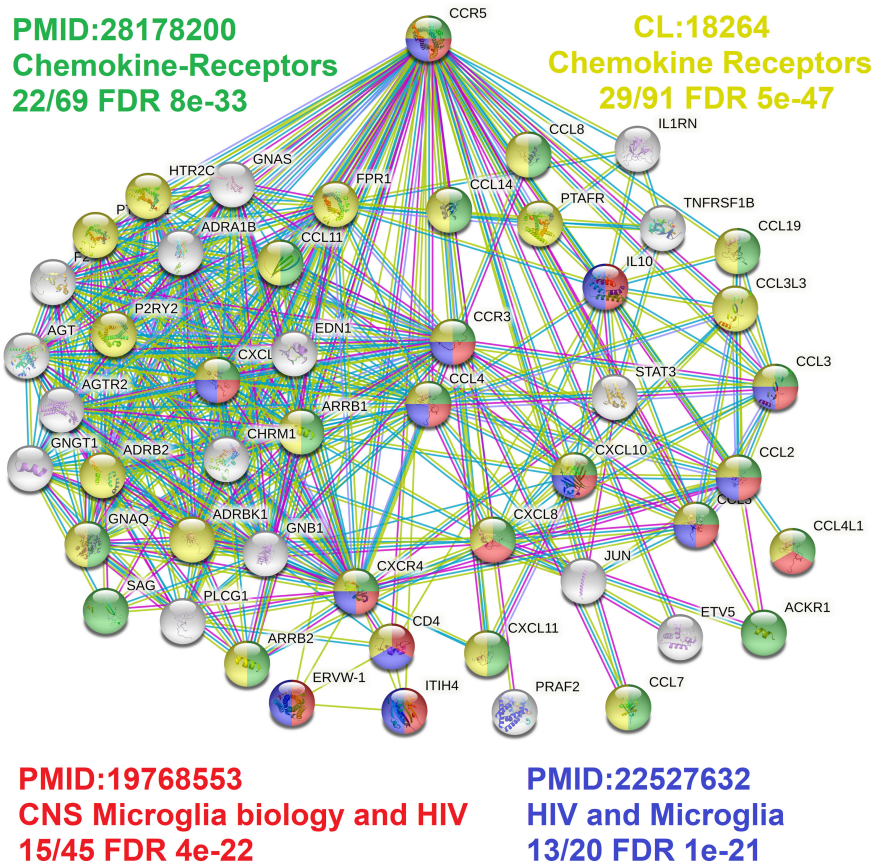

FIGURE 6 | CCR5 protein network highlighting microglia factors. The STRING protein network for the top 50 proteins interacting with CCR5. In green/yellow are various genes annotated to chemokine biology, and in red/blue are genes connected to microglia biology.

In other pathologies, these shared variants that regulate counter genes have been shown for cardiovascular biology and present on the Y-chromosome (92, 93). Viewed in the context of evolution by natural selection, selection on one trait has correlated effects and is often constrained if those correlated effects are themselves deleterious (94, 95). Editing genomes is independent of evolutionary selection over time and could result in unintended medical consequences due to genomic backgrounds used in human editing. Bioinformatic studies of susceptibility loci suggest that pleiotropy, the existence of multiple functions for a single gene, far from being the exception, appears to be the rule, not only in the case of CCR5 but also in the case of susceptibility loci for breast cancer, lung cancer, coronary artery disease and other severe diseases (96). Thus, understanding the effects of CCR5 removal in diverse genome backgrounds is critical before introducing the variants in diverse human backgrounds. These studies are very complex, requiring extensive data, experimentation, and a great deal of financial resources and time.

An additional set of ethical, legal, and social implications has been brought to light by the He Jiankui affair, in which CRISPR-Cas9 gene editing was used in an attempt to edit the CCR5 gene of two embryos to the Δ32 variant with the hope of conferring immunity to HIV. The germ line was crossed, and twin baby girls, Nana and Lulu, were born. This universally condemned ethical breach leaped over several ethical safeguards. Before translating germline gene-editing to the fertility clinic, it will be necessary to carefully assess the ethical and legal conditions for its permissibility and move toward finer line-drawing in determining these conditions (97). For example, there is a significant biological and ethical difference between a "correction" of a rare, disease-associated mutation to a widespread non-pathogenic allele as is being investigated for HBB and MYBPC3 in the case of β-thalassemia and hypertrophic cardiomyopathy respectively, and attempted edits to CCR5 with its complex, multiple phenotypic effects and potentially pathogenic off-target effects (97, 98). Germline gene-editing carries with it its own set of ethical issues, most notably extreme uncertainty about the effects on the edited individual, as the effects of edits—whether those intended, errors, or off-target—to the genome early in life ramify unpredictably throughout embryogenesis and later development, as well as concerns about long-term intergenerational effects that are difficult to study and whose risks cannot be easily assessed.

Genetic and genomic reductionism takes many forms, and the terms are variously used in the philosophy of biology (99). The particular form of genetic reductionism under criticism here

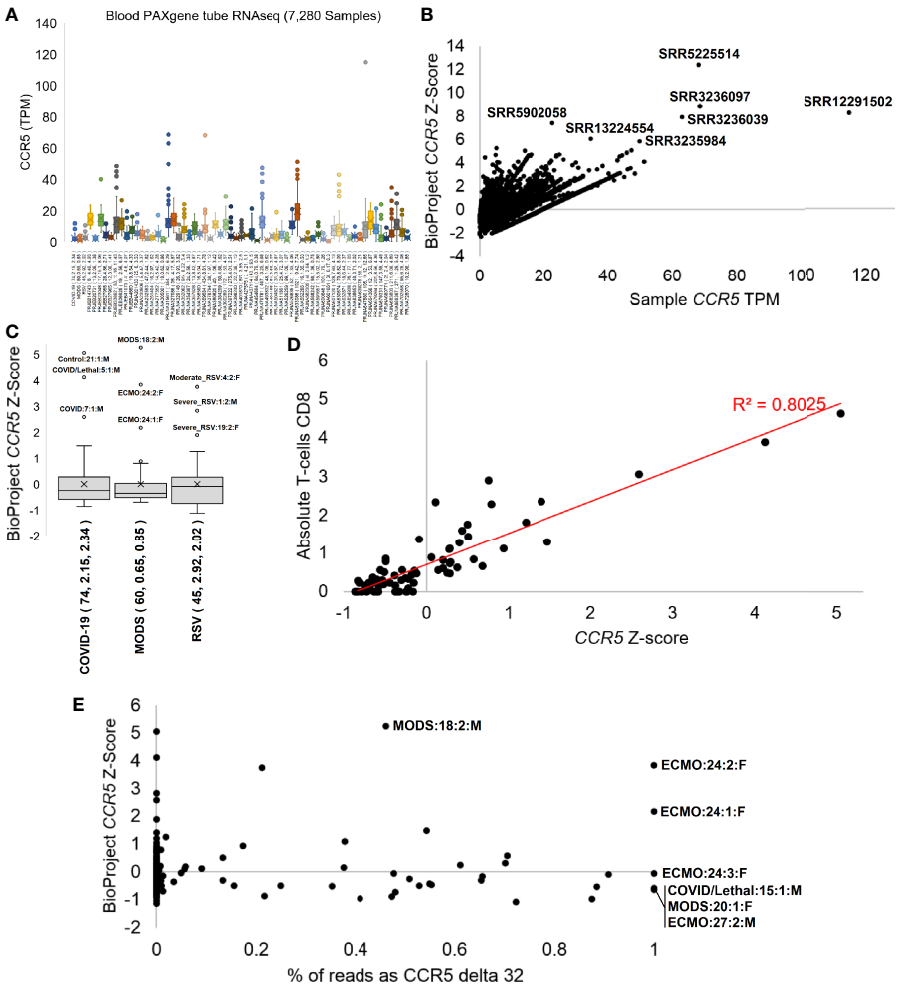

FIGURE 7 | CCR5 expression in blood PAXgene tube RNAseq. **(A)** Box and whisker plots for the expression (TPM) of *CCR5* from various NCBI BioProjects that were generated by Illumina paired-end RNA-Seq from human blood collected PAXgene tube samples. Listed next to each BioProject code is the number of samples, average *CCR5* expression, and standard deviation of CCR5 expression. **(B)** The TPM expression for each sample (x-axis) of panel **(A)** relative to the BioProject normalized Z-score (y-axis). The top seven samples based on Z-score are labeled. **(C)** The Z-scores for our three pilot precision transcriptome datasets, with outlier samples labeled. **(D)** The COVID-19 study analysis of *CCR5* normalized expression relative to CIBERSORTx absolute CD8 T-cell values. **(E)** The percent of transcripts containing the delta 32 variant relative to wild type (x-axis) for samples of panel **(C)** relative to the BioProject normalized Z-Score. All homozygous samples for delta 32 are labeled as is the heterozygous sample with the highest study Z-score.

is the mistaken belief that a given gene stands in a strict one-to-one relationship to a given character state, whereas a one-to-many relationship is the rule (99). With rare exceptions, causal pathways in biology are complex. Yet, in the case of genomics, there is good reason to believe that a reductionistic tendency has been inherited from a long-standing biomedical model (100). Reductionism has played an especially important role in the history of understanding pathogenicity, with the locus classicus being Koch's postulates, whereby it can be experimentally demonstrated that a single species of microorganism is the cause of a particular disease state (100). Based on the prevalence of "gene-for-X" publications in the genomics literature, the reductionistic biomedical model that underlay Koch's postulate throughout the expansion of the paradigm of the germ theory of disease appears to have been quite widely adopted in genomics research, despite acknowledgment of the complexity and context-dependence of gene expression, pleiotropy, and other effects that belie reductionism (96, 100). Genetic reductionism is best understood as a research strategy from which much is learned in its failures, which point to additional elements of the overall biological context necessary for fuller understanding (101, 102). In other words, methodological reductionism should be understood as a heuristic device for uncovering ontological complexity (101).

An explicit ethical framework, modeled on informed consent, is necessary to undergird a careful, thoughtful approach to assessing and considering the ethical, legal, and social implications of genomic research in which CODE can play a crucial role. In the same way that it has been urged that students of biology need a thorough understanding of the tools and

principles of ethics (103), the public, as stakeholders in assessing the ethical, legal, and social implications of genomic research and genomic medicine, need an understanding of the complexities of molecular biology to the extent that this can be achieved. The importance of providing tools for a broad range of educational encounters to build a more complex and realistic understanding of genomics is necessary if the public is to be consulted on research oversight, policy, and clinical application. As research proceeds in the study of CCR5 and other potential intervention targets for germline gene editing, a complex conversation is unfolding in which scientists, when consulted on the need for ethical boundary policing and a cautious approach to moving from basic to translational research to potential application, are pointing to the need to include the public in deliberations over where to draw the line in gene therapy more broadly and more specifically in interventions and genomic edits that cross the germ line (104). An analogy can be drawn with informed consent, a pillar of biomedical ethics, whereby if the public's voice is to play a role in providing ethical guidance for genomics research and genomic medicine, it is necessary for the public to be informed. CODE can serve as a platform for providing the sorts of educational tools to be deployed in various settings to improve understanding of the science of genomics.

Complicating research and public understanding, assessing the quality of large public databases has been challenging. During the middle of 2019, a Nature Medicine study brought to light the possibility that delta32 decreases life expectancies, yet the results were retracted due to errors in genotyping. This retraction has had consequences of potential public misinformation, suggesting that delta32 has no negative impact, even though the study only addressed a few associations, and public stories rarely discuss the known risks of delta32 (27), something to which our blood RNA-seq also lends support. The removal of CCR5 is not the only possible solution to developing HIV resistance, with the FDA fast-track work on compounds like Leronlimab to antagonize CCR5 binding by HIV. If these compounds are successful, then genomic modification may be unnecessary, with medications offering the safer approach for the patient. More importantly, all potential treatments need to be considered in the light of all available data, including their role in modulating neurodevelopment and sepsis outcomes. These discussions have diverged from the central question of what CCR5 does within cells, why it is important, and the severity of variants like delta32, which removes multiple transmembrane helices and thus prevents the correct proteins production and cellular localization. Further discussions are critically needed into potential gain-of-function roles of misfolded CCR5 delta32 (105) and our interesting observation of the broad distribution of blood CCR5 delta32 RNA-based allele frequencies in heterozygous individuals and accumulation of RNA in one individual that was homozygous (**Figure 7E**). Thus, the development of educational material for projects like CCR5 is critical for a more robust view of the protein, its variants, and its diverse physiological functions outside of what is emphasized in popular media.

Further complicating the role of CCR5 in human biology is the interplay of cytokine biology with CCL3, CCL4, and CCL5 (primary ligands for CCR5). The role of these chemokines in biology and disease have been well studied, and they are known to play a role in asthma, viral infections, dengue fever, acute kidney disease, and multiple sclerosis, with the oscillation of their role between benefit and harm depending on circumstance and insult (106–110). Although seen as inflammatory mediators, these chemokines are induced by inflammatory cytokine in multiple models, including neural inflammation. Typically treated as a site of immune privilege, resident macrophages, or glial cells within the brain, play an integral role in formulating a proper inflammatory response in a state of infection or injury. Microglia have been shown to secrete the inflammatory cytokines IL-1β and TNF-α, resulting in increased secretion of CCL3 and CCL4 (111), further supporting the role of CCR5 in microglia function and feedback.

Similarly, treatment of human brain endothelial cells with IL-1β, TNF-α, and IFN-γ resulted in a significant increase in the secretion of CCL3, CCL4, and CCL5, likely resulting in an increased capacity for leukocyte extravasation through integrin activation (110, 112). The interplay of cytokines in this paradigm is not monodirectional. CCL5 can skew the host's CD4 T cell response to pathogen from a TH2 phenotype to a TH1 phenotype, altering cytokine secretion. This modulation results in the release of proinflammatory cytokines such as TNF-α and IFN-γ, further potentiating the skewing (108). The biological complexity of CCR5, its ligands, and the interplay of the immune response as a whole demonstrates the need for a more nuanced discussion of our understanding of the available data.

Microglia, the resident macrophages of the central nervous system, express CCR5 as discussed. Microglia contribute to the blood-brain barrier (BBB) accompanied by a meshwork of endothelial cells, astrocytes, pericytes, and a basement membrane (113). In early development, microglia contribute to the pruning of neurons, with dysregulation of this process known to contribute to autism spectrum disorders (114). The ligands for CCR5 (CCL3, CCL4, and CCL5) display expression profiles from astrocytes, endothelial cells, microglia, and within specific subpopulations of neurons (112, 115). CCR5 expression and activation by its respective ligands promote CCR5+ leukocyte adhesion and transmigration through the blood-brain barrier (116). In response to CNS insults, CCR5 is upregulated, prompting an inflammatory response (117). In stroke, CCR5 knock-out is established to increase the severity of brain injury (118). However, this function of CCR5 in pathological states is either beneficial or detrimental depending on the inciting event. Studies with infectious agents such as *Toxoplasma gondii* (119), Herpes Simplex Virus type 1 (120), and Herpes simplex virus type 2 (121) have shown that knocking out *CCR5* contributes to enhanced infection and increased disease severity, likely due to blunting of the immune response.

On the contrary, malarial infection with cerebral involvement in *CCR5* knock-out mice resulted in neuroprotection by decreasing lymphocyte migration and destruction of the CNS (122). Much like our blood *CCR5* expression analysis, this data

suggests a balance of infection to outcomes based on CCR5 in the brain. Despite the prominent immunological role of CCR5, both peripherally and centrally, downregulation of *CCR5* does not reduce immune microglial transmigration in the CNS during pathological insults (123), indicating that there are other alternative pathways able to compensate for a dampened immune response. Constitutive basal levels of *CCR5* expression within the CNS in the absence of pathology indicate ancillary roles for this chemokine receptor outside of the known immune system component-with studies supporting its involvement in neural development and physiological functioning.

The complex role CCR5 plays in the central nervous system beyond the immune system has not been fully fleshed out; however, it has been demonstrated to play a role in fetal CNS development, neuronal differentiation, and neuronal survival. In a study by Westmoreland et al., it was found that *CCR5* was expressed by primate fetal cells with increasing expression from birth to 9 months of age (124). Further studies have shown that CCR5 activation results in neuronal differentiation and neuronal survival during apoptotic states (125, 126). The expression of chemokine receptors, such as CCR5, plays a role in neural progenitor cell migration and neuronal connections (127). Collectively, it appears that CCR5 plays a role in neural cell migration, differentiation, and survival during the postnatal period of CNS development, correlating to the timing of microglial pruning. This conclusion is also supported by the fact that many CNS tumors upregulate CCR5 during their rapid growth, including glioblastoma multiforme (128) and primary CNS lymphoma (129). Further insight into CCR5's role in the CNS is needed to determine the specific mechanisms at which it is involved outside of the known immune functions.

CODE projects lower the barriers to engaging in bioinformatics research by placing genomic analysis within the grasp of a broad and diverse audience. A typical project path mirrors the approach taken by clinical research analysts, beginning with database research, followed by modeling and simulations, and culminating in data analysis. Students first identify a genetic variant of interest identified through genomic research at HudsonAlpha, Michigan State University, or from a publicly available genome database like ClinVar (human). Students use publicly available databases and relevant software to learn more about the gene containing the variant and its functional product. Students compare the genomic sequence with similar segments from other organisms to study the evolutionary conservation of both DNA and protein. This information helps in making predictions regarding the functional consequence of the DNA variant.

Students next employ molecular modeling to study how variants may alter the protein. This type of molecular visualization provides essential support for reasoning on and formulating hypotheses related to molecular structure. Current software tools such as YASARA, PyMOL, and UCSF Chimera allow students to build a 3-D model of a protein, insert a variant, and visualize whether the variant changes protein folding and structure. Students can then study the DNA variant in a computationally-derived cellular environment, running simulations to predict how the variant-containing protein might behave inside a cell. Much like this study, that molecular level knowledge can be combined with analysis of multiple expression databases to allow the students to identify the cell types and tissues where the variants might alter function, known and genotype-to-phenotype insights. This type of analysis can facilitate understanding of the variant's effect and even provide insight into interventions to offset potentially damaging impacts. Students document their work and findings of their genetic variant in written reports, poster presentations, and research talks at their schools and scientific conferences and contribute to published research in relevant journals. As a byproduct, the students often generate educational resources such as 3D models, videos, and worksheets that can inform clinical analysis and broader understanding of genetic variants, such as shown here for CCR5.

This report on the impact of studies fueled by academic resources shed light on the need for and potential impact of applying education resources to cases in genomics. In the growing era of precision medicine, the need for tools to define the effects of variants (90) and propagate the information to patients and the public has grown, especially to combat genomic reductionism. CODE provides undergraduate students at diverse institutions with an improved understanding of genetics and developing educational material. As knowledge continues to grow for CCR5, several of the CODE students can continue engaging in molecular-level research, such as simulating the recently solved CCR5 ligand-bound structures (130) and integrating the knowledge into our tools. We have expanded these tools to clinical variants from the HudsonAlpha genomic sequencing projects to help explain complex Variants of Uncertain Significance (VUS), including MED13 (131) and RALA (132) that can be found at prokoplab.com/educational-resources/. At the outbreak of the SARS-CoV-2 pandemic, we also used this same workflow to establish critical and rapid insights on the viral encoded proteins and their interactions with host proteins utilizing CODE as a distributed research network to gain faster insights of proteins (133, 134). Expanding these tools to additional projects and collaboration will open a door for educational information in the needed area of genomics and genomic medicine.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/supplementary material.

AUTHOR CONTRIBUTIONS

JB, RS, RO, JM, AE, CS, SC, and JP contributed to the generation of CCR5 amino acid knowledgebase or expression analysis. MM, LB, CLS, DH, and JP contributed to the generation of CCR5 educational material. JB, RS, RO, JM, AE, JZ, NH, BC, SR, CB,

and JP contributed to the analysis and interpretation of 3 house RNAseq studies. JH contributed philosophical oversight of genomic reductionism. MM, CLS, SC, BC, SR, CB, and JP supervised the completion of all work. JB, MM, JZ, and JP wrote the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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CCR5Δ32 in Brazil: Impacts of a European Genetic Variant on a Highly Admixed Population

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The genetic background of Brazilians encompasses Amerindian, African, and European components as a result of the colonization of an already Amerindian inhabited region by Europeans, associated to a massive influx of Africans. Other migratory flows introduced into the Brazilian population genetic components from Asia and the Middle East. Currently, Brazil has a highly admixed population and, therefore, the study of genetic factors in the context of health or disease in Brazil is a challenging and remarkably interesting subject. This phenomenon is exemplified by the genetic variant CCR5Δ32, a 32 base-pair deletion in the *CCR5* gene. CCR5Δ32 originated in Europe, but the time of origin as well as the selective pressures that allowed the maintenance of this variant and the establishment of its current frequencies in the different human populations is still a field of debates. Due to its origin, the CCR5Δ32 allele frequency is high in European-derived populations (~10%) and low in Asian and African native human populations. In Brazil, the CCR5Δ32 allele frequency is intermediate (4-6%) and varies on the Brazilian States, depending on the migratory history of each region. CCR5 is a protein that regulates the activity of several immune cells, also acting as the main HIV-1 co-receptor. The CCR5 expression is influenced by CCR5Δ32 genotypes. No CCR5 expression is observed in CCR5Δ32 homozygous individuals. Thus, the CCR5Δ32 has particular effects on different diseases. At the population level, the effect that CCR5Δ32 has on European populations may be different than that observed in highly admixed populations. Besides less evident due to its low frequency in admixed groups, the effect of the CCR5Δ32 variant may be affected by other genetic traits. Understanding the effects of CCR5Δ32 on Brazilians is essential to predict the potential use of pharmacological CCR5 modulators in Brazil. Therefore, this study reviews the impacts of the CCR5Δ32 on the Brazilian population, considering infectious diseases, inflammatory conditions, and cancer. Finally, this article provides a general discussion concerning the impacts of a European-derived variant, the CCR5Δ32, on a highly admixed population.

Keywords: CCR5, CCR5delta32, Brazil, cancer, inflammation, infectious disease, pathogen, population genetics

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INTRODUCTION

Genetic Aspects of the Brazilian Population

Until the year 1500 CE, Brazil was inhabited only by Native Americans belonging to different linguistic groups, distributed along the coast and hinterland of the country. This scenario changed dramatically after the arrival of the Portuguese explorers in the Brazilian territory that year, affecting many cultural and biological aspects of the native populations. The European colonization of Brazil and the associated influx of Africans had a strong influence on the genetic makeup of the Brazilian population. In Brazil, as well as in other countries colonized by the Europeans, the Native American population deeply declined after colonization (contracted around 90% in the Americas) (1–3). The remaining native population underwent a strong process of genetic miscegenation. However, the processes of population change continued throughout Brazilian history, even in more recent times. Over the past 200 years, Brazil has received a large influx of European immigrants from various countries, also described as the last migration pulse, which added another layer to the genetic makeup of the Brazilian population (1–4).

In general terms, the genetic background of current Brazilians has Amerindian, African, and European components in different proportions (2, 3, 5–7), depending on the Brazilian region under investigation (North, Northeast, Center-West, Southeast, or South). For example, the genetic makeup of Brazilians in the southern region of Brazil was strongly influenced by migratory flows from Europe in the 19th and 20th centuries; although in the Northeast of the country, the African genetic component is high (1, 2, 8). Of note, the European component is preponderant in different Brazilian regions when the Amerindian, African, and European components are compared, but even observing some regional peculiarities as those mentioned above, the genetic composition of the Brazilian population is rather uniform in its miscegenation in different regions of the country (1).

Throughout history, Brazil also received migrants from other countries beyond those from Europe and Africa, including countries from Asia and Middle East (7, 9). The intense migration within the national territory (10) allowed the exchange of genetic information between Brazilians from different regions, ethnic and genetic groups. As a result of the interactions of these different groups, the Brazilian population is currently highly miscegenated, a characteristic evident in the rich genetic and phenotypic diversity observed among the Brazilian population (2, 6, 11, 12). Considering the scenario mentioned above, the Brazilian population can be considered genetically heterogeneous and admixed, in addition to being relatively uniform throughout the country (1). Interestingly, admixed Brazilian populations are probable "reservoirs" of the diverse Native American genetic component (3), currently the least prevalent genetic component in the population (1, 8).

Y-chromosome haplogroup analysis corroborates the high genetic miscegenation observed in the Brazilian population. Abe-Sandes et al. (13) investigated the frequency of different haplogroups in Brazilian individuals from different ethnicities. A significant frequency of typical European haplotypes in Afro-Brazilians was found, for example, in the Quilombola community of São Gonçalo, Bahia state, northeastern Brazil. Abe-Sandes et al. (13) also found the E-SRY4064 haplotype, usually observed in populations from Sub-Saharian Africa and almost absent in populations from Europe and Asia, in white Brazilians, in a notable frequency (13). Marrero et al. (14) also reported evidence of admixture in Native American populations, showing the presence of non-Amerindian haplotypes in Kaingang and Guarani peoples (14). Finally, numerous studies analyzing Y-chromosome haplogroups reinforce the miscegenation addressed in this article, pointing to European, Amerindian, African and Asian haplogroups in different ethnicities and population groups from different Brazilian regions (13–28).

In the same direction, evaluation of mitochondrial DNA in different populations of Brazil showed the presence of diverse haplogroups characteristic of African, European, Native American and Asian populations, again evidencing the high level of miscegenation in the Brazilian population (14, 29–37). Of note, Cardena et al. (38) assessed a population from São Paulo, southeastern state of Brazil, specifically evaluating mtDNA haplogroups and comparing such data with self-declared ethnicity. Interestingly, a significant parcel of the individuals classified as whites showed a high percentage of African mtDNA (37.6%), with less participation of Amerindian (31.6%) and European (30.8%) origins. When analyzing other genomic loci of the same individuals, a higher European contribution was noticed (63.3%), evidencing a considerable African participation of maternal origin in individuals simultaneously presenting high non mtDNA European ancestry (38, 39).

Pivotal Information Regarding the CCR5Δ32 Variant

The CCR5Δ32 polymorphism (reference SNP ID number: rs333) is a genetic variant that originated in the European population (40), and therefore can be used as an ancestry-informative marker in studies involving population genetics and genome ancestry (41, 42). This variant represents a 32-base pair deletion in the *CCR5* gene (chromosome 3; 3p.21.31), a fundamental component of the immune system responsible for encoding the CCR5 protein, which acts mainly in the regulation of inflammatory cell migration. It is unclear what selective pressures (considering positive selection) were responsible for fixing CCR5Δ32 in the human genome. Smallpox, bubonic plague, and other infectious diseases have already been suggested, but there is no consensus on this aspect (40). Neutral evolution is also a possibility (43). What is somehow certain is that the variant probably originated in the European population at 700-5,000 years ago (43, 44), potentially even earlier than 5,000 years (45, 46), and later spread heterogeneously across the world.

The CCR5Δ32 allele presents a higher frequency in northern Europe (greater than 15% in Norway, Latvia, and Estonia), being less frequent in countries located in the south of the European

continent. For example, the frequency of the CCR5Δ32 allele is 8.1% in Spain, 6.9% in Portugal, 6.2% in Italy, and 5.1% in Greece. The allele frequency is very low or even absent in most Asian and African countries: for example, 0.4% in China, 2.2% in Korea, 0.7% in Cameroon, 0.26% in Eritrea, and 2.9% in Egypt (47). A recent study reports the absence of the CCR5Δ32 allele in the Nepalese population (48). Similarly, CCR5Δ32 is rare in Native American groups, showing an overall CCR5Δ32 allele frequency of 0.2%, mostly probably due to miscegenation (42). In the contemporary Brazilian population, the overall frequency of the CCR5Δ32 allele usually ranges from 4 to 6% but showing significant variations between different Brazilian regions and ethnic groups (42, 49), as will be discussed in the next sections of this article.

The main function of the CCR5 is coordinating leukocyte migration during inflammatory reactions through interaction with different chemokines, especially CCL3, CCL4, and CCL5 (40). Of note, these chemokines were historically called "MIP-1α", "MIP-1β" and "RANTES", respectively, but that denomination has fallen into disuse (50, 51). The CCR5 protein is expressed on the cell surface and has seven transmembrane domains connected by three extracellular loops and three intracellular loops. Leukocytes are the main cells that express the CCR5 (40), although the protein is also detected in other cell types, such as human embryonic neurons (52), adipocytes (53), and several types of cancer cells and tissues (54–58), indicating that CCR5 performs immune functions that go beyond coordinating the migration of inflammatory cells.

Carriers of the wild-type *CCR5* gene have CCR5 expression constitutively, with some variation between individuals. CCR5Δ32 causes important phenotypic effects, affecting the interaction of the CCR5 with chemokines. Due to the induction of a change in the *CCR5* gene reading frame, the CCR5Δ32 produces a truncated protein that is not expressed on the cell surface, presenting a gene-dosage effect. In brief, the presence of the CCR5Δ32 allele in heterozygous causes a reduction in the expression of CCR5 at the membrane. The presence of the CCR5Δ32 allele in homozygosis culminate in virtually no expression of CCR5 molecules on the cell surface (59–63). The CCR5Δ32-derived molecules are not phosphorylated and remain retained in the endoplasmic reticulum (64). Interestingly, it was suggested that in addition to the gene-dosage effect associated to CCR5Δ32, the CCR5Δ32-derived truncated protein could promote the sequestration of the CCR5 and CXCR4 proteins, both HIV-1 co-receptors, from the cell surface (65, 66).

These changes in the expression of CCR5 associated to CCR5Δ32 culminate in a disrupted CCR5-mediated immune response, which can be beneficial in some situations or harmful in others (67) since the 'chemokine system' is not completely redundant. The absence of CCR5 can impact the cell signaling coordinated by CCL3, CCL4 and CCL5, thus perturbing the proper CCR5-mediated immune responses (68). Disruptions in the chemokine system can significantly alter the susceptibility and progression of different diseases. For instance, COVID-19 severe cases are associated with uncontrolled receptor-ligand interactions and consequent inflammatory dysregulation, which characterizes the cytokine storm frequently observed in such severe disease cases (69, 70). Recently, CCR5Δ32 deletion was identified as a protective factor in Czech First-Wave COVID-19 subjects (71). Different *CCR5*-editing techniques are currently available and can be used to test *in vitro* the impacts of the CCR5 absence in different conditions, simulating the consequences of CCR5Δ32 on the immune system and disease conditions (72, 73). However, it is essential to emphasize that the *CCR5*-editing in human embryos raises many ethical concerns and may have deleterious consequences (67, 74).

Looking at the desirable effects, CCR5Δ32 protects against HIV infection, since the homozygous state of the variant impairs the proper expression of CCR5, preventing the interaction of CCR5 (the main HIV co-receptor) with the virus on the cell surface, thus avoiding infection of the host (75, 76). As mentioned above, CCR5Δ32-derived molecules (CCR5 truncated proteins) can also have an important protective effect against HIV by sequestrating CCR5 and CXCR4 from cell surface (65, 66). The discovery of this effect was truly relevant because it gives support to the use of CCR5 blockers for the clinical control of HIV infection. The best example of this case is maraviroc, a noncompetitive CCR5 antagonist that prevents the proper interaction between the HIV envelope glycoprotein and the CCR5. Currently, other CCR5 blockers (e.g., cenicriviroc, leronlimab) are being tested to treat HIV infection and other inflammatory conditions, and maraviroc emerges as a potential drug to treat other diseases involving CCR5, especially some types of cancer (77). In Brazil, CCR5 blockers represent a good choice for HIV treatment, since most of the circulating viral strains show CCR5 tropism (78–80). Based on the scenario presented above, **Figure 1** shown an alluvial diagram representing the classic outcomes associated with the CCR5Δ32, including "desirable" and "undesirable" effects.

Another major achievement involving CCR5Δ32, and HIV infection was the sustained remission of the infection in the 'Berlin Patient', reported in 2009 (83) and confirmed in 2011 (84), and in the 'London Patient', reported in 2019 (85) and confirmed in 2020 (86). Both individuals were HIV positive and developed hematological malignant diseases (acute myeloid leukemia and Hodgkin's lymphoma, respectively), requiring allogeneic hematopoietic stem-cell transplantations. After receiving cell transplantations from CCR5Δ32 homozygous donors, both showed sustained remission of HIV infection. Other cases like Berlin and London patients are being followed up, such as the 'Düsseldorf patient' (87). The success of this strategy, although involving few cases, shows that sustained remission of HIV is possible to be achieved and subsequently maintained free of antiretroviral therapy. The Berlin patient, Timothy Ray Brown, passed away on September 29, 2020, due to the recurrence of acute myeloid leukemia, not HIV infection (88, 89). In addition to having collaborated enormously to advance research involving HIV, T. R. Brown created the Timothy Ray Brown Foundation and contributed significantly to the field of HIV/AIDS research, with a big and admirable impact on global society as an HIV activist (89–91).

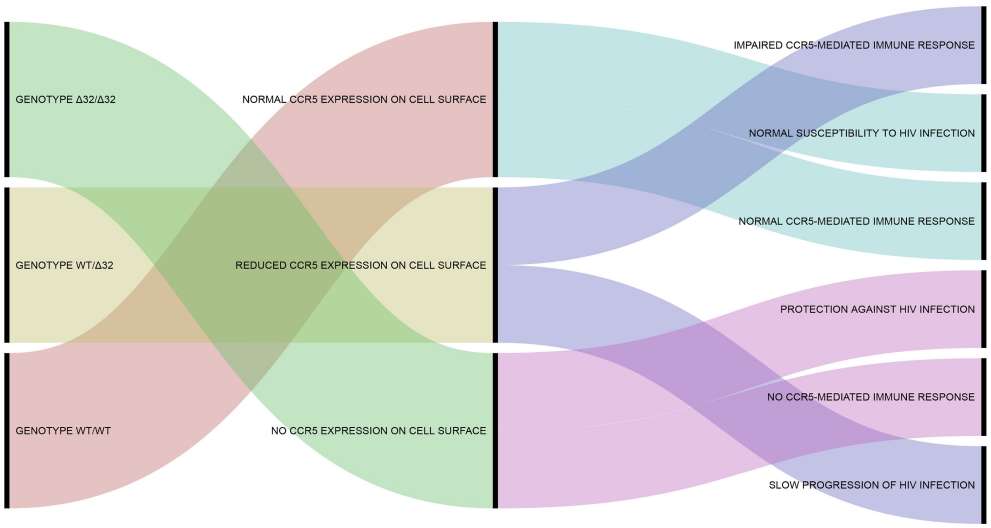

FIGURE 1 | Alluvial diagram representing the classic outcomes associated with the CCR5Δ32. The CCR5Δ32 genotypes are shown in the left part of the diagram. The phenotypic effects of each genotype are shown in the center. The more classical consequences associated with each phenotype are shown in the right part of the diagram. Additional information concerning the phenotypic effects of the CCR5Δ32 on human cells and immune system can be found in previous studies of our group (40, 68, 81). This figure was created using RAWGraphs (https://rawgraphs.io/) (82).

Currently, it is known that the influence of CCR5 and CCR5Δ32 goes beyond protection against HIV infection and is much broader than previously believed, influencing the susceptibility and outcome of different conditions, such as other different viral, bacterial, and parasitic diseases (40, 92), as well as non-infectious inflammatory conditions (93–96). This occurs because the lack of CCR5 expression, in humans naturally due to CCR5Δ32, interferes with multiple aspects of inflammatory responses, including expression of immune system genes, levels of inflammatory markers, and activity of immune cells (97–103). On the other hand, now looking at the undesirable aspects of CCR5Δ32, this genetic variant increases the risk of serious complications caused by the West Nile virus and Tick-borne encephalitis virus (104–109).

Although Brazilians form a population of more than 210 million individuals, genetic studies in this population are still limited, with most genetic studies focusing on populations with European ancestry (6, 9). The Brazilian population can serve as a study case to understand the impact of genetic admixture on the frequency of genetic variants, such as CCR5Δ32, and its impacts on different conditions and pharmacogenomics (7). Understanding the extent to which the CCR5Δ32 variant influences the health of different populations is critical since it indicates which individuals and ethnic groups are more likely to benefit from therapies focused on modulating CCR5 in the context of cancer, infections, and inflammatory diseases. Focusing on HIV, knowing the frequency of CCR5Δ32 in different human populations is the initial step to guide potential new attempts at sustained remission of HIV infection through stem cell transplantation with CCR5Δ32 homozygous genotype. Moreover, it is also essential to understand how CCR5Δ32 impacts the health of the Brazilian population.

Considering that (I) the frequency of CCR5Δ32 is quite varied among Brazilians from different country's regions and that (II) the role of CCR5Δ32 in various pathological conditions is an emerging topic with several knowledge gaps, the primary objective of this article is to review the effects of the genetic variant CCR5Δ32 on the Brazilian population, considering several diseases and clinical conditions. The secondary objective of this article is to discuss the impacts of a European-derived variant, the CCR5Δ32, on a highly mixed population.

METHODS

For the initial selection of articles, the terms "CCR5", "CCR5 delta 32", "CCR5Δ32" and "rs333", used in combination with "Brazil" or "Brazilian", were searched on PubMed (https://pubmed.ncbi.nlm.nih.gov/). Subsequently, the same search strategy was used on Scientific Electronic Library Online - SciELO (https://scielo.org/). The articles were initially selected based on the title and abstract. Only articles addressing CCR5Δ32 in Brazilian populations were included in this review. Articles published in English and Portuguese were considered in the evaluation, without restriction concerning the date of publication. On some specific occasions, the reference list of selected articles was also used as an additional source of published works involving CCR5Δ32 in the Brazilian population. Additional unstructured searches were performed on PubMed to select the articles cited in the introduction section and additional points of the review.

CCR5Δ32 FREQUENCY IN BRAZIL

A study published in 2016 by Silva-Carvalho and collaborators (49) presented a very complete meta-analysis regarding the

CCR5Δ32 frequency in Brazil. In addition to original data from those authors, the meta-analysis included 29 articles reporting the CCR5Δ32 frequency in Brazil, encompassing populations from ten Brazilian States. The study found an overall allelic frequency of 4% in the country (49). The frequencies of the CCR5Δ32 allele in the Brazilian States, including data compiled by Silva-Carvalho et al. (49), are summarized in **Figure 2**. Henceforward, we expand the information concerning the CCR5Δ32 frequency in Brazil, highlighting studies not included in the meta-analysis by Silva-Carvalho et al. (49), and including data obtained from studies with indigenous populations and quilombola communities, as discussed below.

Leboute et al. (112) reported the absence of the CCR5Δ32 allele in a sample of 300 Amerindians from four indigenous populations of the Brazilian Amazon region, namely: Tikuna (n = 191), Baniwa (n = 46), Kashinawa (n = 29), and Kanamari (n = 34). Based on such data, we can argue that, at least until the date of publication of that work, the studied Amazonian tribes probably did not have a significant degree of miscegenation at a level sufficient for the introduction of the CCR5Δ32 allele into those indigenous groups. Alternatively, the allele could already be circulating in the groups, but it may not have been detected due to the small sample size (112).

Carvalhaes et al. (113) also described the frequency of the CCR5Δ32 allele in different ethnic groups of the Brazilian Amazon region, specifically from Pará State. The sample groups investigated were composed of 394 individuals from Belém (capital of Pará), 67 Afro-Brazilian individuals, 89 Amerindian individuals, and 111 Japanese immigrants. The CCR5Δ32 allele was not observed in Amerindian individuals and Japanese immigrants. In the sample of Afro-Brazilian individuals, only one individual carrying the allele in heterozygous was found, with the allele frequency, in this case, being 0.75%. In the sample of random individuals from Belém, one homozygous individual for the gene deletion and 22 heterozygous individuals were found, resulting in a CCR5Δ32 allele frequency of 3.04% (113).

Hünemeier et al. (110) evaluated the frequency of the CCR5Δ32 allele in Native American populations in Brazil and Paraguay: five Amazonian groups (Tiriyo, Mura, Cinta Larga, Gavião, and Zoró); a group from the Paraguayan Gran Chaco (Lengua); one from the Paraguayan forest (Aché); and one from

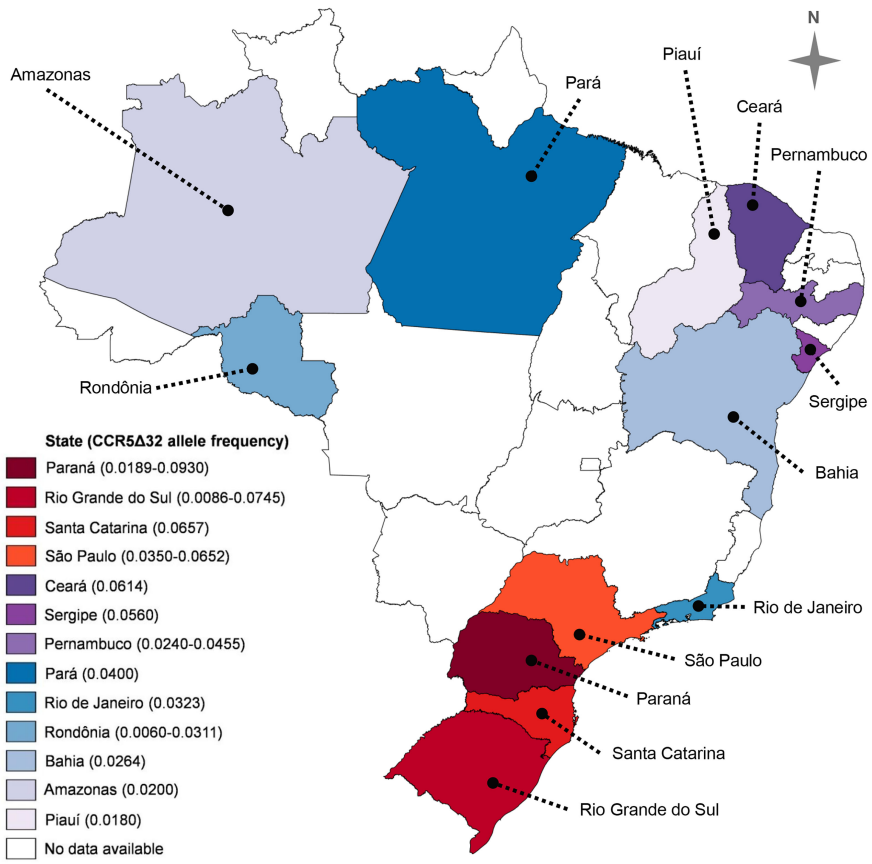

FIGURE 2 | CCR5Δ32 allele frequency in thirteen Brazilian states. Two values in parentheses represent the lowest and the highest frequency observed in a given state. Data from Silva-Carvalho et al. (49), Hüneimeier et al. (110) (Mura population; Amazonas State), Carvalho et al. (41) (Mocambo community; Sergipe State), and Ferreira-Fernandes et al. (111) (Piauí State). The map was created with the help of MapChart (https://mapchart.net/), licensed under a Creative Commons Attribution-ShareAlike 4.0 International License.

southern Brazil (Kaingang). The CCR5Δ32 allele was found only in two groups: Mura (2%) and Kaingang (3%). The presence of the CCR5Δ32 allele in the samples of these two groups may be due to gene flow, which is explained by previous data showing that both populations have a degree of miscegenation. Thus, the CCR5Δ32 allele may have been introduced in American-native populations due to European miscegenation (110).

Vargas et al. (42) investigated the distribution of the CCR5Δ32 allele in individuals from Alegrete, a city in the western region of Rio Grande do Sul State. The population of Alegrete is highly admixed, with the genetic participation of Spanish, Portuguese, African, and Amerindian peoples. In the study, 103 healthy and unrelated individuals were analyzed, being divided into 'white' (n=59), 'brown' (n=31), and 'black' (n=13). No CCR5Δ32 homozygous individuals were found, and the frequency of heterozygotes was 14% in whites, 13% in browns, and 8% in blacks. Allele frequencies were 6.8%, 6.4%, and 3.8%, respectively (42). In Brazil, the classification of ethnicity performed by the government agency *Instituto Brasileiro de Geografia e Estatística* (Brazilian Institute of Geography and Statistics) is based on skin color, and for this reason many Brazilian studies classify individuals using this criterion. Alternatively, 'white' individuals can be classified as Caucasians, and 'brown' and 'black' can be classified as non-Caucasians.

Ferreira-Fernandes et al. (111) analyzed the CCR5Δ32 frequency in a sample of the population of the Piauí State. The sample consisted of 223 elderly individuals from the Network of Research on Frailty in Elderly Brazilians. The CCR5Δ32 allele was found only in heterozygous in the sample, with an allele frequency of 1.8%. In order to have a more robust investigation, the sample was also stratified according to sex and age (dividing the groups into individuals below or above 73 years old), but the frequencies were not statistically different between groups, ranging from 1.5% to 2.3%. The general CCR5Δ32 frequency observed is in accordance with other data presented by groups also from northeastern Brazil (111).

Carvalho et al. (41) evaluated the CCR5Δ32 frequency in three quilombola communities in the states of Sergipe (Mocambo community) and Bahia (Rio das Rãs and São Gonçalo communities). The groups were founded about 150 years ago by individuals from Sub-Saharan Africa and/or their descendants. The study evaluated individuals born in quilombola communities and recent immigrants, with a total of 100 inhabitants from Rio das Rãs, 71 from Mocambo, and 53 from São Gonçalo. In these communities, 28 were recent immigrants from Rio das Rãs, 18 from Mocambo, and 15 from São Gonçalo. Thus, the total sample size was 224 individuals: 163 born in the quilombos and 61 recent immigrants. In most cases, the oldest person in each family was chosen to participate in the study. The CCR5Δ32 allele was found in the three communities evaluated, but only in heterozygosis, with allele frequencies of 5.6% in Mocambo, 1% in Rio das Rãs, and 0.9% in São Gonçalo. According to the authors, the differences in allele frequencies can be due to several factors, including different proportions of parental populations in the founder's individuals, a founder-effect, and different patterns of inter-ethnic contact (41).

Finally, we summarized in **Figure 2** the frequencies of CCR5Δ32 allele in thirteen Brazilian States, according to data of ten states compiled by Silva-Carvalho et al. (49), and the frequencies observed by Hüneimeier et al. (110) in the Mura population (Amazonas State), by Carvalho et al. (41) in individuals from Mocambo community (Sergipe State), and Ferreira-Fernandes et al. (111) in individuals from Piauí. To the best of our knowledge, there are no data available in the literature on CCR5Δ32 in the other Brazilian States.

CCR5Δ32 IN INFECTIOUS DISEASES

CCR5 plays a critical role in the regulation of the immune response against infectious agents, controlling the traffic of immune cells [e.g., Natural Killer (NK) and T-regulatory (Treg) cells] towards inflammation sites. For instance, a recent study with mice showed that CCR5 has a pivotal role in the recruitment of NK cells to the kidney allowing an adequate neutrophil activity during systemic *Candida albicans* infection, acting as a fundamental molecule for a proper immune response. The absence of CCR5 expression resulted in uncontrolled inflammation and increased renal damage in face of *C. albicans* infection (114). Also, Treg cells play a fundamental role in resolving inflammatory conditions, providing an immunosuppressive activity. During infection by different pathogens (e.g., *Schistosoma* spp.), the poor recruitment of Treg cells to the inflammation sites due to CCR5 absence causes uncontrolled inflammation and related tissue damage (40, 115). On the other hand, during Rocio virus infection, the CCR5 absence was associated with reduced brain inflammation and better prognosis in animals (116). Taking together, imbalances in the CCR5-mediated immune responses due to CCR5Δ32 can cause both reduced and exacerbated inflammation, depending on the type of pathogen responsible for the infection (e.g., fungus, bacteria, virus), the infection site, or the immune cell type affected by the lack or reduction of CCR5 expression (40). In this context, studies addressing CCR5Δ32 and viruses in the Brazilian population will be discussed here, including HIV, Human T-lymphotropic virus (HTLV), Dengue, Influenza A, Hepatitis C virus (HCV), Hepatitis B virus (HBV), and Human papillomavirus (HPV).

As explained in the introduction section, CCR5Δ32 exerts its protective effect against HIV infection through two mechanisms: reduced expression of the *CCR5* gene (gene-dosage effect; probably the most important mechanism) (60, 63) and sequestration of CCR5 and CXCR4 from the cell surface (65, 66). Many studies that evaluated CCR5Δ32 in the Brazilian population corroborated the protective effect of the variant on susceptibility or clinical aspects of HIV infection (e.g., 117–120), although other studies have not evidenced these effects, in some cases probably due to the small sample size (e.g., 121, 122). The main results of the studies involving CCR5Δ32 and HIV infection in Brazil are detailed in **Table 1**.

Experimental evidence indicated that the course of HTLV (type 1 and 2) infection and HIV/HTLV co-infection may be

TABLE 1 | Impacts of the CCR5Δ32 on HIV infection.

Population	Sample	Main findings	Reference
Brazilian HIV+ individuals	177 ARV-naive individuals	Heterozygous individuals for CCR5Δ32 have a better response to ARV treatment than wild-type homozygotes	Accetturi et al. (117)
Brazilian individuals from different regions	1162 individuals (133 with HIV+ status)	CCR5Δ32 heterozygous cells (PBMCs) showed partial resistance to R5-HIV-1 *in vitro*; No significant differences in CD4+ T-cell counts between HIV+ individuals heterozygous and wild-type homozygous for CCR5Δ32; HIV load in heterozygous individuals are significantly lower than in wild-type individuals	Grimaldi et al. (123)
Individuals from São Paulo State, Brazil	129 HIV+ individuals and 26 blood donors	CCR5Δ32 heterozygous genotype was associated with reduces RANTES/CCL5 levels	Mikawa et al. (124)
Individuals from São Paulo State, Brazil	183 HIV+ individuals and 115 controls	The frequency of the CCR5Δ32 heterozygous genotype was lower in HIV+ individuals (11.5%) than in controls (13.0%)	Munerato et al. (125)
Individuals from Pará, Brazil	110 HIV+ and 139 uninfected individuals	Similar frequencies of the CCR5Δ32 allele were observed in the two groups: 2.7% in HIV+ individuals and 2.2% in the controls	Carvalhaes et al. (121)
Children from Pernambuco State, Brazil	106 HIV+ and 70 uninfected children exposed to infection risk and 104 controls	No significant influence of the CCR5Δ32 in the risk of HIV vertical transmission	Souza et al. (126)
HIV+ children from São Paulo State, Brazil	51 HIV+ children divided into rapid, moderate and slow progressors	No influence of the CCR5Δ32 in disease progression (limited sample size)	Angelis et al. (127)
Individuals from southern Brazil	134 blood donors; 145 HIV-exposed seronegative individuals; 152 HIV+ asymptomatic individuals; 478 HIV+ individuals with AIDS	CCR5Δ32 homozygous genotype was significantly associated with reduced risk of HIV infection	Vissoci Reiche et al. (118)
Individuals from São Paulo State, Brazil	200 HIV+ (155 on pre and post-ART) and 82 uninfected individuals	CCR5Δ32 heterozygous genotype was associated with better CD4+ T cell recovery after ART initiation	Rigato et al. (119)
Injecting drug users from Rio de Janeiro State, Brazil	48 HIV+ and 558 uninfected injecting drug users	No significant impact of the CCR5Δ32 on susceptibility or protection to HIV infection	Teixeira et al. (128)
Individuals from Bahia State, Brazil	506 HIV+ individuals (155 divided into rapid, typical and slow progressors)	CCR5Δ32 allele was more frequent in typical than in rapid progressors (without statistical significance)	Abe-Sandes et al. (122)
HIV+ individuals from Rio Grande do Sul State, Brazil	249 HIV+ individuals	CCR5Δ32 heterozygous genotype was associated with reduced risk of CD4+ T cell depletion (univariate analysis) and with increased risk of death after AIDS diagnosis (multivariate analysis; potentially due to the emergence of CXCR4-tropic HIV strains); CCR5Δ32 was a protective factor on disease progression in survival curve analysis	Vieira et al. (129)
Serodiscordant couples from Santa Catarina State, Brazil	9 HIV-exposed seronegative individuals; 9 ART-treated HIV+ individuals; 12 healthy controls	The CCR5Δ32 heterozygous genotype was observed in two HIV-exposed seronegative individuals, two ART-treated HIV+ individuals, and one control; In one serodiscordant couple, both individuals had CCR5Δ32 heterozygous genotype and the CXCR4 viral tropism was observed in the infected individual	Santos et al. (130)
Individuals from Roraima State, Brazil	117 HIV+ individuals	CCR5Δ32 heterozygous genotype was found in 11 individuals (9.4%); CCR5Δ32 allele frequency estimated at 4.6%	Corado et al. (131)
Individuals from Pernambuco State, Brazil	213 HIV+ and 234 uninfected individuals	CCR5Δ32 frequency was reduced in HIV+ individuals compared to controls; Stratification of data according to CCR5Δ32 genotypes did not modify the results of *TRIM5* polymorphisms observed in the study	Celerino da Silva et al. (132)
Individuals from São Paulo State, Brazil	66 HIV+ individuals with recent infection	CCR5Δ32 heterozygous genotype was detected in two individuals (one infected by R5-tropic HIV strain and other by CXCR4-tropic HIV strain); No significant association between CCR5Δ32 and tropism switch	Arif et al. (133)
Individuals from Paraná State, Brazil	35 individuals with HIV/HBV or HIV/HCV co-infection	CCR5Δ32 allele was not observed in the sample	Avanzi et al. (80)
Individuals from Pará State, Brazil	30 HIV+ individuals (divided into viremia controllers and non-controllers)	CCR5Δ32 heterozygous genotype was detected in one non-viremia controller	Gomes et al. (134)
Individuals from Paraná State, Brazil	81 perinatally infected HIV+ adolescents and young adults (61 genotyped for CCR5Δ32)	CCR5Δ32 heterozygous genotype was detected in one individual (1.6%); This patient was infected by an R5 HIV strain	Martin et al. (135)
Individuals from Pernambuco State, Brazil	266 HIV+ and 223 uninfected individuals	CCR5Δ32 frequency was reduced in HIV+ individuals compared to controls (without statistical difference); CCR5Δ32 along with other polymorphisms did not show statistically significant influence on plasma viral load	Celerino da Silva et al. (136)

(Continued)

TABLE 1 | Continued

Population	Sample	Main findings	Reference
Individuals from Rio Grande do Sul State, Brazil	294 uninfected individuals and 206 HIV+ individuals (divided into 40 rapid progressors and 166 non-rapid progressors)	Plasma viral load was lower among CCR5Δ32 heterozygous individuals as compared to wild-type homozygous individuals	Valverde-Villegas et al. (120)
Individuals from Pernambuco State, Brazil	248+ individuals divided into immunological recovery profiles during ART (222 of the 248 HIV+ individuals were genotyped for CCR5Δ32)	CCR5Δ32 heterozygous genotype was statistically associated with immunological recovery failure (result from logistic regression analysis)	Carvalho-Silva et al. (137)

ART: antiretroviral therapy.

affected by CCR5 expression patterns, which can be modulated by such viruses (138, 139). The CCR5 and its ligands can also influence the course of Dengue infection (140, 141). CCR5Δ32 was associated with an increased risk of fatal Influenza virus infection in Spanish individuals (142). However, CCR5Δ32 has a limited impact on these infections in the Brazilian population. Studying HTLV-1 infection, no statistically significant association was found between CCR5Δ32 and susceptibility or presence/absence of a symptomatic infection (143). Only one study was found regarding this evaluation in a non-Brazilian population. Hisada et al. (144) investigated the CCR5Δ32 frequency in Jamaican HTLV-1-infected individuals and healthy controls. However, the frequency found was too low to further conclusions. That said, no study found an association between the variant and HTLV-1 infection (144). Also, no statistically significant association was observed when the frequencies of CCR5Δ32 were compared between severe Dengue cases and controls (145). A similar study carried out in an Australian population also found no association between the CCR5Δ32 allele and DENV infection (146). The CCR5Δ32 was not associated with hospitalization in individuals infected by Influenza A virus (2009 pandemic H1N1 strain) (147). Subsequently, a study addressing the same virus also reported no significant effect of CCR5Δ32 on H1N1 infection severity (148). A study conducted in a Spanish population identified an association between the CCR5Δ32 allele and fatality due to Influenza A (H1N1) infection (142). Also, an association of the variant with disease severity was observed in a Canadian population (149). Therefore, further studies evaluating the role of this polymorphism in Influenza virus infection are needed.

HCV and HBV are associated with the development of hepatocarcinoma and other liver diseases (150). CCR5 could affect both susceptibility to these viruses and associated diseases due to its regulatory role in inflammatory reactions. Our group evaluated the influence of CCR5Δ32 on susceptibility to HCV infection and HCV/HIV co-infection. In the same study, we also accessed the potential impact of the CCR5Δ32 on HCV-related fibrosis, cirrhosis, and hepatocarcinoma. In total, 1352 individuals were included in the study. No statistically significant associations of CCR5Δ32 with the evaluated criteria were observed (151). Looking at data reported in other populations [see discussion in reference (151)], we highlight that the association between the CCR5Δ32 variant and HCV infection can show important biases in some populations, and other studies corroborate our results showing a lack of association between the variant and HCV infection. Importantly, our work had the largest sample evaluated in the context of HCV infection (151).

More recently, we evaluated the influence of CCR5Δ32 on susceptibility to HBV infection and HBV/HIV co-infection in a study involving 1113 individuals. We found no significant effect of CCR5Δ32 on susceptibility to HBV mono-infection. On the other hand, the CCR5Δ32 allele exerted a protective influence on HBV/HIV co-infection. Of note, this result was potentially due to the known protective effect of CCR5Δ32 on HIV infection (92). In a study in the Indian population, the heterozygous genotype (WT/Δ32) was associated with a higher susceptibility to HBV infection, whereas in a study in the Iranian population, the variant was a protective factor against the infection (152, 153). Other studies carried out in different populations reported a lack of association between HBV infection and the CCR5Δ32 variant (154–156), which is in agreement with the major finding observed in our previous study (92).

HPV is strongly associated with the development of cervical cancer (157) and it was suggested that CCR5 could play a role in the context of HPV infection and related diseases. Nevertheless, Mangieri et al. (158) observed no significant effect of CCR5Δ32 on susceptibility to the infection or cervical lesions (158). Also, the CCR5Δ32 was not associated with infection by a particular HPV genotype (159). In contrast, in a Swedish population, the homozygous genotype for the variant was associated with an increased risk of HPV infection (160). Given the limited amount of data and the contradictory results concerning the involvement of CCR5 in HPV infection, further evaluation concerning the potential role of the CCR5Δ32 variant in the context of HPV infection and related diseases in Brazilian and other populations are needed.

The influence of CCR5Δ32 on parasitic diseases was also investigated in the Brazilian population, including Chagas disease, leishmaniasis, and toxoplasmosis. CCR5 can have two opposite effects on Chagas disease, a disease caused by *Trypanosoma cruzi* infection. CCR5 mediates the control of acute infection, assuming a favorable role for the host. In opposition, the increased expression of CCR5 during Chagas disease is associated with exacerbated inflammation and related cardiac complications (161). Thus, the levels of CCR5 expression are critical in the outcome of Chagas disease. However, two other studies found no association between the CCR5Δ32 variant and

cardiac or digestive manifestations on chronic Chagas disease (162, 163). In a Peruvian population, the frequency of the Δ32 allele was not high enough to allow an analysis of association with *T. cruzi* infection, and a study with individuals from Venezuela did not find an association of the variant with the presence of disease symptoms (164, 165). Therefore, the potential CCR5Δ32 allele role in Chagas disease is still under discussion.

Brajão de Oliveira et al. (166) and Ribas et al. (167) reported no statistically significant difference between Leishmania-infected individuals and controls concerning CCR5Δ32 frequencies (166, 167). In the study performed by Brajão de Oliveira et al. (166), the CCR5Δ32 allele carriers showed a less severe spectrum of clinical manifestations, but without statistical significance (166). Ribas et al. (167) observed a higher frequency of the CCR5Δ32 polymorphism among a subgroup of patients with recurrent lesion, but this specific result was based on an exceedingly small cohort (167). Also, a study performed in a Pakistani population showed no association between the CCR5Δ32 variant and cutaneous leishmaniasis (168).

The CCR5Δ32 wild-type genotype in association with AA or AG genotypes (from the *CCR5* rs1799987 polymorphism, an intron A/G SNP) was associated with increased risk of ocular toxoplasmosis, potentially due to the persistent CCR5-mediated inflammation in individuals with normal CCR5 expression (169). Also evaluating Brazilians, Vallochi et al. (170) found no association between the CCR5Δ32 and ocular toxoplasmosis (based on a brief description; detailed data not described by such authors) (170). No other studies evaluating the role of this variant in the context of ocular toxoplasmosis in non-Brazilian populations were found.

Based on the studies discussed above, apart from the protective effect of CCR5Δ32 on HIV infection, the impacts of CCR5Δ32 on viral and parasitic infections in Brazilian populations seem quite limited (details of each study are presented in **Table 1** and **Table 2**). However, considering the recognized role of CCR5 in the regulation of inflammation, it is possible that potential influences of CCR5Δ32 on non-HIV infections have not been detected due to the small number of studies carried out in Brazil on these topics, many of them involving a small sample size.

Finally, the impact of the CCR5Δ32 on fungal infections is unknown in Brazilian populations and quite sparse in other human populations, and therefore research in this field is needed. Of note, Brazil is affected by several endemic mycoses, such as Dermatophytosis, Paracoccidioidomycosis, Histoplasmosis, and Cryptococcosis, among others (171). Understanding whether and how the CCR5Δ32 influences the susceptibility or clinical progression of these diseases can provide insights into the potential use of CCR5-based therapies for these diseases.

CCR5Δ32 IN INFLAMMATORY CONDITIONS

Considering the critical role of CCR5 in the regulation of the inflammatory response, several authors have been investigating the effect of CCR5Δ32 on conditions that have their susceptibility or clinical course affected by different types (e.g., systemic, local) and intensity of inflammation. In this topic, we review the role of CCR5Δ32 on the following inflammatory diseases or inflammation-related clinical conditions: multiple sclerosis, systemic lupus erythematosus, preeclampsia, rheumatoid arthritis, juvenile idiopathic arthritis, periodontitis, osteomyelitis, transplant rejection, and sickle cell disease. Details of each study are described in **Table 3** and discussed below.

Multiple sclerosis is an autoimmune, chronic, and inflammatory disease showing heterogeneity in clinical findings. Chemokines and chemokine receptors are molecules involved in the pathogenesis of multiple sclerosis (172, 194), and the CCR5Δ32 can influence different aspects of this disease, as shown in studies with non-Brazilian individuals (195–197). A meta-analysis carried out in 2014 evaluated the role of this variant in multiple sclerosis in different populations, and concluded that the CCR5Δ32 is not associated with susceptibility to the development of multiple sclerosis in Europeans, calling attention to the need for further studies involving other populations (198). In Australian individuals, this variant also did not show a protective role to multiple sclerosis (199). However, other studies have shown an association of the Δ32 allele with treatment response, disease severity, and susceptibility to multiple sclerosis (196, 200–202). In Brazil, only two papers explored the possible impact of the CCR5Δ32 on multiple sclerosis. Based on magnetic resonance imaging, Kaimen-Maciel et al. (172) observed a decreased disease progression in patients bearing the CCR5Δ32 allele (172). Subsequently, Troncoso et al. (173) described a statistically significant higher CCR5Δ32 allele frequency in Euro-Brazilian controls (7.4%) compared to Euro-Brazilian patients (3.3%), suggesting a protective role of the variant on the development of multiple sclerosis. Besides, the frequency of the CCR5Δ32 was higher in Euro-Brazilian patients with progressive multiple sclerosis than Euro-Brazilian patients with relapse remitting multiple sclerosis (173). Both studies carried out in Brazil show that the CCR5Δ32 variant can influence both the susceptibility and the clinical outcome of multiple sclerosis.

Systemic lupus erythematosus is a chronic inflammatory autoimmune disease characterized by the large production of autoantibodies, triggering generalized tissue damage. This disease has different clinical manifestations and a complex genetic influence, and chemokines and their receptors, such as CCR5, are implicated in the pathogenesis of lupus (96, 185, 203, 204). The CCR5Δ32 variant has already been studied in this context, being previously associated to protection against lupus development and, albeit in a contradictory manner, this polymorphism was also associated to susceptibility to nephritis in lupus patients (203, 204). In Brazil, two studies evaluated the CCR5Δ32 variant in lupus.

Schauren et al. (185) investigated the role of the CCR5Δ32 in healthy patients and controls of Rio Grande do Sul State (185). A lower frequency of the CCR5Δ32 allele was found in Euro-Brazilian patients (2.7%) compared to Euro-Brazilian controls (7.5%), suggesting a protective role of the variant against the development of systemic lupus erythematosus. However, in the

TABLE 2 | Impacts of the CCR5Δ32 on infectious diseases.

Disease/ Infection	Population (Brazilian state)	Sample	Main findings	Reference
HTLV-I infection	Individuals from Minas Gerais State, Brazil	229 blood donors (50 HTLV-I seronegative individuals; 179 HTLV-I-infected individuals)	No statistically significant association was observed concerning CCR5Δ32 and HTLV-I infection	Pereira et al. (143)
Cutaneous leishmaniasis (*Leishmania* infection)	Individuals from Paraná State, Brazil	100 individuals with cutaneous leishmaniasis and 100 healthy controls	No statistical significant difference regarding CCR5Δ32 frequency between the two groups	Brajão de Oliveira et al. (166)
Cutaneous leishmaniasis (*Leishmania* infection)	Individuals from Paraná State, Brazil	111 individuals with cutaneous leishmaniasis and 218 controls	No statistically significant difference of the CCR5Δ32 frequency was observed between cases and controls	Ribas et al. (167)
Dengue virus infection	Individuals from Rio de Janeiro State, Brazil	87 severe children cases of Dengue and 326 controls	No statistical significant difference regarding CCR5Δ32 frequency between the two groups	Xavier-Carvalho et al. (145)
Chagas disease (*Trypanosoma cruzi* infection)	Individuals from São Paulo State, Brazil	85 Chagas disease patients with normal left ventricular systolic function; 43 Chagas disease patients with mild to moderate left ventricular systolic dysfunction; 40 Chagas disease patients with severe left ventricular systolic dysfunction	No statistical significant association between CCR5Δ32 and Chagas disease-related left ventricular systolic dysfunction	Oliveira et al. (162)
Chagas disease (*Trypanosoma cruzi* infection)	Individuals from São Paulo State, Brazil	109 patients with digestive form of Chagas disease; 131 patients with cardiac form of Chagas disease; 172 controls	No statistical significant influence of the CCR5Δ32 on digestive or cardiac form of Chagas disease, including left ventricular systolic dysfunction	Oliveira et al. (163)
Influenza A infection (2009 pandemic H1N1)	Individuals from northern and northeastern regions of Brazil	174 non-hospitalized Influenza-infected individuals and 156 hospitalized Influenza-infected individuals	No statistical significant impact of the CCR5Δ32 on infection severity	Maestri et al. (147)
HPV infection	Individuals from Pernambuco State, Brazil	139 HPV-infected women with cervical lesions and 151 HPV-infected women without cervical lesions	No statistical significant influence of the CCR5Δ32 on HPV-related cervical lesions or infection by specific HPV genotype	Santos et al. (159)
HCV infection, HCV/HIV co-infection and HCV-related hepatic diseases	Individuals from Rio Grande do Sul State, Brazil	674 HCV-infected individuals (stratified between 124 individuals without hepatic manifestation, 268 individuals with fibrosis, 190 individuals with cirrhosis and 92 individuals with hepatocarcinoma); 104 HCV/HIV co-infected individuals; 300 HIV-infected individuals; 274 controls	No statistical significant influence of the CCR5Δ32 on susceptibility to HCV infection, HCV/HIV co-infection or HCV-related hepatic manifestations	Ellwanger et al. (151)
Ocular toxoplasmosis (*Toxoplasma gondii* infection)	Individuals from São Paulo State, Brazil	160 individuals with ocular toxoplasmosis; 160 individuals with non-ocular toxoplasmosis; 160 controls	In association with AA or AG genotypes (from *CCR5* 59029 A/G SNP - rs1799987), the CCR5Δ32 wild-type genotype was associated with increased risk of ocular toxoplasmosis (based on multivariate logistic regression analysis)	Faria Junior et al. (169)
HPV infection	Individuals from Paraná State, Brazil	164 HPV-infected women and 185 control women	No statistically significant influence of the CCR5Δ32 on susceptibility to HPV infection or cervical lesions associated with HPV infection	Mangieri et al. (158)
Influenza A infection (2009 pandemic H1N1)	Individuals from South, Southeast and Northeast Brazilian regions (nine states in total)	153 individuals with influenza like illness; 173 individuals with severe acute respiratory infection; 106 fatal influenza-infection cases	No significant effect of the CCR5Δ32 on severity of Influenza virus infection or Influenza-linked mortality	Matos et al. (148)
HBV infection and HBV/HIV co-infection	Individuals from Rio Grande do Sul State, Brazil	335 HBV-infected individuals; 144 HBV/HIV co-infected individuals; 300 HIV-infected individuals; 334 controls	No significant effect of the CCR5Δ32 on susceptibility to HBV mono-infection; CCR5Δ32 was a protective factor on HBV/HIV co-infection	Ellwanger et al. (92)

same study, patients with the CCR5Δ32 allele had a greater predisposition to the development of class IV nephritis than patients without the allele, which suggests a more severe clinical outcome associated with the genetic variant (185).

Baltus et al. (96) evaluated the frequencies of the CCR5Δ32 in patients and controls in the Paraná State, also southern Brazil. Unlike the first study, the frequency of the CCR5Δ32 allele was statistically higher in patients (6.8%) than in controls (1.9%), suggesting the variant as a risk factor for systemic lupus erythematosus. Also, by stratifying the sample according to ethnicity, the researchers identified that Euro-Brazilian individuals carrying the CCR5Δ32 were more likely to develop systemic lupus erythematosus than Afro-Brazilian patients carrying the variant. In another analysis of the study, CCR5Δ32 carriers had a lower age of systemic lupus erythematosus onset and higher levels of anti-dsDNA antibodies. Thus, the CCR5Δ32 allele was associated with increased susceptibility to the development of systemic lupus

TABLE 3 | Impacts of the CCR5Δ32 on inflammatory conditions.

Disease/ Condition	Population (Brazilian State)	Sample		Main findings	Reference
		Cases	Controls		
Multiple sclerosis (MS)	Paraná State	124 MS patients	127 healthy individuals	There was no statistically significant difference regarding the CCR5Δ32 allele between patients and controls, and no association was also found regarding clinical course and *CCR5* variants; A decreased disease progression was observed in patients bearing the CCR5Δ32 allele, with carrier presenting lower Expanded Disability Status Scale (EDSS) values	Kaimen-Maciel et al. (172)
	São Paulo State and Rio Grande do Sul State	261 MS patients	435 healthy individuals	Considering only Euro-Brazilians, the CCR5Δ32 allele frequency was significantly higher in healthy individuals than in MS patients (p=0.013). Also, there was a higher frequency of Δ32 homozygous and heterozygous individuals in controls than in patients (p=0.033)	Troncoso et al. (173)
Juvenile idiopathic arthritis (JIA)	Rio Grande do Sul State	101 JIA patients and 203 rheumatoid arthritis patients	104 healthy individuals	The frequency of the CCR5Δ32 variant was significantly higher (p=0.028) in JIA patients (0.094) than in controls (0.038)	Scheibel et al. (174)
Osteomyelitis	Ceará State	39 bone trauma with osteomyelitis cases	114 bone trauma without osteomyelitis cases	The frequency of the CC5Δ32 variant did not vary significantly, but patients with type I or type II fractures that carried the allele did not develop the disease	Souza et al. (175)
Periodontitis	São Paulo State	197 chronic periodontitis cases and 91 aggressive periodontitis cases	218 healthy individuals and 193 chronic gingivitis cases	The frequency of the CCR5Δ32 variant was significantly higher in patients with chronic gingivitis (0.11) than in chronic (0.058) (p=0.01) or aggressive periodontitis (0.055) (p=0.03)	Cavalla et al. (176)
Preeclampsia	Rio Grande do Sul State and Rio de Janeiro State	155 preeclampsia pregnancies	144 healthy pregnancies	The frequency of the CCR5Δ32 variant was significantly higher (p=0.047) in healthy women (0.14) than in pre-eclamptic women (0.07)	Telini et al. (177)
	Minas Gerais State	156 preeclampsia pregnancies	213 healthy pregnancies	The frequency of the CCR5Δ32 variant was significantly higher (p=0.047) in healthy women (0.045) than in pre-eclamptic women (0.016)	Kaminski et al. (178)
Rheumatoid arthritis (RA)	Rio Grande do Sul State	92 RA patients	160 healthy individuals	The frequency of the CCR5Δ32 variant did not vary significantly between the groups	Kohem et al. (179)
	Pará State	186 RA patients	206 healthy individuals	The frequency of the CCR5Δ32 variant was significantly higher in healthy individuals (0.075) than in RA patients (0.040) (p=0.016)	Toson et al. (180)
	Rio Grande do Sul State	361 RA patients	233 healthy individuals	The frequency of the CCR5Δ32 variant was significantly higher in healthy individuals (0.034) than in RA patients (0.011) (p=0.022)	
	Pernambuco State	104 AR patients	154 healthy individuals	The frequency of the CCR5Δ32 variant did not vary significantly between groups	
	São Paulo State	89 AR patients	83 healthy individuals	The frequency of the CCR5Δ32 variant did not vary significantly between groups	
Sickle cell disease (SCD)	Rio Grande do Sul State and Pernambuco State	79 SCD patients	112 healthy afro-Brazilian individuals and 102 healthy euro-Brazilian individuals	The comparison of the CCR5Δ32 frequency between afro-Brazilian healthy individuals (0.013) and SCD patients (0.051) was of borderline significance (p=0.05)	Chies and Hutz (181)
	Rio Grande do Sul State	73 SCD patients	58 healthy individuals	The frequency of the CCR5Δ32 variant did not vary significantly between groups	Vargas et al. (182)
	Pernambuco State	483 pediatric SCD patients and 312 adult SCD patients	247 healthy individuals	The frequency of the CCR5Δ32 variant did not vary significantly between the groups	Lopes et al. (183)
	Bahia State	20 SCD patients	–	The CCR5Δ32 variant was not found in any patient evaluated	Nascimento et al. (184)
Systemic lupus erythematosus (SLE)	Rio Grande do Sul State	280 euro-Brazilian SLE patients and	235 euro-Brazilian healthy	The frequency of the CCR5Δ32 variant was significantly higher in healthy euro-Brazilian controls (0.075) than in euro-Brazilian SLE patients (0.027) (p=0.002); Patients carrying the CCR5Δ32 variant were predisposed to the development of class IV nephritis (p=7E-6)	Schauren et al. (185)

(Continued)

TABLE 3 | Continued

Disease/ Condition	Population (Brazilian State)	Sample		Main findings	Reference
		Cases	Controls		
		87 afro-Brazilian patients	individuals and 200 afro-Brazilian healthy individuals		
	Paraná State	169 SLE female patients	132 female healthy controls	The frequency of the CCR5Δ32 variant was significantly higher in patients (0.068) than in healthy controls (0.019) (p=0.0047). Euro-Brazilian individuals carrying the allele had a higher predisposition to the development of SLE than in afro-Brazilian individuals carrying the same variant (p=0.0286). Patients with heterozygous genotype presented a lower age of SLE onset and higher levels of anti-dsDNA antibodies when compared to individuals homozygous for the wild type allele (p=0.0293 and p=0.0255, respectively).	Baltus et al. (96)
Transplant rejection	Paraná State	86 kidney transplant patients with rejection episodes	160 kidney transplant patients without rejection episodes	No statistically significant difference was found in the CCR5Δ32 frequency between the groups (8.3% for individuals with rejection episodes; 6.3% for transplant recipients without rejection)	Cilião et al. (186)

erythematosus and severity in clinical outcomes (96). Studies performed in different populations have found no association between the variant and the development of systemic lupus erythematosus (205–208). Such divergence involving the results mentioned above deserves attention and, therefore, more studies in other populations are required.

Preeclampsia is a hypertensive gestational complication and an important cause of maternal-fetal mortality in Brazil. Relevant clinical findings of the disease, such as edema and proteinuria after the 20th week of pregnancy, are intricate with an excessive inflammatory process and endothelial dysfunction. In preeclampsia, increased systemic production of pro-inflammatory chemokines was observed, highlighting the role of the chemokine-ligand system in this condition (177, 178, 209). Two studies evaluating the CCR5Δ32 variant in preeclampsia were carried out in Brazil, both published by our group, but evaluating samples from different Brazilian regions. Firstly, Telini et al. (177) evaluated the frequency of the CCR5Δ32 in Brazilian women who developed preeclampsia and women who did not develop this condition during their pregnancies. The group of healthy women had a higher frequency of the CCR5Δ32 allele (14%) when compared to the group of women who developed preeclampsia (7%). The analysis revealed a protective role of the variant on preeclampsia development (177). More recently, Kaminski et al. (178) also investigated the role of CCR5Δ32 in women who developed preeclampsia and in women with healthy pregnancies (178). In accordance with the results of Telini et al. (177), healthy pregnant women also showed an increased CCR5Δ32 allele frequency (4.5%) compared to the group of pregnant women with preeclampsia (1.6%). Thus, the study corroborated the protective role of the CCR5Δ32 variant on preeclampsia development, endorsing the hypothesis that a reduced inflammatory millieu may contribute to a lower risk of developing preeclampsia (177, 178). A study conducted in a Turkish population found similar results, strengthening the conclusion here presented (210).

Rheumatoid arthritis is a systemic autoimmune disease characterized by progressive damage to the joints caused by chronic inflammation in the synovial fluid. Given the intense migration of immune cells to the inflammation sites, the role of CCR5 in rheumatoid arthritis appears to be of great importance (179, 180). In Brazil, two studies investigating the role of the CCR5Δ32 variant in rheumatoid arthritis were published. Kohem et al. (179) evaluated the frequency of the allele in healthy patients and controls from the Rio Grande do Sul State, and no statistically significant difference was found between the groups. Of note, the sample group was relatively small, with 92 patients and 160 healthy controls (179). Toson et al. (180) performed a similar study but evaluating the frequency of the CCR5Δ32 variant in different Brazilian populations, considering four different regions (south, southeast, northeast, and north). Two of the four sample groups, from southern and northern regions, showed a statistically significant difference between rheumatoid arthritis patients and healthy controls (4% vs. 7.5%; 1.1% vs. 3.4%, respectively), being precisely the groups with the largest sample sizes. The difference concerning the northeast region sample was not statistically significant but followed a similar trend to the groups in southern and northern. Only the southeastern sample deviated from the trend, with the small sample size possibly being the reason for the lack of statistical association. In sum, the study suggests a protective role for the CCR5Δ32 variant against the development of rheumatoid arthritis (180). A meta-analysis carried out in 2012 concluded that the variant may play a role in protection to rheumatoid arthritis in European populations, corroborating the data found in Brazil (211).

Juvenile idiopathic arthritis is a chronic inflammatory condition characterized in the synovial joints of young people up to 16 years of age (174, 212). Scheibel et al. (174) investigated the potential association of the CCR5Δ32 variant with juvenile idiopathic arthritis subtypes in a sample from Porto Alegre, southern Brazil. A statistically significant difference was found

between patients (9.4%) and healthy controls (3.8%), especially considering the group of patients of the systemic juvenile idiopathic arthritis subtype (25%). The researchers conclude that the CCR5Δ32 variant, although not a risk factor for the development of juvenile idiopathic arthritis, contributes to the progression and clinical status of patients (174). Interestingly, the meta-analysis previously mentioned (211) also explored the role of the Δ32 allele in juvenile idiopathic arthritis, and concluded that the variant was a protective factor for this condition as well (211). A further study comprising children from different populations found an association between the heterozygous genotype and mild disease course, but no influence on susceptibility to disease development (213). That said, these controversial results evidence the importance of novel studies investigating the CCR5Δ32 variant in juvenile idiopathic arthritis.

Periodontitis is an oral disease characterized by a chronic infection accompanied by inflammatory processes, causing irreversible and progressive destruction of dental support structures. The CCR5-mediated immune responses affect multiple aspects of periodontitis. For instance, not only CCR5 and its ligands are important in the context of disease protection, but also influence periodontal destruction and bone resorption (176, 214–217). Cavalla et al. (176) investigated the CCR5Δ32 variant and its possible influence on periodontitis development. The CCR5Δ32 allele was significantly more frequent in individuals classified in the group of chronic gingivitis (11.1%) than in individuals with chronic periodontal disease (5.8%) or aggressive periodontal disease (5.5%). This result suggests a protective role of the variant concerning periodontitis (176). Other studies carried out in Taiwan and Germany found no association between the variant and periodontitis (218, 219). Considering the conflicting results, it is interesting to carry out further studies in other populations to better understand the role of CCR5 in the development of periodontitis.

Osteomyelitis is an infectious-inflammatory condition that can occur after bone trauma often following *Staphylococcus aureus* infection (175, 220). Souza et al. (175) evaluated the CCR5Δ32 frequency in patients who were admitted to a hospital in Fortaleza, northeastern Brazil, with bone trauma. The patients were prospectively studied to assess a possible development of osteomyelitis. There was no statistically significant difference between individuals who developed and those who did not develop the disease, but all patients with closed fractures (type I or type II) and who carried the CCR5Δ32 variant did not developed the condition. The researchers conclude that the lack of statistical significance observed in their study was probably due to the low sample size (175). No other studies regarding the potential role of the CCR5Δ32 in osteomyelitis were found in the literature.

The immune response and inflammatory processes that occur after an organ transplant are critical in the process of tissue rejection. Genetic variants related to the immune system can therefore influence the response to transplantation (186, 221–223). Studies carried out in non-Brazilian populations observed no association between the CCR5Δ32 allele and kidney transplant rejection (224–228). A study in a multicentric sample from Europe showed a higher survival rate after kidney transplantation in individuals with the CCR5Δ32 homozygous genotype (222). In Brazil, Cilião et al. (186) evaluated the CCR5Δ32 frequency in transplanted individuals who had episodes of rejection comparing to individuals who did not have such episodes. A sample of 246 patients was collected in a referral hospital in Londrina, Paraná State. However, the frequency of the CCR5Δ32 variant did not vary significantly between the groups (186).

Sickle cell disease is an inherited disorder caused by a single nucleotide substitution in the beta-globin gene. This mutation originated in Africa and is, therefore, more common in African populations and Afro-descendants. Sickle cell disease can be understood as a chronic inflammatory condition, which may be the cause of associated secondary complications. In this sense, high levels of inflammation in sickle cell disease patients are related to disease morbidity (181–184). A study in a population from Egypt found no association between the variant and sickle cell disease (229). In Brazil, four studies investigated the influence of the CCR5Δ32 variant in sickle cell disease, all detailed below.

Chies and Hutz (181) assessed the potential role of the CCR5Δ32 in severe and recurrent infections that could contribute to differentiated survival of sickle cell anemia patients. The study involved individuals from different ethnic groups and the frequencies of the CCR5Δ32 allele found were 4.4% in Euro-Brazilian controls, 1.3% in Afro-Brazilian controls, and 5.1% in sickle cell anemia patients. When comparing these frequencies between the different groups, no statistically significant difference was found. However, it is important to note that, considering the same ethnic background of the groups of patients and Afro-Brazilian controls, a difference in the allele frequency was evidenced, being the CCR5Δ32 allele three times more present in the group of sickle cell anemia patients. Given the low frequency of the allele in the sample of Afro-Brazilian controls, a 3-fold increase in the group of patients is quite important. The researchers suggested that the CCR5Δ32 allele was more frequent in the group of patients for conferring some advantages concerning the clinical course of the disease (181). As mentioned previously, sickle cell anemia can be considered a chronic inflammatory disease (93), and patients with the CCR5Δ32 allele would benefit from developing inflammatory responses at low levels. According to this hypothesis, the CCR5Δ32 allele was associated with an improvement in the general health status of the patients (93, 181).

Subsequently, Vargas et al. (182) evaluated CCR5Δ32 in sickle cell anemia patients from Porto Alegre, Rio Grande do Sul State. No statistically significant difference was observed in the study but, interestingly, the CCR5Δ32 allele was present only in the group of patients with a severe clinical course (when the pain rate was considered). Such data may indicate a trend towards the development of a severe clinical course associated with the CCR5Δ32 allele in sickle cell anemia patients (182). Lopes et al. (183) compared the CCR5Δ32 frequencies of two groups of patients (pediatric and adult) and between sick adults and healthy controls from Pernambuco, northeastern Brazil. There were no statistically significant differences in any of the comparisons made in the study (183). Finally, Nascimento et al. (184) evaluated the CCR5Δ32

frequency in sickle cell anemia patients from Bahia State. However, the CCR5Δ32 allele was not found in the study (184).

CCR5Δ32 IN CANCER

Chemokines and chemokine receptors have fundamental participation in both antitumor response and pathogenesis of cancer. The migration of regulatory immune cells to tumor sites can create an immunosuppressor environment proper for cancer development. Also, cancer cells can subvert the anti-tumor action of chemokine-ligand interactions (187–191). Of note, CD4+ T cells are important modulators of the immune response, acting as drivers for the action of effector cells. Some CD4+ regulatory T cells express the CCR5 molecule, being this a key receptor of the cellular response against tumor development. The presence of the CCR5Δ32 variant can impair the action of CCR5+/CD4+ T cells, influencing the risk of cancer development. In brief, chemokine receptors can assume multiple roles in different tumoral processes, and more investigation is needed to unravel the connections between CCR5 and cancer (101, 192). Two meta-analyses published in 2014 evaluated the possible role of the Δ32 allele in cancer. Ying et al. (230) found no association of the variant with risk of tumorigenesis, while Lee et al. (205) found an association of the allele with susceptibility to cancer in Indians, specifically concerning breast cancer (205, 230). Further studies found associations of the CCR5Δ32 variant with improved metastasis-free survival in breast cancer patients and, contradictorily, also with an increased risk for developing breast cancer (231–233). In Brazil, the possible role of the CCR5Δ32 variant in cancer has been addressed (**Table 4**) and the available data will be presented below.

The action of CD8+ cytotoxic T cells is important in the antitumor immune response. The use of immunomodulators in antitumor treatment is increasingly common, with carboxymethyl-glucan (CM-G) being one of the best-described immunostimulators (192, 234). Magnani et al. (192) evaluated the CD3+, CD4+ and CD8+ cell populations of patients with advanced prostate cancer and compared this data with the *CCR5* genotype, associating it with the administration of oral CM-G for 28 days. The CCR5Δ32 variant was found only in a heterozygous genotype, in six patients, at an allelic frequency of 10%. Five patients reported a family history of prostate cancer, two of whom had affected first-degree relatives. Both patients carried the CCR5Δ32 allele. In general, CCR5Δ32 non-carriers had higher counts on CD3+ and CD4+ cells when comparing respectively after and before treatment with CM-G, as well as higher counts of CD8+ cells when comparing to CCR5Δ32 carriers only after treatment with CM-G. In addition, the average CD4+/CD8+ cell ratio showed a worsened antitumor response after treatment in CCR5Δ32 allele carriers (192). Zambra et al. (193) also evaluated the CCR5Δ32 frequency in Brazilian prostate cancer patients, comparing to individuals affected by benign prostatic hyperplasia and healthy subjects. No association was found considering the variant and risk to both conditions, nor with clinical outcomes (193).

Aoki et al. (188) assessed the CCR5Δ32 frequency in individuals with breast cancer and healthy women. However, no significant difference was observed between groups. The impact of *p53* genotypes, a known tumor suppressor gene, together with the CCR5Δ32 genotypes, was also evaluated revealing a higher frequency of individuals with the p53 Arg homozygous genotype and the CCR5Δ32 wild-type genotype amongst controls as compared to patients (188). Banin-Hirata et al. (189)

TABLE 4 | Impacts of the CCR5Δ32 on cancer.

Cancer type	Population (Brazilian state)	Sample	Main findings	Reference
Acute lymphoblastic leukemia (ALL)	Paraná State	79 ALL patients and 80 healthy controls	No statistically significant differences regarding CCR5Δ32 between ALL patients and controls	Oliveira et al. (187)
Breast cancer (BC)	Paraná State	72 BC patients and 90 healthy women	The allelic frequency estimated in patients was of 3.47% and 7.78% in healthy women; However, no statistically significant difference was found between these groups	Aoki et al. (188)
Breast cancer (BC)	Paraná State	118 BC patients and 180 healthy women	No statistically significant differences between groups regarding susceptibility, clinical outcome, or treatment response.	Banin-Hirata et al. (189)
Breast cancer (BC)	Paraná State	94 samples from 47 BC patients (47 tumoral tissues and 47 adjacent tissues)	No impact of CCR5Δ32 on CCL5 levels considering tumoral or normal tissues	Derossi et al. (190)
Cervical intraepithelial neoplasia (CIN)	Pernambuco State	290 HPV+ women (151 without cervical lesions and 139 with cervical lesions, divided in 12 women with cervical cancer (CC), 40 women with CIN I and 87 with CIN II or III)	No statistically significant differences regarding CCR5Δ32 between CIN or CC patients and HPV+ women without lesions	Santos et al. (159)
Neuroblastoma (NB)	Paraná State	28 tissue samples from NB patients and 80 cancer-free children	CCR5Δ32 was more frequent in the group of NB patients than in healthy controls ($p<0.05$)	Vieira-Filho et al. (191)
Prostate cancer (PCa)	Paraná State	30 advanced PCa patients	Significant increase in CD3+ and CD4+ cells was observed in CCR5Δ32 non-carriers; The average CD4+/CD8+ cell ratio decreased in CCR5Δ32 non-carriers after treatment	Magnani et al. (192)
Prostate cancer (PCa)	Rio Grande do Sul State	119 healthy individuals, 136 PCa patients and 130 benign prostatic hyperplasia (BPH)	CCR5Δ32 allele was not statistically associated with risk of developing BPH or PCa or clinical outcomes of both conditions	Zambra et al. (193)

also evaluated whether the CCR5Δ32 variant was associated with susceptibility, response to treatment, and clinical course of breast cancer. No association was found between CCR5Δ32 and the features analyzed (189). In accordance, Derossi et al. (190) did not found an association between the CCR5Δ32 and CCL5 levels in breast cancer (190).

HPV infection is the main cause of cervical cancer. However, factors other than HPV infection, including genetic, immune, and environmental factors, also affect tumorigenesis (159, 235, 236). In this context, Santos et al. (159) evaluated the CCR5Δ32 frequency in HPV+ women with and without cervical neoplastic lesions. No association was found between the variant and the presence of cancer or lesions severity (159).

In addition to the multiple roles of CCR5 in tumorigenesis and antitumor response, this molecule is also an important modulator of neuroinflammation (237–239), potentially affecting the development of brain-related diseases. In this sense, Vieira-Filho et al. (191) found an association between the presence of the CCR5Δ32 allele and susceptibility to neuroblastoma (191). Lastly, Oliveira et al. (187) investigated the role of the CCR5Δ32 variant in acute lymphoblastic leukemia, but no association was found between the variant and the disease development (187). In conclusion, the CCR5 has varied influences in different types of cancer.

IMPACTS OF CCR5Δ32 ON A HIGHLY ADMIXED POPULATION – A CRITICAL LOOK

At a population level, the effects of CCR5Δ32 on European populations may be different than those potentially observed in highly admixed populations. However, the population-specific effects of CCR5Δ32 are not only due to its frequency, but also due to its interaction with different alleles. There are nine widely known *CCR5* haplotypes, which are formed by combinations of eight *CCR5* polymorphisms (including CCR5Δ32) and one polymorphism located in the *CCR2* gene (40, 70). The impact of the *CCR5* haplotypes on HIV disease progression differs between African Americans and Caucasians since the effects of the CCR5Δ32 can be modulated by other alleles heterogeneously distributed among the populations (240). In a broader perspective, this information indicates that the effect of the CCR5Δ32 observed in Europeans (or other non-Brazilian populations) may be modified by further genetic traits circulating in Brazilians, which may also vary in different regions of the country. In fact, the detection of the real effect of CCR5Δ32 on different health and disease conditions in the Brazilian population is not a simple task. Of note, gene-disease association studies performed with admixed populations can be difficult due to differential linkage disequilibrium patterns (241).

Pharmacogenomic approaches, including the use of CCR5 modulators based on the CCR5Δ32 genotyping, must be considered at an individual level, especially in highly admixed populations, where the frequency of polymorphisms may be quite different from those observed in populations with greater genetic homogeneity (7). The CCR5Δ32 genotyping could be considered in pharmacological treatments involving CCR5 blockade in the context of inflammatory diseases or types of cancer. The use of CCR5 modulators in individuals with the CCR5Δ32 genotype probably has a limited effect due to the natural absence of CCR5 expression on the cell surface. Although the number of individuals with this genotype is exceptionally low in an admixed population such as the Brazilian population, the cost-benefit of this strategy must be considered on a case-by-case basis. Despite the limitations, the area of pharmacogenomics involving CCR5Δ32 genotyping is expected to progress in the next years, especially considering the increasing use of CCR5 modulators to treat other diseases not associated with HIV infection. Some important advances have already been made. For instance, the CCR5Δ32 genotyping can help clinicians to predict the progression of human enteroviral cardiomyopathy, also helping the decision making concerning the early use of antiviral interferon-β therapy in such condition (242).

CONCLUSIONS

The CCR5Δ32 allele frequency is quite variable in Brazil, being extremely low in some regions (e.g., 0.6% in Rondônia), but high in others (e.g., up to 9.3% in Paraná and 7.4% in Rio Grande do Sul). In Native American populations, the allele is absent or occurs at low frequencies. In Brazil, CCR5Δ32 is not uncommon in non-Caucasian populations, because of the miscegenation that has occurred in the country.

Many studies corroborated the protective effect of the CCR5Δ32 on susceptibility or clinical aspects of HIV infection in the Brazilian population. On the other hand, there is no evidence pointing to a relevant role for CCR5Δ32 on Cutaneous leishmaniasis, Chagas disease, HTLV-1, Dengue virus, Influenza A, HPV, HBV and HCV infections, or HCV-HIV co-infection in Brazilians. Limited evidence indicates a potential involvement of CCR5Δ32 wild-type genotype in ocular toxoplasmosis and a protective effect of the variant on HBV/HIV co-infection.

Considering inflammatory conditions, the CCR5Δ32 can influence both the susceptibility and the clinical outcome of multiple sclerosis. Of note, CCR5Δ32 reduces the risk of preeclampsia and periodontitis development, potentially due to the CCR5Δ32-associated reduced inflammation. Moreover, CCR5Δ32 can reduce the risk of rheumatoid arthritis, but contributes to the progression and clinical status of juvenile idiopathic arthritis patients. CCR5Δ32 can also influence sickle cell anemia-related immune conditions. However, the impact of CCR5Δ32 on systemic lupus erythematosus is controversial. Concerning tumoral development, the CCR5Δ32 has varying influences on the development of different types of cancer, including prostate cancer and breast cancer. It is not possible to generalize the impact of the variant on cancer development, especially in the Brazilian population.

Understanding the real impact of the CCR5Δ32 variant in different conditions is essential to indicate in which diseases the use of CCR5 modulators may be relevant. This knowledge is fundamental for the advancement of CCR5-based therapies,

especially in populations with a complex genetic structure. Finally, CCR5Δ32 influences should be assessed within the context of each population, since genetic admixture and interactions with other alleles may alter the expected phenotypic effects attributed to CCR5Δ32.

AUTHOR CONTRIBUTIONS

BK-L and JE wrote the first version of the manuscript. JC revised and edited the text. All authors contributed to the article and approved the submitted version.

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Reduced CCR5 Expression and Immune Quiescence in Black South African HIV-1 Controllers

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Unique Individuals who exhibit either suppressive HIV-1 control, or the ability to maintain low viral load set-points and preserve their CD4+ T cell counts for extended time periods in the absence of antiretroviral therapy, are broadly termed HIV-1 controllers. We assessed the extent to which black South African controllers (n=9), differ from uninfected healthy controls (HCs, n=22) in terms of lymphocyte and monocyte CCR5 expression (density and frequency of CCR5-expressing cells), immune activation as well as peripheral blood mononuclear cell (PBMC) mitogen-induced chemokine/cytokine production. In addition, relative CD4+ T cell CCR5 mRNA expression was assessed in a larger group of controllers (n=20) compared to HCs (n=10) and HIV-1 progressors (n=12). Despite controllers having significantly higher frequencies of activated CD4+ and CD8+ T cells (HLA-DR+) compared to HCs, CCR5 density was significantly lower in these T cell populations (*P*=0.039 and *P*=0.064, respectively). This lower CCR5 density was largely attributable to controllers with higher VLs (>400 RNA copies/ml). Significantly lower CD4+ T cell CCR5 density in controllers was maintained (*P*=0.036) when HCs (n=12) and controllers (n=9) were matched for age. CD4+ T cell CCR5 mRNA expression was significantly less in controllers compared to HCs (*P*=0.007) and progressors (*P*=0.002), whereas HCs and progressors were similar (*P*=0.223). The levels of soluble CD14 in plasma did not differ between controllers and HCs, suggesting no demonstrable monocyte activation. While controllers had lower monocyte CCR5 density compared to the HCs (*P*=0.02), significance was lost when groups were age-matched (*P*=0.804). However, when groups were matched for both CCR5 promoter haplotype and age (n=6 for both) reduced CCR5 density on monocytes in controllers relative to HCs was highly significant (*P*=0.009). Phytohemagglutinin-stimulated PBMCs from the controllers produced significantly less CCL3 (*P*=0.029), CCL4 (*P*=0.008) and IL-10 (P=0.028) compared to the HCs, which was largely attributable to the controllers with lower VLs (<400 RNA copies/ml). Our findings support a hypothesis of an inherent (genetic) predisposition to lower CCR5 expression in individuals who naturally control HIV-1, as

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has been suggested for Caucasian controllers, and thus, likely involves a mechanism shared between ethnically divergent population groups.

Keywords: HIV-1 control, CCR5 expression, immune activation, CCR5 ligands, IL-10

INTRODUCTION

People living with HIV (PLWH) who are able to naturally control HIV infection are likely to possess genetic or immunological attributes that could provide important insights for the development of therapeutic agents and to inform HIV cure strategies and vaccine design. However, despite the high burden of disease (1), studies assessing protective mechanisms in sub-Saharan PLWH who exhibit good control of HIV-1 infection/disease are more limited.

HIV-1-infected progressors and non-progressors have been described to differ in their host gene complement, viral strains as well as their immunological responses (2). Although non-progression in HIV-infected individuals has been attributed to infection with attenuated viruses in a minority of reports (3, 4), other studies report non-progression in individuals infected with fully replication-competent HIV-1 viruses (5–7), lending support to the idea that host factors play a large role in delayed disease progression. The role of CCR5 coreceptor density in the susceptibility of an individual to HIV-1 has been well established. The *CCR5Δ32* allele has been reported as over-represented within groups of patients infected with HIV-1 who progress to disease at slower than normal rates (8–10). This deletion results in truncation of the expressed protein and prevents the expression of CCR5 on the cell surface (11). Furthermore, high CCR5 expression on CD4+ T cells associates with high viral loads (VLs) and accelerated disease progression (12, 13). However, although the *CCR5Δ32* allele is virtually absent in sub-Saharan populations (14, 15), *CCR5* promoter haplotypes have been demonstrated to affect CCR5 surface expression in cohorts of South African individuals (16, 17). This has been demonstrated in individuals with and without HIV-1 infection.

It is well established that immune activation is a hallmark of pathogenic HIV-1 infection. Immune activation levels serve as the best predictors of disease progression to AIDS and death, independently of HIV-1 VL (18–20). Several lines of evidence point to CCR5 functioning as a molecule that enhances T cell activation. Antibody-mediated blockade of the CCR5-CCR5 ligand axis has been demonstrated to result in lower expression of IL-2, IFNγ and CD25 - molecules that serve as markers of cellular activation (21–23). CCR5 expression influences IL-2 and CD25 expression through regulation of the intracellular levels of NFAT (nuclear factor of activated T cells) (23). During T cell stimulation, CCR5 molecules are sequestered to the immunological synapse where they are stimulated and deliver costimulatory signals (24, 25). In contrast to CXCR4-utilising strains, those that utilise CCR5 enhance CD4+ T cell activation, thus favouring HIV replication and spread (26). In addition, the function of CCR5 as a costimulatory molecule is dependent on the level of CCR5 cell surface expression. CCR5 density correlates with, and is predictive of, the immune activation levels of HIV-1-infected individuals independently of VL (27). CCR5 density on naïve CD4+ T cells is unaffected by neither the initiation of antiretroviral therapy (ART) nor ART treatment interruption, despite the respective decrease and increase in the proportion of activated CD8+ T cells ($CD38^{hi}$) - i.e., the baseline level of CCR5 density is a determinant of the intensity of immune activation (27). Gornalusse et al. (28) showed an inverse correlation between the DNA methylation status of the *CCR5 cis*-regulatory regions and CCR5 levels on T cells, and that T cell activation induced demethylation of these regions, leading to upregulation of CCR5 expression. Furthermore, they showed that polymorphisms in *CCR5 cis*-regulatory regions that associated with increased and decreased HIV/AIDS susceptibility were also associated with increased and decreased sensitivity to activation-induced demethylation, respectively (28).

We previously reported that the cell surface density of CCR5 and proportions of CCR5-expressing cells differ significantly between white and black South African individuals who are HIV-1 uninfected (29). Generally, white individuals displayed higher CCR5 cell density, whereas black individuals had higher proportions of CCR5-expressing cells, which correlated positively with the proportions of activated cells (29). To our knowledge, no studies have directly assessed the role of CCR5 expression on natural control of HIV-1 in a sub-Saharan population.

MATERIALS AND METHODS

Study Cohorts

The majority of the work reported in this study (CCR5 expression and cytokine production) has been conducted on a small group of well characterized black South African HIV-1 controllers (n=9) and a group of black South African healthy control donors (n=22) - termed cohort 1. The HIV-1-infected controllers in this cohort comprised 9 black South African individuals infected with HIV-1 with long-term follow-up that had been prospectively recruited. These individuals were a mixture of those with suppressive viral control (i.e. elite controllers) or with low viral set points (viraemic controllers and/or long-term non-progressors). Criteria for selection were the sustained control of disease in the absence of antiretroviral treatment (ART) for a period of ≥6 years and/or consistently high CD4+ T cell counts. This group comprised six females and three males and had a median age of 38 years (range: 32-54 years) at the time at which the experiments were conducted (**Table 1**). Among the group of controllers, two individuals (TG11 and Pru1) met the criteria of elite controllers i.e., patients with plasma HIV RNA levels of <50 copies/ml (30). At the time of this study, the median number of years of infection without treatment for this cohort was 9 years (range: 6-14), and subsequent to this

TABLE 1 | Characteristics of HIV-1 controllers (cohort 1).

Patient ID	Age (years)	Gender	Viral load (RNA copies/ml)	CD4+ T cell count (cells/μl)	Time since diagnosis for current study (years)	Time since diagnosis without ARVs[1] (years)	*CCR5* genotype
TG1	38	M	6 070	334	9	11	HHA/HHF*2
TG2	47	F	5 780	400	6	7	HHA/HHF*2
TG4	35	M	183	910	9	14	HHA/HHA
TG9	46	F	<400	327	9	12	HHE/HHF*2
TG11	32	F	<40	693	7	12	HHA/HHC
Pru1	54	F	<40	>2000	14	20	HHC/HHC
Pru2	43	M	1 155	637	14	20	HHF*1/HHG*1
Pru3	36	F	1 410	775	11	16	HHA/HHC
Pru4	38	F	124	379	13	19	HHC/HHD

[1]*The time since diagnosis in the absence of antiretroviral drugs (ARVs) up to the latest follow-up point or up to the initiation of ARVs – experiments in current study were thus performed on samples collected at earlier time points.*

Bold patient IDs indicate the 5 controllers that were included in the CCR5 mRNA expression (CD4+ T cell) experiments (cohort 2)

study (last recorded data), the median number of years of infection without treatment was 14 years (range: 7-20; **Table 1**).

The group of 22 healthy black South African individuals, without HIV-1 infection (HCs), has been previously described (29). The age and gender of the HC participants are listed in **Supplementary Table 1**. Although attempts were made to age and sex match the HIV-1 controllers and the HCs, the HCs had a trend of lower age compared to the controllers (medians: 32.5 vs. 38 years, respectively; P=0.05). There was no difference in the male:female ratio between the two groups (P=1).

A second cohort (cohort 2) was used to compare CCR5 mRNA expression in CD4+ T cells between a larger group of black South African HIV-1 controllers (n=20), a different group of black South African healthy controls (n=10) and a group of black HIV-1-infected progressors (n=12).

The characteristics of cohort 2 are described in **Supplementary Table 2**. The HIV-1 controllers (controllers-2) included 6 individuals that met the criteria for elite controllers and included 5 of the 9 controllers from cohort 1 described above (TG4, TG11, Pru1, Pru2 and Pru3). HIV-1 infected progressors were recruited based on CD4+ T cell counts <250 cells/μl and VL >10,000 RNA copies/ml plasma, and were subsequently initiated on ART. The three groups [controllers-2, healthy controls (HCs-2) and progressors] did not differ significantly in age (P≥0.05 across all group comparisons), and although the progressors had markedly less females (58%) compared to the controllers-2 (85%) and HCs-2 (70%), the groups did not differ significantly (progressors vs. controllers-2, P=0.20; progressors vs. HCs-2, P=0.68; controllers-2 vs. HCs-2, P=0.63).

This study was approved by the University of the Witwatersrand Committee for Research on Human Subjects, and informed written consent was obtained from all of the participants.

Plasma Viraemia Quantification and CD4+ T Cell Determination

HIV-1 RNA levels were quantified using one of two methods: (i) the COBAS®AmpliPrep/COBAS®TaqMan® HIV-1 test v2.0 (Roche Diagnostic Systems, Indianapolis, IN, USA) with a lower detection limit of 20 HIV-1 RNA copies/ml or (ii) the Roche Amplicor RNA Monitor Assay (Roche) with a lower detection limit of 400 HIV-1 RNA copies/ml. CD4+ T cell counts were determined using the commercially available FACSCount System (Becton Dickinson, Franklin Lakes, NJ, USA).

CCR5 Genotyping

The full-length *CCR5* gene sequence (~9.2 kb) was determined for the 9 controllers (cohort 1) as described previously (31). A real-time assay was used for the detection of the *CCR2-V64I* polymorphism (17), thereby allowing genotyping of individuals according to the haplotypes described by Gonzalez et al. (32).

CCR5 Quantification

EDTA-anticoagulated whole blood obtained from each of the study participants (cohort 1) was stained within one hour of blood collection. Four antibody panels were used for each sample to assess CCR5 expression on T, B and natural killer (NK) cells as well as granulocytes and monocytes. Furthermore, HLA-DR was included as a marker in a fifth panel to assess the extent of cell activation (i.e., percentage of HLA-DR-expressing cells) – this was carried out on all controllers and a subset (16/22) of the HCs. The detailed staining/flow cytometry method has been previously described (29). Briefly, the CCR5 antibody used was conjugated to phycoerythrin (PE) at a ratio of 1:1, thereby allowing for CCR5 quantification, as the mean number of CCR5 molecules per cell (CCR5 density), in addition to the percentage of CCR5-expressing cells within a cell subset. Quantification was carried out using the QuantiBRITE system (BD BioSciences) which is a set of four precalibrated beads to calibrate the fluorescence 2 (FL2) axis in terms of PE molecules.

Soluble CD14 Quantitation

Plasma separated from EDTA-anticoagulated whole blood was diluted 1:1000 with phosphate buffered saline (PBS). The levels of sCD14 were quantified using the Human CD14 DuoSet ELISA Development System (R&D Systems), with a 62.5 pg/ml limit of detection, as per the manufacturer's recommendations.

Cytokine Production Measurement

Cytokine production assays were performed as previously described (33). Equal numbers of isolated peripheral blood

mononuclear cells (PBMCs) were incubated for 20 h with or without phytohemagglutinin (PHA, 12.5 μg/ml). Concentrations within the harvested culture supernatants of the cytokines interleukin (IL)-2, IL-4, IL-6, IL-7, IL-8, IL-10, IL-12p70, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon γ (IFN-γ) and tumour necrosis factor α (TNF-α) in addition to the CCR5 chemokine ligands Chemokine (C-C motif) ligand 3 (CCL3), CCL4 and CCL5 were determined either by ELISA (DuoSet ELISA Development Systems; R&D Systems, Minneapolis, Minnesota, USA) or Cytometric Bead Array (CBA) (BD BioSciences, San Jose, CA, USA). The samples were compared with protein standards.

CCL3, CCL4 and IL-8 were quantified in unstimulated and PHA-stimulated PBMCs by means of ELISA as described previously (33). The minimum detection levels were <10, 15.6 and 31.25 pg/ml for CCL3, CCL4 and IL-8, respectively. The remaining cytokine concentrations were determined by means of CBA. The CBA immunoassays were conducted as three separate multiplexes: (i) GM-CSF, G-CSF, IL-10 and IL-12p70; (ii) IL-7, IL-4 and IL-2 and (iii) TNF-α, IFN-γ and CCL5. The detection limits for the cytokines measured by means of CBA were as follows: TNF-α and CCL5, 1.25 pg/ml; IFNγ, 1.8 pg/ml; IL-4, IL-7, IL-12p70 and GM-CSF, 2.5 pg/ml; G-CSF and IL-10, 10 pg/ml; and IL-2, 11.2 pg/ml. All CBA immunoassay samples were analysed using a FACSCalibur (BD BioSciences) instrument and FCAP Array v1.0 software (SoftFlow, Hungary). Prior to analysis, the cytometer was calibrated using set-up beads according to the manufacturer's instructions. All samples with concentrations below the minimum limit of detection (assay-specific for each cytokine) were assigned a value of zero, whereas those above the maximum detection limit, i.e. >2500 pg/ml, were repeated at an appropriate dilution. The cytokine production values were calculated as follows: cytokine production of PHA-stimulated PBMCs minus the cytokine production of unstimulated PBMCs, if within the assay detection levels, from the same individual.

Absolute Counts of Cells in Blood Samples

TruCOUNT™ Tubes (BD Biosciences, San Jose, CA) were used to determine the absolute counts of lymphocytes and monocytes in blood. Assay conditions were as recommended by the manufacturer. Briefly, monoclonal antibodies were added with 50 μl whole blood to the lyophilized pellet containing a known number of fluorescent beads and the samples were prepared using a lyse/no wash procedure. Flow cytometric acquisition was performed on a FACSCalibur system (BD Biosciences). Data were analysed using FlowJo 7.6.1 (Tree Star, San Carlos, CA). Lymphocytes and monocytes were gated on a CD45 versus SSC dot plot, while beads were gated on a FL1 versus FL2 dot plot. Absolute counts (cells/μl) were calculated by the product of: [the number of events in the cell-containing region divided by the number of events in bead-containing region] and [number of beads per test (lot specific) divided by the test volume (50μl)].

T, B and NK cell counts were determined based on the proportions of total lymphocytes the respective cell subsets comprised, as determined from appropriate antibody panels used to quantitate CCR5 expression. Absolute counts of monocytes and lymphocyte subpopulations in the PBMC cultures (1.4×10^6 cells total) were calculated based on the monocyte:lymphocyte ratio determined from the absolute counts.

Relative CD4+ T Cell CCR5 mRNA Expression

CD4+ T cells were positively isolated from Ficoll-isolated PBMCs (cohort 2) using MACS® cell separation technology with CD4+ Microbeads and MS columns (Miltenyi Biotec, Germany), according to manufacturer's instructions. Cells were stored in 150 μl of *RNAlater*® solution (Life Technologies, California, USA) at -80°C until time of RNA extraction. Ice cold PBS (150 μl) was added to the *RNAlater*®-cell suspension and the tube was then centrifuged for 6 minutes at 9000 g to pellet cells prior to extraction. RNA was extracted using the mirVana miRNA Isolation Kit (Ambion®, Life Technologies, California, USA), according to manufacturer's instructions. Post extraction, RNA quality was assessed using the Agilent RNA 6000 Nano Kit and the Agilent 2100 Bioanalyzer system (Agilent Technologies, California, USA). All RNA samples had an RNA Integrity Number (RIN) greater than 7. The total RNA amount used in the cDNA synthesis was standardized to the sample with the lowest concentration. cDNA was synthesized using the Invitrogen Superscript III first strand synthesis system (Thermofisher Scientific, Massachusetts, USA), using both oligo-dT primers and random hexamers. A DNase digestion step was not included since our quantification assay probes all spanned exon-exon boundaries.

Synthesized cDNA was used as the template for gene-specific amplification using a predesigned gene expression hydrolysis probe assay for *CCR5* (Life Technologies: Hs00152917_m1). Two reference genes were used for normalization: ribosomal protein large, PO (*RPLPO*) (Life Technologies: Hs04189669_g1), and beta-actin (*ACTB*) (Life Technologies: Hs01060665_g1). Reactions (10 μl final volume) were performed in triplicate for each sample and were set up in 96-well plates, with each well containing 0.5 μl of the respective 20x Taqman Gene Expression Assay, 5 μl of 2x Taqman Gene Expression Mastermix (Life Technologies), 1 μl of cDNA and 3.5 μl of nuclease-free water (Ambion). Amplification was carried out on an Applied Biosystems 7500 Real-Time PCR system. The amplification settings included an initial holding stage at 95°C for 10 minutes and cycling stages (40 cycles) of 95°C for 15 seconds and 60°C for 40 seconds. A no template control (NTC) was included for each assay. Relative gene expression was calculated using the $2^{\Delta Cq}$ method, subtracting the average target gene C_q from the average reference gene C_q for each individual to get the ΔC_q value.

Statistical Analysis

Fisher's exact tests were performed using the Simple Interactive Statistical Analysis software (34) to test for differences in single nucleotide polymorphism (SNP) and haplotype frequencies found in the controllers and those found in a previously described cohort of healthy black South Africans (31) expanded by the recruitment of an additional six individuals

(n=41). Two-sided tests were used and statistical significance was considered if $P<0.05$. Data presented as continuous variables, i.e., expression or production levels of the test molecules, were compared using a Mann-Whitney *U*-test. Spearman's non-parametric correlations of select cell group CCR5 densities and a number of parameters (age, spontaneous PBMC CCR5 ligand production) were carried out. Mann-Whitney U-tests and Spearman's correlations were conducted using GraphPad Prism v4.02 (GraphPad Prism Software, Inc, La Jolla, California, U.S.A.).

RESULTS

CCR5 Gene Polymorphism Distribution

Assembled sequences of the *CCR5* gene including promoter, coding and 3' untranslated region (UTR) regions, from 9 HIV-1 controllers were analysed for DNA polymorphisms, single nucleotide polymorphisms (SNPs) and indels. Across the entire 9.2 kb region sequenced, 36 SNPs and 4 indels were identified. No polymorphisms were found within the *CCR5* open reading frame (ORF). All polymorphisms identified had been previously reported in South African populations (31). The frequencies at which polymorphisms were found in controller individuals, along with the background population (an expanded HC cohort; n=41) frequencies are indicated in **Supplementary Table 3**. Four of the SNPs identified within the group of controllers (-4257A/C, -3886C/T, -1060C/T and +1823C/T) had previously been thought to be absent in black South African individuals (31). Among the identified polymorphisms, one SNP located in the 3′ UTR, rs3188094 (+2458A/C), was found at a significantly higher frequency within the controller group (27.8%) compared to the background population (8.5%) (P=0.038), with two controllers (Pru1 and Pru4; **Table 1**) being homozygous for the minor allele – homozygosity was not detected in the background population. Interestingly, this SNP does not form part of previously identified *CCR5* haplotypes (31, 32).

Individuals within the controller cohort were assigned to previously described haplogroups (32). No controllers were found to possess the *CCR5Δ32* (HHG*2) allele (**Table 1**). All haplotypes, with the exception of HHB, found in our background population were also found in the group of controllers. Haplotypes HHA, HHC, HHD, HHE, HHF*1, HHF*2 and HHG*1 were present at frequencies of 33% (6/18), 28% (5/18), 6% (1/18), 6% (1/18), 6% (1/18), 17% (3/18) and 6% (1/18), respectively. Haplotype frequencies did not differ between controllers and the background population.

CCR5 Cell Surface Expression

HIV-1 Controllers Have Lower CCR5 Density on CD4+ T Cells and Monocytes Compared to HCs

Cell surface CCR5 expression of HIV-1 controllers was compared to that of HCs (29). CCR5 density was significantly lower on CD4+ T cells and on monocytes of controllers compared to HCs (P=0.039 and P=0.020, respectively, **Figures 1A, E**). Furthermore, there was a trend towards controllers expressing CCR5 at lower densities than HCs on CD8+ T cells (P=0.064, **Figure 1B**). It is interesting to note that the CCR5-density range on the CD8+ T cells of controllers was very narrow in comparison to HCs (1422-2035 versus 1055-5339 CCR5 molecules/cell, respectively). No differences in CCR5 density were noted in NK cell subsets between the two study groups (data not shown).

We next stratified our controller cohort into two groups according to the HIV VL: i) controllers with low VLs, i.e., <400 HIV-1 RNA copies/ml (n=5, range: <40 – 183 HIV-1 RNA copies/ml), and ii) controllers with higher VLs, i.e., >400 HIV-1 RNA copies/ml (n=4, range: 1155 – 6070 HIV-1 RNA copies/ml). Surprisingly, the CCR5 surface density was lower among controllers in the higher VL category. CCR5 density on CD4+ T cells in the VL<400 group was similar to that of the HCs, however, CCR5 density on CD4+ T cells of individuals with VL>400 was significantly lower than that of the HCs (P=0.017, **Figure 1A**). There was also a trend towards the VL>400 controllers expressing CCR5 at lower densities on CD4+ T cells compared to the VL<400 controllers (P=0.064, **Figure 1A**). The lower CD8+ T cell CCR5 density was mainly due to the VL>400 group of controllers (**Figure 1B**).

Controllers with VL<400 had similar CCR5 expression levels on monocytes to those observed in the VL>400 controllers (P=0.91, **Figure 1E**). No differences in CCR5 density on any cell subset relative to HCs were observed when controllers were stratified according to CD4+ T cell count < and >500 cells/μl (**Supplementary Figure 1**).

HIV-1 Controllers Have Higher Proportions of CCR5-Expressing CD8+ T Cells Relative to HCs

The proportion of CCR5-expressing CD4+ T cells did not differ between the controllers and HCs (P=0.88, **Figure 1C**). Similarly, the proportion of CCR5-expressing CD4+ T cells did not differ between the low and higher VL controller groups (P=1.00, **Figure 1C**). However, controllers had a strong trend of higher proportions of CCR5-expressing CD8+ T cells in comparison to HCs (P=0.07, **Figure 1D**). This relationship could be attributed to the VL<400 individuals, who had significantly higher frequencies of CCR5-expressing CD8+ T cells compared to that of the HCs (P=0.009, **Figure 1D**). Controllers with VL>400 however, had similar percentages of CCR5-expressing CD8+ T cells to the HCs (P=0.97, **Figure 1D**). Controllers with CD4+ T cell counts >500 cells/μl (CD4>500) had significantly higher proportions of CCR5-expressing CD8+ T cells compared to HCs (P=0.023, **Supplementary Figure 1**), however this was less significant than the VL<400 controller group comparison and is likely due to the three controllers with the highest frequency of CCR5-expressing CD8+ T cells (>80%) overlapping between the two groups - in fact the most significant difference in frequency of CCR5-expressing CD8+ T cells was seen when comparing the three controllers with VL<400 and CD4>500 to HCs (P=0.007, data not shown).

The percentage of CCR5-expressing monocytes did not differ between the controllers and HCs (P=0.23, **Figure 1F**), and did

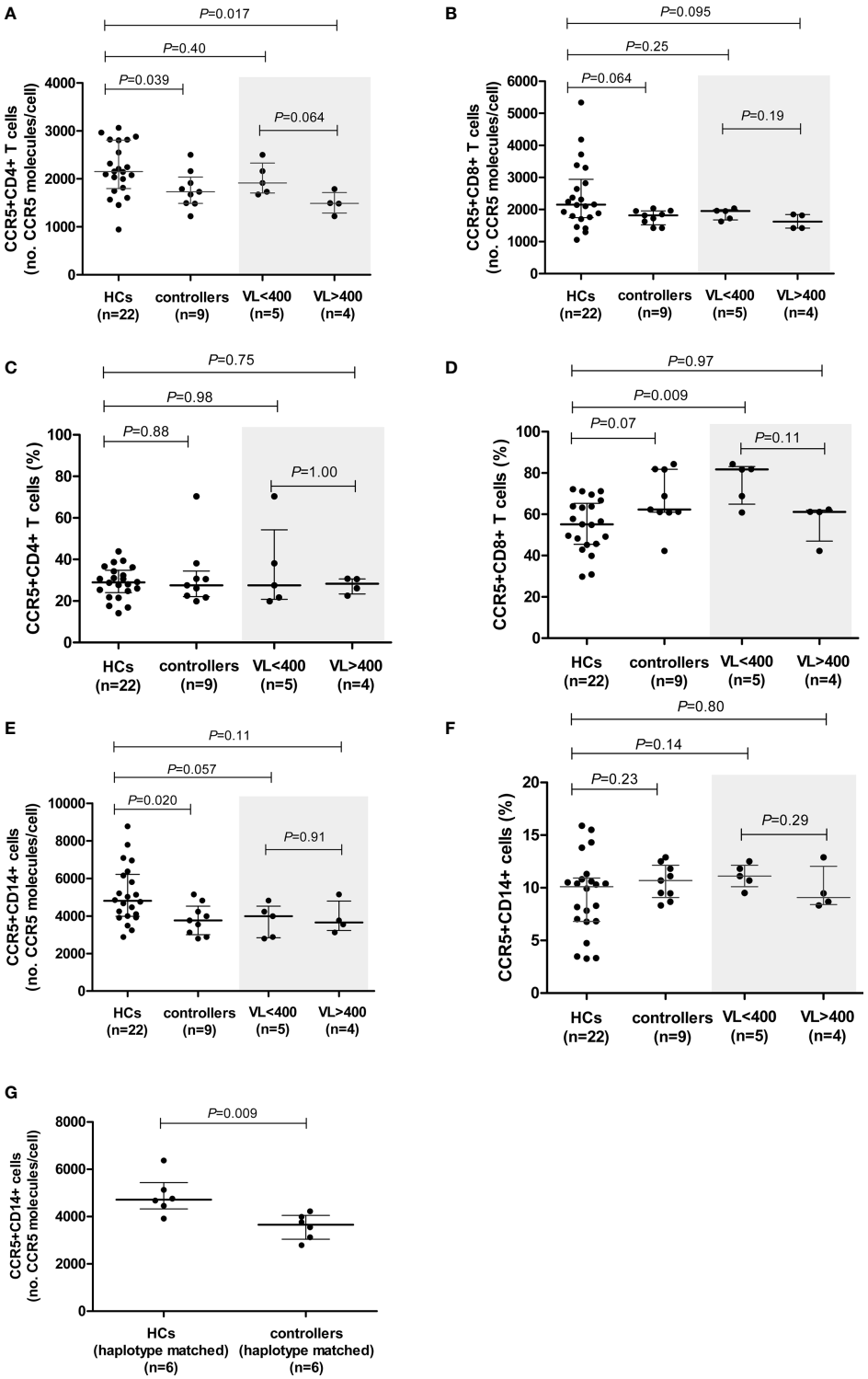

FIGURE 1 | CCR5 expression (density and percentage CCR5-expressing cells) on CD4+ **(A, C)** and CD8+ **(B, D)** T cells and monocytes **(E, F)** in healthy controls (HCs) and HIV-1 controllers (cohort 1). CCR5 expression for controllers, stratified according to viral load (VL), VL<400 and VL>400 (RNA copies/ml), is shaded in grey. CCR5 density on monocytes in HCs and HIV-1 controllers matched for *CCR5* genotype **(G)**. Mann-Whitney *U*-tests were conducted to test for significance. The medians and interquartile ranges are shown by horizontal bars. *P* values and the number of individuals in each group are indicated.

not differ either upon VL or CD4+ T cell count stratification (**Figure 1F** and **Supplementary Figure 1**, respectively).

Lower CCR5 Density on Monocytes of Controllers in Comparison to the HCs Remains Significant When Matched for CCR5 Promoter Haplotype and Age

We had previously reported a significant negative correlation between age and monocyte CCR5 density in the HCs (29). Since there was a strong trend of lower age in the HCs compared to the controllers (P=0.05), we selected a smaller, age-matched subgroup of the HCs (n=12) and again compared CCR5 density in monocytes, as well as CD4+ and CD8+ T cells (**Supplementary Figure 2**). Statistical significance was lost when monocyte CCR5 density between controllers and HCs (P=0.804), suggesting that age was likely a contributing factor to the significant lower density in controller monocytes in our original evaluation. Although the CD8 + T cell median for controllers was still lower than that of the HCs, the trend was lost (P=0.189). What is interesting however is that the CD4+ T cell significance was maintained and was slightly stronger (P=0.036).

To control for the possible influence of individual *CCR5* haplotypes and/or genotypes, we selected and compared a subgroup of individuals from the HC cohort that shared *CCR5* promoter genotypes with the controllers (only 6/9 controllers had genotypes that were present in the HC group). These subgroups consisted of the following genotypes (controllers:HCs): HHA/HHA (1:1); HHA/HHC (2:1); HHA/HHF*2 (2:1) and HHC/HHD (1:3). These subgroups did not differ with respect to age (P=0.748). The significant difference in CD4+ T cell CCR5 density was lost when CCR5 genotype-matched subgroups were compared; however, a weak trend was maintained (P=0.093, **Supplementary Table 4**). This could possibly be attributed to the small number of individuals in each group (n=6 in each). In contrast, the difference seen in monocyte CCR5 density between the controllers and HCs became more significant when age and genotype-matched subgroups were compared (P=0.009, **Figure 1G** and **Supplementary Table 4**). Given that these genotyped-matched groups did not differ in age, it is interesting that CCR5 density was so significantly reduced on controller monocytes relative to HCs, and suggests that although age is a determining factor for monocyte CCR5 density, there may be other mechanisms at play when one controls for age as well as CCR5 promoter strength (genotypes).

HIV-1 Controllers Have Significantly Less CD4+ T Cell CCR5 mRNA Expression Compared to HCs and HIV-1 Progressors

Results of relative CD4+ T cell CCR5 mRNA expression from a larger cohort of controllers (cohort 2) encompassing 5/9 controllers from cohort 1, compared to CD4+ T cell CCR5 mRNA from a different group of healthy controls (HCs-2, n=10) as well as HIV-1 progressors (n=12), are shown in **Figure 2**. Controllers had significantly less CCR5 mRNA expression compared to HCs (P=0.007) as well as to progressors (P=0.002). Interestingly, HCs and progressors did not differ significantly in terms of CD4+ T cell relative CCR5 mRNA expression (P=0.223). The 5 controllers that overlapped between cohort 1 and cohort 2 are shown in **Figure 2** as different coloured (pink) dots and were all situated below the medians for HCs and progressors.

Cell Activation Was Higher In HIV-1 Controllers, Compared to the HCs in T Cell but Not NK and Monocyte Cell Subsets

Cell activation levels, as measured by the percentage of cells expressing HLA-DR, were significantly higher on CD4+ and CD8+ T cells in controllers compared to the HCs (P=0.002 and P=0.0001, respectively, **Figures 3A, B**). This is expected as a result of HIV-1 infection. However, no differences in cell activation were observed in NK cells (P=0.356, data not shown).

In controllers, cell activation levels were higher in CD8+ cells compared to CD4+ T cells. The median percentage of HLA-DR-expressing CD4+ T cells in controllers was 23.1%, whereas this value was considerably higher in CD8+ T cells, i.e., 62.8% (P<0.0001). When analysed according to VL-stratified groups, both controller groups demonstrated significantly higher CD4+ T cell cellular activation levels compared to HCs (P=0.043 for VL<400 and P=0.0046 for VL>400, **Figure 3A**). Similarly, both VL controller subgroups expressed HLA-DR on CD8+ T cell subsets at significantly higher levels than the HCs (P=0.002 for VL<400 and P=0.0039 for VL>400, **Figure 3B**). There was no difference in CD4+ or CD8+ T cell activation levels between the two controller VL subgroups (P=0.286 and P=0.413, respectively, **Figures 3A, B**). Similarly, stratification of controllers according to CD4+ T cell count showed both groups having significantly higher CD4+ T cell cellular activation levels compared to HCs (P=0.006 for CD4>500 and P=0.047 for CD4<500, data not shown) and both groups having significantly higher CD8+ T cell cellular activation levels compared to HCs (P=0.002 for CD4>500 and P=0.004 for CD4<500, data not shown). There was no difference in CD4+ or CD8+ T cell activation levels

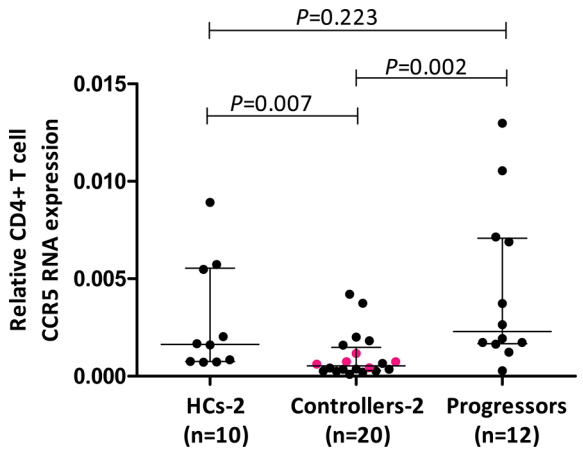

FIGURE 2 | Comparison of relative CD4+ T cell CCR5 mRNA expression in healthy controls (HCs), HIV-1 controllers and HIV-1 progressors (cohort 2). Mann-Whitney *U*-tests were conducted to test for significance. The medians and interquartile ranges are shown by horizontal bars. *P* values and the number of individuals in each group are indicated. The pink coloured dots in the controller group indicate 5/9 controllers that were studied in cohort 1.

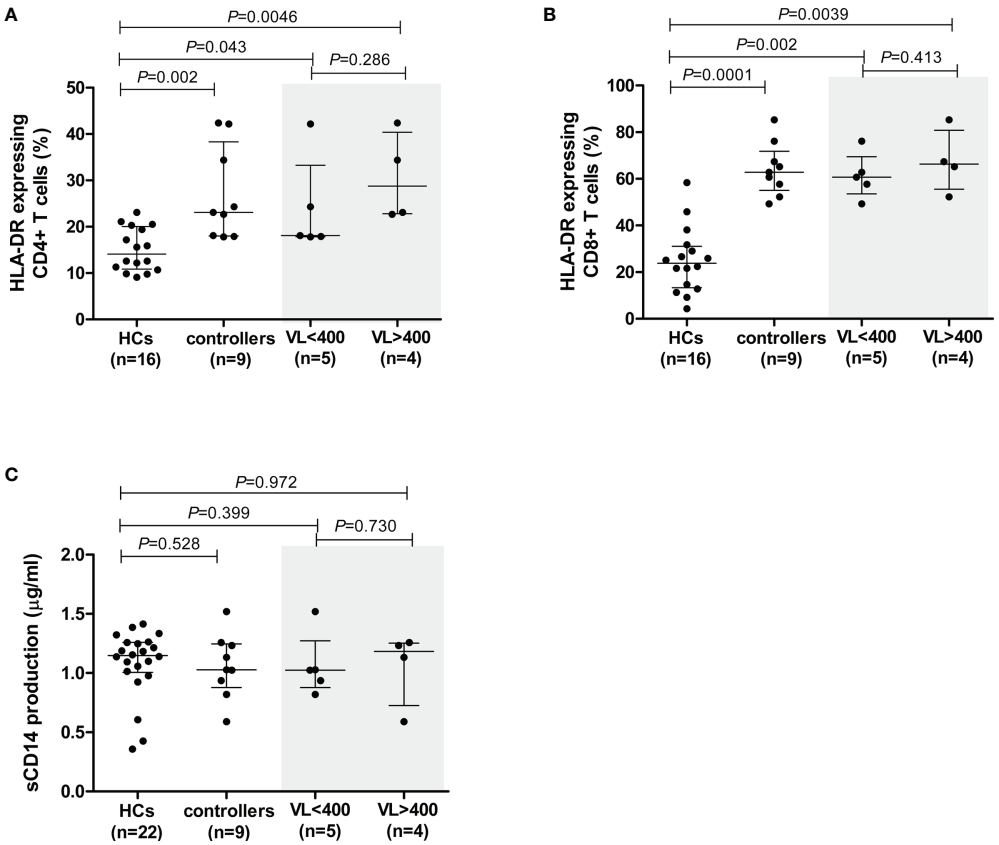

FIGURE 3 | Cell activation levels in healthy controls (HCs) and HIV-1 controllers (cohort 1), as measured by the percentage of cells expressing HLA-DR for CD4+ T cells **(A)** and CD8+ T cells **(B)**, and sCD14 measured in plasma - a measure of monocyte cell activation **(C)**. Mann-Whitney *U*-tests were conducted to test for significance. The medians and interquartile ranges are shown by horizontal bars. *P* values and the number of individuals in each group are indicated. For the HLA-DR comparisons **(A, B)**, only 16 of the 22 HCs were included due to later incorporation of this marker in the study. Controllers stratified according to viral load (VL), VL<400 and VL>400 (RNA copies/ml) are shown within the grey shaded boxes.

between the two controller CD4+ T cell subgroups (P=0.413 and P=0.730, respectively, data not shown).

Elevated plasma sCD14 in chronic HIV infection is associated with impaired immune restoration in response to ARV (35) as well as disease progression in both HIV-1 and HIV-2 infection (36–38). The sCD14 levels, measured in plasma samples from all study participants, were comparable between controllers and HCs (P=0.528, **Figure 3C**). Similarly, sCD14 production did not differ between controllers stratified according to VL (P=0.73, **Figure 3C**) or CD4+ T cell count (P=0.596, data not shown).

PHA-Induced Production of the CCR5 Ligands Was Lower in HIV-1 Controllers Compared to the HCs

The chemokines CCL3, CCL4 and CCL5 were quantified in the supernatants of unstimulated and PHA-stimulated PBMCs following incubation at 37°C for 20 h. Spontaneous production of CCL3 and CCL4 was not different between controllers and HCs (**Figures 4Ai, Bi**), although there was a trend (P=0.052) towards lower CCL4 production in controllers, and stratification of controllers according to VL revealed that the VL<400 group had significantly lower production of CCL4 compared to the HCs (P=0.044, **Figure 4Bi**). Stratification according to CD4+ T cell count did not show any significant differences (**Supplementary Figure 3**).

Within the controller cohort, there was one individual (TG4) whose PHA-induced CCL3 and CCL4 production was well above that of the HCs and controller cohorts combined (CCL3: 44.95 ng/ml compared to 6.52 – 35.55 ng/ml in HCs and 3.33 - 18.24 ng/ml in the remaining controllers; CCL4: 82.77 nl/ml compared to 9.27 – 70.27 ng/ml in HCs and 8.06 – 27.24 ng/ml in the remaining controllers). We thus considered TG4 as an outlier and excluded him from the PHA-induced CCL3, CCL4 and CCL5 analyses (**Figures 4Aii–Cii**).

The PHA-induced production of CCL3 and CCL4 by controllers was significantly lower compared to the HCs (P=0.029 and P=0.008, respectively, **Figures 4Aii, Bii**). Stratification of controllers according to VL revealed that it was the VL<400 controllers that were driving the significant relationships seen, with these individuals having significantly

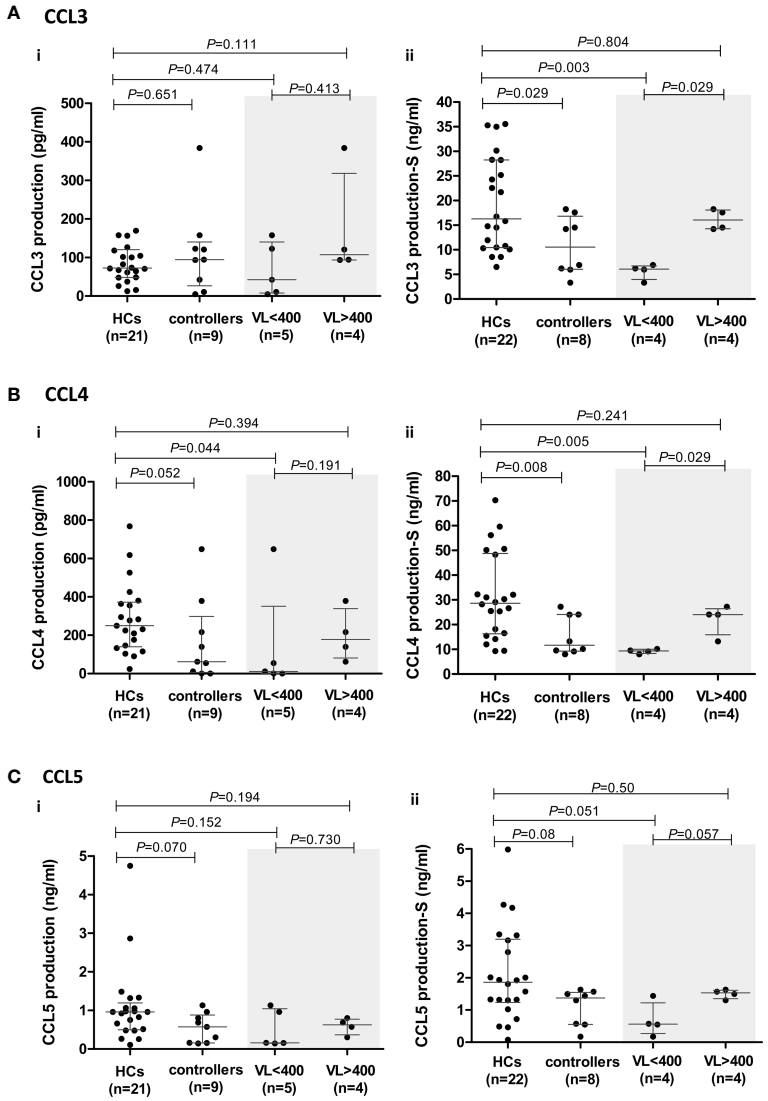

FIGURE 4 | CCR5 ligand production by peripheral blood mononuclear cells (PBMCs) isolated from healthy controls (HCs) and HIV-1 controllers (cohort 1): CCL3 **(A)**, CCL4 **(B)** and CCL5 **(C)**. Overall cytokine production is shown for unstimulated (spontaneous) (i) and PHA-stimulated (S) (ii) PBMCs. Mann-Whitney *U*-tests were conducted to test for significance. The medians and interquartile ranges are shown by horizontal bars. *P* values and the number of individuals in each group are indicated. Due to insufficient sample, a single control individual was omitted from the unstimulated production assays. A Controller (TG4), considered an outlier, was omitted from the PHA-stimulated comparisons (hence, n=8). Chemokine production from controllers stratified according to viral load (VL), VL<400 and VL>400 (RNA copies/ml) are shown within the grey shaded boxes.

lower CCL3 and CCL4 production compared to the HCs (*P*=0.003 and *P*=0.005, respectively, **Figures 4Aii, Bii**). When we stratified controllers based on CD4+ T cell count no significant differences were noted with CCL3, but the CD4>500 controllers had significantly lower CCL4 production compared to HCs (P=0.036, **Supplementary Figure 3**), however this was less significant than the VL<400 comparison.

Similarly, controllers produced lower levels of CCL5; however, this was not statistically significant (*P*=0.08, **Figure 4Cii**). This difference was more evident in unstimulated cultures where the median CCL5 production in the controllers (571.3 pg/ml) was lower than that of HCs (960.1 pg/ml, *P*=0.07, **Figure 4Ci**). PHA-stimulated PBMCs from VL<400 controllers also produced CCL5 at lower levels than HCs (*P*=0.051, **Figure 4Cii**) and PBMCs from controllers with VL>400 showed a strong trend of greater CCL5 production compared to the VL<400 group following PHA stimulation (*P*=0.057, **Figure 4Cii**). Stratification according to CD4+ T cell count did not show any significant differences (**Supplementary Figure 3**).

PHA-Induced Production of Additional Cytokines

To investigate whether the lower chemokine production by PBMCs from controllers, compared to that by HCs, was

restricted to the CCR5-ligand axis, we quantified the production of other cytokines: pro-inflammatory (IL-8, IFN-γ, TNF-α, G-CSF and GM-CSF), hematopoietic (IL-7, G-CSF and GM-CSF), T cell homeostatic (IL-2 and IL-4) and anti-inflammatory (IL-10) cytokines.

Spontaneous (unstimulated) PBMC production of only TNF-α and IL-8 were detectable in the assays used. Although controllers produced more TNF-α compared to HCs, this was not statistically significant (*P*=0.172, **Supplementary Figure 4A**). However, controllers with higher VLs (VL>400) produced significantly more TNF-α than the HCs (*P*=0.0095, **Supplementary Figure 4A**). A similar pattern was seen with IL-8, although not significant. The VL>400 subgroup produced more IL-8 compared to the HCs (*P*=0.129, **Supplementary Figure 4A**), and significantly higher than the VL<400 subgroup of controllers (*P*=0.029, **Supplementary Figure 4A**) – the TG4 outlier was excluded from the IL-8 analysis. These results suggest an association between VL and these two proinflammatory cytokines. No significant differences were seen when controllers were stratified according to CD4+ T cell count (data not shown).

The levels of PHA-induced IL-7 were below detection levels for both cohorts. PHA-induced PBMC production of the cytokines IL-2, IL-4, IL-10 and IFN-γ was lower in controllers compared to the HCs (**Figures 5A, B, D, F**, respectively), attaining statistical significance only for IL-10 (*P*=0.028, **Figure 5D**). IL-2 showed a strong trend, which became strongly significant following the removal of the TG4 controller outlier (*P*=0.064 and *P*=0.014, respectively, **Figure 5A**). However, given that the amount of PHA-induced IL-2 produced by TG4 (2.2 ng/ml) fell within the range produced by the HCs (0.15 – 2.8 ng/ml), this result should be viewed with caution. Controllers with VL<400 showed a strong trend (*P*=0.070, **Figure 5B**) of lower PHA-induced IL-4 levels compared to the HCs (stronger than the total controller group comparison, *P*=0.085). In addition, a strong trend of lower PHA-induced IFN-γ production by controllers compared to HCs was observed (*P*=0.071, **Figure 5F**), which was also attributable to the VL<400 controllers; removal of the TG4 outlier resulted in a significantly lower production of IFN-γ by the VL<400 controllers relative to the HCs (*P*=0.021, **Figure 5F**) but, similar to IL-2, the PHA-induced IFN-γ produced by TG4 (1.88 ng/ml) fell within the range produced by the HCs (0.14 -2.31 ng/ml) and thus should also be viewed with caution. IL-12 was the only cytokine that showed a trend of higher PHA-induced production in controllers relative to the HCs (*P*=0.071, **Figure 5E**). No differences in PHA-induced production of IL-8, G-CSF, GM-CSF and TNF-α were noted between the two study groups (**Figures 5C, G, H, I**, respectively). Stratification of controllers according to CD4+ T cell count did not show any significant or stronger trends than stratification according to VL, with the exception of IL-10 where controllers with CD4<500 showed a strong trend of lower IL-10 compared to HCs (*P*=0.06, **Supplementary Figure 5**) and IL-12, where controllers with CD4>500 showed a strong trend of higher IL-12 production compared to HCs (*P*=0.057, **Supplementary Figure 5**).

HIV-1 Controller PBMCs Had Higher Proportions of T Cells and Lower Proportions of NK Cells Compared to HC PBMCs

PBMCs are comprised of monocytes and lymphocytes, including T (CD4+ and CD8+), B and NK cells. We calculated the expected number of each of these cell types within the PBMCs used in chemokine/cytokine production assays by extrapolating from absolute cell counts determined from whole blood. **Figure 6** shows comparisons of both the absolute counts (**Figure 6A**), the calculated total number of cells used in the PBMC cultures (**Figure 6B**), and a comparison of the mean percentage of different cell types within the PBMC cultures (**Figure 6C**).

Controllers had similar absolute CD4+ T cell counts to HCs (P=0.948, **Figure 6A**), and although not statistically significant, controllers had fewer CD4+ T cells compared to the HCs in the total number of cells used in the PBMC cultures (*P*=0.064, **Figure 6B**). This CD4+ T cell deficit was offset by the significantly higher number of CD8+ T cells compared to the HCs, in both the absolute count and PBMC culture comparisons (*P*<0.001 and P<0.0001, **Figures 6A, B**, respectively). CD8+ T cell expansion is expected in individuals infected with HIV-1. Controllers were also found to have lower numbers of NK cells (CD56+, CD16+CD56+ and $CD56^{dim}$) than HCs, evident as both absolute counts and NK cell numbers in PBMC cultures (*P*=0.016, *P*=0.058, *P*=0.018 and *P*=0.0016, *P*=0.016, *P*=0.009, **Figures 6A, B**, respectively). No differences were noted between the two groups in either absolute monocyte counts in blood or in PBMC cultures (*P*=0.845 and *P*=0.145, **Figures 6A, B**, respectively). **Figure 6C** indicates the proportional representation of different cell types within PBMCs from controllers and HCs – differences seen in the production of specific cytokines may be in part attributed to differences in representation of particular cell types.

DISCUSSION

The vast variation in the rates of HIV-1 disease progression among individuals can be attributed to viral, genetic and immunological factors. A number of host genetic factors associated with delayed disease progression have been identified [Reviewed in (39, 40)]. Among the genetic factors known to associate with delayed disease progression are genetic polymorphisms of *CCR5*, the principal HIV-1 coreceptor, and gene copy number variation of its ligands, CCL3L and CCL4L (41–43). In this study, we compared features important in the CCR5 coreceptor-ligand axis between two groups of black South African individuals—HIV-1 controllers who are able to control HIV-1 infection for extended periods of time in the absence of antiretroviral treatment and healthy controls. We included measures of CCR5 cell surface density, immune activation (proportions of CD4+ and CD8+ T cells expressing CCR5 and HLA-DR, and plasma levels of sCD14) and the capacity of mononuclear cells to produce chemokine/cytokines in response

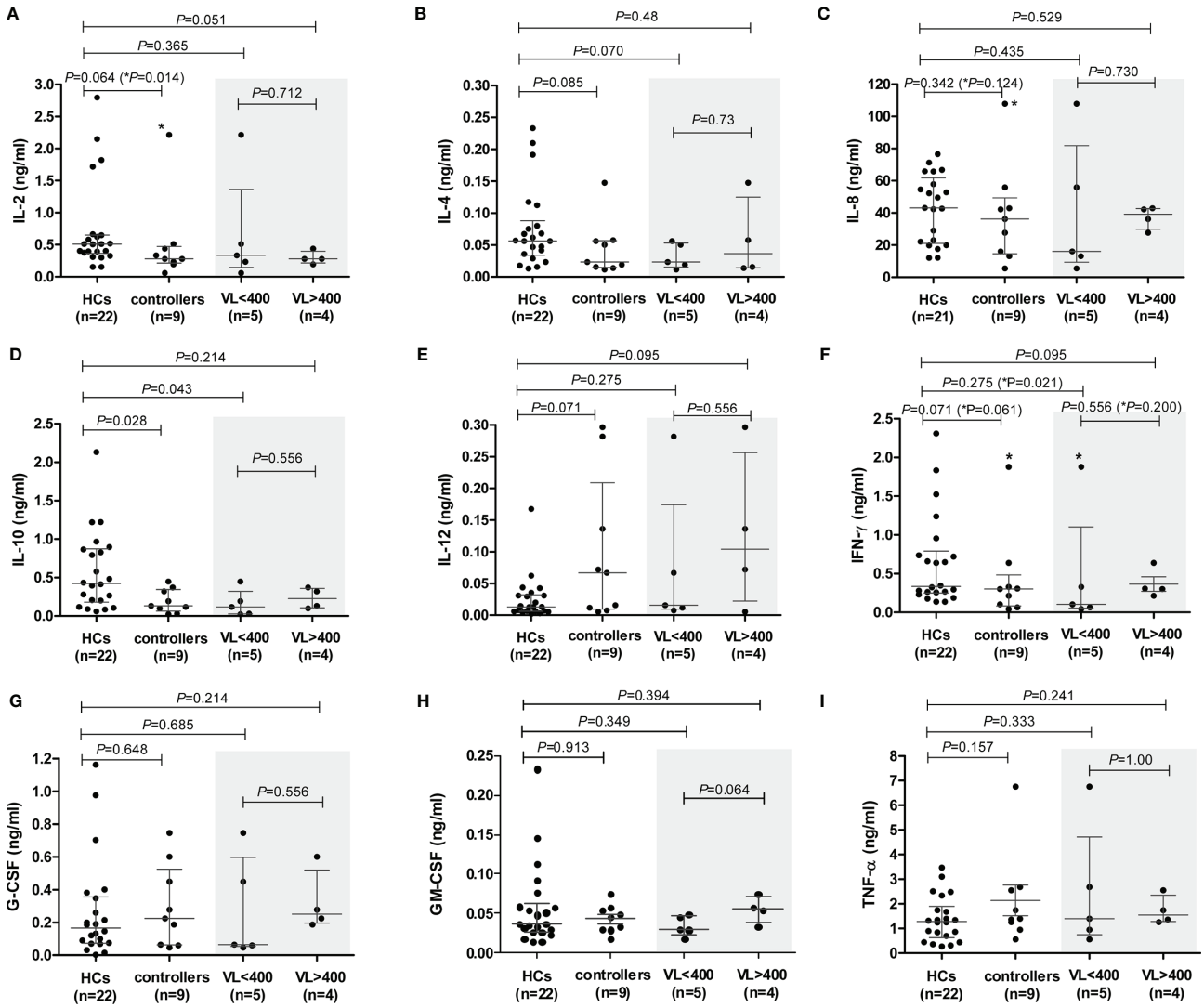

FIGURE 5 | PHA-induced cytokine production by peripheral blood mononuclear cells (PBMCs) isolated from healthy controls (HCs) and HIV-1 controllers (cohort 1): IL-2 **(A)**, IL-4 **(B)**, IL-8 **(C)**, IL-10 **(D)**, IL-12 **(E)**, IFN-γ **(F)**, G-CSF **(G)**, GM-CSF **(H)** and TNF-α **(I)**. Mann-Whitney *U*-tests were conducted to test for significance. The medians and interquartile ranges are shown by horizontal bars. *P* values and the number of individuals in each group are indicated. Chemokine production from controllers stratified according to viral load (VL), VL<400 and VL>400 (RNA copies/ml) are shown within the grey shaded boxes. *P* values following the removal of the TG4 controller outlier as indicated with an asterisk (*) in **(A, C, F)**.

to PHA. Despite the small number of controllers used in these comparisons, these individuals have been closely monitored over many years and 8/9 controllers have been able to control disease in the absence of ART for ≥11 years, with two of these individuals for at least 20 years. In addition a larger cohort of HIV-1 controllers (including 5 of the 9 controllers studied in detail) was assessed for CCR5 mRNA expression in CD4+ T cells and compared to both healthy controls and HIV-1 progressors.

Although 36 SNPs and four indels were identified within the controllers (cohort 1), these were restricted to the noncoding regions of the *CCR5* gene and all had previously been identified. In a recent study, we investigated various *CCR5* regulatory genetic variants in a larger cohort of HIV-1 controllers (which included 8 of the 9 controllers from this study) and HIV-1 progressors (44). While select promoter haplotypes and variants were significantly over-represented in HIV-1 progressors relative to the controllers in that study, these same variants did not differ between this smaller cohort of controllers and healthy controls in the current study (HCs were not included in the Koor et al. (44) study). A 3' UTR SNP (rs3188094; +2458A>C) was significantly more prevalent in the controllers compared to the HC cohort in this study (27.8% versus 8.5%), with two controllers being homozygous for the minor allele. However, representation of this SNP in the larger controller cohort (9.86%) is more aligned with the HC representation (44). It is interesting to note however that this +2458A>C SNP is a relatively rare SNP and data from the

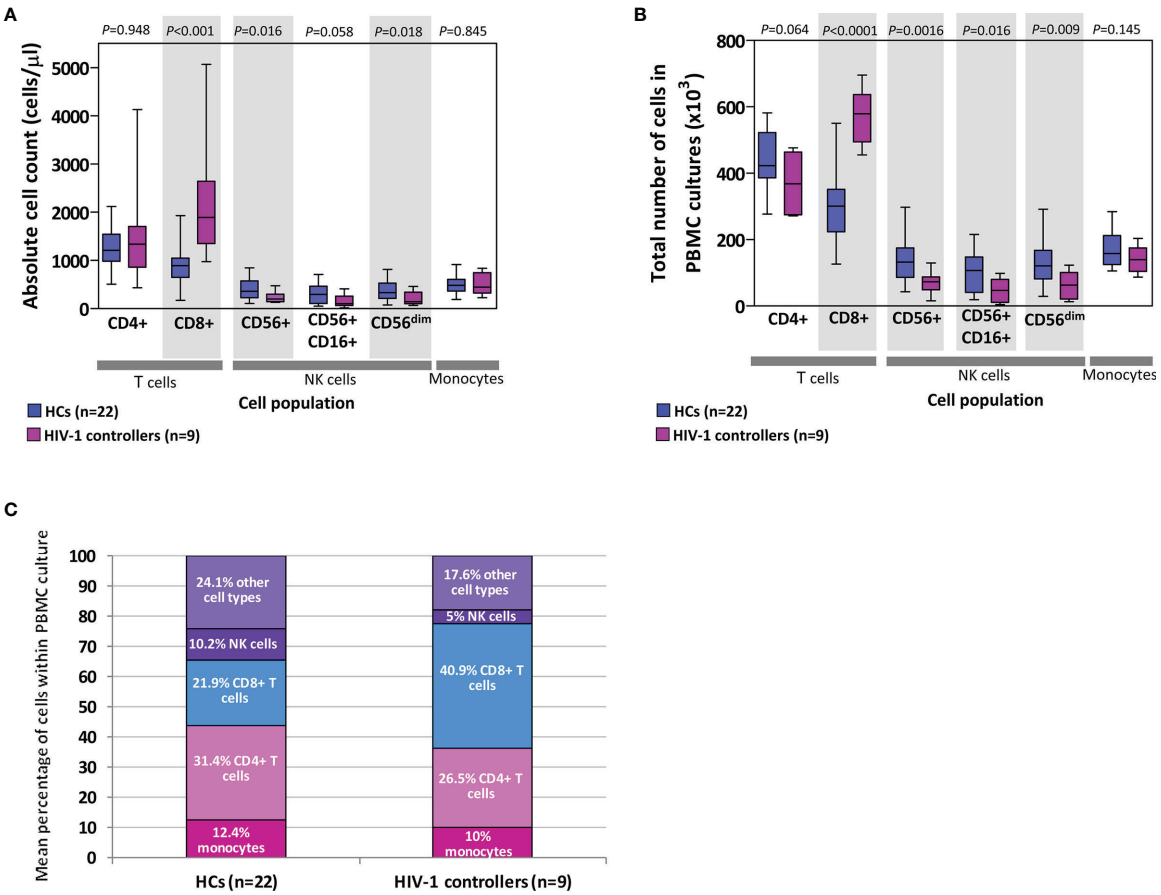

FIGURE 6 | Absolute counts of cells (lymphocytes and monocytes) in blood **(A)** as well as extrapolated number of cells used in peripheral blood mononuclear cell (PBMC) cultures **(B)** in healthy controls (HCs) and HIV-1 controllers (cohort 1). Shaded grey boxes indicate significant differences between the two groups with *P* values as indicated (Mann-Whitney *U* tests). Box-whisker plots depict the median (horizontal black line), 25th and 75th percentiles (margins of the box) and the 10th and 90th percentiles (whiskers). Proportional representation of different cell types within PBMCs isolated from HCs and controllers determined using the mean values from the respective groups **(C)**. Cells referred to as "other cell types" include B cells, double-negative T cells, basophils and dendritic cells.

1000 Genomes Project (45) shows that it is a predominantly African-based variant with 4% representation in the total African population group. Furthermore homozygosity for the minor allele is extremely rare (0.002% in the total African population – only one individual out of 661 individuals) – thus the black South African population exhibits a relatively high representation of this variant. Although not significant, representation of the +2458A>C SNP was also found to be lower in black South African HIV-1 infected progressors (4.7%) compared to controllers (44) and may be worth investigating further in terms of its role in CCR5 expression or function – preliminary analysis using a miRNA target prediction tool (http://www.targetscan.org/) revealed the major allele of the +2458A>C SNP to sit within the predicted binding site of the hsa-miR-376b-3p miRNA in the CCR5 3'UTR (data not shown).

HIV-1 infection is associated with increased CCR5 density on T cells, particularly CD4+ T cells (46, 47); however, we observed the opposite in that HIV-1-infected controllers expressed CCR5 at lower densities compared to HCs within these same cell subsets. Furthermore, controllers also exhibited significantly lower relative abundance of CD4+ T cell CCR5 mRNA compared to both HCs and HIV-1 progressors. The beneficial effects of reduced CCR5 expression on CD4+ T cells has been highlighted in simian immunodeficiency virus (SIV) infection models, where natural SIV hosts that do not develop AIDS-like symptoms express CCR5 on remarkably lower proportions of CD4+ T cells in blood, lymph nodes and mucosal tissues compared to humans and non-natural SIV hosts such as rhesus macaques (48).

The role of CCR5 expression in the natural control of HIV-1 has not been extensively studied. However, there have been some reports, conducted in predominantly European/Caucasian populations, which support our findings in a sub-Saharan black population. A study conducted on 9 slow progressors reported significantly lower CCR5 densities on CD4+ T cells of slow progressors compared to uninfected controls and HIV-1-infected normal progressors as well as rapid progressors (13). Furthermore, and in agreement with our study, no difference was seen between slow progressors and HCs in terms of the

percentage of CCR5-expressing CD4+ T cells (13). Another study conducted on 12 HIV-1 controllers also found lower CCR5 density on CD4+ central memory T cells and a lower percentage of central memory, but not effector memory, CCR5-expressing CD4+ T cells in HIV-1 controllers relative to uninfected donors (49). Interestingly, the lower CCR5 density seen on the CD4+ T cells of our controllers relative to HCs was largely attributed to those with the higher VLs (>400 RNA copies/ml). This suggests that CCR5 density may be a greater contributor to HIV-1 control in the context of higher viraemia, at least in these particular individuals, as seen in the SIV model of infection.

In a more recent and larger study by Gonzalo-Gil et al. (50), a subgroup (n=21) of elite and viraemic controllers, who were identified with *in vitro* CD4+ T cell resistance to R5-tropic HIV-1 (ECs_r/VCs_r), had lower CD4+ T cell CCR5 densities and lower proportions of CCR5-expressing CD4+ T cells following CD4+ T cell stimulation relative to HCs and ECs/VCs who did not express the resistance phenotype (50). In addition, these ECs_r/VCs_r has significantly decreased CCR5 mRNA expression levels in activated CD4+ T cells relative to HCs and ECs/VCs who did not express the resistance phenotype. Although this study differs from the present study in a number of parameters, it serves to corroborate the vital role of lower CD4+ T cell CCR5 expression in HIV-1 control.

Cell surface expression of CCR5 on monocytes plays an important role in HIV-1 infection. CCR5 expression correlates directly with the differentiation of monocytes to macrophages (51, 52). Although controllers had significantly lower monocyte CCR5 density compared to HCs, when we age matched a smaller group of HCs and compared CCR5 density between the two groups, the relationship was lost, however the significant relationship for CD4+ T cells was maintained. Given that we have previously shown a significant negative correlation between age and monocyte density in the HC cohort (29), the lower CCR5 density on monocytes is likely influenced by age and needs to be investigated in larger age-matched cohorts. However, we have also previously demonstrated *CCR5* haplotype-associated differences in CCR5 expression within healthy black South African individuals (17). When we matched controllers and HCs for *CCR5* genotypes (and age), CCR5 density on controller monocytes was distinctly significantly less (P=0.009) compared to HCs - given the strong influence of age on monocyte CCR5 density seen in the larger group comparison, it is difficult to interpret this highly significant relationship, and it is possible that it represents a chance result of smaller groups being compared. Nevertheless, this may be an important finding that should be interrogated in larger, haplotype and age matched cohorts. Very little, to our knowledge, has been reported about the role of monocyte CCR5 expression levels in natural HIV-1 control. In the Gonzalo-Gil et al. (50) study, CCR5 expression in macrophages derived from monocytes (MDMS) – measured as the levels of CCR5-specific RNA and percentage of CCR5-expressing CD14+ cells - was not different from healthy controls in the group of ECs/VCs with CD4+ T cells that displayed resistance to R5 tropic HIV-1. However, this result is not strictly comparable to our study and furthermore, they did not report on CCR5 density in MDMS.

Previous studies suggest that CCR5 receptor expression remains stable in HIV-1-uninfected individuals over multiple time points despite the wide range of variability that exists between individuals (12, 46). Furthermore, in the context of HIV-1 infection, CCR5 cell surface density levels correlate positively with the levels of immune activation (proportions of $CD38^{hi}$-expressing CD8+ T cells), yet initiation or interruption of ART affects levels of immune activation but not CCR5 density (27). These data suggest a constitutive or inherent level of CCR5 cell surface expression within individuals, which is unaffected by the individual's state of immune activation. Overall, high CCR5 density more likely predisposes to the likelihood of greater immune activation (expansion of proportions of HLA-DR, CD38 or CCR5-expressing CD4 or CD8 T cells) and disease progression, rather than the reverse i.e. the cause and consequence argument. As expected, individuals infected with HIV-1 (controllers) demonstrated higher T cell activation levels, as measured by the percentage of HLA-DR-expressing cells, compared to the HCs. Given that increased cell activation is associated with increased CCR5 expression (46, 53–55), it is intriguing that these same individuals expressed CCR5 at densities lower than that of HCs on the same cells. CD38 is widely used as a marker of T and B cell activation. In a cross-sectional study of individuals with normal progressing HIV-1 infection, we previously demonstrated significantly higher percentage of CD38+CCR5+ lymphocytes compared to healthy controls (56). On re-examination of these same data, the percentage of CCR5-expressing CD8+ T cells from HIV-1-infected individuals was found to correlate positively with the percentage of CD38-expressing CD8+ T cells (P=0.005, r=0.54; S. Shalekoff, unpublished data). Therefore, the percentage of CCR5-expressing CD8+ T cells could also be used as a surrogate marker for the extent of cell activation. The expansion of this same cell subset, but not of CCR5-expressing CD4+ T cells, was substantially higher in controllers compared to the HCs, which reflects the persistently higher cellular activation observed in HIV-1 controllers. However, we observed lower CD4+ T cell CCR5 density and mRNA expression in controllers compared to HCs. These findings suggest that, rather than controllers having the ability to downregulate CCR5 expression despite high levels of activation, controllers are comprised of individuals who are inherently low CCR5-expressors. As such, their immune cells are more quiescent and not as "activatable" as other individuals who progress more rapidly. Additional support for this theory is provided by the Gonzalo-Gil et al. (50) study. The CD4+ T cell R5 resistance phenotype of ECs_r/VCsr, associated with the downregulation of ≈500 kb region of Chromosome 3p21 encompassing *CCR5* and *CCR2* (among other genes) was also observed in family members of an index VC. These family members also displayed *CCR5* and *CCR2* downregulation - thereby suggesting an inherited genetic determinant of lower CCR5 expression (50). CCL3, CCL4 and CCL5 are the natural ligands for the CCR5 receptor and are known to inhibit

replication of CCR5-restricted HIV-1 variants (57, 58). While the anti-HIV-1 activity of the CCR5 ligands is mainly attributed to competitive binding to CCR5 (57), a role for these β-chemokines in inhibition of post-entry steps of the HIV-1 life cycle has also been reported (59). Although several studies have investigated the influence of HIV-1 infection and disease progression on circulating levels of CCL3, CCL4 and CCL5, results have been contradictory. There is a lack of consensus on whether individuals infected with HIV-1 produce these chemokines at higher, lower or equivalent levels compared to uninfected individuals, as measured in plasma or serum samples (60–62). Furthermore, there is a lack of consensus on whether or not cellular production and/or circulating levels of the CCR5 ligands correlate with disease progression (60, 62–67). These results are difficult to interpret due to a lack of homogeneity in patient selection as well as differences in study design. In addition, assays for their quantitation do not distinguish between the different chemokines and their isoforms (produced from different genes, present in variable copy numbers and subject to post-translation modifications that alter function/receptor binding), which could mask the true relationships of isoforms that may matter. In the context of mother-to-child HIV-1 transmission, we have shown that elevated levels of mitogen-induced CCL3 production (and to a less extent CCL4) by infant cord-blood mononuclear cells was associated with protection from intrapartum infection (68), suggesting that the levels of these ligands may play different roles depending on HIV acquisition versus disease progression.

If activation levels correlate with β-chemokine production, one might expect spontaneous PBMC production of CCL3, CCL4 and CCL5 to be higher for controllers than HCs; however, CCL3 production was comparable between the two groups, and CCL4 and CCL5 production of the controllers was lower than the HCs. Stimulation with PHA resulted in lower PBMC production of all three CCR5 ligands in controllers compared to that of the HCs, but this was only significantly lower for CCL3 and CCL4. In agreement with our results, activated CD4+ T cells from ECs/VCs that displayed the R5 HIV-1 resistance phenotype discussed earlier (ECs_r/VCs_r) also produced significantly less CCL3 and CCL4 compared to healthy controls (50). Interestingly, PBMCs from controllers with low VLs (VL<400) produced CCL3, CCL4 and CCL5 at lower levels than those from individuals with higher VLs and although not statistically significant, there seemed to be a similar trend in unstimulated cells. The former group also demonstrated slightly lower activation levels than the higher VL group, as measured by the percentage of cells expressing HLA-DR on CD4+ and CD8+ T cells. It could be argued that higher plasma concentrations of the CCR5 ligands could be responsible for increased internalization of the CCR5 receptor and hence lower CCR5 density, and controllers with higher VLs would by virtue of higher activation have higher plasma ligand concentrations and thus lower CCR5 density. However, CCR5 density on CD4+ T cells, CD8+ T cells and monocytes did not significantly correlate with spontaneous CCL3, CCL4 or CCL5 production in HCs or controllers (data not shown). Furthermore the lower CD4+ T cell CCR5 mRNA expression seen in controllers relative to both HCs and progressors points to a transcriptional downregulation rather than receptor internalization as the predominant cause of lower CCR5 density.

CD26/DPP4-mediated proteolysis of CCL3L1 and CCL5 results in a strong affinity of these isoforms for binding to CCR5, which also display potent anti-HIV-1 activity *in vitro* [reviewed in (69)]. In a recent study we conducted, HIV-1 controllers had similar levels of specific CD26/DPP4 activity and percentages of CD26/DPP4+ T cells compared to HCs, but significantly higher levels than HIV-1 progressors (70). Taken together, we postulate that the relative abundance of more effective anti-HIV chemokine isoforms is greater in controllers compared to progressors, due to ineffective CD26/DPP4 proteolysis in the latter, which accordingly is also associated with increased inflammation.

IL-10 was the only cytokine that was produced at significantly lower levels by PHA-stimulated PBMCs from controllers relative to HCs, without removal of outliers, and in fact remained significant post removal of a high-producing IL-10 HC outlier. Numerous reports have highlighted the importance of circulating IL-10 levels in the course of HIV-1 infection. Although high IL-10 levels, associated with *IL-10* single nucleotide polymorphisms, provide protection against acquiring HIV-1, as demonstrated in a study of high-risk South African women who were HIV-1-uninfected when enrolled into the study (71), lower IL-10 levels appear to provide beneficial effects in the chronic phase of HIV-1 infection. Blockade of the IL-10 pathway is capable of restoring HIV-1-specific T cell responses (72, 73). In addition, individuals infected with HIV-1 with *IL-10* genotypes associated with lower IL-10 production, demonstrate a trend towards attenuated CD4+ T cell loss (74). However, the effects of IL-10 on HIV-1 pathogenesis seem to differ according to the stage of infection (71, 72, 74), thus indicating a complex relationship between IL-10 levels and HIV-1 disease progression. Although PHA-induced production of IL-10 by PBMCs has been reported as increased in individuals infected with HIV-1 relative to HCs (75), comparable IL-10 production between HIV-1 long-term non-progressors and uninfected individuals has been reported, whereas individuals with progressive infection maintained significantly higher IL-10 production (76). Furthermore, IL-10 production correlates positively with disease progression (73, 77). Together, this suggests that the clinical progression status of an individual infected with HIV-1 is likely to result in differences in antigen-induced production of IL-10. Furthermore, monocytes are major producers of IL-10 in both individuals with and without HIV-1 infection [Reviewed in (78)]. Plasma levels of sCD14, indicative of the extent of *in vivo* monocyte activation, did not differ between our controllers and HCs. In addition, monocyte numbers in *in vitro* PHA-stimulated cultures did not differ, suggesting that controllers produce less IL-10 independently of monocyte numbers or the level of their activation.

This study has a number of limitations. We only included a small number of controllers, who were also heterogeneous based on definitions of HIV-1 control, in the cell-based experiments. Using larger cohorts that are better matched for age, and including ART-treated HIV-1-infected progressors in future studies will

shed further light on the current findings. We also used HLA-DR alone as a marker of activation - the use of CD38 in addition to HLA-DR would have been more informative, and more detailed analysis of different subsets of activated cells in the context of CCR5 density warrant further investigation. The comparison of CCR5 density directly between activated and non-activated CD4+ and CD8+ T cells would have provided more compelling evidence for the postulated inherent predisposition to lower CCR5 density. We were unable to do this based on the HLA-DR marker being in a separate 4-colour flow cytometry panel.

In summary, in this study we demonstrate reduced CCR5 cell surface density on CD4+ T cells and reduced induced cellular levels of the CCR5 ligands (CCL3, CCL4 and CCL5) and IL-10, in a small group of black HIV-1 controllers compared to HCs. In addition, we show lower CCR5 mRNA expression in CD4+ T cells in a larger group of controllers relative to both HCs and HIV-1-infected progressors. Importantly, this pattern of lower CCR5 expression in CD4+ T cells has also been shown for Caucasian HIV-1 controllers, is independent of the presence of the *CCR5Δ32* deletion (13, 50), and is likely to involve a transcriptional downregulation of a large region of the chromosome encompassing *CCR5*, which appears to be genetically predetermined (50). A genetically predetermined lower CCR5 expression is in keeping with our findings and thus probably involves a mechanism that is shared among ethnically divergent population groups – this is an important finding and supports continued investigation into the underlying mechanism responsible for this phenomenon, which could inform future HIV cure efforts, particularly in sub-Saharan Africa, where cure interventions are most needed.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

This study was approved by the University of the Witwatersrand Committee for Research on Human Subjects. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AP: Conducting experiments, analysis and writing. MP: Analysis and writing. GWK: Conducting experiments. AB: Conducting experiments. SS: Conducting experiments, review and editing. RL: Conducting experiments, review and editing. PI: Resources (cohort). CTT: Conceptualization, funding acquisition, writing. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.781263/full#supplementary-material

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Filling the Gaps in Antagonist CCR5 Binding, a Retrospective and Perspective Analysis

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The large number of pathologies that position CCR5 as a central molecular determinant substantiates the studies aimed at understanding receptor-ligand interactions, as well as the development of compounds that efficiently block this receptor. This perspective focuses on CCR5 antagonism as the preferred landscape for therapeutic intervention, thus the receptor active site occupancy by known antagonists of different origins is overviewed. CCL5 is a natural agonist ligand for CCR5 and an extensively studied scaffold for CCR5 antagonists production through chemokine N-terminus modification. A retrospective 3D modeling analysis on recently developed CCL5 mutants and their contribution to enhanced anti-HIV-1 activity is reported here. These results allow us to prospect the development of conceptually novel amino acid substitutions outside the CCL5 N-terminus hotspot. CCR5 interaction improvement in regions distal to the chemokine N-terminus, as well as the stabilization of the chemokine hydrophobic core are strategies that influence binding affinity and stability beyond the agonist/antagonist dualism. Furthermore, the development of allosteric antagonists topologically remote from the orthosteric site (e.g., intracellular or membrane-embedded) is an intriguing new avenue in GPCR druggability and thus a conceivable novel direction for CCR5 blockade. Ultimately, the three-dimensional structure elucidation of the interaction between various ligands and CCR5 helps illuminate the active site occupancy and mechanism of action.

Keywords: CCR5, CCL5, antagonist, binding, rational design

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INTRODUCTION

CCR5 is a chemokine receptor belonging to the GPCR superfamily. CCL5 is a proinflammatory chemokine, largely studied for the pathophysiological implication of CCR5 engagement. The CCR5: CCL5 axis proved to be a crucial crossroad for a large number of infections and inflammatory conditions (including HIV-1 and *Staphylococcus aureus* infections, cancer and atherosclerosis). In most of these pathologies, CCR5 blockade is a promising therapeutic avenue (1, 2). CCR5 antagonist occupancy of the receptor active site has a dual advantage: it stabilizes the receptor in an inactive conformation, and it allows competitive binding with the natural agonist ligands (e.g., CCL3, CCL4 and CCL5) or the microorganism ligands (e.g., HIV-1 gp120 and *S. aureus* LukED toxin). Interestingly, both gp120 and LukED pathogenic engagement of CCR5 has been reported not to

activate the receptor (3, 4). However, the sequence variability presented by the diversity of HIV-1 strains is reflected in the molecular determinant for CCR5 binding (i.e., the V3 loop), possibly generating an array of binding modes (including agonist binding) and CCR5 conformation occupancy (5). CCR5 antagonism is a mandatory pharmacological intervention for inflammatory conditions caused by receptor activation and a rational approach to combat HIV-1 infection. The availability of maraviroc (MVC), a small chemical compound approved as drug for HIV-1 entry inhibition, allows the prompt investigation of CCR5 biochemical blockade beyond HIV-1 infection (6–8). Pertaining to HIV-1 inhibition, a large body of research has been focusing on the development of potent N-terminally-modified chemokine ligands of CCR5, mostly based on CCL5. Initially, CCL5 variants retained CCR5 agonist activity, later followed by efforts to attain a switch to antagonism (9). Last generation CCL5 derivatives acting as CCR5 antagonists could be a valid alternative to MVC, as they present *in vitro* anti-HIV-1 activity largely superior to MVC (10, 11). Monoclonal antibodies (mAbs) acting as CCR5 antagonists have been the subject of intense investigation (12) leading to promising therapeutic perspectives (13, 14). However, to date there is no information on the structural details of the interaction between these mAbs and CCR5. Hence, although extremely interesting, the structural understanding of their CCR5 blockade is to date relatively limited. Rational drug design approaches show that MVC is also prone to molecular improvements (15). The dualism on small chemical compound *versus* protein based CCR5 antagonists is *de facto* a territory in which information crosstalk may boost the advancement of both molecular classes.

In this study, we investigated both retroactively and in perspective the innovative role of selected point mutations inserted in CCL5 regions distal to the classically targeted N-terminus (11). We also briefly discuss emerging allosteric strategies to block GPCRs alternative to the active site occupancy and that could be of interest to tackle CCR5 in pathology.

METHODS

Modeling Full-Length CCR5 in Complex With CCL5 5P12 5M and Retrospective Analysis

The high-resolution structure of CCR5 in complex with 5P7 CCL5 (a CCR5 antagonist) (PDB ID: 5UIW) (16) was used as template for the modeling of CCL5 5P12 5M, the most potent CCR5 antagonist reported to date (11). Six separate 3D models (T7L, F12Y, A13V, Y27W, F28W and E66S), accounting for the differences between 5P7 CCL5 and CCL5 5P12 5M were built using SWISS-MODEL. To compare the quality of the models, we used I-TASSER, Phyre2 and ModWeb. The model of CCL5 5P12 5M built on the 5UIW coordinates in all the platforms was used as a prototype to validate the most reliable superposition with the 5P7 CCL5 structure, and we decided that SWISS-MODEL provided the best output for the subsequent modeling and analysis.

Next, we modeled on 5UIW the first 15 AA of CCR5 N-terminus from the NMR solution structure of monomeric CCL5 bound to a synthetic doubly sulfated CCR5 N-terminus peptide (PDB ID: 6FGP) (17). We used homology modeling and data-driven flexible refinement to generate the final full-length model.

A retrospective structure-guided analysis was conducted to examine the quality and effect the six AA differences between 5P7 CCL5 and CCL 5P12 5M exerted on the full CCR5 model. Structures were visualized and analyzed using the PyMOL software.

RESULTS

CCR5 Antagonist Active Site Occupancy and Its Role in Pathology

CCR5 stabilization in an inactive conformation by the use of compounds capable to occupy the active site as antagonists may play a significant therapeutic role in several pathologies (**Figure 1**). Ample occupancy of the active site might not seem to be a necessary requirement in inflammatory conditions involving CCR5, however prevention of receptor engagement by natural chemokine agonists (e.g., CCL3, CCL4 and CCL5) needs to be attained to warrant full therapeutic efficacy. The differential efficiency by which MVC and CCL5 derivatives compete with natural chemokines needs to be investigated in detail. Receptor active site full occupancy becomes crucial in HIV-1 entry inhibition as this prevents the insurgence of resistant strains. The deep but limited CCR5 active site area occupied by MVC (**Figure 1A**) (18), as compared to the large surface of interaction covered by gp120 (**Figure 1C**) (3), allows HIV-1 to eventually raise MVC-resistant strains. Conversely, full occupancy of CCR5 active site by CCL5 derivatives (**Figure 1B**) (16) should prevent the emergence of HIV-1 resistant strains (19, 20). The different extent of the CCR5 interaction by MVC, gp120 and the CCL5 variant 5P7 CCL5 (a CCR5 antagonist) is highlighted in **Figure 1F**. It can be appreciated how the presence of 5P7 CCL5 would not allow any penetration of the receptor by gp120, nor any obvious molecular rearrangement leading to HIV-1 resistance could be envisaged. Interestingly, the 3D structure of CCR5 from the three complexes with antagonists is largely overlapping (**Figure 1E**), denoting similar inactive conformations.

Improving CCR5 Engagement by Modifying CCL5 Distal to the N-Terminus

Among the CCL5 derivatives efficiently blocking CCR5, the most potent HIV-1 entry inhibitors reported to date are CCL5 5P12 5M and CCL5 6P4 5M, a CCR5 antagonist and a superagonist, respectively (11). Five mutated hotspots (5M) were selected to enhance different features related to the CCL5:CCR5 interaction. The classic E66S mutation is well known to promote disruption of CCL5 oligomerization (21, 22), a feature that favors an increase in CCR5 engagement by the availability of a larger number of CCL5 monomers in solution (11). F12Y and A13V mutations were introduced to eliminate a proteolytic process

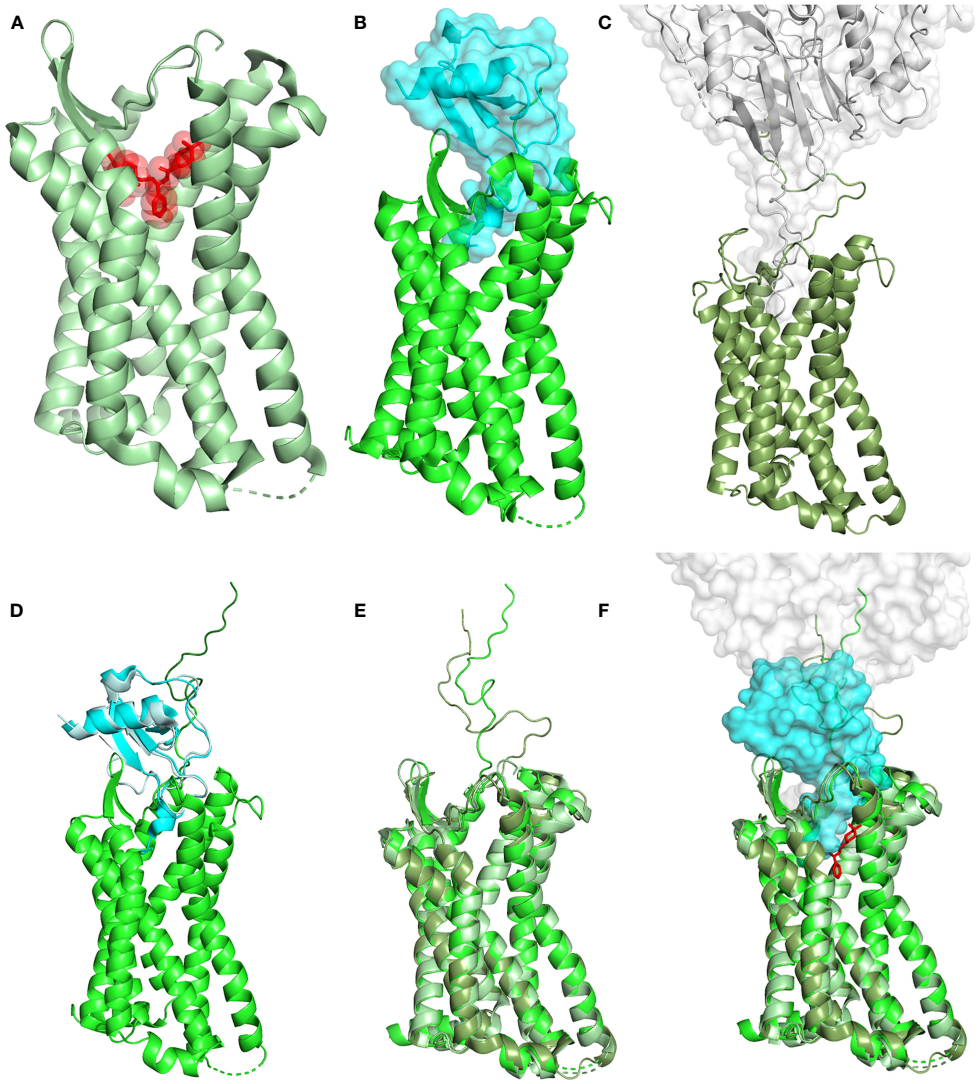

FIGURE 1 | Structural landscape of antagonist CCR5 occupancy. **(A)** Crystal structure of the CCR5 (ribbon, pale green) in complex with the HIV-1 entry inhibitor MVC (transparent surface and sticks, red) (PDB ID: 4MBS). **(B)** Crystal structure of CCR5 (ribbon, green) in complex with high potency HIV-1 entry inhibitor 5P7 CCL5 (transparent surface and ribbon, cyan) (PDB ID: 5UIW). **(C)** Cryo-EM structure of a full-length gp120 (transparent surface and ribbon, gray) in complex with unmodified human CCR5 (ribbon, smudge green) (PDB ID: 6MET). **(D)** Full-length CCR5 (green) (from 5UIW) with the N-terminus (dark green) modeled upon fusion of the first 15 AA from the N-terminal segment of CCR5 in complex with wild-type CCL5 (pale cyan) (PDB ID: 6FGP). CCL5 (from 6FGP) and 5P7 CCL5 (cyan) (from 5UIW) were superimposed to allow reliable modeling. **(E)** Superimposition of CCR5 (ribbon) from 4MBS, 6MET and the modeled full length (5UIW/6FGP); color code as in **(A–C)**. **(F)** Superimposition of **(A, B)**, using the full length CCR5 model in **(D, C)**. CCR5 (ribbon), MVC (sticks), 5P7 CCL5 and gp120 (transparent surface). Color code as in **(A–C)**. Structural representations were generated using PyMOL.

occurring during the production of the CCL5 variants in lactobacilli (23). These positions were also known to be crucial for CCR5 binding, particularly F12 (24, 25), yet both F12Y and A13V mutations led to an increase in anti-HIV-1 activity. Interestingly, the 12-14 AA stretch has been shown to be involved in CCL5 dimerization (22), and the F12Y and A13V might have influenced this feature, although this aspect has not been investigated and the 5P12 N-terminus leads to a monomeric CCL5 derivative (26). The Y27W and F28W mutations were introduced following consistent evidence of their improvement in anti-HIV-1 activity on small CCL5 peptide derivatives (27). The aggregated five mutations generated CCL5 5M, a CCR5 agonist with anti-HIV-1 activity comparable to 5P12 CCL5 and 6P4 CCL5 (11). Finally, the natural N-terminus of CCL5 5M was replaced with the 5P12 and 6P4 amino acid stretches (10), obtaining the CCR5 antagonist CCL5 5P12 5M and the superagonist CCL5 6P4 5M (11).

Retrospective Analysis of CCL5 5P12 5M Interaction With CCR5

The CCL5 5M derivatives have been generated in the absence of the 3D structural details of the CCL5:CCR5 interaction interface,

and were the result of previous findings derived from CCL5 short peptides with anti-HIV-1 activity (27). The crystal structure of CCR5 in complex with 5P7 CCL5 (16) was published after submission of the work on the CCL5 5M variants (11). We therefore propose here a retrospective analysis of CCL5 5P12 5M, modelled upon the available crystal structure of 5P7 CCL5: CCR5, and reveal the insights into the structural details of the previously reported mutations that led to the highly potent anti-HIV-1 activity (**Figure 2**). In order to fully analyze and understand the structural implications of the mutations introduced in CCL5 5P12 5M, we modeled the CCR5 N-terminus on the crystal structure of the complex with 5P7 CCL5. This was made possible by the availability of the NMR structure of the complex between CCL5 and the N-terminus of CCR5 in solution (17). By superimposing CCL5 from 6FGP on 5P7 CCL5 from 5UIW we could complete the missing portion of CCR5 N-terminus (**Figure 1D**). Of interest, Abayev et al. generated a full CCR5 model of the interaction with CCL5 (17).

The CCR5 antagonist 5P12 CCL5 has been selected as lead compound for drug development (28), and, as a follow up, 5P12 was the N-terminus adopted to convert CCL5 5M into an antagonist (11). However, among the CCL5 derivatives obtained in the phage display study (10), 5P7 CCL5 resulted as the most stable CCR5 ligand for crystallization (16). With the

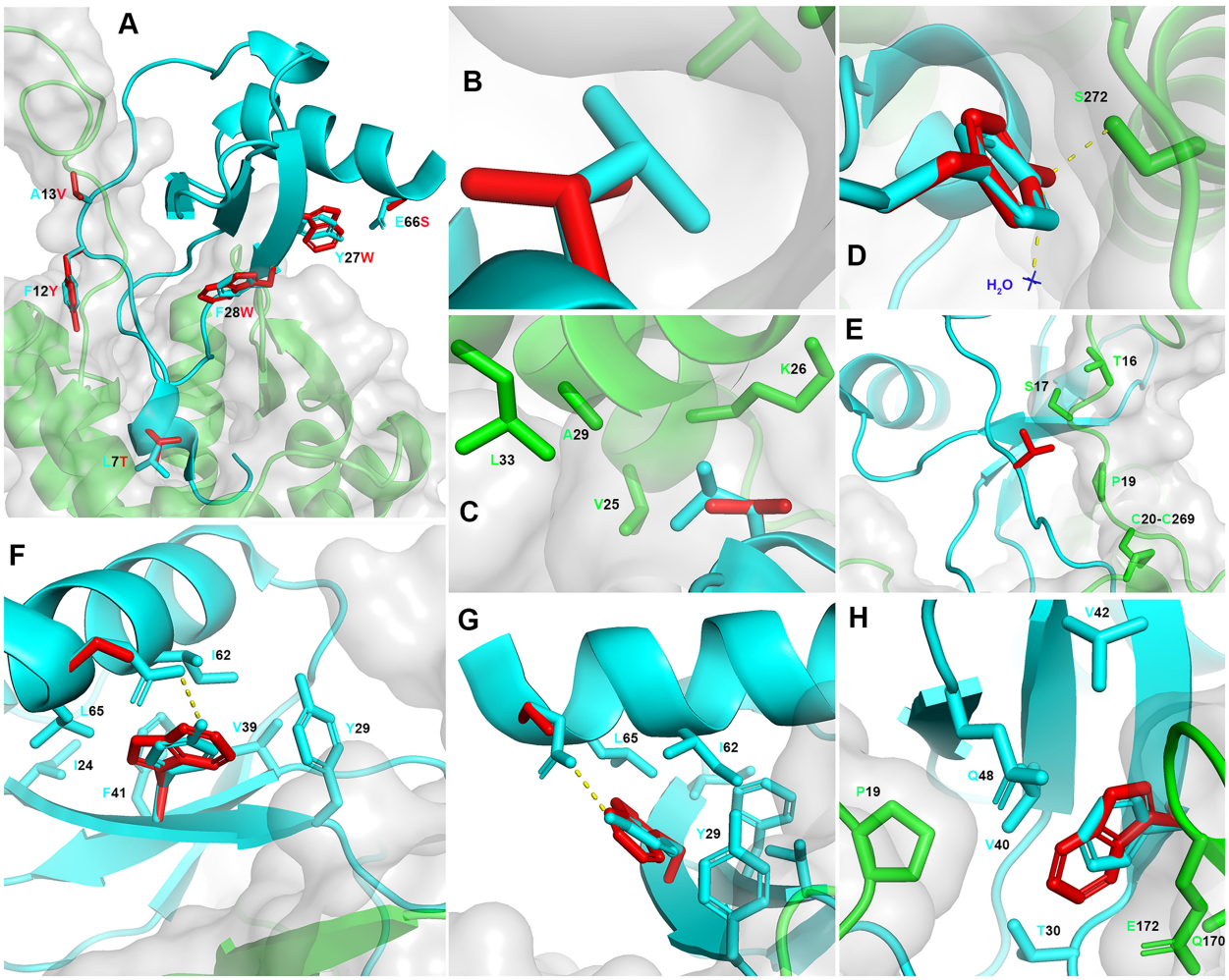

FIGURE 2 | Retrospective structural analysis of CCL5 5P12 5M. **(A)** Overview on CCL5 5P12 5M modeled on 5P7 CCL5 (ribbon, cyan) and complexed with the modeled full length CCR5 (grey transparent surface and ribbon, green). In red, the six CCL5 5P12 5M residue side chains (sticks) that differ from 5P7 CCL5 (original side chains in stick, cyan): L7T, F12Y, A13V, Y27W, F28W and E66S. **(B, C)** Higher occupancy by L7 (cyan) compared to T7 (red) and proximity of V25, K26, A29 and L33 (CCR5). **(D)** Similar occupancy by F12 (cyan) and Y12 (red), with two extra hydrogen bonds with one molecule of water (blue) and S272 (CCR5). **(E)** Larger hydrophobic volume by V13 (red) compared to A13 (cyan), packing to S17 (CCR5) and lock of the CRS1.5 site from the other side of P19 (CCR5). **(F, G)** Y27 and E66 (cyan) form an hydrogen bond, eliminated in W27 and S66 (red). Conversely, W27 occupies more space (partly provided by S66) and packs against Y29, I62 and L65 in the chemokine hydrophobic core and presents more distal packing with I24, V39 and F41, possibly stabilizing the protein fold. **(H)** Substitution of F28 (cyan) with W28 (red) appears to be conservative, yet with an enhancement of a dual role: increase surface of interaction with P19, Q170 and E172 (CCR5) and enhanced chemokine hydrophobic core packing by facing T30, V40, V42 and Q48. Structural representations were generated using PyMOL.

aim of inspecting the differences with 5P7 CCL5 in its interaction with CCR5, the 3D structural model of CCL5 5P12 5M was generated upon the 5UIW coordinates and complexed with the modeled full CCR5 (**Figure 2A**). The 5P7 and 5P12 N-termini differ from each other by a leucine or a threonine in position 7, respectively. Indeed, the CCR5 hydrophobic environment around CCL5 position 7 appears to be better filled by a leucine than a threonine (**Figures 2B, C**). In light of this evidence, we produced a 5P7 version of CCL5 5M (unpublished). F12 has long been considered a crucial residue for the interaction with CCR5 (24, 25), an evidence confirmed by the 3D structure of the 5P7 CCL5:CCR5 complex (16). Nevertheless, the analysis of the F12Y mutation reveals two possible supplementary hydrogen bonds made by the tyrosine hydroxyl group with CCR5 S272 and one water molecule present in the crystal of the complex (**Figure 2D**), likely accounting for the observed increase in anti-HIV-1 activity (11). Compared to wild type alanine, a valine in position 13 appears to pack better with CCR5 S17 and P19 (**Figure 2E**). Taking into consideration the conserved chemokine-receptor pattern of interaction (16), with three chemokine recognition sites (CRS1, CRS1.5 and CRS2), V13 forms a sort of lock from the other side of CRS1.5, defined by the packing of P19 (CCR5) with the chemokine disulfide bond (CCL5 C11-C50). The Y27W and E66S mutations appear to complement each other, with the elimination of the original hydrogen bond between E66 and Y27 replaced by a larger space available for the bulky W27 thanks to the short side chain of S66 (**Figures 2F, G**). Moreover, W27 packs very well within the chemokine hydrophobic core by contacting Y29, I62 and L65, and more distal with I24, V39 and F41. This should possibly stabilize the protein fold and provide an indirect contribution to CCR5 engagement. Interestingly, W27 as well as the original tyrosine do not interact with the receptor. Hence, both S66 and W27 promote higher anti-HIV-1 potency compared to their native counterparts (E66 and Y27) by indirect molecular mechanisms not involving CCR5 binding. Finally, the F28W mutation results in a higher occupancy of the CCR5 space as well as an enhanced hydrophobic core packing of the chemokine. W28 is surrounded by several AA on both sides: CCR5 provides P19, Q170 and the alkyl portion of E172 side chain, while the chemokine face presents T30, V40, V42 and Q48 (**Figure 2H**). The original F28 presents a similar packing, however the larger hydrophobic surface offered by a tryptophan residue justifies the observed increase in anti-HIV-1 potency (11), as this likely reflects a tighter binding to the receptor. Interestingly, CCR5 P19, a crucial component of CRS1.5 appears to be embraced by the longer hydrophobic arms of V13 and W28, as compared to the original A13 and F28. Mutations Y27W, F28W and E66S might also influence the capacity of CCL5 to oligomerize, particularly in the oligomeric form reported in Wang et al. (29). The bulky tryptophan residues lead to a most likely unfavorable condition for oligomerization, given the tight packing observed at the interface for oligomerization (PDB ID: 2L9H), where residues 27 and 28 interact very closely with each other both intramolecularly and intermolecularly (at the dimer of dimers interface).

Further inspection of the CCR5 binding cavity and the extensive occupancy by 5P7 CCL5 allowed the identification of at least two new positions amenable of amino acid substitution. These substitutions have been tested by the SWISS-MODEL generation of 3D models of the CCL5 mutants that indicated a likely further enhancement in receptor occupancy and therefore a possible increase in the potency of the resulting CCL5 mutants (unpublished).

The analysis presented here points to the possibility to improve CCL5 in its interaction with CCR5 by modifying residues distal to the chemokine N-terminus, either by direct receptor affinity increase or by stabilization of the chemokine hydrophobic core.

DISCUSSION AND PERSPECTIVES

Two recent reports shed light on CCR5 activation, with the 3D structure determination of CCL5:CCR5 and CCL3:CCR5 complexes in presence of the G_{i1} protein, as well as a constitutively activated CCR5 coupled to G_{i1}, and the 6P4 CCL5:CCR5 complex also in presence of G_{i1} (30, 31). Differences in CCR5 conformation between inactive and active states can now be appreciated, as well as the chemokine N-terminus determinants that lead to receptor activation, fully opening the area to rational drug design. It is assumed that the major determinant for the affinity towards chemokine receptors is provided by the core of the chemokine, while the N-terminus is responsible for receptor activation (30, 31), however this might not be entirely true for the highly modified N-terminus in CCL5 derivatives such as 5P7 and 6P4. Indeed, the agonist CCL5 5M derivative reached very potent anti-HIV-1 activity in absence of N-terminal modifications (11). An analysis similar to the one reported in **Figure 2** can be envisaged for the CCL5 6P4 5M derivative (11), with largely similar structural implications for the core mutations incorporated in CCL5 5M.

Once aiming at the use of CCL5 derivatives as therapeutics, an important aspect that needs to be considered is the possible loss of tolerance due to the insertion of mutated amino acids in the wild type chemokine. In certain therapeutic settings, this may result in limited efficacy due to elimination by antibodies directed against the modified chemokine. With the availability of the molecular details of the CCR5 complexes with 5P7 CCL5, CCL5 and 6P4 CCL5 (16, 30, 31), the development of a CCR5 antagonist based on a limited modification of CCL5 N-terminus might be conceivable. Complemented by sparse mutations in the chemokine core to improve receptor affinity, this might be a valid strategy to prevent loss of tolerance and create CCL5 variants that fall below the threshold of immune detection.

Changing perspective, away from the orthosteric site, yet remaining focused on the blockade of CCR5, allosteric antagonists have been developed for members of the GPCR

superfamily that engage the receptor from its intracellular side, directly preventing G protein coupling, or even laterally from within the cell membrane, freezing conformational rearrangements leading to accommodation of G proteins (32, 33). GPCR homo and hetero dimerization, as well as oligomerization, extends the complexity of pharmacological intervention (34).

Overall, several possibilities lie ahead in the future of CCR5 therapeutics and the present knowledge of the structural details of this important receptor should provide the platform for their development.

CONCLUSION

In conclusion, the elucidation of the fine structural details of the CCL5:CCR5 interaction, both in agonist and antagonist mode, allows the engineering of core-modified chemokine ligands that may surpass the available CCL5 derivatives in their potency and therapeutic concept. Moreover, new classes of CCR5 blockers may arise from the available 3D structural information and understanding of GPCR complexity.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

LV conceptualized and wrote the original draft of the manuscript. YA and LV prepared the models and figures. LV and YA reviewed and edited the manuscript. All authors contributed to the article and approved the submitted version.

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The Dual Role of CCR5 in the Course of Influenza Infection: Exploring Treatment Opportunities

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Influenza is one of the most relevant respiratory viruses to human health causing annual epidemics, and recurrent pandemics. Influenza disease is principally associated with inappropriate activation of the immune response. Chemokine receptor 5 (CCR5) and its cognate chemokines CCL3, CCL4 and CCL5 are rapidly induced upon influenza infection, contributing to leukocyte recruitment into the airways and a consequent effective antiviral response. Here we discuss the existing evidence for CCR5 role in the host immune responses to influenza virus. Complete absence of CCR5 in mice revealed the receptor's role in coping with influenza *via* the recruitment of early memory CD8+ T cells, B cell activation and later recruitment of activated CD4+ T cells. Moreover, CCR5 contributes to inflammatory resolution by enhancing alveolar macrophages survival and reprogramming macrophages to pro-resolving phenotypes. In contrast, CCR5 activation is associated with excessive recruitment of neutrophils, inflammatory monocytes, and NK cells in models of severe influenza pneumonia. The available data suggests that, while CCL5 can play a protective role in influenza infection, CCL3 may contribute to an overwhelming inflammatory process that can harm the lung tissue. In humans, the gene encoding CCR5 might contain a 32-base pair deletion, resulting in a truncated protein. While discordant data in literature regarding this CCR5 mutation and influenza severity, the association of CCR5delta32 and HIV resistance fostered the development of different CCR5 inhibitors, now being tested in lung inflammation therapy. The potential use of CCR5 inhibitors to modulate the inflammatory response in severe human influenza infections is to be addressed.

Keywords: influenza, chemokine receptor 5, CCR5delta32, CCL5, CCL3

INTRODUCTION

Aside from the onset of Corona Virus Disease 19 (COVID-19) pandemics in 2020, influenza virus is the most relevant respiratory virus for the healthcare system, causing millions of infections worldwide annually with estimates of up to 650 thousand deaths (1, 2). Although it is unpredictable when and to which extent the circulation of influenza among humans will return to pre-COVID-19 levels, the threat is perpetual due to the high genetic variability of the virus and the existence of multiple reservoirs (3). Influenza virus belongs to the Orthomixoviridae family of segmented, negative sense, single stranded RNA viruses (4). The infection of host respiratory epithelial cells occurs through the recognition of glycoconjugates with terminal N-acetylneuraminic acid (sialic acid) in the cell membrane by the viral protein hemagglutinin (HA). The multivalent attachment to sialic acid structures triggers the endocytosis of the virus (5). Influenza A and B are the most medically relevant types among the family causing annual epidemics, whereas only Influenza A might also give rise to pandemics such as the 1918 Spanish Flu and 2009 Swine Flu, both caused by H1N1 strains subtype, that occasioned more than 50 million and 363 thousand deaths respectively (6, 7). Some influenza A avian subtypes, including H5N1 and H7N9, are highly pathogenic to humans and, although human-to-human transmission is still limited, they have been closely monitored as potential new pandemic strains (8). Despite antivirals and vaccine availability, the emergence of pandemic strains is an imminent threat due to the high genetic variability of the virus, the ability to infect birds and swine that act as reservoirs, and a decreased population immunity to new strains (9–12). Therefore, comprehending the disease mechanisms involved in respiratory virus infections and continuous viral surveillance are badly needed as they set the basis for new therapeutics.

Dysfunctional inflammation triggered by influenza infection is related to the clinical manifestations and is orchestrated by different mediators (e.g: leukotrienes, cytokines and chemokines) and cell types (e.g: leukocytes, epithelial and endothelial cells) (13). However, a regulated well-controlled response ensures a proper viral clearance with restoration to tissue homeostasis. Therefore, inflammation has a dual role during influenza infection and disease. Although the chemokine receptor CCR5 does not actively participate in the infection process of influenza, after its activation by the chemokines CCL3/MIP-1α, CCL4/MIP-1β, and CCL5/RANTES, it becomes a key player in the inflammatory milieu that contributes to infection restraint. However, it might also be associated with inflammatory bystander lung damage. Here we discuss this duality of CCR5 activation during influenza infection.

Abbreviations: CCR5, Chemokine receptor 5; CCL3, C-C Motif Chemokine Ligand 3; CCL4, C-C Motif Chemokine Ligand 4; CCL5, C-C Motif Chemokine Ligand 5; COVID-19, Corona Virus Disease 19; BALF, the bronchoalveolar lavage fluid; hAECII, human alveolar epithelial cells; SARS-CoV-2, Severe Acute Respiratory Syndrome Virus-2; NK, Natural Killer; ACKR2, Atypical Chemokine Receptor 2; HIV, Human immunodeficiency virus; SAMHD1, SAM domain and HD domain-containing protein 1; COPD, chronic obstructive pulmonary disease, AF, allele frequency, CXCR4, C-X-C Motif Chemokine Receptor 4; AIDS, Acquired Immune Deficiency Syndrome.

EXPRESSION OF CCR5 AND CCR5 LIGANDS UPON INFLUENZA INFECTION

One of the first reactions of the host after influenza infection is the production of CCR5 ligands by lung resident cells, especially alveolar macrophages and epithelial cells (14–16). CCL5 can be detected in human bronchoalveolar lavage fluid (BALF) samples after 7 days of symptoms onset (17). *In vitro* infection of type 2 human alveolar epithelial cells (hAECII) with either H1N1 or H5N1 virus leads to a significant production of CCL5 by those cells, showing that they may be a principal source of CCL5 during influenza pneumonia. In addition, human alveolar macrophages exposed to both H1N1 and H5N1 viruses produced CCL5. Interestingly, the infection with the H5N1 virus, a more pathogenic subtype, led to stronger CCL5 production in both cell types (15, 18). CCL3 and CCL4, the other CCR5 ligands, are also expressed in response to experimental influenza infection in human volunteers (19). A study of over 15 critically ill patients showed that CCL3 is augmented in lung aspirates of patients, and notably, at the serum level, there was an increment of CCL3 and CCL4 in comparison with mild cases of influenza infection (20). In addition, there is recent evidence showing, at the mRNA level, that peripheral blood monocytes derived from hospitalized patients diagnosed with influenza A or Severe Acute Respiratory Syndrome Virus-2(SARS-CoV-2) infection overexpress CCL3 (21).

In murine models, all CCR5 ligands are produced in lung tissue in response to influenza infection (22, 23). This contributes to the acute recruitment of leukocytes from the innate immunity to the lungs, mainly inflammatory monocytes and neutrophils, but also NK cells, which can induce CCR5 expression in response to the infection (24, 25). The latter recruitment of cells from adaptive immunity is also mediated by CCR5. Indeed, effector cytotoxic Th1 lymphocytes, memory CD8 T cells, and also B lymphocytes express CCR5. Moreover, there is evidence pointing that CCL5:CCR5 interaction contributes to the formation of inducible bronchus-associated lymphoid tissue iBALT in mice (14, 23, 26).

THE ROLE FOR CCR5 IN INFLAMMATION AND IMMUNITY TO INFLUENZA VIRUS

The immune responses that follow influenza infection are crucial to control virus proliferation and for the development of memory responses; however, uncontrolled, or exaggerated activation of the many components of the immune system is associated with severe pulmonary damage and contributes to flu mortality (13). Thus, the immune responses must be finely regulated and coordinated to ensure viral clearance and restoration of lung homeostasis, with minimum bystander damage. As part of the host immune circuits for resistance to infection, CCR5 mediates the recruitment and activation of leukocytes during influenza. Interestingly, CCR5 plays contrasting roles in different inflammatory and infectious

diseases leading to protection against certain pathogens or immunopathology triggered by exacerbated inflammation (27, 28). In this regard, CCR5 activation during different phases of influenza infection might also lead to different outcomes. Indeed, CCR5 activation during the initial stages of influenza infection ensure the proper recruitment of leukocytes and activation of antiviral pathways in the epithelial cells (**Figure 1** left panel) (14, 23, 29, 30). However, sustained or exaggerated CCR5 activation during severe/exacerbated influenza infection might fuel the inflammatory responses leading to increase pulmonary damage and dysfunction (**Figure 1** right panel). The cellular expression of CCR5 dictates what cell type can be recruited by mediators such as CCL3, CCL4 and CCL5, the classical CCR5-associated chemokines.

CCR5 and its cognate chemokines are rapidly induced post-influenza infection in both humand and mice and ensure the prompt recruitment of leukocytes to the airways for an effective response (31). Indeed, the development and use of CCR5 knockout mice shed light on the mechanistic role for CCR5 mediating host protection to influenza. CCR5 deficient mice are highly susceptible to influenza infections and present increased neutrophilic inflammation and lung dysfunction in comparison to wild type mice (23). During influenza infection in mice, neutrophil expression of CCR5 is significantly increased and promotes different ex vivo cell functions (25). Whether CCR5 signaling *in vivo* directly regulates neutrophil activation or recruitment during influenza is yet to be explored; nevertheless, CCL5:CCR5 was shown to promote reprogramming of murine macrophages to pro-resolving phenotypes contributing to resolution of inflammation (32). In addition, CCR5:CCL5 was shown to prevent virus-induced apoptosis of human and mouse macrophages during influenza infection (33). Alveolar macrophages are crucial cells for viral and apoptotic cell clearance during infections preventing further unnecessary inflammatory responses in the lungs and tissue damage (34). Therefore, CCR5 signaling aids to the regulation of macrophage regulatory responses to guarantee restoration of tissue homeostasis during influenza infections.

NK cells are also recruited by CCR5 (24) and play a role in immunity to influenza infections in humans and mice (35, 36). NK cells can interact with influenza-infected cells and with the virus itself leading to secretion of cytokines and cytotoxic granules that restrain viral replication within the early stages of infection (36, 37). On the other hand, influenza virus can directly impair NK function to evade this innate layer of host immunity (38) and exaggerated NK cell activation, rather than being protective, might contribute to lung damage during severe influenza infections (39). Whether CCR5 activation transduces a protective or pathological NK cell response during influenza is yet to be determined. In parallel, the recruitment and activation

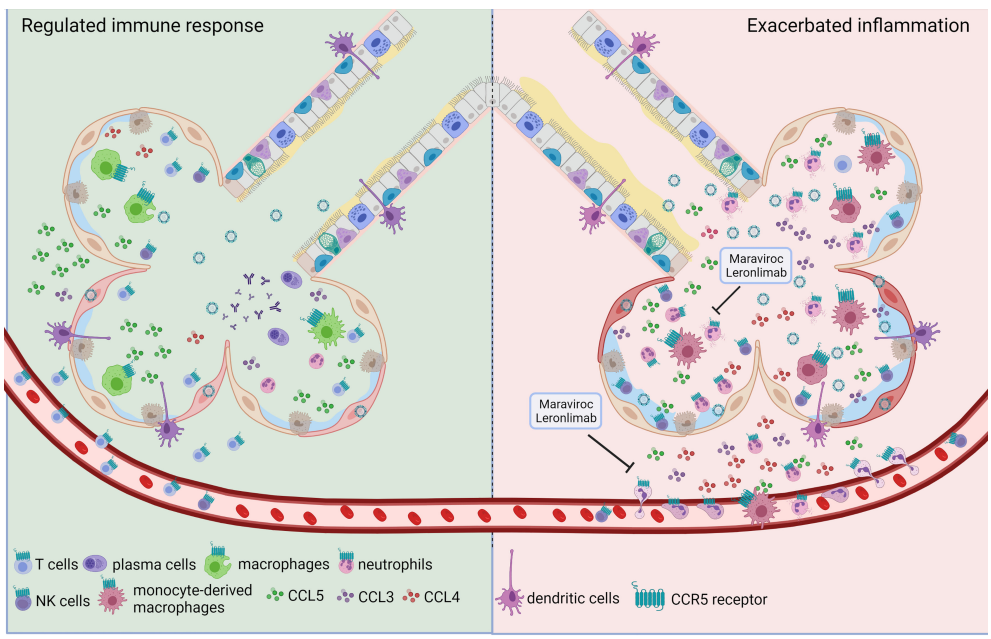

FIGURE 1 | The dual role of CCR5 during influenza infection. Triggered by influenza infection, one of the first reactions of epithelial cells and resident alveolar macrophages is the production of CCR5 ligands. CCL5:CCR5 interaction is necessary for the development of a proper immune response (left side) to restrain viral expansion since it favors resident macrophages survival, promotes reprogramming of macrophages to pro-resolving phenotypes, mediates the recruitment of T lymphocytes and the establishment of iBALT contributing to immunological memory. However, uncontrolled activation of many components of the immune system after influenza infection is associated with severe pulmonary damage (right side). In this scenario, increased recruitment of neutrophils, inflammatory monocytes and natural killer cells can be mediated by CCR5 expression on those cells, and the actual evidence shows that CCL3 may be related to this exacerbated response. In this situation CCR5 inhibition by Maraviroc or Leronlimab, might represent an interesting therapeutic alternative.

of γδ T cells, mainly by CCR5, are important components of the potent antiviral responses to influenza infection in humans (40, 41).

In addition to the above mentioned role in innate immunity to influenza, CCR5 signaling is also necessary for the recruitment and effective response of the components of the adaptive immune system (42). Indeed, the increased pathology and lung dysfunction of CCR5 deficient mice are associated with decreased recruitment of CD8+ T cells during infection (23). Moreover, CCR5 was shown to be important for the development of early CD8+ T cell memory leading to control of virus replication during a secondary infectious challenge in mice (14). Furthermore, CCR5 might also impact B cell activation and recruitment during influenza. Secretion of CCL3 and CCL4 by B cells can lead to the recruitment of activated follicular CCR5+ CD4+ T cells, which enhances interaction between these two cell types and improves humoral immunity (43). Akin with that, mice lacking the CCL5 scavenger Atypical Chemokine Receptor 2 (ACKR2) present increased CCL5 levels, CCR5+CD4+ lymphocyte recruitment to the airways and augmented levels of IgA in the BALF during influenza. The specific phenotype of the T CD4+ recruited *via* CCR5 during influenza is yet to be defined (23). Noteworthy, the antagonism of CCR5 using maraviroc has not impaired the humoral response of HIV patients to the 2009 pandemic influenza A-H1N1 adjuvanted vaccine (44).

Pulmonary epithelial cells, in addition to the leukocytes, are important players driving antiviral responses to influenza (29). More recently, the direct role of CCL5:CCR5 in epithelial antiviral responses was uncovered. CCL5:CCR5 was shown to reduce influenza A replication in human epithelial cells by inducting the antiviral restriction factor SAM domain and HD domain-containing protein 1 (SAMHD1) (30). Keeping with that, the CCR5 agonist gp120 was shown to reduce A(H1N1) pdm09 replication *in vitro* in an IFITM3-dependent manner in human macrophages and human epithelial cervical cancer (HeLa) cells (45). Therefore, CCR5 signaling can impact the antiviral responses mediated by both epithelial and immune cells and, this should be taken into consideration when developing therapeutic strategies targeting this receptor for other inflammatory diseases. Interestingly, a recent study provided strong evidence for the protective role of CCR5 antagonism during Chronic Obstructive Pulmonary Disease (COPD) exacerbations caused by influenza in which CCL3 levels but no CCL5, correlated with an exacerbated inflammatory process (46). Maraviroc treatment during COPD viral exacerbations protected mice from the lethal pulmonary inflammation without affecting viral replication (46). In this regard, understanding the response to the virus and distinguishing between harmful and protective inflammation is crucial. The most relevant findings regarding CCR5 role during influenza infection are summarized on **Table 1**.

CCR5DELTA32 AND DISEASE SEVERITY

The gene encoding CCR5 might contain a 32 base pair deletion within the exon 3 resulting in a truncated protein that cannot be expressed on cell surface and therefore is non-functional (47). This deletion is present at different frequencies on populations around the world, which is related to ancestry. Whereas the allele frequency (AF) of the deletion is more than 15% in some European countries like Norway, Estonia and Latvia, some Asian and African countries present CCR5delta32 AF lower than 1% (48). Delta32 deletion was discovered in individuals multiply-exposed to HIV that were resistant to the infection and carried two alleles of CCR5-delta32 (49). This resistance was observed in CCR5-tropic HIV strains which depend on CCR5 as a co-receptor for cell entry. This process is avoided when a non-functional CCR5 is present in every cell on CCR5-delta32

TABLE 1 | Evidence over CCR5 role on the immune response to influenza virus.

Strategy	Influenza strain	Model	Findings
CCR5 Knockout	A/WS/SS H1N1	Mouse	CCR5 KO and CCL5 KO have higher mortality and increased apoptosis of macrophages at day 9 post-infection (33).
Anti-CCR5 specific antibody	A/WS/SS H1N1	Human macrophages	CCR5 blockage increases the proportion of apoptotic macrophages post-influenza infection *in vitro* (33).
CCR5 knockout	A/HK-x31 H3N2 and A/ Puerto Rico/8/1934 H1N1	Mouse	CCR5 knockout mice have impaired induction of T $CD8^+$ memory cells post-influenza infection and increased viral titers in a secondary viral challenge (14).
Maraviroc	2009 pandemic influenza AH1N1v	HIV patients	Pharmacological blockage of CCR5 does not impact antibody responses triggered by vaccination (44).
CCR5 knockout	A/Puerto Rico/8/1934 H1N1	Mouse	CCR5 knockout mice have diminished numbers of NK cells in the bone marrow, post-infection (35).
HIV glycoprotein gp120	A(H1N1)pdm09	Human epithelial cervical cancer (HeLa) cells	Gp120 acts as an agonist for CCR5 and inhibits influenza replication in HeLa cells (45).
CCR5 Knockout	A/WSN/33 H1N1	Mouse	CCR5 KO mice present increased pulmonary neutrophilic inflammation and damage, and reduced T $CD8^+$ lymphocyte recruitment during influenza infection. (23)
CCR5 agonism (CCL5)	A/Switzerland/9715293/ 2013 H3N2	Human epithelial cell line (A549)	CCL5 binding to CCR5 increases SAMHD1 and prevents viral replication and epithelial cell death *in vitro* (30).
Maraviroc	A/Puerto Rico/8/1934 H1N1	Mouse model of influenza-induced COPD exacerbation	Pharmacological blockage of CCR5 reduced lethality, neutrophilic inflammation, pulmonary damage without affecting viral titers (46).

homozygousity and act as a dominant-negative on the expression of wild type CCR5 and also C-X-C Motif Chemokine Receptor 4 (CXCR4), the other co-receptor of HIV (50).

While CCR5-delta32 homozygosis confers protection to HIV, meta-analysis have shown that HIV susceptibility or perinatal infection are not affected by CCR5-delta32 heterozygosity (51–53). Upon this findings on HIV resistance, CCR5 blockers or antagonists started to be tested against Acquired Immune Deficiency Syndrome (AIDS) and currently the CCR5 antagonist Maraviroc is clinically used (54). Moreover, a patient with acute myeloid leukemia and HIV had the infection controlled by the transplant of stem cells from a homozygous delta32 donor (55).

Besides HIV, CCR5delta32 has been associated with susceptibility (56, 57) or protection to different diseases, including COVID-19 (58–60). Regarding influenza, discordant data are present in literature. After the 2009 H1N1 pandemics, studies on distinct populations evaluated the CCR5delta32 allele frequencies on influenza patients with different outcomes. The first published study, assessing only 20 cases in Canada, found that the CCR5delta32 was a risk factor for the severity of H1N1 infection in white patients (61). In 2013 one Spanish study comparing a mild and a fatal case of the pandemic H1N1 infection found that the fatal case was homozygous for the CCR5Δ32 allele (62). Another Spanish study from 2015, assessing a larger population of 171 influenza patients found a correlation of CCR5Δ32 and mortality (63). On the other hand, three studies, one with 29 European (mostly Italian), other with 330 Brazilians and another with 432 Brazilian influenza patients with different clinical manifestations found no association between CCR5Δ32 and H1N1 severity (64–66). The conflict data might be explained by the global distribution of CCR5Δ32 allele. CCR5delta32 AF in countries where associations with influenza outcomes were found – Canada and Spain – are higher (8.1% and 7%, respectively) than in countries where no association was found – Italy (6.27%) and Brazil (4-5.44%) (48, 67).

CCR5 AS POTENTIAL TARGET TO MODULATE INFLAMMATION IN LUNG

Severe pneumonia following viral infection is principally associated with an overwhelmed production of inflammatory mediators and leukocyte recruitment to lung tissue. For that reason, chemokine receptors are interesting therapeutic candidates for lung inflammation. As aforementioned, CCR5 contribution during influenza infection appears to be crucial for the development of an antiviral response and the proper induction of immunologic memory. On the other hand, CCR5 activation is associated with excessive recruitment of neutrophils, inflammatory monocytes and NK cells in models of severe influenza pneumonia (24, 46, 68). This dual role of a chemokine receptor in the context of lung diseases is not an exclusive characteristic of CCR5 (69). Currently, the information obtained by the use of animal models suggests that while some CCR5 ligands, like CCL5, can play a protective role in influenza infection (23, 33) others, like CCL3, may contribute to an overwhelming inflammatory process which can harm the lung tissue (46, 70). Many pharmacological strategies that aim to impair CCR5 activity and endocytosis have been developed to fight HIV infection and were already tested in humans showing good safety profiles and effective antagonism properties. Nowadays, repositioning strategies based on the well-established CCR5-inhibitory capacities of drugs like Maraviroc, the only CCR5 inhibitor approved for clinical use, and Leronlimab, a CCR5-specific human IgG4 monoclonal antibody, succeed at presenting a good anti-inflammatory performance in the context of lung inflammatory conditions. Indeed, it was recently published that Leronlimab treatment reduced plasma IL-6 and viral load in critical COVID-19 patients (71). Besides, new CCR5 antagonists like cenicriviroc, which also present CCR2 inhibition, and GRL-117C, arise opportunities for the discovery of novel anti-inflammatory treatments focusing on CCR5 in the near future (72, 73). Currently, there is no disclosed clinical trial attempting to assess whether CCR5 antagonism can improve patient outcome during severe influenza pneumonia. However, as the current COVID-19 pandemics brought up challenging times while also emphazised a pre-existing demand for novel treatments to control the inflammatory response in the lungs, at least five clinical trials are being conducted to study CCR5 as potential drug target to treat lung inflammation during SARS-CoV-2 infection (NCT04441385, NCT04475991, NCT04710199, NCT04901676 NCT04901689). Either by CCR5 antagonism with Maraviroc or by its blockage with Leronlimab, these trials attempt to control the excessive inflammatory response by decreasing leukocyte accumulation in the lungs and inflammatory mediators in plasma of COVID-19 patients which is expected to improve patients outcome. By the moment, four of these clinical trials are on recruiting phase and the only completed study has no posted results yet (NCT04710199).

CONCLUSIONS

CCR5 plays important roles during influenza infection (**Figure 1**) by contributing to a suitable immune response *via* CCL5 to cope with the viral infection, but also subsidizing excessive inflammation and tissue damage by mechanisms associated with increased CCL3 production. This ambivalent character of CCR5 on influenza infection is not unique to this chemokine receptor but observed for many others in the dispute between pathological lung inflammation and restoration of physiological state. Thus, the correct use of CCR5 inhibitors as potential anti-inflammatory drugs in severe influenza infections requires a profound knowledge of the different phases in the inflammatory processes to be modulated.

AUTHOR CONTRIBUTIONS

MF, LT, and CG contributed to the conception and design of the study; wrote the manuscript and discussed the content. All

authors contributed to the article and approved the submitted version.

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REVIEW
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Targeting CCR5 as a Component of an HIV-1 Therapeutic Strategy

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Globally, human immunodeficiency virus type 1 (HIV-1) infection is a major health burden for which successful therapeutic options are still being investigated. Challenges facing current drugs that are part of the established life-long antiretroviral therapy (ART) include toxicity, development of drug resistant HIV-1 strains, the cost of treatment, and the inability to eradicate the provirus from infected cells. For these reasons, novel anti-HIV-1 therapeutics that can prevent or eliminate disease progression including the onset of the acquired immunodeficiency syndrome (AIDS) are needed. While development of HIV-1 vaccination has also been challenging, recent advancements demonstrate that infection of HIV-1-susceptible cells can be prevented in individuals living with HIV-1, by targeting C-C chemokine receptor type 5 (CCR5). CCR5 serves many functions in the human immune response and is a co-receptor utilized by HIV-1 for entry into immune cells. Therapeutics targeting CCR5 generally involve gene editing techniques including CRISPR, CCR5 blockade using antibodies or antagonists, or combinations of both. Here we review the efficacy of these approaches and discuss the potential of their use in the clinic as novel ART-independent therapies for HIV-1 infection.

Keywords: antiretroviral drugs, CCR5Δ32, CCR5 monoclonal antibodies, CCR5 small molecule inhibitors, HIV-1 drug resistance, zinc finger nucleases, TALENs, combination therapy

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1 INTRODUCTION

Human immunodeficiency virus type 1 (HIV-1) infection has been a global health problem for over 30 years, affecting more than 37 million people worldwide today (1). The search for a cure is challenged by the inter- and intra-patient diversity of HIV-1 as well as the establishment of latently infected cellular reservoirs that can remain latent for many years (2–8). The advent of antiretroviral therapy (ART), which consists of drugs that inhibit viral replication by targeting different HIV-1 proteins, has enabled control and prevention of newly infected cells. However, ART does not target latently infected cells since they are not actively transcribing HIV-1 genes nor does it resolve many of the immune dysfunctions caused by HIV-1 infection (7, 8). Cessation of ART thus leads to viral rebound or a return to uncontrolled viral replication in the HIV-1-infected individual, an outcome currently only avoided by life-long ART adherence. Due to this as well as the cost, side effects, and possibility of ART-resistant HIV-1 strains emerging, there is a need for novel therapeutics that can more efficiently allow long-term control of HIV-1 infection (7, 8). A therapeutic that can additionally prevent ongoing establishment of latent HIV-1 reservoirs would also make a cure more feasible.

A hope for an HIV-1 cure transpired with news of the Berlin patient, Timothy Ray Brown. Brown was an HIV-1-positive individual who received an allogeneic hematopoietic stem cell transplant as a treatment for relapsed leukemia. The transplant caused his HIV-1 viral load to decrease to undetectable limits (8, 9). The reason for this was found to be that the stem cell donor was homozygous for C-C chemokine receptor type 5 (CCR5) Δ32. This 32-base pair deletion in the CCR5 allele provides a mutation for the CCR5 gene, which encodes the CCR5 that is used as a co-receptor by HIV-1 for attachment and entry into the host cell (8, 9). More recently, another HIV-1 individual, Adam Castillejo, underwent a similar but less toxic version of allogeneic hematopoietic stem cell transplant from a homozygous CCR5Δ32 donor. Thirty months after analytical treatment interruption, the London patient, as he has been designated, has no detectable viral load in any of the examined regions including the peripheral blood, intestinal tissue, CSF, and lymph nodes. This led the authors to conclude that this patient represents a model for HIV-1 cure (10, 11). Targeting of the CCR5 receptor to render host cells less susceptible to infection or possibly resistant to infection may allow for more efficient inhibition of HIV-1 infection, in particular if combined with other anti-HIV-1 approaches.

The cases of the Berlin and London patients led many researchers to investigate other feasible methods for targeting CCR5 and their potential to serve as an HIV-1 cure for many other patients. Studies have investigated the inhibition of extracellular CCR5, through small molecule inhibitors or monoclonal antibodies, as well as the prevention of CCR5 expression, through gene editing techniques such as RNA interference, Transcription Activator-Like Effector Nucleases (TALENS), Zinc Finger Nucleases (ZFN), and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR). Recently, CRISPR has gained more interest as efficacy and lack of off-target effects (edits in other regions of the genome) allowed for a more convenient and sustained prevention of CCR5 expression, providing some benefits over other therapies targeting extracellular CCR5.

However, as a chemokine receptor with important roles in inflammatory signaling pathways, CCR5 is expressed on various immune cell types in addition to CD4+ T cells, the primary host cell targets of HIV-1 (8). While there remain challenges in determining long-term efficacy and safety of CCR5 targeting, investigational studies demonstrated some clinical success in suppressing HIV-1 infection. In this review, we highlight the biological functions of CCR5, summarize methods investigated for ablation of CCR5 in these studies, and evaluate the potential of their approaches to serve as a therapeutic for an HIV-1 cure.

2 EXPRESSION AND FUNCTION OF THE CCR5 RECEPTOR ON WHITE BLOOD CELLS

2.1 Function and Prevalence on Immune Cell Populations

CCR5 is an integral membrane protein expressed on various white blood cells (leukocytes) including cells of the monocytic lineage. When expressed on leukocytes, CCR5 serves as a receptor for inflammatory β-chemokines, which are produced by nearly every cell type during infection or injury and signal through G protein–coupled receptors (GPCRs). The chemokine ligands of CCR5 include Regulated on Activation, normal T-Expressed and Secreted (RANTES; CCL5), Macrophage-Inflammatory Protein-1α (MIP-1α; CCL3), and MIP-1β (CCL4). CCR5 is expressed on macrophages, Dendritic Cells (DCs), and Natural Killer (NK) cells, which are cells of the innate immune response, as well as on T and B cells of the adaptive immune response (12). Expression of CCR5 and chemokine binding exert downstream effects in a cell type-specific manner, which ultimately coordinate the migration of activated leukocytes, lead to secretion of pro-inflammatory cytokines, and stimulate cells of the innate and adaptive immune response (**Figure 1**).

Macrophages are a critical part of the innate immune response that recognize foreign pathogens and secrete antiviral cytokines and type I interferons (IFN-α and IFN-β), which inhibit viral replication by stimulating expression of interferon-stimulated genes (ISGs) that induce an antiviral state within the cell (13). A more immediate response is triggered by activation of CCR5, which was shown to induce expression of inflammatory genes iNOS, COX-2 and IL-1β through activation of nuclear factor kB (NF-κB) and secondary pathways *via* MAPKs ERK, JNK and p38 (13). Additionally, secretion of proinflammatory cytokines TNF-α, IL-1, and IL-6 can also occur. These trigger apoptosis, activation of NK cells, and activation and differentiation of T and B cells, respectively (13). Furthermore, β-chemokine binding to CCR5 is required for directed migration of macrophages (14, 15). This was demonstrated using a mouse model of hepatotoxicity, in which a CCR5 knockout decreased infiltration of macrophages to sites of damage, with production of TNF-α, and iNOs synthesis (16). Together, CCR5 expression on macrophages is an important component of the innate immune system for nitric oxide (NO) production, prostaglandins production, production of proinflammatory cytokines, and activation and coordination of both the innate and adaptive immune response.

For DCs, another cellular derivative of the monocytic lineage, CCR5 is involved in their cell migration to the lymph nodes and subsequent stimulation of naïve T cell differentiation into effector T cells in response to the chemokine CCL4 (17). Activated dendritic cells activate specialized T helper cells and NK cells and induce IFN-γ secretion by IL-12 synthesis and secretion in a CCR5-dependent manner (18, 19). Knockout of CCR5 and treatment with anti-CCL4 antibodies in mice was found to significantly reduce, but not completely abrogate, mobilization of DC precursors into the circulation in response to bacterial infection (20). Consequentially, the monocytic lineage plays a key role in the host defense against pathogens as well as immune regulation among other processes, which reflects the potentially integral function of CCR5 in these diverse processes (21).

NK cells, lymphocytes of the innate immune response, secrete IFN-γ to stimulate macrophages and increase expression of MHC II and chemokines to coordinate antigen-specific CD4+ and CD8+ T cells. NK cells also express CCR5 which is necessary for the control of NK cell trafficking in response to infection and

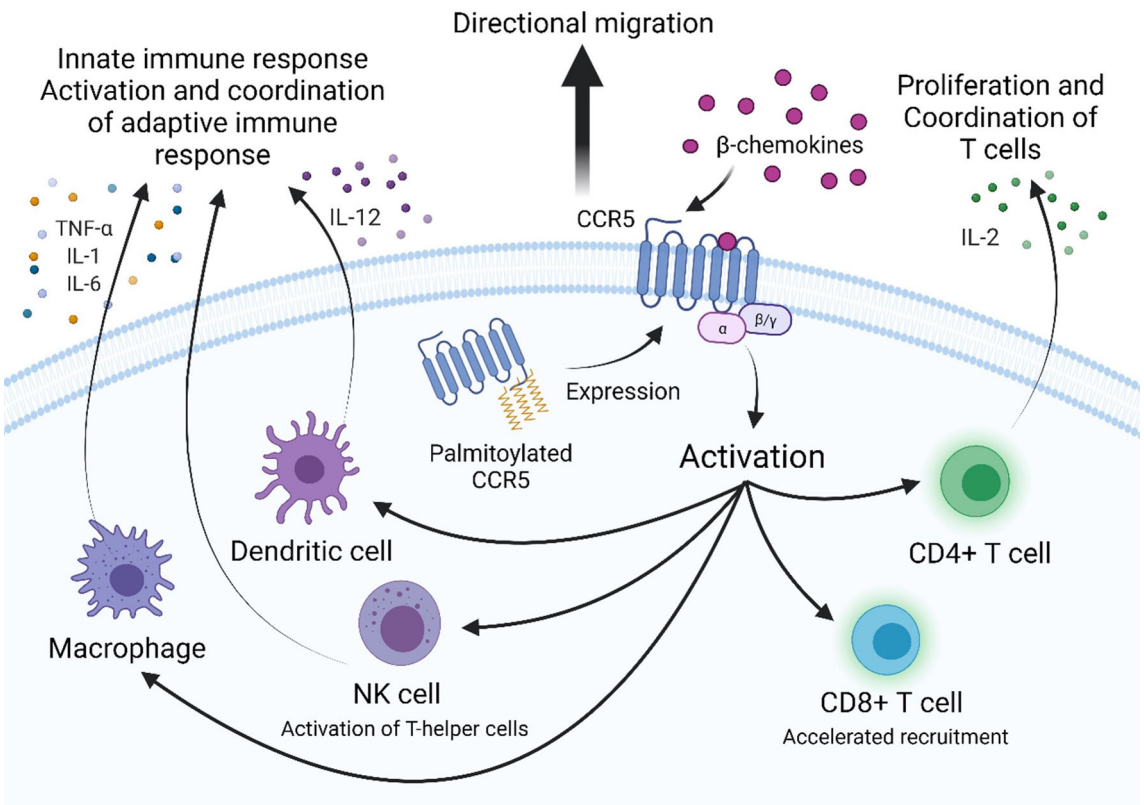

FIGURE 1 | CCR5 is a G-protein coupled receptor that is involved in activation and coordination of the innate and adaptive immune response. Palmitoylation of multiple cysteine residues in the C-terminal domain target CCR5 to lipid rafts in the plasma membrane to participate in extracellular signaling. β-chemokines bind to extracellular domains of CCR5, activating it and inducing downstream signaling. CCR5 expression is required for directional migration and coordination of cells of the innate and adaptive immune response along a chemotactic gradient to sites of infection. CCR5-dependent secretion of pro-inflammatory cytokines by macrophages (TNF-α, IL-1, and IL-6) and dendritic cells (IL-12) activate the adaptive immune response. CCR5-dependent secretion of IL-2 by activated CD4+ T cells induces proliferation and activation of effector, memory and regulatory T cells. CCR5 is required for the accelerated recruitment of effector and memory CD8+ T cells to sites of infection.

coordination of the immune response (22). A study of influenza infection demonstrated that CCR5-deficient mice were more susceptible to infection and exhibited lower levels of NK cells trafficked to sites of viral infection (23). In the adaptive immune response, CD4+ and CD8+ T cells acquire CCR5 during the activation process. In peripheral blood, CCR5 is expressed on circulating memory CD4+ T cells, while in the thymus CCR5 is not expressed on $CD3^-$ immature thymocytes (24–26). Similar to innate immune cells, chemokines coordinate T cell migration into lymph nodes and inflamed tissues. Activated CD4+ T cells orchestrate the immune response by secretion of IL-2, the T cell growth factor, which upregulates CD25 expression, a necessary step in activating and inducing proliferation of effector and memory T cells as well as regulatory T cells. This function is dependent on chemokine stimulated CCR5 intracellular Ca^{2+} transactivation of NFAT and subsequent IL-2 expression, which has been studied in CCR5-deficient mice, biologically relevant cell lines, and primary human T cells (27). Functional expression of CCR5 on antigen-specific memory and effector CD8+ T cells in response to β-chemokines has also been characterized. CCR5 is suppressed during differentiation of CD8+ thymocyte to naïve CD8+ T cells and to resting memory CD8+ T cells but expressed after differentiation to memory CD8+ T cells. CCR5-, but not CXCR3-deficient mice confirm that surface expression of CCR5 is required for the accelerated recruitment of CD8+ T cells to sites of respiratory viral infection to deliver cytotoxic IFN-γ (24, 28). In an LCMV infection of CCR5- and CXCR- deficient mice, CD8+ T cells were still able to infiltrate the CNS, but with a delay, and interestingly augmented generation of effector CD8+ T cells, supporting the thought that the cells can still migrate but not in an accelerated manner (29). The effector and memory CD8+ cells use CCR5 to follow a chemotactic gradient and exert their effect as well as contribute to controlled proliferation and activation. The diverse functions of CCR5 thus help mobilize and orchestrate the inflammatory response which is a necessary process that allows both the innate and adaptive immune system to protect the host against invading pathogens (**Figure 1**).

2.2 Structure and Transcriptional Regulation of Expression

Chemokine receptors are a family of seven transmembrane-spanning GPCRs of which the structure is conserved and

characterized by a N-terminal extracellular region and C-terminal cytoplasmic region as well as seven α-helical hydrophobic membrane spanning domains, and three extracellular (ECL1-3) and intracellular (ICL1-3) loop segments (30). Several conserved amino acids in the extracellular regions compose the active site of CCR5, which is the site of ligand recognition and plays a major role for HIV-1 co-receptor function. They include a tyrosine rich motif in the N-terminal domain (NTD) and amino acids in the first and second ECLs of CCR5 (**Figure 2**) (30, 31). Sulfation of tyrosine residues in the NTD of CCR5 are required for binding ligands and the HIV-1 envelope protein gp120, which has been elucidated by NMR spectroscopy of this important CCR5 domain with RANTES/CCL5 (31, 32). Ligand binding is a two-step process. Site-directed mutagenesis and molecular docking have shown that core domains of CCL5 interact with ECL1, ECL2 and the NTD of CCR5 initially and the N-terminus of CCL5 interacts with the transmembrane helical (TMH) bundle of CCR5 (33). Two disulfide bridges linking together ECL1 and ECL2 (C101-C178) and ECL3 to the N-terminus (C20-C269) are required for maintaining the structural integrity necessary for the TMH bundle to associate closely together and form a binding pocket upon ligand binding, as determined by molecular modeling, ligand docking, and cryo-EM (33–35). CCR5 has been shown to be present in lipid rafts, a site for intracellular signaling; multiple palmitoylation of cysteine residues and a membrane-proximal basic amino-acid rich domain within the cytoplasmic tail facilitate downstream signaling, expression, and targeting to the cellular membrane (36, 37). The C-terminal domain (CTD) is also crucial for interaction with heterotrimeric G-proteins. Ligand binding induces conformational changes and desensitization by PKC/GRK dependent phosphorylation of the CTD and ICL3 followed by internalization of CCR5, and recycling to the surface after ligand removal. Additionally, the conformational changes induce secondary signaling pathways PI3K/Akt and MAPK/ERK *via* release of G-protein subunits and interaction with effector molecules to recruit cytotoxic lymphocytes and activation of antigen-specific T cells (38, 39).

CCR5 expression is activated by transcriptional regulators in response to cell stimulus. The gene encoding CCR5 has two

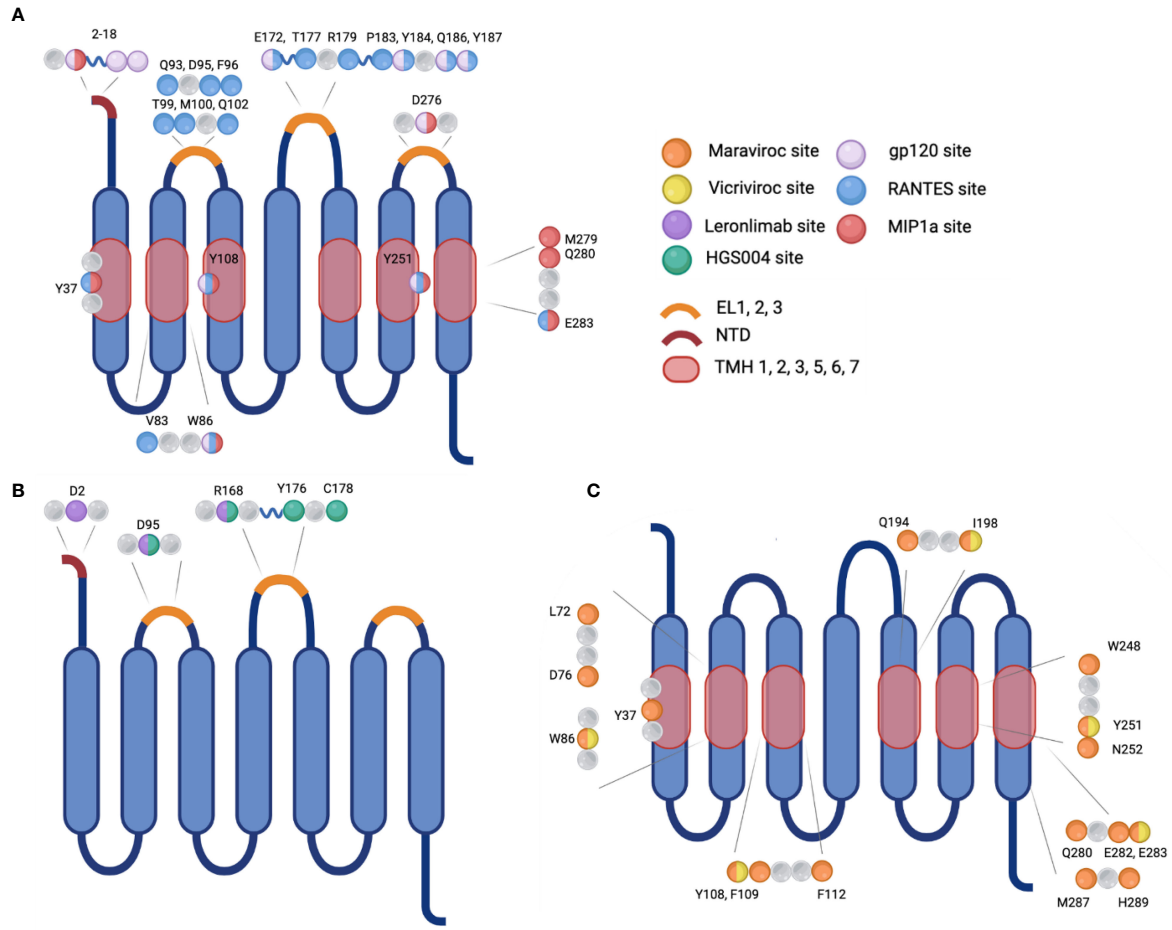

FIGURE 2 | Visualization of the sites of interaction on CCR5 for natural ligands, HIV-1 gp120, monoclonal antibodies, or small molecule inhibitors. **(A)** Binding sites for the natural ligand RANTES or HIV-1 gp120. **(B)** Binding sites for monoclonal antibodies Leronlimab or HGS004, and **(C)** Binding sites for small molecule inhibitors Maraviroc or Vicriviroc. Mutation at selected amino acids inhibit interaction between binding molecule and receptor. EL, extracellular loop; NTD, N-terminal domain; TMH, transmembrane helical bundle.

functional promoter regions termed Pd (downstream) and Pu (upstream), named relative to the location of the transcription start site (40). Distributed among these sites are potential binding sites for several interferon stimulated response elements (ISREs), kB factors, and cAMP-response elements (CRE elements), which bind interferon regulatory elements (IRFs), NF-κB, and the common activator of transcription CREB-1 (CRE-binding protein), respectively (5, 41–47). These promoter elements were shown to bind to their activators *in vitro*, but ultimately the IRF and NF-κB sites were nonfunctional as determined by luciferase reporter assays after stimulation by IFN-γ and TNF-α and LPS, respectively (48–50).

2.3 Redundancy and Impact of Downregulation or Knockout

A 32-bp deletion in the CCR5 coding region (CCR5Δ32) has been reported to protect a homozygous individual from HIV-1 infection and delay disease progression in a heterozygous individual. CCR5Δ32 leads to a frameshift after amino acid 184 in ECL2, disrupting the open reading frame and affecting critical sites of post-translational modifications in the CTD. This results in a loss of critical serines and threonines, which are residues that normally become phosphorylated by kinases and participate in downstream signaling, and loss of cysteine residues, which normally become palmitoylated and are necessary for trafficking the receptor to the cell surface. Disruption of the CTD causes a sequestration of mutant CCR5 in the endoplasmic reticulum and Golgi, which prevents its expression at the plasma membrane on cells of a CCR5Δ32 homozygous individual (37, 51, 52).

Individuals who are homozygous for the CCR5Δ32 allele are reported to be resistant to HIV-1 infection, but these individuals only make up 1% of the human population (53). These individuals experience a slower loss of CD4+ T cells early in infection (54). There is an increased frequency of CCR5Δ32 heterozygotes among people living with HIV-1 who are classified as long-term non-progressors (LTNPs), also known as elite controllers. Transmission studies of CCR5Δ32 show that homozygous individuals have a high level of protection from HIV-1 infection, while those who are heterozygous have partial protection (55). The frequency of the CCR5Δ32 allele was assessed using samples from 87 countries and found to range from the highest allele frequencies (AFs) of 16.41%, 15.63% and 15.09% from Norway, Estonia and Latvia, respectively; while the lowest AFs were from Eritrea (0.26%) and Ethiopia (0%) (56).

The CCR5Δ32 allele has not been the only polymorphism of CCR5 described to influence susceptibility to HIV-1 infection. Polymorphisms in the regulatory, promoter, and coding regions of CCR5 influence transcription factor binding and levels of expression and have been shown to affect the risk of acquiring HIV-1 and the rate of disease progression to AIDS (57–59). These have been grouped into major human haplogroups (HH) based on the combination of cis-regulatory regions A29G, G208T, G303A, T627C, C630T, A676G, and C927T: A, B, C, D, E, F1, F2, G1, and G2 (57, 60). Haplogroup C (HHC) and haplogroup E (HHE) are the most frequent HHs in HIV-1-infected patients across many races and ethnic populations studied (61–63). Both HHG2, which includes the Δ32 allele (rs333), and HHF2, which includes CCR2 V64I (rs1799864), have been associated with resistance and slow progression to AIDS (57, 60, 64). HHE, which includes promoter variants rs2856758 (G29A) and rs1799987 (G303A) is associated with increased promoter activity as well as increased CCR5 expression, susceptibility to HIV-1 infection, and accelerated AIDS progression (57, 60, 63, 64). Indeed, among a cohort of children the 303A/A genotype was correlated with increased rates of disease progression. HHE was also underrepresented in elite controllers as compared to progressors from a black South African ART-naïve HIV-1-infected cohort (58, 65). The HHE 29G and 303G polymorphisms have been linked to decreased surface expression and reduced *in vitro* infectability, determined by flow cytometry of CD4+ T cells and monocytes of exposed seronegative high-risk individuals, though this may be linked to ethnic background as results were not significant for non-Caucasian individuals (66, 67). Among a South American cohort of HIV-1-exposed seronegative (HESN) individuals in serodiscordant relationships, who despite repeated exposure to HIV-1 remain seronegative, CCR5Δ32 was not the protective factor and was found in similar frequencies among HESNs, seropositive individuals and healthy controls of this cohort (61). However, frequencies of SNPs in the promoter, such as A29G was significantly different between controls and seropositive individuals, as well as frequencies of CCR5 haplogroups, HHF1 was found only among healthy controls and HHF2 had a higher frequency among controls compared with seropositive individuals (61). Thus, variants in the promoter of CCR5 have been shown to affect transcript levels and cell surface expression of CCR5 and therefore susceptibility to HIV-1 infection.

These studies on the impact of downregulated or diminished expression of CCR5 in individuals as well as cases such as the Berlin patient, who was infected with HIV-1 and received a hematopoietic stem cell transplant from a CCR5Δ32 homozygote, have suggested the possibility of engineering an HIV-resistant immune system through the suppression of CCR5. However, the impact of CCR5 inhibition on the orchestration of the immune response first needed to be carefully considered before this approach can be deemed feasible. Despite the many diverse functions of CCR5 in the immune response, analyses of whole-genome genotyping and whole exome sequencing data from the UK Biobank and US patient cohorts show that there is no evidence of correlation between mortality and CCR5Δ32 homozygosity (68, 69). These studies were conducted in response to a previous and now retracted study that showed the opposite (70). This may be explained by redundancy in chemokine receptor function. Studies investigating the effect of inhibiting CCR5 expression, through a knockout, elucidated that other receptors may substitute for CCR5 functions. β-chemokines CCL5 and CCL3 can bind to other receptors in the chemokine receptor family such as CCR1 and CCR3. CCL4 can bind CCR8, but CCR1 and CCR3 are not present on T cells and CCR8 is not present on macrophages (71–73). CD8+ T cells can preserve their

functional recruitment to sites of infection without CCR5 through expression of CXCR3 although this is delayed compared to when CCR5 is present (29). Additionally, after CCR5 knockout in mice and induction of hepatotoxicity, macrophages successfully migrated to the liver and those of knockout mice were significantly increased for expression of CCR2 preserving chemokine chemotaxis (16). Infected mice lacking CCR5 exhibited increased and accelerated CD4+ T cell proliferation augmenting disease progression, suggesting that loss of CCR5 negates a protective role of CCR5-mediated CD4+ T cell activation but is also not necessary for recruitment of immune cells (74). In this regard, CCR5 appears to play a complimentary rather than integral role in the immune response and its absence does not compromise the antiviral response due to the redundancy of chemokine receptors and their ligands.

3 ROLE OF CCR5 EXPRESSION IN HIV-1 INFECTION

3.1 Requirement of CCR5 for HIV-1 Entry Into Some Immune Cells

Viral envelope glycoproteins on the surface of the HIV-1 virion utilize the primary receptor CD4 and co-receptors from the chemokine receptor family, CCR5 or CXCR4, to gain entry into target host cells. The envelope glycoproteins are encoded by Env, and associate as trimers at the lipid membrane of the virion as non-covalently bound surface gp120 (SU) and transmembrane gp41 (TM) subunits.

In the first step of viral entry, the gp120 subunit binds to one or more CD4 primary receptors, triggering conformational changes in gp41 and exposing a chemokine receptor binding site which was previously occluded. The V3 loop gp120 residues interact with the residues within the chemokine binding pocket and in ECL1 and ECL2 of the co-receptor, CCR5 or CXCR4, and interacts with the N terminus which also contacts the bridging sheet of gp120 (**Figure 2**) (75–79). Sequential binding to CD4 and a co-receptor bring gp41 and gp120 closer to the target membrane triggering the domains of gp41 to undergo a complex folding to form a fusion intermediate involving a six-helix bundle. This allows gp41 to insert its highly hydrophobic fusion peptide into the lipid bilayer of the target cell membrane with the subsequent fusion of the two membranes and formation of a pore through which the viral capsid can enter into the cytoplasm of the infected cell (80).

HIV-1 gene expression is dependent on host transcription factors, such as NF-κB, Sp, CEBP, CREB, among many other cellular transcription factors (42–44, 47, 49, 81–83). In particular, NF-κB is activated in response to T cell activation upon antigen recognition and leads to enhanced HIV-1 replication and cellular differentiation to effector T cells which release into peripheral blood, a process also known as thymopoeisis. Activated CD4+ T cells are the main cell type that support HIV-1 infection. Direct infection of naïve T cells is less efficient, in part, due to undetectable levels of CCR5 expression (84). A subset of activated cells differentiates to resting memory T cells and some eventually alter their pattern of gene expression and revert to resting memory T cells to enable long-term survival and induce a rapid response after re-exposure to antigen. HIV-1 stably integrated into the host genome of memory T cells or those that have circumvented the fates of activated T cells and reverted to memory T cells, are affected by the lack of transcription and do not express viral RNA, this is termed post-integration latency (85, 86). Pre-integration latency can also occur when HIV-1 infects naïve T cells which are quiescent, blocking the reverse transcription and integration of HIV-1 into the host genome, and later transition to effector or memory cells (87). In either case, HIV-1 transcription and translation can be rescued by activation of naïve cells leading to infected effector and memory cells, or by re-activation of memory cells. Thus, HIV-1 latency and a latent reservoir consist mainly of CCR5-expressing cells and can occur due to (i) infection of activated memory T cells that persist in a memory T cell state, (ii) infection of resting memory T cells, (iii) infection of an activated thymocyte in the transition to naïve T cells, or (iv) infection of activated T cells that transition back to resting memory T cells (5, 14, 85, 88).

3.2 CCR5 Versus CXCR4 Co-Receptor Use Among Variants and Relation to Disease Stage

HIV-1 tropism is classified by the co-receptor used by the variant; R5 viral strains utilize the CCR5 CC-chemokine co-receptor, X4 strains utilize the CXCR4 CXC-chemokine co-receptor, and dual-tropic R5X4 variants have the ability to use both co-receptors though with a greater affinity for CCR5 or CXCR4. Strains that are exclusively R5 predominantly infect monocyte-derived macrophages and memory CD4 cells, which are the prime targets of HIV-1 early in infection, while exclusively X4 strains predominate at a later stage and prefer naïve and resting T cells (44, 84, 89). Early infection is predominantly achieved by R5 tropic viruses because of the relatively high surface expression of CCR5 than CXCR4 on CD4+ memory T cells and immature dendritic cells which determines the efficiency of viral entry, as well as a higher affinity for CD4 (84, 90). HIV-1 transmission by R5 strains is more efficient than X4 strains, as is viral replication. This is supported by studies of people living with HIV-1 (PWH) who are not on antiretrovirals being infected mainly by R5 strains (80-91%), with some dual-tropic (9-20%) and very rarely X4 strains (>1%) (91, 92). In contrast, among PWH on antiretroviral therapy (ART), which clear the pool of infected CD4+ cells, R5/X4 and X4 strains are more common. In approximately 50% of HIV-1 infections, a co-receptor switch by mutation at the site of interaction in variable loops of gp120, especially V3, leading to alteration of N-linked glycosylation sites enables the switching of R5 to X4 tropism (75, 93, 94). X4-utilizing viruses are associated with a more rapid decrease in CD4+ cell count and an accelerated rate of disease progression and mortality in contrast to R5 tropic viruses (91, 95). However, immune activation and progression are not a result of the switch to X4 tropism but rather are a consequence of CD4+ T cell activation depleting host target cell availability, driving the target to naïve T cells allowing X4 strains to predominate later in the course of infection (96, 97). Long-term

non-progressors are a group of PWH able to maintain stable CD4 cell counts and remain asymptomatic without ART. They exhibit lower amounts of CCR5 expression on memory CD4+ T cells compared to normal progressors and healthy controls while CXCR4 expression was similar compared to normal progressors but significantly higher than healthy controls (98, 99). CCR5 and high levels of CCR5 are associated with acute and early HIV-1 infection and rapid disease progression, while low CCR5 expression protects from virus infection (100).

4 MECHANISMS OF TARGETING CCR5 TO INHIBIT HIV-1 DISEASE PROGRESSION

4.1 Extracellular CCR5 Blocking Methods

4.1.1 Small Molecule Inhibitors

Given that CCR5 can be utilized by HIV-1 to enter and infect immune cells, extracellular methods of inhibiting the interaction of gp120 with CCR5 have been developed (**Table 1**). Targeting and preventing this interaction has been mainly done with the use of small molecule inhibitors, which generally work by inducing conformational changes to CCR5 thereby preventing fusion of the HIV-1 envelope with the cellular membrane (108). In contrast to many therapeutics targeting viral proteins, these inhibitors target the various components of the transmembrane CCR5 receptor protein on host cells.

In the early 2000s, several drugs were designed as orally available small molecule CCR5 inhibitors but did not complete stage 3 clinical trials. One of the earliest small molecules, Aplaviroc, was discontinued due to evidence of hepatoxicity in four patients (108). Another CCR5 antagonist, Vicriviroc, showed efficacy in reducing viral loads of treated patients by about one log over 24 weeks, but *in vivo* resistance developed in one patient (**Table 1**). It has not been approved for clinical use because of a potential link to the induction of hematological malignancies in five patients (101).

Later in 2007, the drug candidate Maraviroc was approved for clinical use to act as a non-competitive inhibitor of the CCR5

TABLE 1 | Overview of clinical trial outcomes of selected CCR5 antagonists in HIV-1 infection.

Study	N=	Intervention	Duration or Dose	Outcomes	Notes
Three-Year Safety and Efficacy of Vicriviroc, a CCR5 Antagonist, in HIV-1-Infected, Treatment-Experienced Patients (NCT00082498)	118	Failing Background Therapy + Vicriviroc	5, 10, 15 mg/ day up to 3 years	1) 46% were suppressed <50 copies/ mL after 24 weeks 2) Through the third year 49% did not rebound	• 11% developed malignancies • 29% of patients had mixed tropism • 5.1% developed resistance (101)
Vicriviroc in combination therapy with an optimized regimen for treatment-experienced subjects: 48-week results of the VICTOR-E1 phase 2 trial (NCT00243230)	114	Ritonavir + Vicriviroc or Placebo	20 or 30 mg/ day for 48 weeks	1) Mean viral load change for intervention groups was 1.75, 1.77 log_{10} copies/mL compared to placebo 0.79 log_{10} copies/mL 2) Mean CD4 count increased 102, 136 in treated groups and 63 in placebo	• Four subjects discontinued due to adverse events • Mild elevations in liver tests were observed (102)
Clinical Trial Vicriviroc in HIV-Treatment Experienced Subjects (NCT00523211)	506	Background Therapy + Vicriviroc	30 mg/day for 48 weeks	1) Dual therapy with Vicriviroc achieved suppression more frequently than dual therapy without Vicriviroc 2) At 48 weeks no additional efficacy was seen in patients receiving 3+ drugs	• 60% of patients were on 3 or more antivirals • Adding Vicriviroc did not provide additional efficacy gains • Included only patients with CCR5-tropic infections (103)
Maraviroc as an Immunomodulatory Drug for Antiretroviral-treated HIV Infected Patients Exhibiting Immunologic Failure, Phase 4 (NCT00735072)	45	Maraviroc + Efavirenz or Tipranavir	150, 300, 600 mg twice/day 48 weeks	1) Maraviroc group experienced less of a decline in CD4+ T cell count and an increase in circulating CD8+ cells 2) Low-level viremia decreased on average 48% and 52% in placebo and intervention	• Maraviroc treatment appeared to induce re-localization of activated CD8+ cells from the gut to the periphery (104)
Maraviroc as intensification strategy in HIV-1 positive patients with deficient immunological response (NCT00884858)	100	HAART + Maraviroc	Scaled doses 150-600 mg twice daily up to 48 weeks	1) Maraviroc did not display an advantage in improving CD4+ counts 2) CD8+ counts improved in maraviroc intensified groups	• Study focused on patients with decreasing CD4 counts (105)
Study of PRO 140 by Subcutaneous Administration in Adult Subjects With HIV -1 Infection (NCT00642707)	44	Subcutaneous Leronlimab	62 mg or 324 mg/week for 3 weeks or 324 mg biweekly	1) Log_{10} reduction of 0.23, 1.37 and 1.65 accordingly	• Doses were well tolerated • Serum concentrations were stable through day 8 (106)
A Phase 2a, Randomized, Double-Blind, Placebo-Controlled Study of PRO 140 by Intravenous Administration in Adult Subjects With HIV-1 Infection (NCT00613379)	31	Intravenous Leronlimab	Single 5 or 10 mg/kg infusions	1) Average maximum reduction in viral load was 1.8 log_{10} 2) Receptor occupancy remained above 85% in both groups day 3 through day 29 but change in occupancy was not significant by day 59	• Patients had been off ART for 3 months or more, had viral loads >5000 copies/mL and CD4 counts >300 (107)

These trials reflected common use of the intervention in clinical practice.

receptor (**Table 1**). It is the only CCR5-blocking drug approved for clinical treatment of HIV-1 infection (109). The transmembrane hydrophobic binding site for Maraviroc is not the same used by the major chemokines or gp120. Maraviroc stabilizes a conformation of the CCR5 receptor that is unable to be bound by gp120 (109). HIV-1 is still able to interact with the receptors allosterically bound by Maraviroc but not use them efficiently, leading to suppression of infection. Clinical trials have shown Maraviroc can reduce viral load in treatment naïve patients and patients previously teated with ARRT who are positive for only CCR5-utilizing HIV-1 strains (101). Despite promising clinical trial results, mutations in the highly variable V2 and V3 loop region of viral gp120 have been reported, which result in a recovered CCR5 receptor usage even with the presence of Maraviroc at the binding site (101). Finally, potential changes in viral tropism to utilize CXCR4 as a co-receptor have been of concern, but diagnostic limitations make it difficult to discern novel Maraviroc resistance within the host from the emergence of a pre-existing CXCR4-tropic strain (108, 109). For these reasons, current clinical use trends towards a treatment experienced cohort where ART strategies have failed. In many infected patients, Maraviroc has been added to their regimens as a treatment intensification approach due to low CD4 counts (110). Of note, in a study assessing efficacy and safety of Maraviroc showed slightly increased CD4 counts through 9 months of treatment and appeared to increase naïve CD8+ T cells in the digestive tract, highlighting the potential benefit of restoring immune function by targeting infection-associated inflammation in lymphoid tissues (111).

Other more recent small molecule inhibitors in development include GRL-117C, which demonstrated inhibition of R5-utilizing HIV-1 (108). Interestingly, this study also implicated CCR5 inhibitors in additional benefits for treatment of HIV-1 infection including immunomodulation and even latency reversal. Overall, while small molecule inhibitors confer some protection against HIV-1 infection, results of their treatment usage demonstrate a more feasible therapy is needed that would limit onset of resistant HIV-1 strains as well as be formulated in a way that patients can take easily.

4.1.2 Cases of Natural Antibodies to CCR5

Individuals exposed but uninfected and well-suppressed infected individuals have been shown to have detectable CCR5 antibody. These antibodies have been found in circulation and in mucosal surfaces, a key site for HIV-1 transmission (112). The natural antibodies inhibit HIV-1 infection via binding to the extracellular loop 1 (EL1) of CCR5, inducing receptor internalization (110). Interestingly, CCR5 antibodies were also found in almost a quarter of long-term non progressors, and *in vitro* analysis showed CD4+ T cells from these patients were not susceptible to CCR-5 tropic viruses. Studies have observed no deleterious immune impact in individuals seropositive for anti-CCR5 and these proteins may confer enhanced viremic control *in vivo* (112–114).

4.1.3 Development of Monoclonal Antibodies

In addition to small molecule inhibitors, monoclonal antibodies targeting the CCR5 receptor are being developed and investigated for use in treatment of HIV-1 infection and pre-exposure prophylaxis (PrEP). These antibodies are intended to bind the CCR5 receptor to inhibit gp120 interacting with the co-receptor (**Figure 2**). The drug Leronlimab is a humanized anti-CCR5 IgG4 monoclonal antibody that is delivered subcutaneously or intravenously (**Table 1**) (115). Preliminary studies have shown that contrary to natural antibodies, Leronlimab is able to bind the N-terminal domain of EL2 on the CCR5 receptor, the same binding site used by gp120 (**Figure 2**). This loop is thought to be a well conserved area of CCR5 encoding genes (108). Another CCR5 antibody HGS004 directed at the same area of CCR5 has also show *in vitro* and *in vivo* efficacy in infected patients. However, a linear dose-dependent response was not observed and only about 50% of patients showed a viral load decrease of greater than one log two weeks after a single dose (116).

Studies in rhesus macaques showed dose-dependent protection from CCR5-utilizing infection following injections of Leronlimab subcutaneously. Additionally, 50 mg/kg prevented HIV-1 infection in all sites for all subjects, while just 10 mg/kg prevented infection in rectal tissue in all but one subject (117). In Phase 2 clinical trials in individuals with solely CCR5-utilizing HIV-1 intravenous Leronlimab infusion was well tolerated. Dosage as low as 5 mg/kg elicited maximum antiviral effects around 14 days post injection with greater than 1.8 log viral load reduction (118). In this same study, viral load rebounded to near baseline in all dosages around day 40 post-injection, highlighting a need for sustained treatment. No evidence of resistance or switched tropism was observed while only mild side effects were encountered with this medication, and it has been given a fast track status by the US Food and Drug Administration (FDA) (119).

Of the monoclonal antibodies directed at CCR5 that have been investigated, Leronlimab has achieved the most sustained receptor occupancy. Promising infection prevention and antiviral data has been gathered from clinical trial and macaque studies. Patients exhibited 85% receptor occupancy through day 29 post-infusion of both 5 and 10 mg/kg doses (106). Additionally, while Leronlimab could benefit other neurological diseases, the issue of the viral reservoir will likely not be well addressed by these monoclonal antibody treatments. Studies report 70-75% receptor occupancy in Leronlimab-treated macaques (120). Long-term treatment sustainability and standardized treatment protocols have yet to be determined, though several patients have seen continuous suppression for over two years.

4.2 Alteration of CCR5 Expression as a HIV Therapeutic

4.2.1 RNA Interference

The original concept of RNA interference as a gene editing tool was noticed in *C. elegans* and now includes three distinct tools: short hairpin RNA (shRNAs), short interfering RNA (siRNA), and microRNA (miRNA). While similar in that they each modulate the expression of a gene target, they each have some relevant differences. shRNAs are similar to siRNAs in that they target only one mRNA transcript, but different in that the

shRNA coding sequence is stably integrated into a cell's genome allowing for long-term expression. In contrast, siRNAs are only expressed in the cytosol which is conducive for transient knockdown of the designated mRNA. miRNAs, while initially only found endogenously in cells, have recently become synthesized artificially (121). miRNAs are distinct in their structure, which does not fully compliment the target mRNA, allowing for multiple targets. All three of these have been evaluated in the knockdown of CCR5 for therapy of HIV-1 infection.

Two main cell types have been primarily used in CCR5 knockdown experiments: hematopoietic stem and progenitor cells (HSPCs) and CD4+ T cells. HSPCs provide the advantage of differentiating into macrophages and CD4+ T cells that could be resistant to CCR5-utilizng HIV-1, which contributed to the success of therapy in the Berlin and London patients. However, practical usage of HSPCs in HIV-1-infected individuals is complicated by (i) the damage caused to HSPCs and hematopoietic function in bone marrow from their infection by HIV-1 (122), (ii) the damage to the differentiation potential of HSPCs caused by alteration of these cells (123), and (iii) the rarity of HSPCs and associated difficulty of culturing them *in vitro* (124).

Experiments done in HSPCs have shown significantly better results *in vitro*, compared to *in vivo*. Due to their non-dividing nature, research has focused on de-differentiating hematopoietic stem cells into induced-pluripotent stem cells (iPSCs) to provide a replenishing source of cells. One such study knocked down CCR5 in iPS- derived hematopoietic stem cells using a shRNA, these modified iPSCs then underwent directed differentiation back into hematopoietic stem cells and then end-stage macrophages, while CD4+ T cells were not generated. Above 99% iPSCs were observed to possess shRNA against CCR5, resulting in only 6.7% of macrophages positive for the receptor. These macrophages inhibited HIV-1 infection by more than 2 logs, compared to controls (125). However, CCR5 knockdown and engraftment of edited cells into mice is challenging, while no research to date has been published concerning shRNAs editing HSPCs which are then engrafted into mice. However, miRNAs have been used for this purpose. Myburgh et al. demonstrated more than 70% miRNA transduction into HSPCs using a lentiviral vector. This study's promising results showed 11 of the 15 mice had hCD45+ cell engraftment above 5%, but all 11 of these mice displayed successful CCR5 knockdown below 20% of the control level in CD4+ T cells (126). However, there was evidence of viral escape of the YU-2 CCR5-utilizing infectious molecular clone in one mouse, leading to CXCR4-utilizing virus (126).

In contrast, peripheral CD4+ T cells are significantly more available for experimentation and prolonged proliferation *in vitro*. Additionally, some CD4+ T cell subsets have self-renewal properties similar to stem cells. Stem cell memory T cells, central memory T cells, and effector memory T cells are all capable self-renewal, thus sustaining any modifications made to them. Artificial miRNAs used in primary CD4+ T cells achieved a 39% successful CCR5 knockdown, and a near full reduction in viral load for the length of the eight day experiment (121). While CD4+ T cells and hematopoietic stem cells are the dominant models for gene editing, macrophages are also an important target for HIV-1 infection. In fact, CCR5-utilization is more relevant for macrophages which express high levels of CCR5, as compared to CD4+ T cells, but macrophages express low levels of CD4 and so are more relevant later on in infection when CD4+ T cells are depleted. However, the long-term viability of macrophage transduction with gene editing tools have been challenging, as macrophages are a mostly non-dividing cell type and they phagocytose viral vectors. As a result, there is significantly less research on cells of monocyte-macrophage origin. One effective approach to studying macrophages has been the use of HSPCs or iPSCs that are edited and then differentiated into macrophages. This approach provides a renewable source of CCR5-edited macrophages. Using this strategy, shRNAs were able to achieve above 99% CCR5 knockdown in iPSCs and maintained their modifications past the differentiation into end-stage macrophages (127).

AgoshRNA is a relatively new type of shRNA that is smaller than typical shRNAs and is able to be expressed in monocytes unlike their predecessors. Their smaller size precludes agoshRNAs from being processed by the conventional Dicer, but instead leads to processing by Ago2. Monocytes lack Dicer, thus expanding the cell types available for expression. Anti-CCR5 AgoshRNAs have reduced the number of CCR5-positive cells to less than 20% in the PM1 T cell line and less than 40% in PBMCs. This reduction in CCR5 expression translated to no detectable replication of HIV-1 for the duration of a 25 day experiment, as measured in the PM1 T cell line. In addition, cytotoxicity was found to be negligible (128) (**Figure 3**).

Although a large amount of research exists with respect to the usage of RNAi tools they have largely fallen out of in favor due to their tendency to trigger an innate immune response, their transient nature, minimal ability to penetrate a cell, incomplete knockdown of the genes of interest, and frequent off-target effects. These disadvantages make development of a highly efficient, long-term therapeutic for HIV-1 infection that is based on inhibition of CCR5 expression through RNAi very unlikely, while more robust CCR5 targeting approaches can allow for better therapeutic outcomes.

4.2.2 Zinc Finger Nucleases

In 1985, the zinc finger (ZF) was first identified as possessing an adaptable DNA recognition domain, Cys2His2-ZF, which showed promising DNA-binding results as a gene expression regulator. Cys2His2-ZF is the most common type of DNA-binding motif in eukaryotic transcription factors and constitutes 3% of the genes of the human genome due to its adaptable nature (129, 130). The modular design of ZFs permits numerous combinatorial possibilities for recognizing specific DNA and RNA sequences. ZFs were shown to have applications in biotechnology in 1994, when Choo et al. demonstrated a three-finger protein capable of blocking the expression of a human oncogene (131). Each zinc-finger unit selectively recognizes three base pairs (bp) of DNA and produces

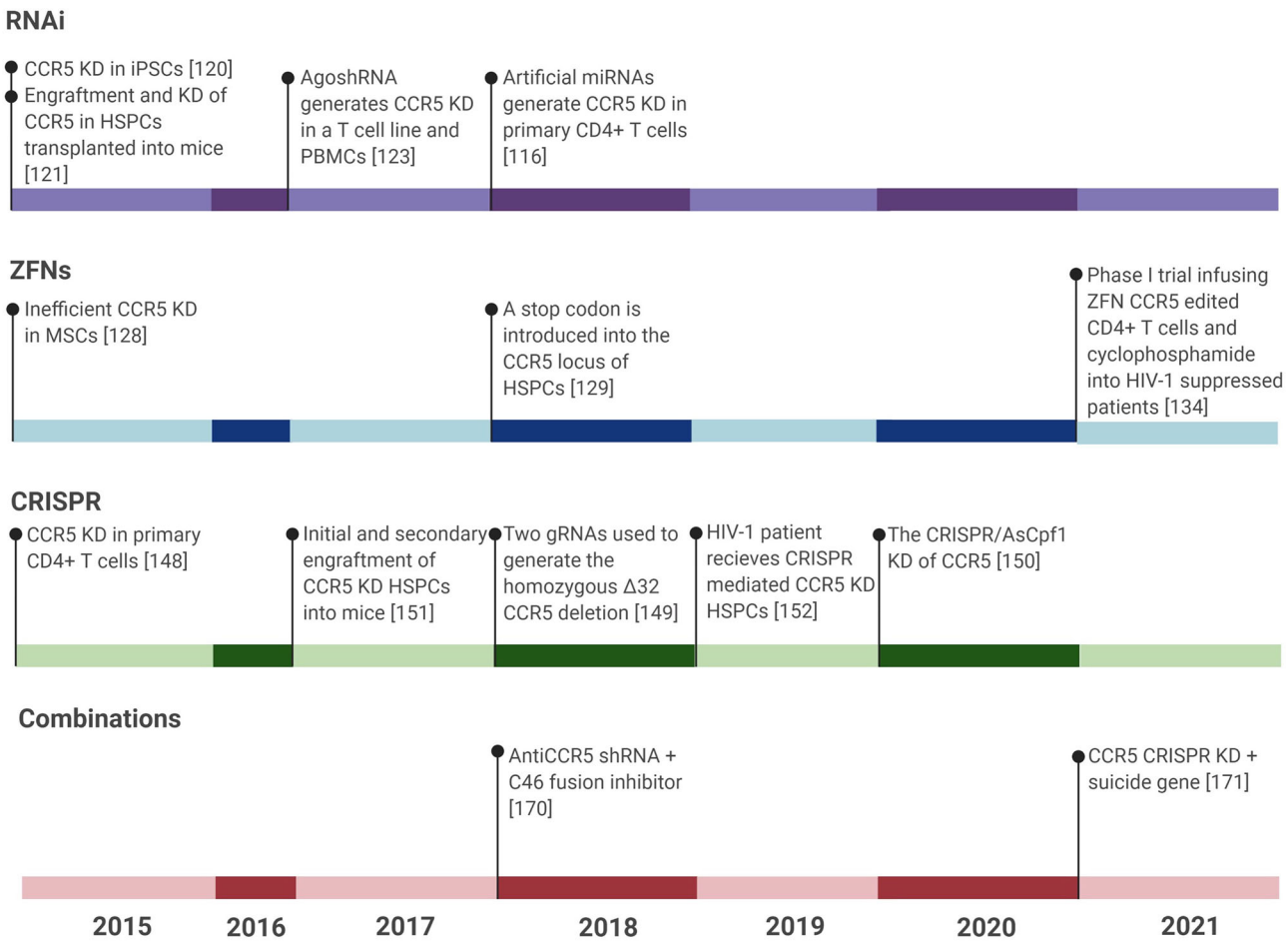

FIGURE 3 | Timeline showing all CCR5 gene editing studies for treatment of HIV-1 infection in the last six years. Gene editing studies include RNAi tools, ZFNs, CRISPR, and combinations of treatments against HIV-1. TALENs were not included due to the publication of only 2 major studies on CCR5 editing using TALENs within the last six years. The last six years have seen the most published research in these areas and so any research prior to this has been left out of this figure. KD, Knockdown.

base-specific contacts through the interaction of its α-helix residues with the major groove of DNA. The zinc finger peptides are linked to the non-specific catalytic domain of the Fok1 endonuclease creating ZF nucleases (ZFN). Cleavage by Fok-I generates two 5′-overhang DNA ends. Because each zinc-finger unit recognizes three nucleotides, three to six zinc-finger units are assembled to generate a specific DNA-binding domain that recognizes a 6- to 18-bp DNA sequence. The target sequence specificity and recognition of ZFNs are influenced by three central aspects: (i) the amino acid sequence of each finger, (ii) the number of fingers, and (iii) the interaction of the nuclease domain. Both the DNA-binding and catalytic domains of ZFNs can be individually adjusted due to the flexible structure of ZFNs, thus facilitating the development of new ZFN designs with the necessary affinity and specificity for selected gene therapy applications (132).

As one of the first gene editing tools, ZFNs have had more time to be developed for clinical use. Taking a somewhat different approach, Manotham et al. demonstrated ZFN-mediated homology directed repair in bone marrow-derived mesenchymal stem cells (MSC). The advantage of MSCs is that they are relatively easily procured through bone marrow aspiration. Further, MSCs have one of the highest proliferation rates of any primary cell culture, contrary to CD4+ T cells and especially HSPC which are difficult by comparison to culture *in vitro*. In theory, editing MSCs using ZFNs should be an effective means to inhibit HIV-1 infection, but in practice editing efficiency is well below optimal. Manotham et al. found that, out of 10,236 cells that they had attempted to edit, only 6 cells were capable of proliferation and contained one allele of the CCR5 gene insertion (133). More recently, the same group attempted to introduce a stop codon into the CCR5 locus of HSPC cells using ZFNs. PCR indicated that only 0.5% of HSPCs contained the stop codon insertions within the CCR5 loci (134).

The translation of this type of research *in vivo* has yielded relatively positive results, with the promise of more to come in clinical trials. Holt et al. demonstrated that 11% of HSPCs engrafted into a mouse model contained the CCR5 disruption,

which is a much higher frequency of edited cells found to engraft into mice compared to the insertion experiments by Manotham et al. which were not engrafted into mice, demonstrating how much more efficient simple cleavage is compared to introduction of a gene. This engineered protection led to undetectable HIV-1 RNA in the small and large intestine of mice at 12 weeks post CCR5-utilizing HIV-1_{BAL} challenge as measured by quantitative PCR (135). These studies have led to translation of this research into clinical trials, for example, the phase I clinical trial run at the City of Hope Medical Center is administering ZFN CCR5 modified autologous SB-728mR-HSPC to HIV-1 CCR5-utilizing infected patients to assess their safety and feasibility at inhibiting infection with CCR5-utilizing HIV-1. This study will conclude in 2022.

Although HSPCs have more long-term potential, the convenience of working with CD4+ T cells has made their use more widespread when using ZFNs. Mice transplanted with CCR5-negative CD4+ T cells from ZFN modification, showed reduction in HIV-1 replication. Perez et al. established that ZFNs effectively disrupt CCR5 in human CD4+ T cells and that this disruption provides sustained inhibition of HIV-1 infection *in vitro* and *in vivo* using the immunodeficient NOD/Shi-*scid*/IL-2Rγ^{null} (NOG) mouse model. Between 50-80% of CCR5 was observed to be mutated in GHOST-CCR5 cells as measured by the Surveyor assay. In primary cells, CCR5 disruption reached 40-60%. When human CD4+ T cells were infused into ten HIV-infected NOG mice, more than 50% of CD4+ T cells in eight of the ten mice contained the CCR5 disruption 50 days after engraftment. This led to a mean viral load of 8,300 copies/ml compared to 60,100 copies/ml in the control (136). Yi et al. expanded on this work in their study using resting T cells in which CCR5 expression was disrupted using ZFN, which were then transplanted into mice (137). The transplantation resulted in a 71% disruption frequency in the CCR5 of these cells in the mice. This modification of CD4+ T cells allowed inhibition of HIV-1 infection and resulted in a significant reduction in viral load, as measured by p24 ELISA and qRT-PCR. Furthermore, this also led to less reduction in CD4+ T cell counts (137).

These successful *in vitro* and *in vivo* studies led to clinical trials, where ZFN CCR5 modified CD4+ T cells were transplanted into HIV-1-infected patients that had predominant CCR5-utilizing virus and initially 13.9% of peripheral CD4+ T cells contained the CCR5 knockdown but at 42 months the concentration of peripheral CD4+ T cells with this modification had reduced to 1.7%. During a 12 week analytical treatment interruption (ATI) that began four weeks post-modified CD4+ T cell infusion, 4 patients had an average of 1.2 $\log_{10}$ decrease in viral load. However, by the end of the ATI, the median circulating CD4+ T cells had declined from 1849 per cubic millimeter at the start of the ATI to 872 per cubic millimeter (138). This same group recently published another phase I trial also using ZFN CCR5-edited CD4+ T cells and cyclophosphamide to increase the engraftment of the modified cells by depleting the presence of immune cells. Infusion of these cells was generally safe and well tolerated with no serious adverse effects throughout the 48 week experiment. Pretreatment with cyclophosphamide had no discernible influence on the time it took to virologic rebound, but a slight trend was observed in improved engraftment in those exposed to cyclophosphamide. One week post-infusion, the median frequency of ZFN CCR5-edited CD4+ T cells was 17%. Similar to previous studies, the overall trend of modified CD4+ T cells was that modified lymphocytes increased then decreased in the peripheral blood, likely due to cell localization to certain tissues or cell death. Notably, no significant increase or decrease in the viral reservoir was detected by intact proviral DNA assay (IPDA) following ATI. In summation, this study demonstrated that CCR5 knockdown CD4+ T cell infusions are safe and may delay viral rebound, but do not have any long-term effects on HIV-1 reservoirs (139) (**Figure 3**).

4.2.3 TALENs

Transcription activator-like effector nucleases (TALENs) have structural similarities to ZFNs as they are heterodimeric nucleases that consist of a fusion between the Fok-I catalytic domain and a transcription activator-like effector (TALE) DNA-binding domain. The DNA- binding domain consists of an array of almost identical repeats of 33–35 amino acids. Each of these repeats independently recognizes one nucleotide through two amino acids called repeat variable diresidues (RVDs), and the recognition specificity is determined by the RVD. TALE modules differ from ZFs in that individual TALE modules seem to recognize DNA mostly independent of their adjacent modules (140, 141). A disadvantage to using TALENs is that the genes encoding the system are approximately three times the size of ZFNs, due to TALE motifs having a comparable size to ZFNs, but TALE motifs only recognize a single base, whereas ZFs recognize three to four bases. Additionally, the consistently repetitive sequences of TALE modules, with the exception of the RVDs, create difficulties in assembling the genes encoding TALENs in *E. coli* for replication. For the same reason, delivery of TALENs into mammalian cells using viral vectors is also difficult (142). Although TALENs were first described in 2010, before TALENs became a sustainable alternative to ZFNs, the CRISPR/Cas9 system was beginning to gain attention.

TALENs have sustained little interest in the gene editing field due to the complexity and expense, especially when compared to cheaper, simpler alternatives such as the many variants of the CRISPR system (143, 144). And for these reasons TALENs have rarely been used to target CCR5. What little research that has been done *in vitro* on CD4+ T cells demonstrates some off target effects leading to low levels of cytotoxicity, as well as high nuclease activity and specificity. No research using TALENs to disrupt CCR5 in HSPC has yet to be published as of this writing. The main TALEN tool developed, CCR5-Uco-hetTALEN, includes a heterodimeric Fok1-cleavage domain and almost completely reduces off-target effects, with the notable exception of the highly homologous CCR2 (145). This technology has advanced so much that it is now automated and can reliably generate the CCR5 knockdown in frequencies above 60% within primary T cells, 40% of which can be biallelic CCR5 mutations (146).

4.2.4 CRISPR

The clustered regularly interspaced short palindromic repeats (CRISPR) system is derived from a microbial adaptive immune system using a combination of a nuclease and a short RNA. Since its discovery in 1987 (109), CRISPR has been redesigned for a number of different gene-editing applications (4, 143, 144, 147, 148). In contrast to the nucleases mentioned above, for which specificity is dependent on protein–DNA interactions, the specificity of the CRISPR system relates to complementary RNA–DNA base pairing. This is "guided" by a single guide RNA' (sgRNA) that contains a 20-nucleotide region designed to be complementary to the genomic DNA target termed the protospacer. Research has shown that partial mispairing is tolerated with the 3' end of this 20 nucleotides being the most crucial (48, 149–151). It has been thought that this may increase the likelihood of off-target cleavage. Indeed, the level of off-target effects varies considerably among different targets, perhaps as a function of sgRNA design. The most commonly used CRISPR system today was derived from *Streptococcus pyogenes* and uses the nuclease Cas9. In contrast to ZFNs and TALENs, cleavage by Cas9 generates blunt DNA ends. CRISPR is 4.8 times more efficient at editing the CCR5 receptor than TALENs, as indicated by FACS followed by Sanger sequencing (152).

As with the other gene-editing strategies, CRISPR editing usually occurs in hematopoietic stem cells or in CD4+ T cells. In CD4+ T cells, CRISPR gene editing efficiency and engraftment has actually had worse efficiency than ZFNs. One of the first studies on CCR5 disruption using CRISPR in primary CD4+ T cells detected more than 30% of cells contained indels within the CCR5 gene using CRISPR/Cas9, compared to 40-60% using ZFNs (136). Furthermore, viral challenge with the R5-utilizing strain HIV-1_{BaL} and the transmitter/founder virus HIV-1_{CH042}, individually, indicated that almost no p24 was produced from primary CD4+ T cells with a CCR5 knockdown at seven days post challenge, approximately 5 ng/ml, in contrast to control cells which demonstrated approximately 80 ng/ml of p24 expression. To validate specificity, the authors performed off-target analysis on the two gRNAs that were individually used in the study, specifically they amplified the 500 bp genomic regions spanning the top 15 sites with the most off-target potential for each gRNA. These amplified genomic regions were subjected to T7E1 analysis, wherein there was no amount of significant cleavage events detected, thus the authors concluded that no off-target effects occurred from these two gRNAs (153). A similar, more recent study using two distinct gRNAs in the CRISPR Cas9 system targeted toward the flanking regions of the CCR5Δ32 mutation locus estimated that less than 11% of primary CD4+ T cells were modified with the homozygous Δ32 mutation. Those that were modified showed almost no expression of p24 six days post HIV-1 challenge. No significant off-target effects were detected by whole genome sequencing (154). More recently, the CRISPR/AsCpf1 system, which is designed for easier multiplexing of gRNAs, multiplexed two gRNAs to knockdown the CCR5 receptor in primary CD4+ T cells *in vitro*. While a lentivirus was insufficient for transfection, an adenovirus achieved up to 28% disruption of CCR5, as determined by T7E1 assay. A p24 ELISA determined the p24 level post-14 days challenge with the CCR5-utilizing HIV-1_{YU-2} to be less than half of the control. As with previous experiments, no off-target effects were observed, and CCR5 disruption had no cytotoxic effects (155).

Studies in mice have fared about as well for CRISPR as they have for ZFNs. A study by Xu et al. demonstrated efficient knockdown of CCR5 in HSPCs which led to prevention of CCR5-utilizing HIV-1 infection when engrafted in mice (156). On average 32% of HSPCs were negative for CCR5, and an average of 8% of HSPCs successfully engrafted onto mice. Further, secondary transplantation of bone marrow cells from these mice onto naïve mice yielded 27% of CD4+ T cells with the CCR5 knockdown. The result of this CCR5 deletion in secondary transplanted mice prevented infection with CCR5-utilizing HIV-1_{BaL-1} infection eight weeks post infection that reduced HIV-1 RNA levels to almost half that of the control (156).

The most relevant example of CRISPR disrupting CCR5 is by Xu et al. which describes a patient that was diagnosed with HIV-1 infection and acute lymphoblastic leukemia in 2016, and immediately underwent ART and standard chemotherapy to treat these disorders (157). Later, in an attempt to cure both disorders, the patient received myeloablative conditioning, using cyclophosphamide and total-body radiation, and an allogeneic hematopoietic stem-cell transplant. The CRISPR/Cas9 system was used to disrupt the CCR5 receptor in HSPCs from a fully matched HLA donor and then transplanted into the patient. Between 5.2-8.28% of circulating bone marrow cells were found to have the CCR5 disruption, and whole genome sequencing of edited cells compared potential off-target sites in the genome of cells to the two gRNAs used, resulting in no DNA cleavage detected at any of these potential sites, thus the authors conclude that no off-target effects occurred after genome editing and at 19 months post-transplantation (157). While at 19 months post-transplantation the acute lymphoblastic leukemia was in full remission, the rapid viral rebound in response to ART interruption at 7 months post-transplantation indicates that this strategy does not successfully cure HIV-1 infection (157).

Cardozo-Ojeda et al. developed a mathematical model that projects the minimum threshold of CCR5 edited cells necessary to achieve a functional cure for HIV-1 infection. It was concluded that two criteria must be met to achieve a functional cure. First, the HPSCs transplanted into a patient must be five times more prevalent than endogenous HPSCs subsequent to total body radiation. The second criterion was that the frequency of transplanted HSPCs homozygous for the CCR5Δ32 allele in a patient must reach 76-94% (158). This model corroborates the ineffective strategy described by Xu et al. whose patient does not meet these criteria.

5 COMBINATORIAL APPROACHES

Considering several factors are involved in the persistence of HIV-1 infection, including the establishment of latent viral reservoirs, it can be expected that a combinatorial approach

will be necessary to achieve at least effective ART-independent control of HIV-1 infection. In addition to ATI with ART along with an investigational CCR5 therapy approach reviewed in Section 4, several novel combinatorial strategies that include inhibition of the CCR5 co-receptor as part of the approach are being investigated. These therapies aim to maximize the prevention of newly infected cells, to allow a better and safer outcome for HIV-1-infected patients that would eliminate the need for continuous ART. Additionally, they would provide the same advantages as coupling ART with CCR5 targeting, which is prevention of the emergence of CXCR4-utilizing virus and development of mutations that render CCR5-utilizing viruses still capable of entry.

The majority of novel therapeutic strategies involving CCR5 targeting combine this approach with CXCR4 inhibition (**Table 2**). This combinatorial approach may more completely prevent infection with the variety of HIV-1 strains within a patient, as well as prevent selective pressure on CXCR4-utilizing viruses. One such approach combined a modified form of RANTES (amino-oxypentane RANTES (AOP-RANTES)) to antagonize CCR5, and a modified Stroma-derived factor 1 beta (SDF-1β) with an added methionine (Met-SDF-1β) to antagonize CXCR4 (159). This study demonstrated that alone, each of these modified forms of the natural ligands RANTES or SDF-1β bound more efficiently to their responding co-receptor yet were suboptimal in inhibiting clinical HIV-1 isolates in PBMCs. However, when combined, their inhibition of infection with the isolates increased to 99% (159). This is just one approach to prevention of HIV-1 replication that involves co-receptor targeting, but which supports that drugs that are suboptimal on their own can have efficient additive or synergistic properties that may be more beneficial in the clinic.

Following this study, it was discovered primarily through other *in vitro* or *ex vivo* studies that antagonism of both CXCR4 and CCR5 was possible using the same compound and several such compounds have been identified (167). These dual

TABLE 2 | Combinatorial approaches utilizing CCR5 targeting techniques for therapy of HIV-1 infection.

Combination Approach	Methods		Study Stage	Model	Outcome
Inhibition of CCR5 and CXCR4	CCR5 inhibition with a modified form of RANTES, aminooxypentane (AOP)-RANTES, and CXCR4 inhibition with Stroma-derived factor 1 beta (SDF-1beta) derivative, Met-SDF-1beta.		*Ex vivo*	PBMCs	Combinations of these compounds inhibited mixed infections with R5 and X4 viruses (95 to 99%), whereas single drugs were less inhibitory (32 to 61%) (159)
	Dual CCR5/ CXCR4 Antagonists	AMD3451	*In vitro and ex vivo*	PBMCs, monocytes, and macrophages	AMD3451 inhibited infection with clinical HIV-1 isolates or a variety of R5, R5/X4, and X4 strains of HIV-1 and HIV-2 at an IC_{50} ranging from 1.2 to 26.5 μM in various T cell lines, CCR5- or CXCR4-transfected cells, PBMCs, and monocytes/macrophages. (160)
		Ingenol derivatives	*In vitro and ex vivo*	MT-4 cells and PBMCs	Ingeol derivatives activated the HIV-1 LTR in MT-4 cells and primary CD4+ T cells with latent virus at 10 nM treatment, inhibited replication of HIV-1 subtuype B and C in MT-4 cells and PBMCs at EC_{50} of 0.02 and 0.09 μM, respectively, and induced downregulation of CD4, CCR5, and CXCR4 (80)
		Cumarin-based ligand GUT-70	*In vitro*	M1-CCR5 T cells	GUT-70 stabilized plasma membrane fluidity, inhibited HIV-1 entry, and down-regulated the expression of CD4, CCR5, and CXCR4. GUT-70 also inhibited HIV-1 replication through the inhibition of NF-κB (161)
		Suramin analog NF279	*Ex vivo*	MDMs infected with pseudoviruses	NF279 suppressed fusion of HIV-1 with MDMs, inhibited Ca^{2+} influx induced by R5 and X4 agonists, and antagonized gp120 mediated activation of CXCR4 (162)
		Pyrazolo-Piperidines	*In vitro*	PBMCs	Different compounds showed IC_{50} values ranging from 0.8 to 25 μM against R5 or X4 HIV-1 strains (163)
		Penicillixanthone A	*In vitro*	TZM-bl cells	Penicillixanthone A inhibited R5 and X4 HIV-1 at an IC_{50} of 0.36 and 0.20, respectively, but had moderate toxicity at 20.6 μM against TZM-bl cells (164)
Gene therapy targeting CCR5 and a suicide gene	Two-vector system: An integrating lentiviral vector expressing an HIV-1 Tat dependent TK-SR3 and a non-integrating lentiviral (NIL) vector expressing CCR5gRNA-CRISPR/Cas9 and HIV-1 Tat protein.		*In vitro*	TZM-bl cells	TZM-bl cells were stably integrated with TK-SR39 and were resistant to R5 HIV-1 (165)
Gene therapy targeting CCR5 in combination with a fusion inhibitor	Cal-1 comprising a short hairpin RNA to CCR5 (sh5) and a peptide that inhibits viral fusion with the cell membrane (C46)		*Ex vivo*	PBMCs	Cal-1 reduced CCR5 expression in PBMCs to CCR5Δ32 heterozygote levels and suppressed virus up to day 12. No escape mutations were present through 9 weeks of challenge. Cal-1 suppressed infection by different R5 viruses and inhibited virion internalization by 70% compared to 13% for C46 (166)

TK-SR39, Thymidine Kinase mutant SR39; LTR, Long Terminal Repeat; MDMs, primary human macrophages (monocyte-derived human macrophages).

antagonists vary greatly in structure and include peptide-based antagonists, pyrazole-based antagonists, bicyclams, and even naturally occurring compounds such as derivatives of ingenol or diterpene and other plant-derived compounds initially intended for treatment of other diseases. Among these, the N-pyridinylmethyl cyclam analog AMD is one of the first bicyclams to be discovered with dual CXCR4 and CCR5 antiviral properties (160). Princen et al. demonstrated this compound can efficiently inhibit infection of a variety of HIV-1 and HIV-2 isolates in various cell lines as well as primary cells with minimal toxicity to these cells, but no clinical study has followed since then (160, 167). Nonetheless, several other studies followed which demonstrated other dual antagonists can successfully inhibit various HIV-1 strains *in vitro* or *ex vivo* while exhibiting low toxicity (80, 161–164, 168).

A decade later in 2014, Abreu et al. demonstrated that ingenol derivatives (ISDs) isolated from *Euphorbia tirucalli* can likewise inhibit X4 and R5 viruses *in vitro* and *ex vivo*. Treatment of PBMCs and MT-4 human T cells with ISDs was shown to inhibit HIV-1 subtype B and C replication at comparable EC_{50}s to drugs used in ART (80). Interestingly, this study also demonstrated potential latency reactivation properties of ISDs. When different reporter cell lines and infected CD4+ T cells from five ART-suppressed patients were treated with ISDs, LTR activation was induced (80). The results of this study provide hope that LTR-driven transcription to reactivate HIV-1 in latently infected cells, along with prevention of HIV-1 infection through downregulation of co-receptors required for entry can both be accomplished using the same modality (80).

Shortly thereafter, other natural products were shown to be dual co-receptor antagonists. GUT-70, a natural product derived from *Calophyllum brasiliense*, was shown to down-regulate the expression of CD4, CCR5, and CXCR4 in M1-CCR5 cells and inhibit entry of X4 and R5 viruses. While the downregulation of these receptors was significantly correlated with reduced infectivity, other mechanisms of GUT-70 action were discovered to greatly contribute to its antiviral effect. These include reducing the membrane fluidity of cells to disrupt viral entry as well as inhibition of NF-κB to prevent viral replication (161, 169). Considering these various cellular components modulated by GUT-70 are necessary for broader biological processes as compared to the inhibition of the co-receptors alone, further understanding of these antiviral mechanisms is necessary to predict potential off-target effects of GUT-70 in HIV-1-infected patients. Moreover, GUT-70 has also previously demonstrated anti-leukemic properties (170). Dual co-receptor antagonism using this compound for treatment of HIV-1 infection can thus provide the additional advantage of simultaneous prevention or treatment of lymphoma or leukemia, which overcomes potential toxicity of drug-drug interactions normally associated with anti-tumor agents and ART (161).

Accordingly, re-evaluation of the antiviral mechanism of even previously characterized anti-HIV-1 compounds has revealed that they also prevent HIV-1 infection through dual co-receptor antagonism. The NF279 was initially reported as an HIV-1 fusion inhibitor that prevents HIV-1 infection by blocking P2X1 channels (171). A recent study, however, demonstrated that it does not inhibit HIV-1 fusion by preventing the activation of P2X1 channels, but by antagonizing CXCR4 and CCR5 signaling through suppression of Ca^{2+} responses in primary macrophages induced by gp120 binding (162). This recent investigation on NF275 is one of the few current studies evaluating the antiviral mechanism of these novel compounds beyond their co-receptor binding properties. Another study evaluated the mechanism of a compound containing pyrazole-piperidine core, which was originally identified through a GPCR-guided screen (163). This compound was found to prevent HIV-1 entry with X4 or R5 strains, but primarily due to its non-nucleoside reverse transcriptase (NNRT) activity as opposed to its co-receptor antagonistic properties (163). While the predominant mode of action of this compound was identified to be its inhibition of HIV-1 RT, its additional dual chemokine antagonism was proposed to delay development of HIV-1 resistance when compared to other NNRTIs (163). Therefore, a novel compound may not strongly inhibit binding of HIV-1 to CXCR4 or CCR5, but may still exert robust antiviral efficacy and protect against infection through additional predominant or complementary mechanisms.

Another compound investigated, penicillixanthone A (PXA), is a natural xanthone dimer derived from the fungus *Aspergillus fumigates* that also exerts dual co-receptor antagonistic effects. This dimer was described to have potent anti-HIV-1 activity due to inhibition of infection with R5-tropic HIV-1 SF162 and CXCR4-tropic HIV-1 NL4-3 in TZM-bl cells, with an IC_{50} of 0.36 and 0.26 μM, respectively (164). However, it exhibited moderate toxicity in TZM-bl cells and thus has a major disadvantage as compared to other dual co-receptor antagonists currently under investigation (164).

While dual targeting of CCR5 and CXCR4 co-receptors appears promising for control of HIV-1 infection, there are concerns that ablating the CXCR4 receptor in certain cell types will lead to detrimental effects. This is likely due to the important role of CXCR4 in maintaining normal function of hematopoietic stem cells (172, 173). In mice, for example, CXCR4 deficiency causes embryonic lethality or malignancy (174, 175). This highlights the persistent challenges faced with development of effective combinatorial approaches—that is, maintaining high antiviral efficacy with low risk of adverse effects in the HIV-1-infected patient.

In addition to CXCR4 inhibition, another combinatorial approach investigated shRNA targeting of CCR5 in combination with the fusion inhibitor C46, a gp41-derived C peptide (166, 176). Not only was this approach demonstrated to be effective against different strains of HIV-1, but mutant viruses were also not detected in infected PBMCs over a week later (166, 176). While the rise of mutants or CXCR4 tropic viruses can still occur months to years later, these recent studies indicate that more assays are being incorporated to assess novel drug or therapy efficacy at the *in vitro* stage to better avoid this outcome.

Alternatively, emergence of CXCR4-utilizing viruses can be avoided using a combinatorial approach that utilizes an HIV-1 protein-dependent suicide gene. This was accomplished by

introducing a CCR5 gRNA-CRISPR/Cas9 system into TZM-bl cells to knockout CCR5 along with an HIV-1 Tat-dependent suicide gene TK-S39 (165). This novel approach allows expression of HIV-1 Tat, which can occur when a CXCR4-utilizng or CCR5 antagonist-resistant virus enters and replicates in a cell, to induce cell death and prevent cell-to-cell spread of HIV-1 in the occurrence of HIV-1 replication despite CCR5 knockdown. The introduction of a suicide gene has in fact been previously argued to be a necessity for CCR5 therapy, which otherwise would fail due to expansion of CXCR4-utilizing viruses and selection of CCR5 antagonist-resistant strains among other factors (177). How clinical efficacy of this or other combinatorial approaches discussed would compare with a more established preventative method like ART adherence during CCR5 therapy has yet to be assessed.

6 DISCUSSION

Several therapeutic strategies targeting CCR5, either through blockade of the co-receptor or through gene editing techniques to inhibit its expression, have demonstrated the potential of CCR5 ablation to inhibit HIV-1 infection, at least temporarily. Accordingly, FDA-approved CCR5 targeting therapeutics such as Miraviroc can be used for patients for which ART may not be suitable (110).

As with all HIV-1 therapeutic strategies being investigated, ART-independent control of HIV-1 infection through CCR5 targeting is promising but major hurdles persist for the development of a cure. Common characteristics of HIV-1 infection such as establishment of latently infected reservoirs, impracticality of therapy delivery to anatomically privileged sites, and the ongoing development of drug resistant viruses continue to challenge efficacies of CCR5 targeting strategies (178). Furthermore, even after optimization of delivery of gene editing tools or of the potency of CCR5 antagonists, there is insufficient data to support that the majority of HIV-1 susceptible cells in a patient can be targeted. This then suggests inhibition of viral replication will still be necessary, which currently is only feasibly accomplished with ongoing ART adherence. Therefore, additional clinical data is needed to support that the therapeutic outcome of individuals undergoing CCR5 targeting therapy will be a functional cure similar to that which occurred for the Berlin or London patients. Instead, CCR5 inhibition alone may serve a more supplementary approach to prevent disease progression and compensate for the shortcomings of ART.

To overcome these limitations of CCR5 monotherapies, many combinatorial approaches have been investigated in recent years and show potential for more efficient inhibition of viral infection with diverse HIV-1 strains as well as avoidance or delay of the development of resistant strains (179). However, in comparison to the vast research on combinatorial methods for HIV-1 therapy or cure, integration of CCR5 targeting appears to be at the beginning *in vitro* stages. Considering these approaches target other cellular functions which may be detrimental to patients, clinical data assessing the safety of these approaches is needed. If evaluated to be safe in patients, many of these approaches including the use of dual co-receptor antagonists, will demonstrate that inhibition of HIV-1 infection without the requirement of life-long ART adherence will at least be feasible.

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REVIEW
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CCR5 as a Coreceptor for Human Immunodeficiency Virus and Simian Immunodeficiency Viruses: A Prototypic Love-Hate Affair

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CCR5, a chemokine receptor central for orchestrating lymphocyte/cell migration to the sites of inflammation and to the immunosurveillance, is involved in the pathogenesis of a wide spectrum of health conditions, including inflammatory diseases, viral infections, cancers and autoimmune diseases. CCR5 is also the primary coreceptor for the human immunodeficiency viruses (HIVs), supporting its entry into $CD4^+$ T lymphocytes upon transmission and in the early stages of infection in humans. A natural loss-of-function mutation CCR5-Δ32, preventing the mutated protein expression on the cell surface, renders homozygous carriers of the null allele resistant to HIV-1 infection. This phenomenon was leveraged in the development of therapies and cure strategies for AIDS. Meanwhile, over 40 African nonhuman primate species are long-term hosts of simian immunodeficiency virus (SIV), an ancestral family of viruses that give rise to the pandemic CCR5 (R5)-tropic HIV-1. Many natural hosts typically do not progress to immunodeficiency upon the SIV infection. They have developed various strategies to minimize the SIV-related pathogenesis and disease progression, including an array of mechanisms employing modulation of the CCR5 receptor activity: (i) deletion mutations abrogating the CCR5 surface expression and conferring resistance to infection in null homozygotes; (ii) downregulation of CCR5 expression on $CD4^+$ T cells, particularly memory cells and cells at the mucosal sites, preventing SIV from infecting and killing cells important for the maintenance of immune homeostasis, (iii) delayed onset of CCR5 expression on the $CD4^+$ T cells during ontogenetic development that protects the offspring from vertical transmission of the virus. These host adaptations, aimed at lowering the availability of target $CCR5^+$ $CD4^+$ T cells through CCR5 downregulation, were countered by SIV, which evolved to alter the entry coreceptor usage toward infecting different $CD4^+$ T-cell subpopulations that support viral replication yet without disruption of

host immune homeostasis. These natural strategies against SIV/HIV-1 infection, involving control of CCR5 function, inspired therapeutic approaches against HIV-1 disease, employing CCR5 coreceptor blocking as well as gene editing and silencing of CCR5. Given the pleiotropic role of CCR5 in health beyond immune disease, the precision as well as costs and benefits of such interventions needs to be carefully considered.

Keywords: CCR5, human immunodeficiency virus, simian immunodeficiency virus, delta 32, red-capped mangabey, sooty mangabey, African green monkey, virus transmission

CCR5 CHEMOKINE RECEPTOR

Role and Function in the Organism

CCR5, a C-C chemokine receptor 5 (formerly known as CC-CKR-5 or CKR5), is primarily involved in immune surveillance, inflammatory response, tumor formation and metastasis (1–3), pathogenesis of inflammatory diseases (4–6), asthma (7, 8), and cancer (2, 3). It plays a key role in the recruitment of the immune cells to inflammation sites by directing immune cell migration (chemotaxis) along the chemokine gradient (9, 10). CCR5 regulates trafficking and effector functions of memory/effector T lymphocytes, macrophages, and immature dendritic cells (11). Beyond its direct involvement in mediation of the immune processes, it acts as a suppressor of learning, memories and synaptic connections in the brain (12).

CCR5 Receptor and Its Native Ligands

CCR5 is a seven-transmembrane, G protein-coupled receptor (GPCR), a member of the family of class A GCPRs. As a GPCR, CCR5 comprises of seven transmembrane α-helices, three extracellular loops, three intracellular loops, an amino-terminal domain and a carboxyl-terminal domain (13).

The natural ligands for CCR5 include chemokines (small chemoattractant cytokines) involved in innate immunity, which are natural suppressors of HIV-1 infection (14–17): macrophage inflammatory proteins CCL3 (MIP-1 α) and CCL4 (MIP-1 β), CCL5 (RANTES - regulated on activation, normal T-cell expressed and secreted) and CCL3L1, the most potent among the agonists of CCR5 and HIV-1-suppressant (18). CCL7 (MCP-3) is, on the other hand, the main antagonist ligand of the CCR5 receptor (19). Activation of the CCR5 receptor by its agonist ligands stimulates cell migration and mediates inflammatory responses.

CCR5 Receptor Lifecycle

CCR5 activation occurs upon binding its agonist ligands and leads to stimulation of pertussis toxin-sensitive heterotrimeric $\alpha\beta\gamma$G protein by catalysing the exchange of GTP for GDP in the Gα subunit that triggers intracellular pathways involved in chemotaxis and activation of leukocytes (20). Upon ligand binding, CCR5 receptor undergoes rapid phosphorylation in the carboxy-terminal region that promotes desensitization and internalization regulated by β-arrestin, an adaptor protein causing sequestration of the receptor to clathrin-coated pits. Upon clathrin-mediated endocytosis, CCR5 receptor moves to endosomes and Golgi network, and then is recycled back to plasma membrane (21–23). The conformation of the CCR5 receptor is dynamically impacted through this process and dependent on cellular localization (24).

CCR5 Expression

Cell and Tissue Expression

CCR5 is expressed on a wide array of bone-marrow-derived cells, including lymphocytes, monocyte/macrophages, granulocytes, T cells, and specialized immune cells including natural killer (NK) cells and regulatory T (Treg) cells, located in primary and secondary lymphoid organs, including thymus and spleen, nonhematopoietic peripheral tissues, such as epithelium, endothelium, vascular smooth muscles, fibroblasts, and in central nervous system in neurons, astrocytes, microglia (15, 25–29). In the normal adult brain, CCR5 is highly expressed in microglia, yet it is undetectable in neurons (30).

CCR5 Expression in Relation to Inflammation

Increased levels of CCR5 expression on mononuclear cells is characteristic to chronically inflamed tissues, suggesting that $CCR5^+$ cells are recruited to the inflammatory sites (25). In addition to the lymphoid tissues, CCR5 expression is induced in the cortical neurons and transiently lowered in microglia/macrophages in response to stroke (30).

Alterations of CCR5 Expression Through Genetic Deficiencies

While CCR5 appears an essential player in various aspects of immune health, knockout alleles of this gene, leading to the loss of function of the CCR5 coreceptor, are present in different primate species at high frequency and with occurrence of null homozygous genotypes (**Figure 1**).

The CCR5-Δ32 Genetic Deficiency in Humans

CCR5 coreceptor expression on the cell surface can be prevented by a natural genetic variant, a 32-bp deletion (Δ32) observed in human populations. This mutation is localized in the region encoding the second extracellular loop of the receptor and results in a frameshift in the protein coding sequence leading to premature truncation of the normal CCR5 protein and abrogating its availability on the cell surface. The loss of function of the CCR5 gene modulates the risk for HIV transmission and counteract the pathogenesis of HIV infection (33–35).

Global Distribution of CCR5-Δ32 Allele in Human Populations

The CCR5-Δ32 allele is primarily observed in populations of European descent, where its average frequency is ~10%, while

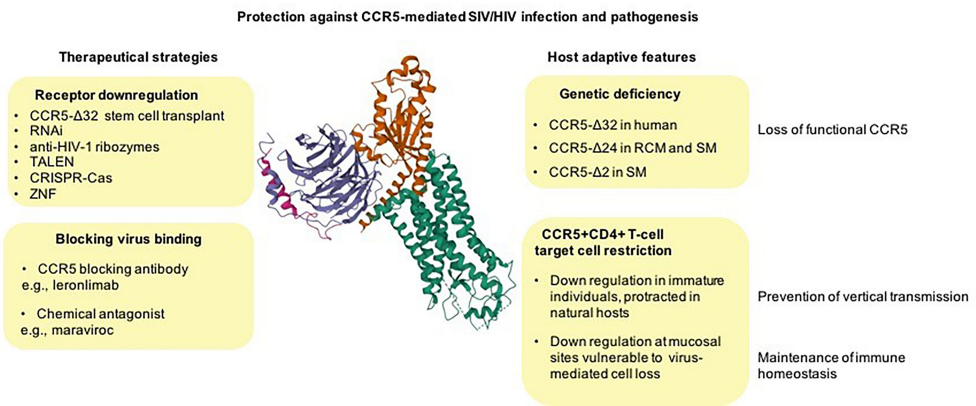

FIGURE 1 | From natural control of CCR5 activity to therapeutic approaches against HIV disease. CCR5 null alleles, preventing CCR5-mediated virus entry, naturally emerged and raised to high frequency in different primate species most likely as a result of host adaptation to lethal pathogens (unknown ancient pathogen in humans and SIV in natural hosts) (top right). CCR5 downregulation of CCR5 on CD4+ T-cells may be an adaptive feature of natural hosts protecting against the vertical transmission of the virus (*via* breast feeding), and it may also represent an evolutionary adaptation to spare essential CD4+ T-cell subsets from killing by the virus (bottom right). Examples of approaches for therapeutic disruption of CCR5 expression include natural *CCR5* gene knockouts (resulting in functional cure), programmable nucleases, and gene silencing (top left) and approaches to blocking the virus fusion with cell membrane *via* chemical CCR5 receptor antagonists or antibodies (bottom left). Cryo-EM structure of the chemokine receptor CCR5 (green) in complex with RANTES and guanine nucleotide-binding protein Gi subunits alpha-1 (red), beta-1 (blue), and gamma-2 (magenta) was acquired from https://www.rcsb.org/3d-view/7F1R/1 wwPDB: Worldwide Protein Data Bank (13, 31, 32).

being virtually absent in SubSaharan Africans, Asians and Native Americans (34–37). Migrations have likely contributed to the global distribution of the CCR5-Δ32 allele, as, despite its predominant presence in Europe, high frequencies of CCR5-Δ32 were also observed in specific populations of European ancestry outside Europe, e.g., in South Africa (13%) and Chile (12%) (38). The CCR5-Δ32 is also present in African Americans, yet at low incidence (2%) (35) and in some Jewish populations, with the highest frequency in the Ashkenazis (11-20%) (39), where it probably emerged through admixture with people of European descent. The presence of this variant mainly in Euroasian populations suggests that the mutation occurred after their separation from the founders/ancestors of African, Asian, and Native American populations (40).

In Europe, the distribution of the CCR5-Δ32 variant shows marked clines, North-South and East-West. The highest frequency of the CCR5-Δ32 variant was observed among Northern Europeans, for example, in Finland and Russia (16%), Iceland (15%), Sweden (14%), Denmark (13%), Northern France (14%), and Norway (10%) (36, 37, 41, 42), while its lowest frequency was seen in Southern European and Mediterranean populations, such as Spain (7%), Italy (5.6%), Portugal (5.2%), Sardinia (4%), with the lowest prevalence being observed in Corsica (0.9%) (36, 37, 41).

Origins and Age of the Variant

The spatial distribution of the CCR5-Δ32 mutation, with the highest frequency in the Nordic countries suggests a Northern European origin of this variant (37, 41) and subsequent spreading out from the Scandinavian peninsula across Europe by the Vikings through their raids in the 8^{th}-10^{th} centuries (41). While the role of the long-range dispersal consistent with the Viking mediated spread was demonstrated, other models raise the possibility that the allele arose outside of Scandinavia and moved into the region *via* dispersers from the South (43) or from the Finno-Ugrian tribes of Russia, where the mutation is frequent nowadays (37).

Several features of the CCR5-Δ32 mutation suggest that it had a unitary origin, subsequently becoming a subject to positive selection, as supported by: (a) its high frequency, (b) its virtual absence in other populations than those of European descent, (c) a striking gradient of this variant across Europe, as well as (d) a long-range disequilibrium in this CCR5 locus (36, 37, 44). Age estimates of the ancestral CCR5-Δ32 variant based on microsatellite analysis suggested that the deletion arose relatively recently, yet in a time frame that varies widely: about 700 years ago with an estimated range of 275-1,875 yrs (based on haplotype coalescence) (36); 3,400 years ago (based on the recombination frequency near the CCR5 locus); 1,400 years ago (based on the microsatellite mutation rate); or 2,000-2,200 years ago (based on mutation and crossover events) (37). Other studies suggested that the original single mutation occurred 2,500 years ago (44).

Neutral Drift vs. Selection in Favor of CCR5-Δ32

The relatively recent origin of the mutation, ranging between the Neolithic period (37) and Middle Ages (36), suggests that a positive selection or a selection acting on heterozygotes associated with selective advantage, rather than random drifts, could have driven the allele to the currently high frequency in the populations of European descent (37, 41). In comparison to the calculations based on the linkage disequilibrium, mutation rate, and the spatial allele distribution, which indicated the age of CCR5-Δ32 variant between several hundred to several thousand

years, the age estimate for this variant was 127,500 years (i.e., two orders of magnitude higher), when its frequency was determined assuming neutral drift (36). This discrepancy points to intense natural selection, rather than random drift, as a force shaping the frequency of CCR5-Δ32 allele, suggesting an existence of local environmental factors, such as major pathogens, that have exerted a marked selective pressure on this locus in the historical time (43, 45).

Potential Selective Factors

Pathogens and infectious diseases represent major selective forces that shape the frequencies of alleles involved in protective immune mechanisms. Nowadays, the CCR5-Δ32 variant plays an important role against the HIV transmission in the human populations. An ancestor of HIV-1 originated from a cross-species transmission from chimpanzee to humans at the beginning of the 20th century (46) and then it spread out in the European populations only in the 1980s (47, 48). Meanwhile, several cross-species transmissions of the SIVsmm that naturally infect the sooty mangabeys are at the origin of HIV-2 (49–52). These cross-species transmissions also occurred during the 20th century (53). Since HIVs were passed to humans only recently, they have not had sufficient time to exert such profound selective effect on the allele frequency. Instead, several other pathogens and resultant diseases were proposed to drive the CCR5-Δ32 mutation to the contemporarily high frequencies.

The first proposed selective factor was the plague, which had a high mortality rate and was confined to Europe, where it persisted for ~300 years, from 1347 to 1670 (36). The plague hypothesis, which still remains one of the most "popular" concepts, despite some contradicting observations, proposed that macrophages infected with *Yersinia pestis* (54) were introduced in the human bloodstream by bites by fleas travelling on black rats in the Middle Ages (36), spreading the disease that killed ~40% of the population of Europe during epidemics, such as the Black Death of 1348–1350. *In vitro* studies showed, however, that although the *Ccr5*-deficient macrophages have a drastically reduced uptake of *Yersinia pestis* (an isolate from a fatal human case of plague), they experience a similar mortality with the wild-type *Ccr5*-expressing macrophages (55), suggesting that, if this model is representative to humans, the CCR5 deficiency did not have a protective effect against plague in people (56). The plague hypothesis was also challenged by the observation that plague may have not generated sufficient selective pressure for increasing the CCR5-Δ32 allele to contemporary frequencies (57). Instead, the selective raise of the CCR5-Δ32 allele was proposed to be attributed to smallpox *(Variola major)* caused by the poxvirus, based on population genetic analysis, which considered the temporal pattern and age-dependent nature of the diseases (57). The smallpox hypothesis, on the other hand, was opposed by the argument that the lethal form of smallpox emerged only recently (in England ~1628), not long before the introduction of variolation ~1750 and vaccination ~1800 (44) that gave the pathogen a narrow time window to push the mutant allele frequency to the current level (less than estimated 600 years needed for that) (57). As a selecting factor, other models considered recurrent epidemics of viral hemorrhagic fevers ("hemorrhagic plague") affecting the eastern Mediterranean region since at least 1500 BC (44) or a pathogen spread during the time of the expansion of Roman Empire (58). A potential influence of other microbes, such as *Shigella*, *Salmonella*, and *Mycobacterium tuberculosis*, on the frequency of CCR5-Δ32 variant was also proposed (36).

Sabetti et al. reported that the variation of the CCR5-Δ32 was consistent with the pattern of neutral selection and estimated that the ancestral haplotype carrying CCR5-Δ32 variant might have arisen more than 5,000 years ago, with a certain probability that some selection has occurred thereafter (40).

Common CCR5 Deficiencies in Nonhuman Primates (NHPs) That Are Natural Hosts of SIV

The case of CCR5-Δ32 allele in humans resembles deletions in the CCR5 gene present in the African monkeys of the genus *Cercocebus*, preventing CCR5 coreceptor-mediated SIV entry.

CCR5-Δ24 Mutation in Red-Capped Mangabey (RCM, *Cercocebus torquatus*) and Sooty Mangabey (SM, *C. atys*)

West African natural host species of SIVs, particularly the *Cercocebus* species, such as the RCM and the SM often carry a 24-bp deletion mutation in the *CCR5* gene (CCR5-Δ24) that causes an in-frame deletion of eight aminoacids in the fourth transmembrane region, abrogating the cell surface expression and coreceptor function of CCR5 for SIV entry (59, 60). The CCR5-Δ24 mutation in RCMs has a high frequency (86.6%) (60), exceeding that of the CCR5-Δ32 allelic variant in the human populations (44). It was observed in geographically distant RCM populations, from Gabon and Nigeria (and mangabeys in the US zoos), which demonstrates that its frequency is not due to local founder effects, but rather attributable to an old age and selective advantage of the variant (60, 61). The CCR5-Δ24 allele was also observed in SMs, yet at lower frequencies (4.1%) (60). The geographic ranges of RCMs and SMs are not overlapping at present days, as RCMs inhabit the swamps, mangroves and riverine forests along the Gulf of Guinea shore of Nigeria, Cameroon, Equatorial Guinea, Gabon, and the Gabon-Congo border (62), while the habitat of the SMs ranges in the forests at the Atlantic coast from Senegal to the Ivory Coast (63). The exact time of origin of the CCR5-Δ24 mutation in these two *Cercocebus* species is not known. The timing of divergence for these species is estimated at 2.29 MYA (64), suggesting either an ancient age of mutation before the split of these species, or its emergence following recurrent events. Unlike HIVs, which on the pandemic scale have been in the human populations for ~60 years, SIVs have been present in African NHPs for a much longer evolutionary time scale (65, 66) and potentially could have been a selective factor behind the high frequency of null CCR5 alleles. However, this hypothesis has yet to be confirmed.

CCR5-Δ2 Mutation in SM

In addition to the CCR5-Δ24 allele (that they carry at a frequency of 3%), SMs are also frequently (26%) carrying a 2-bp deletion in the *CCR5* gene (CCR5-Δ2), which, like the CCR5-Δ24 allele,

encodes a truncated molecule that is not expressed at the cell surface (59).

The presence of these common deletion alleles of CCR5 in different primate species (CCR5-Δ32 in humans, CCR5-Δ24 in RCMs and SM, and CCR5-Δ2 in SM) suggests that the emergence and high frequencies of these alleles may represent a convergent evolution, yet it remains unclear what pathogens were driving these adaptations, most likely different for humans and African NHPs.

CCR5 ROLE IN HIV/SIV INFECTION

To infect $CD4^+$ T cells in humans, HIV-1 utilizes CCR5 (mediating entry of R5 viruses) or CXCR4 (mediating entry of X4 viruses), or both entry coreceptors (67). CCR5 coreceptor is mostly expressed on memory $CD4^+$ T cells, while CXCR4 is expressed on both memory and naive cells. The change in coreceptor usage towards CXCR4-tropism during the later stage of HIV-1 infection may contribute to accelerated disease progression (68–70). Meanwhile, in addition to CCR-5, HIV-2 uses GPR15 (BOB) and CXCR6 (BONZO) (71).

CCR5 density on the surface of $CD4^+$ T cells is a key regulator of cell infectability and virus production, and a critical determinant of the HIV-1 disease progression (72). The extent of cell death correlates with the virus replication, and the capacity of HIV to induce cell death depends on the level of CCR5 expression on the surface of the $CD4^+$ T cells (73). The density of CCR5 receptors on target cells is logarithmically correlated with HIV-1 viremia (72) and disease progression (74). *In vitro* studies suggested a dual role of CCR5 in determining HIV-1 production: as an entry coreceptor, it acts as a critical factor for infection, yet exerts only a moderate influence on the magnitude of viral loads, while as a postentry regulator of the HIV-1 life cycle, particularly at reverse transcription stage, it accounts for the logarithmic relation between the viremia and CCR5 density (75).

HIV Binding

HIV entry *via* CCR5 receptor occurs through a series of processes, depending on the conformational state of both viral envelope protein and cellular receptor (24). CCR5 stabilizes the CD4-induced conformation of Env protein and anchors the virus near the cell surface (76). Chemokines that are native CCR5 ligands naturally restrict HIV-1 infection sterically, by masking the viral envelope glycoprotein gp120 binding sites and promoting CCR5 endocytosis, reducing the CCR5 cell surface level (77, 78). The second extracellular loop and amino-terminal domain of CCR5 receptor are critical for interacting with HIV Env protein and binding natural chemokine ligands, such as CCL4 and RANTES. While these molecules bind different regions, they both compete with the virus for the binding site (79, 80).

Biological activity of CCR5 depends on its conformations (81), which influences interaction with HIV gp120 and native chemokines (82). Some receptors have low binding affinity for native CCR5 chemokines and therefore chemokines are weak inducers of CCR5 endocytosis (82).

CCR5-Δ32 Genetic Variant Has a Protective Action Against HIV Infection

The CCR5-Δ32 variant generates a nonfunctional entry coreceptor for HIV that does not support fusion between the virus and the target-cell membrane, thus preventing infection and pathogenesis. The homozygous CCR5-Δ32bp genotype (Δ32/Δ32) carriers (about 1% of Europeans) are highly protected from HIV-1 infection (33–35, 83–85), yet this protection is not complete, as rare cases of HIV infection were reported in the homozygotes (86–88). The Δ32bp knock-out of the *CCR5* gene was observed in cohorts of multiple HIV-exposed seronegative (HESN) individuals (33, 35), and resistance of circulating cells to HIV infection *in vitro* was reported (34).

The WT/Δ32 heterozygotes exhibited a reduced ability to support HIV-1 replication compared to the wild type homozygotes (WT/WT) (33–35), had reduced viral loads (33, 34, 36, 84), a slower rate of $CD4^+$ T-cell depletion (84), resulting in a 2-3 years delayed progression to AIDS (33–35, 84, 85, 89), and improved virological response to antiretroviral therapy (90). CCR5-Δ32bp heterozygosity also appeared to be associated with reduced susceptibility to HIV-1 infection (91), yet this observation was not universally confirmed (92). The CCR5 WT/Δ32 genotype was also associated with protection from AIDS-related lymphoma, a non-Hodgkin's B cell malignancy that is common in patients with AIDS (93, 94).

An increased prevalence of heterozygotes for the CCR5-Δ32 mutation was found in some, but not all, cohorts of HIV long-term nonprogressors (LTNP, i.e., HIV infected individuals with little or no clinical signs of progression), but it does not appear either essential or sufficient for protection against disease progression (87, 95). In elite controllers (ECs, which spontaneously control HIV replication to undetectable viral loads and maintain stable $CD4^+$ T-cell counts), the prevalence of CCR5-Δ32 heterozygotes appears somewhat elevated compared to the general population, yet this difference is not striking (96).

Molecular Mechanism of Protection Conferred by the CCR5-Δ32 Allele

When compared to homozygotes with both normal copies of CCR5 WT/WT, CCR5 heterozygotes WT/Δ32 associate a >50% reduction in cell surface expression of CCR5, and display a lower infectability of blood cells by the M-tropic HIV-1 *in vitro* (97, 98). The abrogation of cell surface expression of CCR5 coreceptor is caused by the interruption of CCR5 transport to the cell membrane. While normal CCR5 protein can undergo both post-translational phosphorylation and/or cotranslational multimerization, the mutant CCR5-Δ32 can only form multimers and is incapable of being phosphorylated. In the CCR5 heterozygotes, the heterodimers between the normal CCR5 and mutant CCR5-Δ32 proteins are retained in the endoplasmic reticulum causing reduced cell surface expression of the functional CCR5 coreceptor (98).

Genetic Variation in CCR5 and Its Ligands May Influence CCR5 Functionality

The delayed progression to HIV disease was associated with other types of genetic variation in the *CCR5* locus. For example, variants

located within the CCR5 promoter (99–102) showed regulation of CCR5 cell surface expression and of $CD4^+$ T-cell apoptosis, as well as a correlation with HIV disease progression (103). The CCR5 promoter variant 59029 G/A reduced the activity of the CCR5 promoter by ~45% and resulted in ~4 years delayed progression to AIDS in the carriers of this mutation (104). Downregulation of active transcription of CCR5, paralleled with reduced cell surface expression of CCR5, was observed in a subset of elite and viremic controllers with an R5-resistance phenotype (105). The transcriptomic downregulation of CCR5 (9-fold) was associated with downregulation of multiple genes, including CCR2, in the 500 kb block around the CCR2-CCR5 locus on the chromosome 3p21 (105).

Also, genetic variation in genes coding ligands of CCR5 may influence the functionality of this receptor. For example, CCL3L, a HIV-1 suppressive chemokine, shows a copy number variation that is associated in a dosage dependent manner with susceptibility to HIV infection; lower number of copies of CCL3L are associated with an increased risk of HIV (106).

The effect of the CCR5 deficiency (Δ32/Δ32) in conferring nearly complete prevention of HIV-1 infection, was achieved through experimental manipulation that blocked HIV-1 entry into cells with an anti-CCR5 reagent (97). Given that the genetic variation lowering the CCR5 expression has an advantageous effect on taming HIV pathogenesis and did not seem to be associated with a deleterious phenotype in humans (33), interventions blocking or reducing the CCR5 expression emerged as promising approaches to the prevention and treatment of the HIV disease (107–115). However, while the initial studies suggested that the loss of function due to the CCR5-Δ32 does not bear marked impact on health, there is increasing evidence that CCR5 plays a complex role in organism homeostasis, and is not completely dispensable (45, 116–118).

CCR5 Role in Infectious Diseases

Beyond SIV/HIV infection, CCR5 plays multiple roles in viral diseases (119), bacterial and parasitic infections (120). It is anticipated that CCR5-deficiency may exert several different, some mutually opposing, effects: (a) prevent infection with CCR5-tropic pathogens, (b) weaken the immune response to some pathogens, leading to increased susceptibility to infection, and (c) reduce CCR5-mediated inflammation, which can either hamper protective inflammatory response, or reduce problems related to excessive inflammation.

CCR5 is a key protective factor against some pathogens. For example, it promotes survival during infection with the West Nile virus (WNV), which can cause fatal encephalitis, by promoting leukocyte trafficking to the brain during the infection (116). However, genetic deficiency of CCR5 (Δ32/Δ32) confers a strong risk of symptomatic WNV infection associated with a fatal outcome (117, 121). In influenza patients, CCR5 deficiency causes a four-fold increased mortality (122). These findings warrant a question regarding the safety of some HIV therapies employing null CCR5 alleles and motivates a development of strategies blocking virus binding CCR5 while preserving the functionality of CCR5 as a chemokine receptor (118).

On the other hand, CCR5 is implicated in infections with CCR5-tropic pathogens, such as Dengue virus (123) and *Staphylococcus aureus* (124). CCR5-Δ32 mutation showed a protective effect against community acquired pneumonia caused by *Streptococcus pneumoniae* (125) and against a severe form of COVID-19 (126).

The CCR5-Δ32 variant plays a complex pro- and antimicrobial role in *Mycoplasma pneumoniae* infection, showing an association with development of chronic infection, yet also with a reduced risk of asthma development in infected children, when compared to children with a nondeleted version of CCR5 (127). Analogically, CCR5 null allele plays a protective effect against toxoplasmosis (*Toxoplasma gondii*) infection (128, 129), while the functional CCR5 receptor is an essential regulator of the inflammatory response following this parasitic infection (10, 130).

Role of CCR5 Beyond Response to Microbes

CCR5-Δ32 mutation was implicated as a factor modulating the risk of neurodegenerative dementias (131, 132) and recovery after stroke and traumatic brain injury (30). CCR5 link with Alzheimer disease was suggested by several studies, yet contradicting association results were reported in the others (131). In the human populations, the CCR5-Δ32 allele was not significantly associated with neurodegenerative dementias, however, an earlier age of onset of neurodegenerative disease was observed in carriers of the CCR5-Δ32 allele, suggesting that the deletion may have a detrimental effect in the context of neurodegeneration (132). Humans that carry CCR5-Δ32 have better outcomes after stroke, with an enhanced motor recovery and reduced cognitive deficits (30). Based on that observation, CCR5 was proposed as "a translational target for neural repair in stroke and traumatic brain injury" (30). This is consistent with the observation in a mouse model that inhibition of CCR5 signaling enhanced neuroplasticity processes, learning and memory, while overexpression of CCR5 led to learning and memory deficits (12).

A Nonprogressing SIV Infection in Natural Hosts

Many African NHP species (e.g., SM, RCM, African green monkey-AGM, mandrill-MND) carry species-specific SIVs, a family of viruses from which HIV evolved. Yet, in contrast to the progressing hosts, such as humans and macaques, the African species do not typically develop immunodeficiency despite many years of infection and high levels of viral replication (133–135).

Host-Pathogen Coevolution

This lack of disease progression in the natural hosts is attributed to the long-term host-pathogen coevolution spanning between hundreds of thousands to possibly millions of years (65, 66), which allowed for the development of protective mechanisms, including lower levels of immune activation upon infection (136–142). Such nonpathogenic SIV infections in natural hosts, some of which utilize specific CCR5 regulations to minimize pathogenesis, provide an insight into adaptive mechanisms protective against the disease (143).

Differences in Infection Between Natural and Non-Natural Hosts

SIV infection in its respective natural hosts is usually nonprogressive and presents the following main features: a) only a transient depletion of peripheral $CD4^+$ T cell, b) absence of intestinal dysfunction and its deleterious consequences, allowing the maintenance of integrity of gut barrier, and c) resolution of immune activation after acute infection. These features are in stark contrast to the pathogenic SIV/HIV infection in a non-natural host, which is characterized by a) progressive $CD4^+$ T-cell loss, b) disruption of the intestinal barrier leading to severe gut dysfunction, and c) chronic inflammation and immune activation (144).

Despite the fact that progressing and nonprogressing hosts display stark differences in the course of infection and pathogenesis, they share several common features of the lentiviral infection, such as the high virus replication rates and fast turnover of infected cells (145–147). Natural SIV infections are therefore different from HIV-1 long-term nonprogressors and SIV-infected RMs, in which the deleterious impact of HIV/SIV infection is minimized through a control of viral replication. Instead, it resembles more to the viremic HIV controllers, a small fraction of HIV-infected individuals that control disease progression by keeping at bay chronic inflammation and T-cell immune activation, in the context of a very active viral replication (148).

Target Cell Availability Shapes Susceptibility to Infection and the Extent of SIV Pathogenesis

The SIV's usage of CCR5 coreceptor to infect its target cells (149) renders the cells coexpressing CCR5 and CD4 (T-cells, the monocytes/macrophages, dendritic cells) the main targets for SIV/HIV infection.

Tissue Expression

The natural hosts of SIV, both uninfected and SIV-infected, are characterized by markedly lowered abundance of $CCR5^+$ $CD4^+$ T cells at the mucosal sites, as well as in peripheral blood, lymph nodes and in bone marrow compared to pathogenic hosts, human and macaques (134). In addition to the $CD4^+$ T cells, CCR5 expression on monocytes is lower in the natural host than in humans and macaques, yet to a lesser extent (134). Intermediate levels of CCR5 expression on the $CD4^+$ T cells were observed in the chimpanzee, a non-natural host, which acquired its species-specific SIVcpz more recently than the African monkey hosts. Still, chimpanzees were infected in the wild for considerably longer periods than humans and macaques. It was therefore postulated that chimpanzees did not have sufficient evolutionary time to adapt well to the virus and thus remain vulnerable to its pathogenic effects (149). Indeed, studies in wild chimpanzees reported that they can progress to AIDS-like disease and develop $CD4^+$ T cell depletion, also their mortality rate was 10-16-fold increase compared to uninfected chimpanzees (150, 151).

In nonprogressing hosts, downregulation of CCR5 expression on $CD4^+$ T cells is associated with lower levels of infection than in non-natural hosts (e.g., SMs vs RMs) (152). In SMs, $CD4^+$ T cells, in particular central memory cells, did not upregulate CCR5 in response to *in vitro* stimulation, and the low CCR5 expression on central memory cells was associated with reduced susceptibility to infection (152). This specific regulation of CCR5 expression on different cell types may protect from SIV infection (and subsequent death) the $CD4^+$ T cell subsets critical to a mild course of infection, while the virus replicates in less dispensable cells (152). It was postulated that long-standing selective pressure of SIV has led to the adaptive shift toward immune functions less dependent on the $CD4^+$ $CCR5^+$ T cells in natural hosts (134).

Age-Related Regulation of CCR5 Expression on T Cells

The $CD4^+$ T cells expressing CCR5 on their surface are the main targets for HIV/SIV infection in both natural and non-natural hosts. The CCR5 surface expression shows a distinctive ontogenetic pattern characterized by an increase of CCR5 expression with the host maturation; as a result, availability of $CCR5^+$ $CD4^+$ target T cells increases with age. In general, the levels of target cells are very low in newborns compared to adults, in both natural hosts of SIV (i.e., AGMs) and in non-natural vulnerable hosts, i.e., macaques and humans (153–156). This pattern, however, markedly varies between progressing and nonprogressing hosts with respect to the timing. The target cell maturation is programmed distinctively among different primate species: (a) rapid in RMs, reaching $CCR5^+$ $CD4^+$ T-cell levels comparable to those in adults by the age of 9 months i.e., at the end of lactation (154, 155, 157); (b) intermediate in humans, with a gradual increase, reaching the adult level by 5-6 years i.e., long after weaning, yet still during childhood (158); (c) slow in natural hosts, in which at the end of the lactation period, the levels of target cells are not significantly different from newborns and much lower than in adults, and an increased CCR5 expression on $CD4^+$ T cells only occurs at sexual maturity (144, 153, 159).

Delayed Maturation of Target Cells Protects Against Mother-to-Child Transmission (MTIT)

Vertical transmission of HIV and SIV can occur *in utero*, *intrapartum*, and postnatally, through breastfeeding (BFT) (160). Humans and NHPs show striking species-specific differences in the timing of maturation of the CCR5 expression on $CD4^+$ T cells (i.e., onset of $CD4^+$ $CCR5^+$ T target cells) in immature individuals: early in RM, intermediate in humans, and later in AGMs. The increasing age of target cells availability was paralleled by the rates of BFT: 60% in RMs (161), 29% in humans (162), and 0-5% in AGMs (66, 153, 159) suggesting that the delayed maturation of the SIV target cells in natural hosts compared to pathogenic hosts may be the factor behind the lack of/low SIV BFT (144).

These observations are concordant with the age-related increase of SIV transmission in natural populations of AGMs in West Africa (sabaeus) and South Africa (pygerythrus/vervet) (66, 159) and ontogenetic changes in the abundance of $CCR5^+$ $CD4^+$ T cells in the blood of sabaeus AGMs. Among the uninfected monkeys, the levels of $CCR5^+$ on circulating $CD4^+$ T cells are low in infants and juveniles, and markedly increased in adults, that usually become infected at the age of sexual maturity (159). While there is no significant difference in the mean levels of target cells between SIV infected and uninfected adults, in immature individuals the availability of target cells is positively associated with the SIV infection status (159). A

convergent observation was made in infants of rhesus macaques experimentally exposed to SIV treated with maraviroc - the maraviroc treatment prevented vertical SIV transmission only in individuals with naturally low levels of CCR5 on the $CD4^+$ T cells prior to the treatment (163). The susceptibility to infection is proportional to the target cell availability at mucosal sites (164) and therefore the natural restriction of CCR5 expression in young individuals may represent a strategy to protect target cells from infection.

Restriction of CCR5 Expression and $CD4^+$ $CCR5^+$ T Cells at Mucosal Sites

In comparison to non-natural hosts, natural host show much lower expression of CCR5 on $CD4^+$ T cells (but not $CD8^+$ T cells) at mucosal sites, in particular in the gut, that leads to overall low numbers of $CCR5^+$ $CD4^+$ T target cell (134, 153), especially memory cells, at these locations (134). These adaptive roles of $CCR5^+$ $CD4^+$ target cell restriction suggest that interventions mimicking the natural phenomenon of limited abundance of target cells at critical developmental periods and tissues may protect against transmission and excessive cell death, respectively.

Despite the difference in availability of target cells supporting viral replication, both natural and non-natural hosts show high viremia. It brings up a question, if not $CD4^+$ $CCR5^+$ T cells, which other cells facilitate virus replication. A plausible explanation is that SIVs can infect cells in natural hosts through other coreceptors than CCR5, and in this way protect target cells and specific anatomical sites from SIV pathogenesis, and prevent vertical SIV transmission (153).

SIV Usage of CCR5 and Alternative Coreceptors in Natural Hosts

SIVs predominantly use the canonical CCR5-mediated cell entry pathway (165), yet they can also use other coreceptors, usually in addition to CCR5: CXCR4, CXCR6 (AKA BONZO, STRL33), orphan receptors GPR1 and GPR15, as demonstrated through *in vitro* studies (165–169). A unique alternative coreceptor usage, CCR2, was developed by SIVrcm and SIVmm (59, 61, 170, 171).

SIVrcm Counters the CCR5 Deficiency by Altered Coreceptor Use

As a result of the high frequency of deletion alleles, some RCMs and SMs are homozygous for nonfunctional CCR5 variants and therefore completely lack the functional CCR5 receptor on the cell surface. The frequency of null homozygotes in RCMs exceeds 70% (60). However, the CCR5 null genotype in RCMs and SMs is not sufficient to protect them from SIV infection *in vivo* (59–61, 172–174) in contrast to homozygotes of CCR5-Δ32 in humans that are nearly completely protected from HIV infection. This suggests that SIVs infecting these species bypassed the inactivated CCR5-mediated entry pathway by developing the ability to use alternative receptors (**Figure 2**).

SIVrcm Naturally Infecting RCMs Displays a Unique CCR2-Tropism

In spite of being frequently CCR5-Δ24 homozygous and thus widely protected against infection with a CCR5-tropic SIV, the RCMs are naturally infected with a species-specific SIVrcm. The SIVrcm strains collected from naturally-infected RCMs from distant geographic locations, Gabon and Nigeria, are members of the same lineage of SIV (60, 61). SIV prevalence in RCMs is significant, about 22% (175, 176). The explanation for this successful SIVrcm spread in the RCM populations is that the virus utilizes CCR2b as the main coreceptor for virus entry, unlike the vast majority of SIV and HIV strains that use CCR5 (60, 177). The high allelic frequency of the CCR5-Δ24 in RCMs paralleled with the unique among primate SIVs R2b-tropism of SIVrcm suggest that the CCR2 coreceptor usage may have been

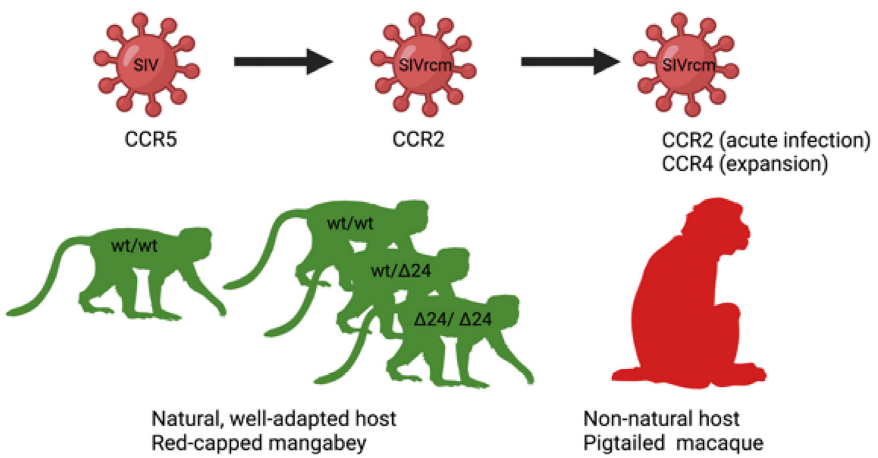

FIGURE 2 | Host-pathogen co-adaptations in a natural (left) and non-natural host (right). On the left, a putative origin of a unique among SIVs CCR2-tropic SIVrcm infecting red-capped mangabey RCM: A high frequency of CCR5-Δ24 mutation, disrupting CCR5 function and protecting from infection with CCR5-tropic SIV, led to the virus adaptation *via* changing the usage of CCR5 to CCR2b for cell entry (60). On the right, SIVrcm adaptation of coreceptor usage to a new host: experimental SIVrcm infection of pigtailed macaque, a natural host, showed a CCR2 usage in early infection and expansion of coreceptor usage to CCR4 demonstrating that lentiviral adaptation may occur rapidly through strain selection (170).

acquired by SIVrcm as an adaptation to CCR5 genetic defects in its host.

CCR2 is mainly expressed on monocytes (long lived cells) and nearly absent on T lymphocytes (short lived cells), and therefore it could be anticipated that, upon experimental infection, SIVrcm would show *in vivo* tropism toward monocytes (170). Yet, such an experimental infection of pigtailed macaques led to a surprising pattern of viral replication characteristic for short lived cells and a significant $CD4^+$ T cell loss in the intestine and blood, in particular effector memory $CD4^+$ T cells, and only a minimal monocyte depletion (170). These pathogenic features were explained by an *in vivo* expansion of the SIVrcm tropism upon infection of the pigtailed macaques upon expanded coreceptor use to CCR4. This coreceptor use expansion led to a selective depletion of CCR4-expressing memory $CD4^+$ T cells (170). CCR4 was indeed reported to be expressed mainly on lymphocytes and only at very low levels on monocytes (178).

Alternative Pathways for $CD4^+$ T Cell Entry: SIVsab in AGMs and SIVsmm in SM

Blocking of CCR5 coreceptor *in vitro* did not prevent SIV infection in circulating lymphocytes of SMs or AGMs (171) suggesting an existence of alternative entry pathways. In both cases, in addition to the CCR5 coreceptor, CXCR6 was an efficient entry pathway of SIV in *in vitro* experiments. Thus, SIVagm and SIVsmm utilize, in addition to CCR5, CXCR6 and GPR15 (171, 179). Additionally, SIVsmm utilizes GPR1 less frequently (59). *In vitro*, CCR5 appears nonessential for SIVsmm infection in SMs as SIVsmm glycoprotein can interact with GPR15 and CXCR6 supporting a similar level of infection as that mediated *via* CCR5 (59).

Alternative pathways are exclusively responsible for SIVsmm replication in animals that genetically lack functional CCR5, while both CCR5 and alternative coreceptors may be used in hosts where both CCR5 and alternative pathways are available (180). It was postulated that the usage of alternative non-CCR5-mediated pathways in natural hosts may be a counter measure to minimize pathogenicity of infection, yet still maintain high virus replication levels by directing the virus to different cell subsets, less critical to the maintenance of immune homeostasis (59).

Note, however, that these *in vitro* experiments were carried out using SIV clones, some of them derived after passage through pathogenic hosts, which might have impacted their *in vitro* tropism. *In vivo*, SIVsab was shown to preferentially infect and deplete central and effector memory cells (181), being thus possible that, *in vivo*, the transmitted-founder viruses preferentially infect CCR5-expressing $CD4^+$ T cells, thus recapitulating the pathogenesis of HIV-1 transmission, when transmitted founder HIV strains infect exclusively CCR5-expressing cells and display specific phenotypes (182–184). It is very likely that, as infection progresses, SIVs confronted with a low availability of the $CCR5^+$-expressing cells, expand their *in vivo* tropism towards the other coreceptors described above (59, 171, 179, 180).

This idea is also supported by reports of coreceptor expansion for SIVsmm to efficiently infect naïve cells. While typically SM do not experience chronic $CD4^+$ T-cell loss or clinical signs of disease, a small subset of SMs showed a profound $CD4^+$ T-cell depletion associated with carrying SIVsmm variants with an expanded use of SM-derived coreceptos (180) and human coreceptors, including CXCR4 (180, 185, 186), and generally expanded tropism. These coreceptors may support virus replication in SMs that have restricted CCR5 expression and lack functional CCR5 due to loss of function mutations.

Loss of Ability to Use CXCR6 and Switch Towards Virtually Exclusive Use of CCR5 by Pathogenic Lentiviruses

SIVcpz infecting chimpanzees and HIV-1 infecting humans are members of the same virus lineage and are both pathogenic (**Figure 3**). Both can use CCR5 as a principal entry coreceptor, but cannot use CXCR6, which was a coreceptor used for the entry of the ancestor of HIV-1 originated from cross-species transmission of SIVcpz infecting chimpanzees (46). In chimps, SIVcpz emerged from a *Cercopithecus* lineage of SIV (188), which *env* gene has a recombinant origin SIVgsn/mus/mon from greater spot-nosed monkey (*Cercopithecus nictitans*), mustached monkey (*C. cephus*), and mona monkey (*C. mona*) (189, 190). Contemporary SIVmus, similarly to SIVsmm and SIVagm, uses both CCR5 and CXCR6 for cell entry (187), suggesting the CXCR6 usage as a major coreceptor is attributable to SIVs with nonpathogenic course of infection in their respective hosts MUS, SM, and AGM. In the SIVcpz and HIV-1 lineage, the ability to infect *via* CXCR6 was lost and the viruses shifted their tropism exclusively towards CCR5. CXCR6 is expressed on $CD4^+$ effector memory T-cells, yet on a subpopulation distinct from those with expression CCR5 (187) and therefore the switch in coreceptor use resulted in the change of target cells, probably to more vulnerable cell subsets what can lead to pathogenesis (187).

Note, however, that the main limitation of this hypothesis that SIV pathogenicity in different species is dependent on the coreceptor usage is that SIVagm and SIVsmm can actually be highly pathogenic in macaques upon direct cross-species transmission (137, 191). In macaques, both SIVsmm and SIVsab target preferentially memory cells and not naïve cells (137, 191, 192). Therefore, in order to confirm this theory, comparative pathogenesis studies in the AGMs/SMs versus macaques should be carried out to show that the viruses restrict coreceptor usage to exclusively CCR5 upon cross-species transmission.

Thus, the SIV coreceptor expansion may enable the viruses to circumvent the lowered CCR5 expression in the natural hosts, making SIV infection in natural hosts less dependent on the use of a CCR5 coreceptor (193). In more recent hosts, high and unaltered CCR5 expression appears indispensable for HIV-1 infection in humans and SIVmac infection in RMs (33–35, 84). Differential cell targeting between natural and non-natural hosts could contribute to different outcomes between infection in natural and non-natural hosts (194), but *in vivo* studies are needed to confirm these *in vitro* observations and infection of naïve cells in natural hosts of SIVs.

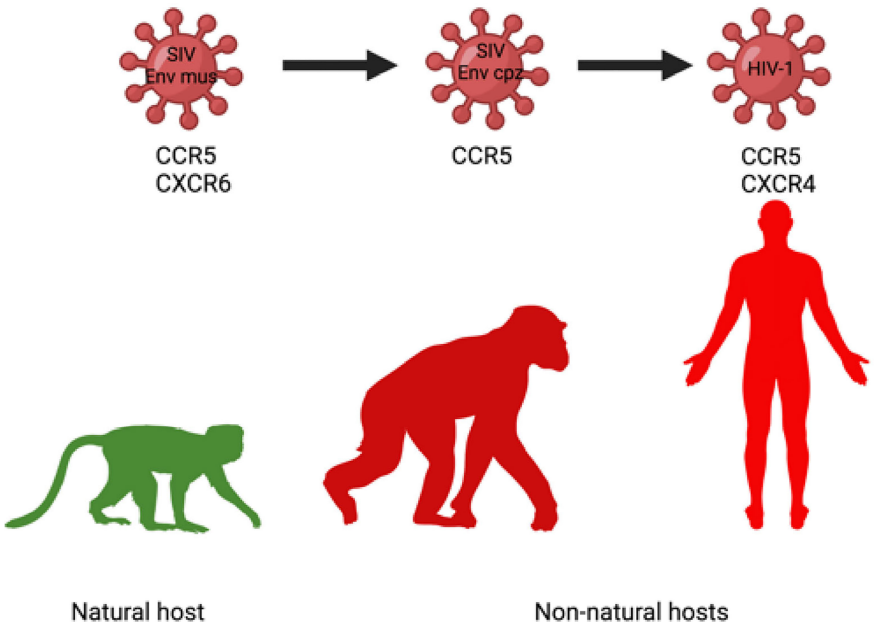

FIGURE 3 | Change of coreceptor tropism (loss of CXCR6 usage) in the SIVcpz/HIV-1 lineage may contribute to increased use of CCR5 as an entry coreceptor, more widespread infection of target cells and enhanced pathogenicity of SIVcpz/HIV-1 in chimpanzee and human, respectively (187).

A more promiscuous coreceptor usage allows the virus to expand their spectrum of target cells. These new target cell subsets are less essential, with short life span, and their infection has minimal impact on immune health. This can explain why low levels of CCR5 expression on $CD4^+$ T cells in natural hosts does not reduce the infection of the $CD4^+$ T cells, permit high viremia, and result in infection of cells with short lifespan in natural host in comparison to non-natural host. Restricted expression of CCR5 coreceptor may thus protect essential cells from infection (in particular memory $CD4^+$ T-cells) and preserve immune homeostasis. Note, however, that, in pathogenic HIV infections, high pathogenicity and disease progression is associated with an expansion in coreceptor usage rather than a more restrictive coreceptor use.

IMPACT FOR THERAPIES

The impact of natural CCR5 loss-of-function mutations and the phenomenon of target cell restriction *via* downregulating cell surface CCR5 expression on preventing infection or minimizing SIV/HIV pathogenesis pointed to the central role of CCR5 in the process of natural protection against SIV/HIV. It led to development of therapeutic approaches to inactivate or block the function of CCR5 gene or protein, such as chemical or antibody-based blocking of CCR5 receptors, or generation of CCR5 cells which are deficient or downregulate CCR5.

CCR5 Blockade - Targeting the Interaction of CCR5 With HIV

CCR5 blockers mimic the effects of naturally occurring CCR5-Δ24 mutation, at least in part, with respect to inhibiting HIV-1 utilization of cell surface CCR5 for cell entry.

Maraviroc (MVC), a nonpeptidic small molecule, causing a pharmacological blockade of CCR5 signaling, was the first CCR5 antagonist approved by FDA for the treatment of patients infected with R5-using HIV-1 virus (195). MVC blocks binding of viral envelope, gp120, to CCR5 to prevent the membrane fusion and viral entry, through allosteric inhibition (i.e. without occupying the binding site for chemokines and the HIV envelope glycoprotein gp120) and without affecting CCR5 cell surface levels or associated intracellular signaling (196–198). Maraviroc administration, originally devised for HIV treatment, may have applications beyond HIV/AIDS. Rodent models either treated with maraviroc or with CCR5 knockout showed an induced recovery after traumatic brain injury (30), however, maraviroc blockade of infant macaques only marginally impacted the rate of oral SIVmac transmission (163).

Leronlimab, a CCR5-blocking monoclonal antibody, binds to the external domains of CCR5 and through a competitive mechanism prevents HIV and SIV from binding to the CCR5 coreceptor, entering the cell and replication (199, 200). Beside the CCR5 masking from SIV/HIV, leronlimab CCR5 binding does not downregulate CCR5 expression or deplete CCR5-expressing cells (199), but prevents CCL-5-induced activation and migration of inflammatory CCR5-expressing monocytes and T lymphocytes along a chemical gradient (199). In this context, leronlimab appears a an excellent prospect for treatment of diseases, in which the CCL5-CCR5 pathway is involved in the pathogenesis. Given the role of CCR5 in immune cell migration and inflammation, CCR5 blockade with leronlimab was applied in critical COVID-19 patients, and led to the reduction of the IL-6 levels, restoration of CD4/CD8 ratio, and resolution of SARS-CoV-2 burden, thus implicating CCR5 as a potential therapeutic target for COVID-19 (201).

Antibody conjugates (ACs) comprising of an antibody carrier and small molecule CCR-5 antagonist were developed to enhance the CCR5-dependent therapies, specifically, to increase their clinical effects, reduce off-target effect and toxicity, and extend the pharmacokinetic profile of the attached molecule (202). To increase the potency of CCR5 targeted therapies, anti-CCR5 monoclonal antibodies were conjugated with a CCR5 small molecule antagonist, targeting nonoverlapping epitopes (203) and with a fusion inhibitor (204). The neutralization activity of CCR5 antagonists, such as MVC, can be further extended by formulating them with long-lived carriers - chemically programmed antibodies (cpAbs) and PEGylated derivatives. Such compounds containing MVC had significantly extended pharmacokinetic profiles (205).

Gene-Based Therapeutic Approaches

CCR5-Deficient Transplants

Sustained remission of HIV infection was achieved using stem-cell transplantation from donors homozygous for *CCR5* null allele Δ32/Δ32, lacking functional expression of the CCR5 coreceptor and showing HIV resistance in two patients treated for acute myelogenous leukemia (Berlin patient) and refractory Hodgkin lymphoma (London patient) (206, 207). The Berlin patient received two rounds of total body irradiation and allogeneic hematopoietic stem-cell transplantation (allo-HSCT) and the London patient underwent one HSCT procedure.

The Berlin patient achieved long-term post treatment control of HIV (206, 208, 209) and the London Patient has been in HIV-1 remission for at least 30 months with no detectable replication-competent virus in blood, CSF, intestinal tissue, or lymphoid tissue. Both these cases potentially represent cases of HIV-1 cure (207, 210–212).

While the allogenic transplantation of CCR5 deficient cells demonstrated a feasibility of cure, finding HLA-matched donors with naturally occurring homozygous CCR5 deletions is a limiting factor of this approach. Therefore, various genomic manipulations have been attempted to disrupt CCR5 function.

Gene editing and silencing technologies have been implemented to block the CCR5 function, including (i) modifications of naturally existing anti-HIV-1 ribozymes (108), (ii) gene silencing using RNA interference to suppress CCR5 (110), (iii) programmable nucleases, such as zinc-finger transcription activator-like effector nucleases (TALENs) and (iv) clustered regularly interspaced short palindromic repeat (CRISPR)-Cas (113), and engineered zinc-finger nucleases (ZFNs) (107, 112, 213, 214).

Editing the CCR5 gene *via* CRISPR-Cas9 technology was also applied to genetically modify Mauritian cynomolgus macaque embryos as the foundation for developing a model system of SIV resistance for studying SIV/HIV disease and development of therapies. Through this technology, a disrupted gene CCR5, containing homozygous deletions in CCR5 (including a 24-bp deletion region, which does not occur spontaneously in macaques) was introduced into macaque embryos and edited cells (115).

Germline editing using CRISPR-Cas9 technology was also used to introduce a null genotype of CCR5 in human embryos from an HIV discordant couple, which is similar, yet not identical to CCR5-Δ32. Twin girls with this genetic alteration were born in 2018. While the intention behind the germline inactivation of CCR5 in the human embryo appears to be protection from HIV infection in later life, this intervention evoked questions regarding the necessity of such permanent gene inactivation, while other preventive methods are available. It also evoked discussion about readiness of germline editing technology (its safety and control of target off effect) and still limited knowledge of pleiotropic function of immune genes in health, and therefore difficulty to precisely predict the effects of the introduced alterations (215).

The pleiotropic role of CCR5, which makes this chemokine receptor a promising target for therapies of various diseases, needs to be closely studied in relation to potential undesirable effects of CCR5-targeted therapies (the receptor blockage or disruption). While they can prevent the cell-to-cell spread of HIV/SIV and reduce chronic T-cell immune activation and inflammation, the inactivation of natural CCR5 function may have various unintended consequences. For example, there is increasing evidence on the prominent role of CCR5 in the differentiation, activation and migration of the $CD8^+$ T cells to the sites of inflammation (216, 217), and these processes are impaired by CCR5 deficiency or blockade (218, 219). CCR5 is also highly expressed in virus-specific $CD8^+$ T cells during various viral infections, including HIV-1, suggesting a role of CCR5 in the $CD8^+$ T cell responses to viral infections (216, 220–222). CCR5-expressing $CD8^+$ T cells display an effector memory phenotype, age-related increase in rhesus macaques, and a marked reduction during the progression of SIV disease (223). MVC treatment reduced the *in vitro* activation of $CD8^+$ T-cells from SIV-infected macaques (223). This effect could be beneficial as it may reduce disease-related chronic immune inflammation; on the other hand, it may limit the $CD8^+$ T-cell responses to the virus, and potentially increase a risk of the virus latency (223). The complex role of CCR5 in immune health highlights the need for studies of the CCR5-directed therapies on $CD8^+$ T cell and immune health in general.

CONCLUSIONS

CCR5 is central to HIV pathogenesis. Targeting this receptor was successfully used as an antiretroviral therapy and could be successfully expanded to either other infections or to medical areas which are unrelated to infectious diseases. Natural hosts of SIVs adapted, over an evolutionary history of millennia, to counter SIV infection by limiting the expression of the CCR5 receptor on the target cells. Meanwhile, viruses naturally infecting natural hosts of SIVs found escape routes to counter replication restrictions due to low CCR5 expression. As such, the CCR5-SIV relation represents a perfect illustration of the red queen principle which proposes that species must constantly adapt, evolve and proliferate in order to survive in contact with the opposed species (224). "Now, here, you see, it takes all the running you can do, to keep in the same place." (225). This remarkable ability of the SIVs and their species-specific hosts to continuously adapt calls for a careful evaluation of the cure approaches targeting CCR5 expression.

AUTHOR CONTRIBUTIONS

AJJ, IP, and CA designed, wrote and edited the manuscript. All authors contributed to the article and approved the submitted version.

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The CCR5 Gene Edited CD34$^+$CD90$^+$ Hematopoietic Stem Cell Population Serves as an Optimal Graft Source for HIV Gene Therapy

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Transplantation of allogenic hematopoietic stem and progenitor cells (HSPCs) with C-C chemokine receptor type 5 (CCR5) Δ32 genotype generates HIV-1 resistant immune cells. CCR5 gene edited autologous HSPCs can be a potential alternative to hematopoietic stem cell transplantation (HSCT) from HLA-matched CCR5 null donor. However, the clinical application of gene edited autologous HSPCs is critically limited by the quality of the graft, as HIV also infects the HSPCs. In this study, by using mobilized HSPCs from healthy donors, we show that the CD34$^+$CD90$^+$ hematopoietic stem cells (HSCs) express 7-fold lower CD4/CCR5 HIV receptors, higher levels of SAMHD1 anti-viral restriction factor, and possess lower susceptibility to HIV infection than the CD34$^+$CD90$^-$ hematopoietic progenitor cells. Further, the treatment with small molecule cocktail of Resveratrol, UM729 and SR1(RUS) improved the *in vivo* engraftment potential of CD34$^+$CD90$^+$ HSCs. To demonstrate that CD34$^+$CD90$^+$ HSC population as an ideal graft for HIV gene therapy, we sort purified CD34$^+$CD90$^+$ HSCs, treated with RUS and then gene edited the *CCR5* with single sgRNA. On transplantation, 100,000 CD34$^+$CD90$^+$ HSCs were sufficient for long-term repopulation of the entire bone marrow of NBSGW mice. Importantly, the gene editing efficiency of ~90% in the infused product was maintained *in vivo*, facilitating the generation of CCR5 null immune cells, resistant to HIV infection. Altogether, CCR5 gene editing of CD34$^+$CD90$^+$ HSCs provide an ideal gene manipulation strategy for autologous HSCT based gene therapy for HIV infection.

Keywords: CCR5, hematopoietic stem cells, gene therapy, gene editing, HIV, long-term engraftment

INTRODUCTION

Human immunodeficiency virus-1 (HIV-1) infection leads to acquired immunodeficiency syndrome (AIDS) and more than 30 million people are affected by it worldwide (1). Conventionally, viral replication in patients is suppressed by lifetime anti-retroviral therapy (ART). However, long term ART is associated with risks such as drug resistance, immunological non respondence, organ damage and age-related health complications (2, 3). In addition, interruption of ART leads to HIV-1 rebound in patients.

HIV infects the immune cells through the receptors such as CD4, CCR5 and CXCR4, which are predominantly expressed on the host immune cells such as T cells, macrophages and dendritic cells (4). These receptors are the potential targets to block HIV invasion and destruction of critical immune cells (5, 6). Particularly, the CCR5 receptor is an attractive target owing to a naturally occurring CCR5 null variant (CCR5 delta32/delta32), which confers resistance to R5-tropic HIV-1 (7). HSPCs from CCR5 null homozygous individuals have been shown to impart functional cure to AIDS patients on allogeneic transplantation (7, 8). Importantly, CCR5 receptor is dispensable for survival and function of immune cells (9). The limited availability of HLA matched CCR5 null donors, more specifically in African and south-Asian populations with high prevalence of HIV, poses major challenge to this approach as a regular therapy (10).

To this end, gene editing tools such as Zinc Finger Nucleases (ZFN), Transcription activator like effector nucleases (TALEN) and Clustered regularly interspaced short palindromic repeats/CRISPR associated protein 9 (CRISPR/Cas9) provide alternate option for generating CCR5 edited immune cells (4). CCR5 gene editing in CD4 T cells demonstrated the protection of edited cells from HIV mediated T cell lysis in the patients (11, 12). HIV-1 resistance was also reported using CCR5 edited HSPCs in mice models (13). Although both strategies were proved safe in clinical studies, they failed to provide functional cure (14).

Identifying the ideal target cells for CCR5 gene-editing is a key step towards the success of HIV gene therapy. CCR5 gene editing in CD4 T cells protects only the T cells and harvesting high quantity and quality of CD4 T cells from the patients can be a challenge (15). In addition, cellular senescence may deplete the frequency of modified T cells *in vivo* and may require repetition of the procedure (16, 17). Though CCR5 gene editing in HSPCs can generate CCR5 null lineages, it is also associated with certain challenges (4). Unlike, gene manipulation of autologous HSPCs for genetic disorders, HSPCs obtained from infectious disease patients may also contain infectious particles. The presence of HIV pro-viral DNA present an added risk of viral activation during *ex vivo* culture and gene editing procedure (18, 19). The recent non-human primate model transplantation studies have clearly defined the immunophenotypic definition of HPCs and HSCs as $CD34^+CD90^-$ and $CD34^+CD90^+$ cells, respectively but how HIV infects any of these defined subpopulation remains elusive (20). There are also considerable hurdles associated with HSPC gene therapy that can negatively affect the outcome of the manipulation, such as, low frequency of HSCs in the HSPC graft, its reduction during *ex vivo* culture and gene editing, lack of bi-allelic gene editing in HSCs, the need to manipulate large number of HSPCs and the drop in gene editing efficiency post transplantation (4).

In this study, we show that $CD34^+CD90^+$HSC fraction of HSPCs have reduced CD4/CCR5 receptors and increased antiviral restriction factors to limit the HIV-1 infection and demonstrate that they are the potential target cells for CCR5 gene editing. We also show that 100,000 $CD34^+CD90^+$HSCs are sufficient to repopulate the entire mouse bone marrow in order to generate CCR5 null immune cells.

MATERIALS AND METHODS

TZM-bl Cell Lines

TZM-bl cells (JC53BL-13 derived from HeLa, Cat No: ARP-8129) were received through the NIH AIDS Reagent Program, (Division of AIDS, NIAID, NIH). The cells were cultured in DMEM medium with 10% FBS and 1X antibiotic and antimycotic solution (Cat No:15240062, Thermo Fisher scientific). Cells were cultured for at least two passages prior to gene-editing. The HIV infection in TZM-bl was measured by luciferase reporter expression which is under the control of HIV-Tat regulatory element (21).

Purification of $CD34^+$HSPCs, $CD34^+CD90^+$ HSCs and $CD34^+CD90^-$ HPCs

Granulocyte colony stimulating factor (G-CSF) mobilized peripheral blood was collected from healthy donors after obtaining approval from Institutional Review Board (IRB). Peripheral blood mononuclear cells (PBMNCs) were separated using ficoll density gradient centrifugation. $CD34^+$ cells were isolated from PBMNCs using EasySep CD34 positive selection kit (Cat No: 17896, STEMCELL Technologies) as per the manufacturer instructions. The purified $CD34^+$ cells were pre-stimulated with Serum free essential medium (SFEM) containing hematopoietic stem cell specific cytokines such as SCF (240ng/ml), FLT3 (240ng/ml), TPO (80ng/ml) and IL-6 (40ng/ml) and small molecule cocktail of Resveratrol, Stem Reginin-1 and UM729 (RUS) as described in our previous work (22). All the donors were screened for delta32 genotype before conducting CCR5 gene editing (**Supplementary Figure 3A**). For $CD34^+CD90^+$ and $CD34^+$CD90- cell sorting, the purified $CD34^+$ cells were briefly cultured with above mentioned cytokines (6-12 hours) and stained with CD90 antibody (8µg/$1x10^6$ cells) for 20 min at room temperature. After brief washes with PBS, $CD90^+$ and $CD90^-$ cells were sorted using BD FACS Aria III in purity mode. The purity of sorted cells was re-assessed by staining with CD34 and CD90 antibodies.

Transwell Migration Assay

0.1 million HSCs were seeded on the upper chamber of the transwell plate with 100µl of basal medium and the lower chamber was loaded with 500µl of SFEM II cytokines (SCF (240ng/ml), FLT3 (240ng/ml), TPO (80ng/ml) and IL-6 (40ng/ml) medium containing 100 ng/mL of SDF-1α ligand. The plate was incubated at 37°C for 24 hours. On the next day, cells in the

lower chamber were counted using trypan blue dye exclusion method. The counts were normalized with non-SDF-1α containing media. Percentage of migration was calculated with the number of cells in the bottom chamber divided by number of cells placed in the upper chamber.

Colony Forming Units (CFU) Analysis

Based on the experimental requirements, 300 to 500 HSCs were seeded in 3ml of semi-solid methylcellulose medium (Cat No: #04044, Stem cell technologies). After 14-16 days, hematopoietic progenitor colonies were enumerated under microscope. Based on the morphology, colonies were categorized as Burst forming unit-erythroid (BFU-E), Colony forming unit-erythroid (CFU-E), Colony forming unit-granulocyte-monocyte progenitor (CFU-GM) and Colony forming unit- granulocyte, erythrocyte, monocyte, megakaryocyte (CFU-GEMM).

RNA Isolation and Real-Time PCR Analysis of Antiviral Restriction Factors

Total RNA was isolated from sort purified HPCs and HSCs using RNeasy mini kit (Cat No:74104, Qiagen, Hilden, Germany) as per the manufacturer's instructions. 1μg of total RNA was reverse transcribed into cDNA using PrimeScript 1ststrand cDNA synthesis kit (Cat No: 6110A, Takara) and analyzed by Real-Time PCR (RT-PCR) with TB Green *Premix Ex Taq* ^{TM}II (Cat No: #RR820A, Takara) as per the manufacturer instructions. RT-PCR data were analyzed using the standard $2^{-\Delta\Delta CT}$ method and presented as the fold expression normalized to the reference gene Ubiquitin C. Primers used for RT-PCR are listed in **Supplementary Table 2**.

Purification and Culture of CD4 Cells

CD4 cells were isolated from peripheral blood of the healthy donor using CD4 positive selection kit (Cat No: #17852, stem cell technologies) as per the manufacturer's instructions and the purity was analyzed using CD3 and CD4 FACS antibodies. Isolated cells were cultured using human XF T cell expansion medium (Cat No: 10981, stem cell technologies) along with CD3/CD28/CD2 T cell activator cocktail (Cat No: 10970, Stem cell technologies). CCR5 gene editing was performed on day 4 of expansion.

CCR5 Gene-Editing

The two sgRNAs targeting CCR5 gene (E2C5- UGACAUCAA UUAUUAUACAU (GRCh38.p10, Chromosome 3- 46372915 – 46372973), E3C5-CAGCAUAGUGAGCCCAGAAG (GRCh38.p10, Chromosome 3- 46373133 – 46373191) were designed based on the common hits identified using Synthego (https://design.synthego.com/#/), CHOPCHOP (https://chopchop.cbu.uib.no/) and benchling (https://www.benchling.com/crispr/) tools. Cas9-Ribonucleoprotein (RNP) was electroporated using Lonza 4D nucleofector with program DZ100 for HSCs, CN114 for TZM-bl cells and DN100 for CD4 cells. After 72 hours, cells were genotyped by sanger sequencing. The chromatographs obtained from the sequencing were analyzed by Inference of CRISPR Edits (ICE) tool from Synthego (https://ice.synthego.com/#/) The primers used for the region-specific amplification is listed in **Supplementary Table 2**. The deletion induced by dual sgRNA system was analysed using gap PCR and quantified with ImageJ software (https://imagej.nih.gov/ij/download.html).

Macrophage Differentiation

Macrophage differentiation of HSCs was carried out using the published protocol with minor modifications (23). Briefly, control and edited HSCs were plated in non-tissue culture treated polystyrene plates with macrophage differentiation medium (SFEM-II, SCF (100ng/ml), Flt3-L (50ng/ml), IL-6 (10ng/ml), IL-3 (10ng/ml), GM-CSF (10ng/ml) and M-CSF (10ng/ml). Non-adherent cells were collected every 72 hours and reseeded in the macrophage differentiation medium. Adherent cells were cultured using RPMI medium containing 10% FBS along with GM-CSF (10ng/ml) and M-CSF (10ng/ml). After 14-16 days, adherent cells were observed under microscope for morphology, harvested with Accutase (Material Number: 561527, BD Biosciences), stained for CD4 PE, CD14 FITC, CCR5 APC, CXCR4 APC, CD14 BV421, CD80 FITC, CD206APC, CD64 PE, CD163-PE CF594 and CD71 FITC antibodies and analyzed using BD FACS Aria III flow cytometer. Phagocytic potential of generated macrophages was validated using pHrodo Red *E. coli* BioParticles conjugate (Cat No, P35361, Thermo scientific) as per the manufacturer's instructions. The proportion of phagocytosis was calculated by enumerating the phagocytosis positive and negative cells.

HIV Production and Challenge Assay

All the HIV related experiments were carried out under BSL-2 facility using BSL-3 practices after the approval of Institute Biosafety committee. HIV-1 p49.5 R5-tropic molecular clone (Cat No: ARP-11389) was obtained through NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH. The clone was contributed by Dr. Bruce Chesebro. For HIV production, plasmid was transfected into HEK293T cells using standard calcium phosphate method. 72 hours of post transfection, media containing viral particles was collected and filtered with 0.45μm filter and stored at -80°C as multiple aliquots. HIV production was monitored by measuring p24 antigen using ELISA kit, obtained from R&D biosystems (Cat No: DHP240B). Infectivity of the HIV-1 stock was determined using TZM-bl cells. HIV challenge assay with gene edited TZM-bl cells and macrophages was done using 25ng and 150ng of p24 respectively for 6 hours in growth medium containing polybrene (8μg/ml). HIV infectivity assay in HSPCs and its subsets were performed as described (24). In brief, 0.25 million HSPCs/HPCs/HSCs were seeded in 250μl of SFEM-II medium (with 8μg/ml of polybrene) in 48-well Retronectin coated plates and R5-tropic HIV-1 (200ng of p24) was added. The plate was subjected to spinfection for 30 mins at 900g and incubated at 37°C for overnight with 5% CO_2. On the next day, cells were washed with PBS by centrifugation, cultured for 4 to 6 days and stained with HIV Gag antibody. (KC 57- FITC, Beckman coulter).

HIV Proviral DNA Amplification

Total genomic DNA was isolated from equal number of infected and non-infected HSPCs/HPCs/HSCs and subjected to PCR amplification using HIV specific primers listed in **Supplementary Table 2**. The PCR was conducted using HotstarTaq master mix as per the standard PCR protocol using 50ng of DNA template.

NBSGW Transplantation Studies

All animal experiments were conducted after obtaining approval from institute animal ethical committee, Christian Medical College, Vellore, Tamil Nadu, India. The Nonirradiated NOD, B6. SCID Il2ry$^{-/-}$ Kit W$^{41/41}$ (NBSGW) mice (Jackson laboratory) were bred in inhouse animal facility. Depending on the experimental requirements, 0.5-1x10^5 gene-edited HSCs were infused into busulfan conditioned 7-8 weeks old female NBSGW mice *via* tail vein injection. After 16 weeks of infusion, mouse bone marrow, peripheral blood and splenic cells were harvested and analysed for human cell engraftment using human CD45 and mouse CD45.1 antibodies. The percentage of human cell engraftment was calculated using the formula: = (% hCD45)/(% hCD45 + % mCD45.1) x 100. The gene editing in the engrafted cells was analysed by extracting the DNA using Quick DNA Extract (Cat No: QE0905T, Lucigen) and subjected for sequencing with human CCR5 specific primers and ICE analysis. Multilineage repopulation was analyzed with lineage specific antibodies listed in **Supplementary Table 1**.

Statistical Analysis

All the statistical analysis was performed using PRISM GraphPad 8 package (GraphPad Software Inc., San Diego, CA, USA). Data analysis was done using two tailed unpaired t-test and multiple T-test using Holm-sidak method as indicated in figure legends. Error bars denotes ± SEM. Number of independent experimental replicates (n), number of donors used are indicated in the figure legend. P value < 0.05 is considered as statistically significant.

RESULTS

Mutagenesis by CRISPR-Cas9 InDels Generates HIV-1 Resistance Similar to Deletion of CCR5 Coding Region

Naturally occurring deletion (Δ32) in the coding region of CCR5 is shown to ablate CCR5 expression. Generating CRISPR-Cas9 mediated CCR5 Δ32bp genotype or deletion of coding region in HSPCs needs incision by two sgRNAs of similar efficiency and this procedure increases the chance of off- target editing and chromosomal rearrangements (13, 25, 26). To test the impact of small InDels on CCR5 expression, we used single sgRNA (E2) that targets coding region and compared it with dual sgRNA (E2 and E3) approach that deletes 246 bp in TZM-bl cells (**Supplementary Figure 1A**). High frequency of InDels (>80%) with single sgRNA and deletion (>85%) with dual sgRNA were detected by ICE analysis of Sanger sequencing reads and by gap PCR, respectively (**Figure 1A**). Both the single sgRNA and dual sgRNA approaches resulted in reducing CCR5 expression to <12% and <3%, respectively, compared to un-edited control. (**Figures 1B–D**).

Next, we challenged the single and dual sgRNA edited TZM-bl cells with R5-tropic HIV. While the control cells showed cell death (**Figure 1F**), and high frequency of HIV infection as indicated by luciferase reporter expression (**Figure 1G**) and by intracellular HIV gag p24 staining (**Figures 1E, H**), all the edited conditions showed complete HIV resistance. These observations suggest that single sgRNA mediated InDels are sufficient to provide HIV resistant phenotype similar to deletion of CCR5 coding region.

Limited Expression of CD4/CCR5 Receptors on CD34$^+$CD90$^+$ HSCs Contributes to the Reduced Susceptibility for R5-Tropic Infection

HIV infection in HSPCs has been reported and this can potentially limit the use of autologous HSPCs for CCR5 gene editing as the transplanted cells can later serve as reservoir for HIV infection (27, 28). HIV receptor-CD4 and co-receptor-CCR5 are crucial for R5-tropic HIV infection and therefore, we examined the expression of CD4/CCR5 on the G-CSF mobilized HSPCs from the healthy donors. In agreement with previous findings (29, 30), HSPCs expressed HIV receptors. While 25% of HSPCs expressed only CD4 (CD34$^+$CD4$^+$CCR5$^-$ cells) around 6% of HSPCs had both the receptors (CD34$^+$CD4$^+$CCR5$^+$ cells) (**Figure 2A** and **Supplementary Figure 1C**). To identify the subpopulation of HSPCs that expresses both CD4 and CCR5 (CD4/CCR5) receptors, we sorted HSPCs as CD34$^+$CD90$^-$ HPCs and CD34$^+$CD90$^+$ HSCs (31) (**Supplementary Figure 1B**). The CD34$^+$CD90$^+$ HSCs contained 7-fold reduced CD4$^+$CCR5$^+$ receptors than CD34$^+$CD90$^-$ HPCs (8.4% *vs* 1.2%) (**Supplementary Figure 1C** and **Figure 2B**).

To validate this finding, we infected the CD34$^+$ HSPCs, CD34$^+$CD90$^-$ HPCs and CD34$^+$CD90$^+$ HSCs with R5-tropic HIV. Consistent with the pattern of HIV receptor expression, the CD34$^+$CD90$^+$ HSCs displayed lesser HIV gag p24 staining than the HPCs and HSPCs (3-fold and 2-fold, respectively) (**Figures 2C, D**). Next, on examination of HIV-1 proviral DNA integration, the HSPCs and CD34$^+$CD90$^-$ HPCs had proviral DNA but not the CD34$^+$CD90$^+$ HSCs (**Figure 2E**). To further confirm that CD34$^+$CD90$^+$ HSC fraction effectively resist R5- tropic HIV infection, HIV infected HSPCs, HPCs and HSCs were cultured in monocyte stimulation medium for 7 days. While, both HSPCs and CD34$^+$CD90$^-$ HPCs had 2-fold increase in viral outgrowth post-stimulation, such increase was not detected in CD34$^+$CD90$^+$ HSCs (**Figure 2F**).

The presence of high levels of antiviral restriction factors have shown to restrict HIV-1 infection in resting CD4 T cells (32–34). To examine whether such phenomenon exists in the CD34$^+$CD90$^+$ HSCs, we investigated the expression of anti-viral restriction factors. Real time PCR quantification indicated the high expression of IFITM2, and SAMHD1 in the CD34$^+$CD90$^+$ HSC fraction when compared with the CD34$^+$CD90$^-$ HPCs. In particular, SAMHD1 which is shown to restrict HIV infection

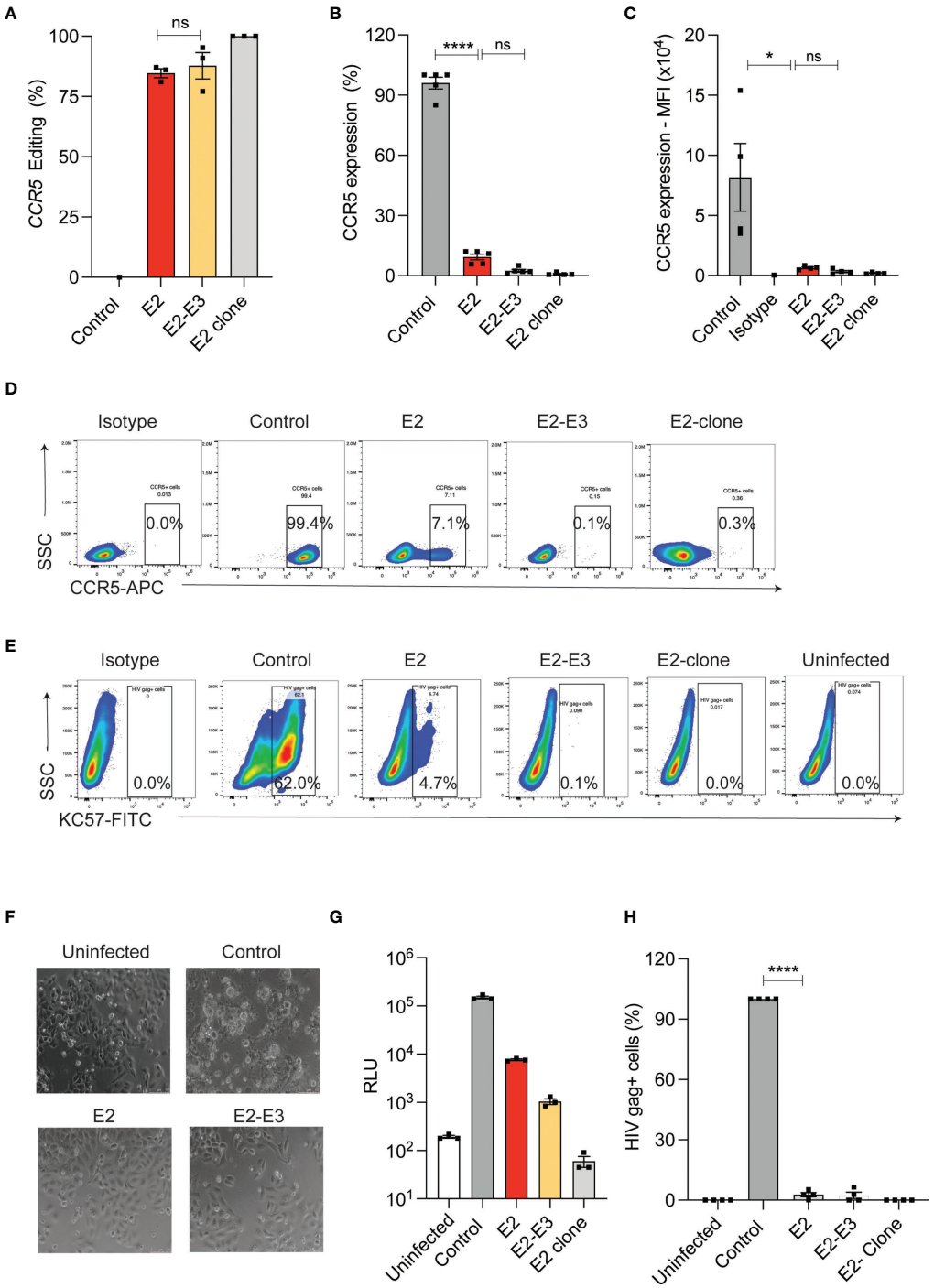

FIGURE 1 | InDel mutagenesis in *CCR5* provides HIV resistance in TZM-bl cells. **(A)** Percentage of *CCR5* gene-editing with single (E2) and dual (E2, E3) sgRNA in TZM-bl cells. (n = 3). E2-clone: homozygous InDel clonal cell line generated from E2-sgRNA edited TZM-bl cells. **(B)** Percentage of CCR5 expression in control and *CCR5* gene-edited TZM-bl cells. CCR5 expression was analyzed by flowcytometry (n = 5). **(C)** Mean Fluorescence Intensity (MFI) of CCR5 in control and CCR5 edited TZM-bl cells (n = 4). **(D)** Representative FACS plot showing CCR5 expression in control and CCR5 gene-edited TZM-bl cells. **(E)** Representative FACS plot showing HIV Gag+ cells in control and CCR5 gene-edited TZM-bl cells after 48 hours of HIV infection. **(F)** Representative phase contrast micrograph of control and CCR5 gene-edited TZM-bl cells at 10x magnification. TZM-bl cells were infected with 25ng of HIV and 48 hours post infection, the cell morphology was analysed. Scale bars were indicated at the right corner of the image. **(G)** Luciferase expression measured as relative light unit (RLU) after 48 hours of HIV infection in control, CCR5 edited TZM-bl cells, (n = 3). **(H)** Percentage of HIV gag positive cells in control and CCR5 edited TZM-bl cells. (n = 4). HIV infection (Gag+ cells) in control TZM-bl cells was normalized to 100. Error bars denotes mean ± SEM, ns; non-significant. *p ≤ 0.05, ****p ≤ 0.0001. Statistical analysis was performed using multiple t-test (holm-Sidak method).

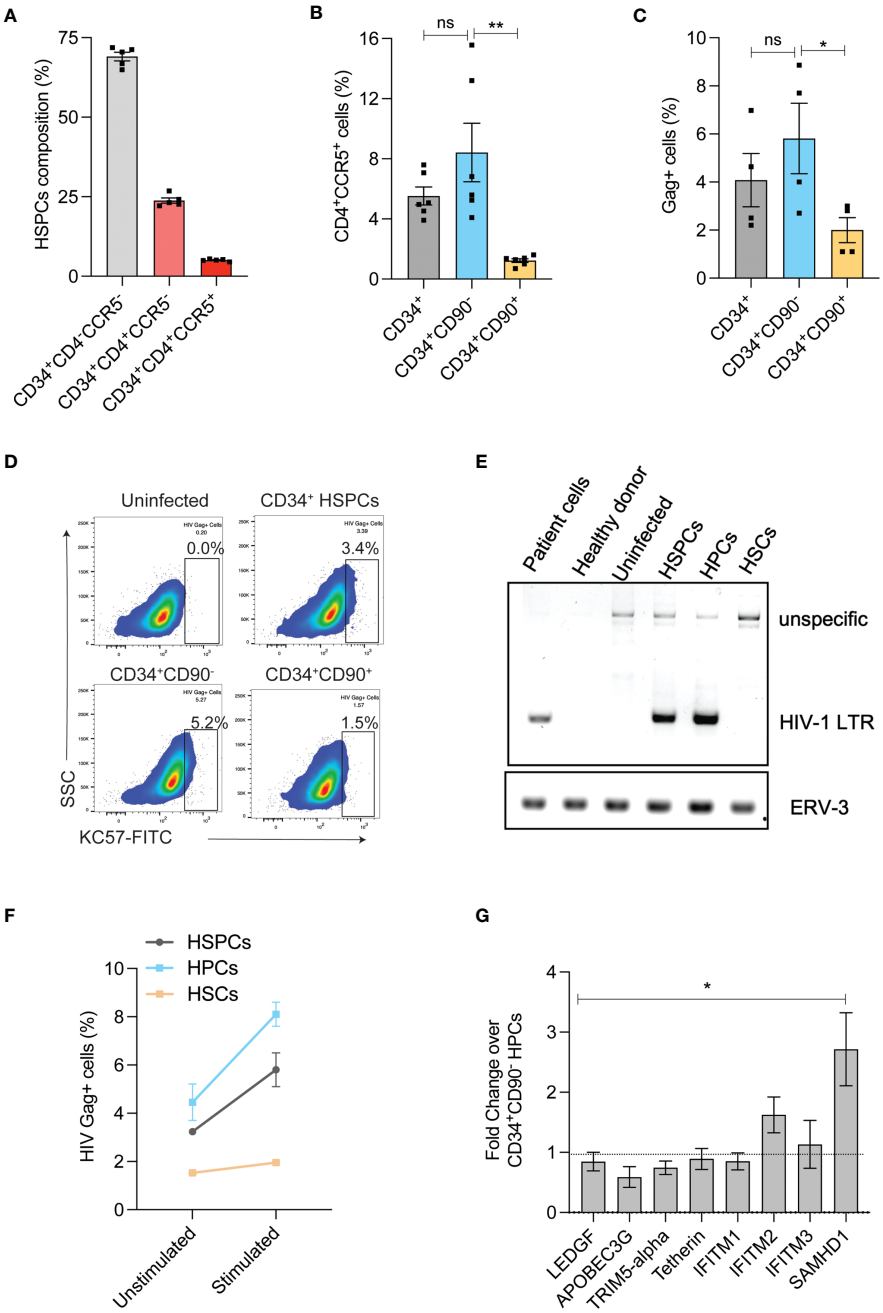

FIGURE 2 | HIV-1 infection in HSPC sub-populations. **(A)** Expression of HIV-1 receptors in HSPCs. Flow cytometric analysis of HIV receptors was conducted immediately after the purification of HSPCs (uncultured cells). (n = 5), Donors: 3. **(B)** Percentage of $CD4^+CCR5^+$ cells in $CD34^+$ HSPCs, $CD34^+CD90^-$ HPCs and $CD34^+CD90^+$ HSCs. (n = 6), Donors: 3. **(C)** Percentage of HIV-1 infection in $CD34^+$ HSPCs, $CD34^+CD90^-$ HPCs and $CD34^+CD90^+$ HSCs. Cells were infected with 200ng of R5-tropic HIV and 7-days post infection, intracellular flowcytometric staining of Gag+ cells were analyzed. (n = 4), Donors: 2. Error bars denotes mean ± SEM, *$p \leq 0.05$ (Unpaired t-test, Two-tailed). **(D)** Representative FACS plot showing HIV Gag+ cells in $CD34^+$HSPCs, $CD34^+CD90^-$HPCs and $CD34^+CD90^+$ HSCs, 7 days post HIV infection. **(E)** Agarose gel electrophoresis image showing PCR analysis of HIV proviral DNA in $CD34^+$ HSPCs, $CD34^+CD90^-$ HPCs and $CD34^+CD90^+$ HSCs. The genomic DNA was extracted from HIV-1 patient PBMNCs, healthy individual cells, uninfected HSPCs and HIV-1 infected cells, and analysed with primers that amplify a region covering HIV-1 LTR and gag (Labelled as LTR), the PCR also produced an unspecific band at 1.5kb. Human endogenous retroviral sequence ERV3 used as a loading control. (n = 2), Donor: 2. **(F)** Viral outgrowth from HIV infected $CD34^+$ HSPCs, $CD34^+CD90^-$ HPCs and $CD34^+CD90^+$ HSCs and before and after stimulation with monocyte differentiation medium for 7-days. Percentage of HIV-1 infection was measured by intracellular flowcytometric staining of Gag+ cells. (n = 2), Donor: 1. **(G)** Expression levels of antiviral restriction factors in $CD34^+CD90^+$HSCs compared to $CD34^+CD90^-$ HPCs, as measured by Real-time PCR analysis (Ubiquitin-C is used as internal control). (n = 4) Donors: 4. Error bars denotes mean ± SEM, *$p \leq 0.05$, **$p \leq 0.01$. [Statistical analysis was performed using multiple t-test (holm-Sidak method)]. ns, non-significant.

(33) was expressed 2.5-fold higher in $CD34^+CD90^+$ HSCs (**Figure 2G**). All these findings suggest that reduced expression of HIV receptors and presence of higher levels of antiviral restriction factors are mediating the resistance of $CD34^+CD90^+$ HSCs to HIV infection. These findings also suggest that $CD34^+CD90^+$ HSCs are ideal target cells for CCR5 gene manipulation for HIV gene therapy.

The Engraftment Potential of CCR5 Edited $CD34^+CD90^+$ HSCs Is Augmented by RUS Treatment

Recent study showed that the gene editing of BCL11A binding site in $CD34^+CD90^+$ HSCs reduced the requirement of target cell population by 10-fold for gene-manipulation and resulted in durable engraftment in non-human primates (35). We reported that sort enriched $CD34^+CD90^+$ HSCs can be preserved with a small molecule cocktail of Resveratrol, UM-729 and SR-1 (RUS) (22).

To test whether RUS treatment could retain the stemness of sort-enriched $CD34^+CD90^+$ HSCs for CCR5 gene editing, the purified HSPCs were sorted for $CD34^+CD90^+$ HSCs. The $CD34^+CD90^+$ HSCs sorting procedure enriched the most primitive HSCs, marked as $CD34^+CD133^+CD90^+CD45RA^-CD38^-CD49f^+$ cells (22), by >80% (**Supplementary Figure 2A**). The sorted cells were pre-stimulated with cytokines for 48 hours with or without RUS before gene editing with Cas9 RNP targeting *CCR5*. The CCR5 gene edited cells cultured with RUS displayed increased retention of $CD34^+CD90^+$ cells (**Supplementary Figure 2B** and **Figure 3A**) and high frequency of $CD34^+CD133^+CD90^+CD45RA^-CD38^-CD49f^+$ HSCs (**Figure 3B**). Consistent with surface expression analysis, the colony formation assay (CFU) showed high frequency of GEMM colonies (**Supplementary Figure 2C**). The RUS treated $CD34^+CD90^+$ cells showed 2-fold increase in CXCR4 expression, a factor crucial for stem cell homing in the bone marrow (**Supplementary Figure 2D**) and thereby resulted in 2-fold greater response towards SDF-1α cytokine ligand (**Figure 3C**). Additionally, sodium nitroprusside (SNP) treatment mediated activation of nitric oxide signaling is shown to increase the CXCR4 expression, transwell migration and homing of HSPCs isolated from umbilical cord blood (36). Remarkably, RUS treatment suffices to improve the SDF1-α mediated transwell migration of the $CD34^+CD90^+$ HSCs at comparable levels as that of SNP treated $CD34^+CD90^+$ HSCs (**Figure 3C**). RUS treatment also showed modest increase in the editing frequency (79% *vs* 60% in vehicle) (**Figure 3D**).

To confirm that RUS treated $CD34^+CD90^+$ HSCs have superior engraftment potential *in vivo*, we transplanted the $CD34^+CD90^+$ HSCs that were cultured for 48hrs with cytokines and with or without RUS into NBSGW mice. The RUS treated cells displayed a higher human cell chimerism in bone marrow (**Figure 3E**), spleen (**Figure 3F**) and peripheral blood (**Figure 3G**) than the vehicle treatment. Strikingly, infusion of 50,000 RUS treated $CD34^+CD90^+$ cells was sufficient to repopulate approximately 56% of mouse bone marrow (**Figure 3H**). The RUS treatment did not alter the multi lineage repopulation capacity of the $CD34^+CD90^+$ cells and we detected lineages such as HSPCs (CD34), T cells (CD3), myeloid cells (CD13), B cells (CD19) and monocytes (CD14) (**Figure 3I**). These results demonstrate that RUS supplementation during culture of HSCs preserves stemness and provides an improvised culture condition for CCR5 gene editing.

Efficient CCR5 Gene Editing in $CD34^+CD90^+$ HSCs Provides HIV Resistance

The CD4 T-cells and $CD34^+$HSPCs are the currently explored grafts for CCR5 gene editing. To utilize the $CD34^+CD90^+$ HSCs for CCR5 gene editing, The RUS treated $CD34^+CD90^+$ HSCs were edited for *CCR5* with different concentrations of Cas9-RNP complex and >90% InDels were observed with 100 pmol of RNP (**Figure 4A** and **Supplementary Figure 3B**). The *CCR5* edited cells produced similar number and pattern of multilineage colonies as the control cells in the CFU assay (**Figure 4B**). The edited $CD34^+CD90^+$ HSCs were single cell sorted in methocult medium and the analysis of clonal colonies showed that >85% cells were bi allelic edited cells (**Figure 4C**).

In-vitro macrophage differentiation of CCR5 edited $CD34^+CD90^+$ HSCs generated comparable macrophage cell yield (**Supplementary Figure 3C**), expression of respective lineage markers CD14, CD71, CXCR4, and CD4 (**Figure 4D**) and subsets of macrophages (M1 and M2) to that of control (**Figure 4E**). Importantly, 90% of the macrophages lacked the CCR5 expression (**Figure 4D**). The lack of CCR5 expression has not affected the phagocytosis, an important function of macrophages (**Supplementary Figures 3D, E**).

Next, we infected the macrophages with the R5-tropic virus. We observed an active R5-tropic HIV infection in control macrophages whereas >80% of CCR5 modified cells were resistant to infection as determined by HIV gag p24 staining (**Figure 4F** and **Supplementary Figure 3F**). Of note, InDel patterns generated during CCR5 editing of $CD34^+CD90^+$ HSCs were conserved in the TZM-bl cells, CD4 T-cells, HSPCs, and the macrophages (**Figure 4G**). Such InDel pattern in CD4 T cells resulted in CCR5 null CD4 cells suggesting the uniform functional outcome (**Figure 4H**). All these suggest that CCR5 editing in $CD34^+CD90^+$ HSCs generates CCR5 null lineage cells which are HIV resistant and functionally intact.

A Low Dose of CCR5 Edited $CD34^+CD90^+$ HSCs Is Sufficient to Produce HIV Resistant Immune System

As RUS treatment preserves the engraftment potential of $CD34^+CD90^+$ HSCs, we hypothesized that a low dose of RUS treated CCR5 edited $CD34^+CD90^+$ HSCs could repopulate the mouse bone marrow. We tested this by sorting the $CD34^+CD90^+$ HSCs, cultured it for 48hours with RUS cocktail and gene edited $1x10^5$ $CD34^+CD90^+$ HSCs with Cas9-RNP targeting CCR5 loci or Cas9- tracrRNA as a control. The crRNA less Cas9- tracrRNA control will not induce DNA double strand breaks and thus helps to better understand any gene-editing associated engraftment defect.

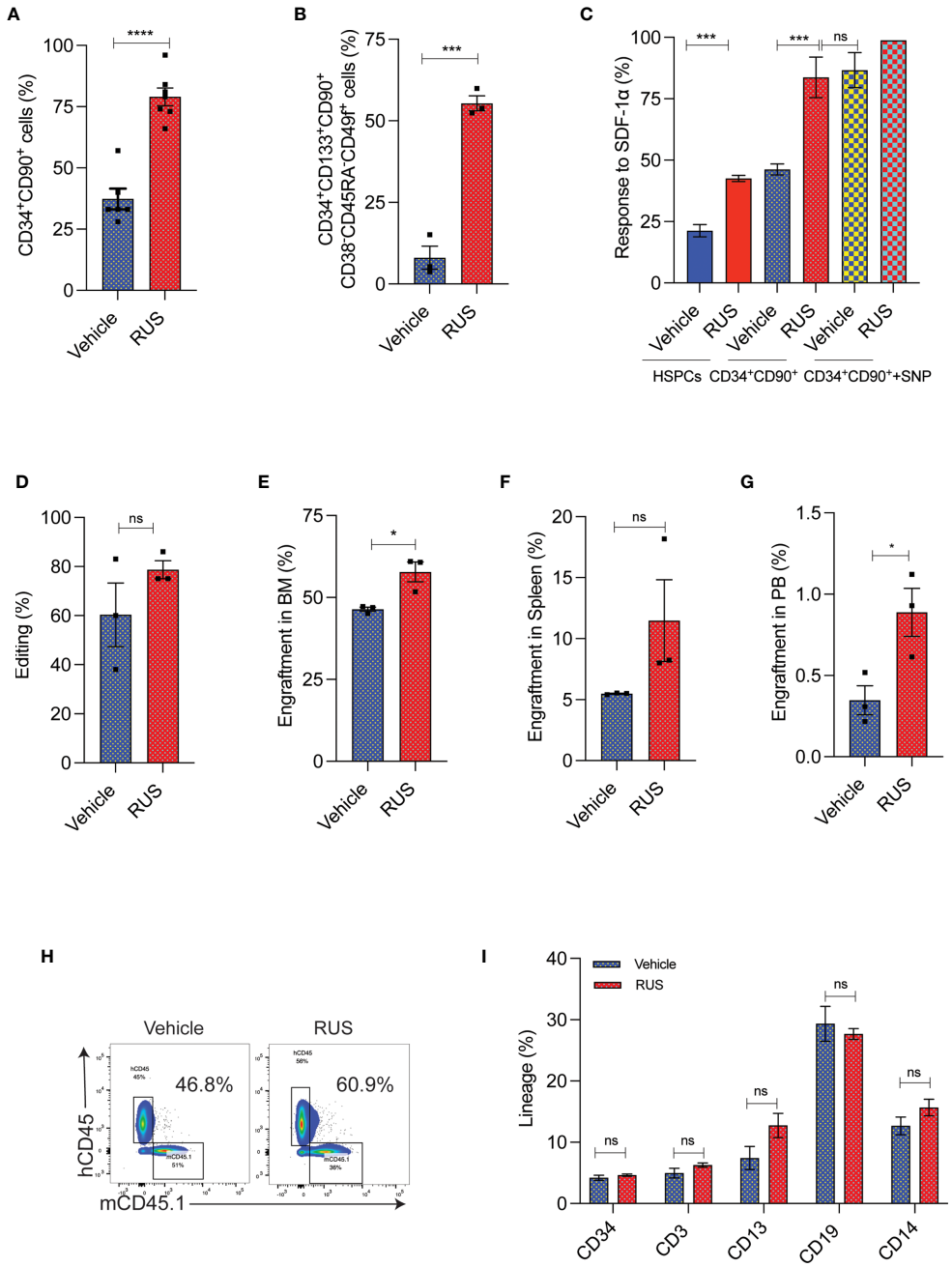

FIGURE 3 | RUS treatment improves the engraftment potential of *CCR5* gene edited $CD34^+CD90^+$ HSCs. Experimental outline for **(A, D)** The FACS purified $CD34^+CD90^+$ HSCs were cultured with vehicle or RUS for 48 hours and subjected for CCR5 editing with 50pM of RNP. Cells were collected 24 hours post editing for the following analysis. Error bar denotes mean ± SEM. ns; non-significant. ***p ≤ 0.001, ****p ≤ 0.0001 (Unpaired t test, two-tailed). **(A)** Percentage of $CD34^+CD90^+$ cells as analysed by flow cytometry. (n = 7), Donors: 4. **(B)** Percentage of $CD34^+CD133^+CD90^+CD38^-CD45RA^-CD49f^+$ cells as analysed by flow cytometry. (n = 3), Donors: 2. **(C)** Percentage of HSCs responding to SDF1-α in the lower chamber in the transwell migration analysis. The $CD34^+CD90^+$+SNP group was treated with 10μM of Sodium Nitroprusside (SNP) for 16 hours prior to trans well migration assay. (n = 4), Donors: 2. **(D)** Percentage of CCR5 edited HSCs as measured by ICE analysis of sanger sequencing reads. (n = 3), Donors: 2. Experimental outline for (**E–I**) The FACS purified $CD34^+CD90^+$ HSCs were cultured with vehicle or RUS for 48 hours and 50000 cells were transplanted into 7-8 weeks old female NBSGW mice. 16 weeks post transplantation, different tissues of mice were collected and analysed for the engraftment. Each dot indicates a NBSGW mice. Error bar denotes mean ± SEM. ns; non-significant. *p ≤ 0.05 (Unpaired t test, Two tailed). **(E)** Percentage of human cell engraftment in bone marrow. **(F)** Percentage of human cell engraftment in spleen. **(G)** Percentage of human cell engraftment in peripheral blood. **(H)** Representative FACS plot showing the percentage of mice and human cells in NBSGW mice bone marrow. The percentage in the inset refers to engraftment percentage of human cells calculated as described in *Methods*. **(I)** Multilineage reconstitution [HSPCs (CD34) T cells (CD3), myeloid cells (CD13), B cells (CD19) and monocytes (CD14)] by control and CCR5 edited cells in bone marrow.

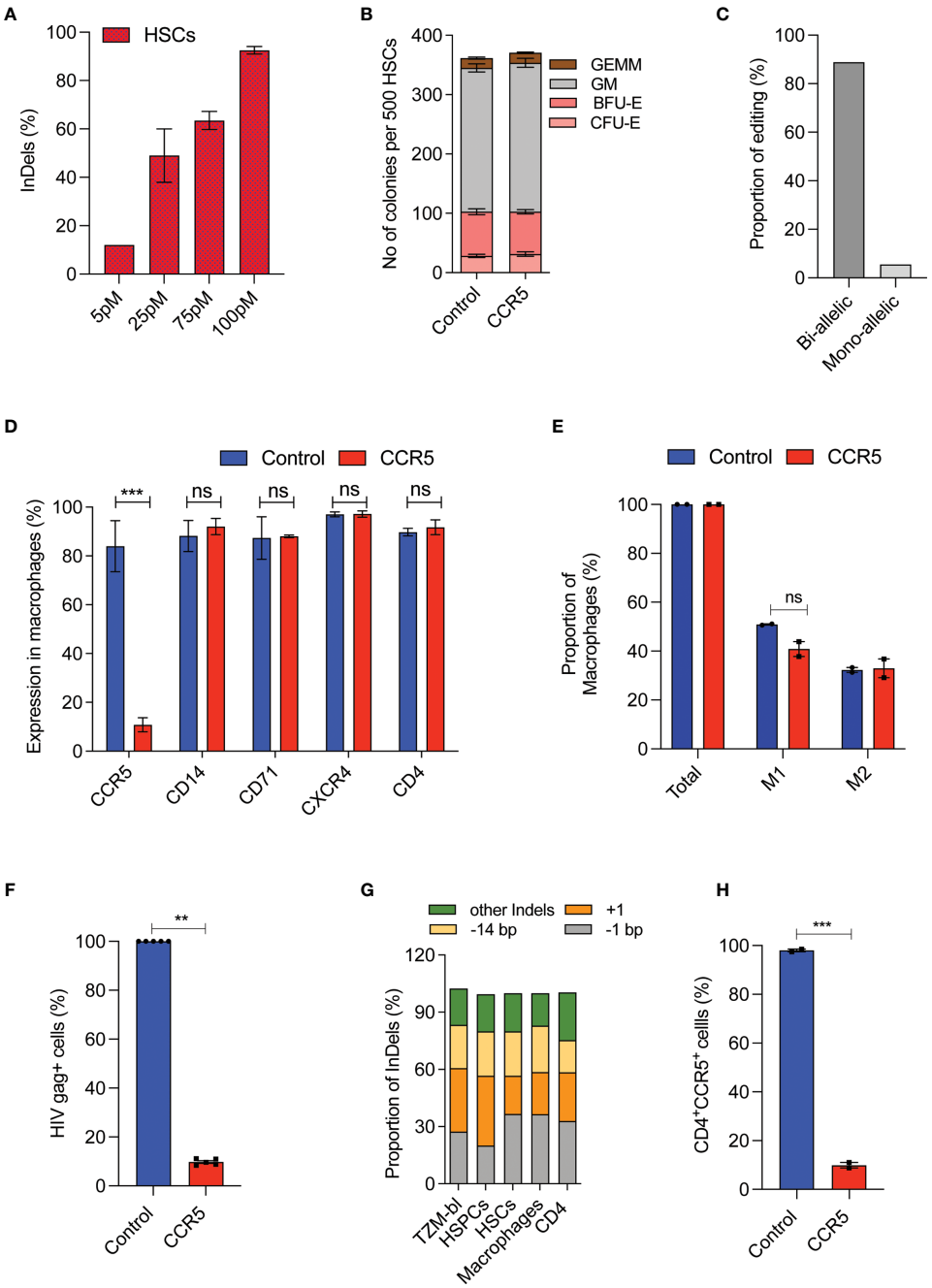

FIGURE 4 | High-frequency *CCR5* editing in CD34$^+$CD90$^+$ HSCs provide HIV resistance.**(A)** Percentage of *CCR5* editing in HSCs with different doses of Cas9-RNP. (n = 4), Donors: 3. **(B)** Colony forming analysis of control and *CCR5* edited HSCs. n = 6, Donors: 3. **(C)** Percentage of mono and biallelic editing in methocult colonies. CCR5 edited HSCs are single cell sorted in the macrophage differentiation medium and the colonies generated were genotyped. No of colonies analysed: 20. **(D)** Expression pattern of macrophage receptors. The control and *CCR5* edited HSCs were *in vitro* differentiated into macrophages and analysed by flow cytometry for the expression of different markers. (n = 5), Donors: 2. **(E)** Percentage of M1 and M2 macrophage subsets. The control and CCR5 edited HSCs were *in vitro* differentiated into macrophages and analysed by flow cytometry for the expression of total (CD14), M1 (CD14$^+$CD80$^+$) and M2 (CD14$^+$CD206$^+$CD163$^+$). (n = 2), Donor: 1. **(F)** Percentage of HIV-1 infection in macrophages. The control and *CCR5* edited HSC derived macrophages were infected with HIV-1. HIV Gag$^+$ cells were analyzed 6 days post infection using flowcytometry (n = 4), Donors: 2. HIV infection (Gag+ Cells) in control macrophages was normalized to 100. **(G)** Pattern and proportion of Cas9-RNP induced InDels at *CCR5* in TZM-bl cells, HSPCs, HSCs, macrophages and CD4 T cells. (n = 3), Donors: 2. **(H)** CD4 T cells expressing CD4 and CCR5 receptors post gene editing with control or CCR5. (n = 2), Donor: 1. Error bar denotes mean ± SEM. ns, non-significant. **p ≤ 0.01, ***p ≤ 0.001 (Unpaired t test, Two tailed).

At 16th week post transplantation, into NBSGW mice, we observed that the CCR5 edited cells engrafted as efficiently as the control cells with the mean human cell chimerism in bone marrow (**Supplementary Figure 4A** and **Figure 5A**), spleen (**Figure 5B**) and peripheral blood (**Figure 5C**) of about > 70%, 60% and 20% respectively. This analysis indicates that CCR5 gene editing does not affect the engraftment of $CD34^+CD90^+$ HSCs and a dose of $1x10^5$ $CD34^+CD90^+$ HSCs is sufficient to repopulate 70% of the bone marrow. Multilineage analysis in bone marrow showed the formation of myeloid and lymphoid lineages with no lineage bias in the CCR5 edited group (**Figure 5D**).

Tissue trafficking of $CCR5^-$ cells is crucial to eradicate the established HIV-1 reservoirs. To test whether CCR5 edited cells can lodge in to different mouse tissues, we analysed the CCR5 editing frequency in the cells recovered from different tissues. InDel analysis showed reconstitution of CCR5 edited cells in bone marrow, spleen, peripheral blood and brain. Importantly, the frequency of gene-editing was maintained from that of the infused product (**Figure 5E**). InDel pattern analysis confirmed that the HSPCs with the prominent +1 and -1 InDels were retained on long-term repopulation, while the cells with 14bp deletion diminished, suggesting that HSPCs with 14bp deletion is not competent for long-term repopulation (**Figure 5F**). Similar loss of 13bp deletion has been reported with gene editing of γ-globin promoter (35).

To test the functional proficiency, macrophages were generated from the engrafted human cells and that showed > 90% of cells lacking CCR5 expression in the CCR5 edited group with no defects in macrophage generation or maturation when compared with the control (**Figure 5G** and **Supplementary Figure 4B**). When these macrophages were challenged with infectious R5-tropic HIV, > 80% of cells showed resistance in the CCR5 edited group, consistent with the genotype and CCR5 expression (**Figure 5H**).

DISCUSSION

In this study, we show that single sgRNA mediated CCR5 gene editing in RUS treated $CD34^+CD90^+$ HSCs is an ideal approach to generate HIV resistant immune system based on the following important observations:

InDels induced by single sgRNA gives HIV resistance as similar as dual sgRNAs approach

Reduced receptors for HIV infection and increased expression of antiviral restriction factors in the $CD34^+CD90^+$ HSCs;

A high frequency of bi-allelic editing in $CD34^+CD90^+$ HSCs;

Infusion of a low dose of RUS treated $CD34^+CD90^+$ HSCs is sufficient to repopulate the bone marrow.

While CCR5 Δ32 allogenic stem cell transplantation has demonstrated the ART independence and undetectable level of viral genome per cell (8), such an effect has not been achieved yet with gene edited autologous HSPCs. The hematopoietic progenitor cells of the HIV infected patients are reported to have various defects including decreased numbers, altered functional characteristics, and defects in the lymphoid lineage (28, 30, 37–39). The defects are probably a result of HIV-1 infection in the HPCs. Studies have shown that HPCs can be infected with HIV-1 and they harbor HIV genome at a frequency similar to $CD4^+$ T cells (18, 30). *In vitro* experiments demonstrated that HIV-1 infection in the HPCs triggers apoptosis (28). This provides a possible reason for the peripheral blood cytopenia in the AIDS patients (40). In addition, if HSPCs are used for gene manipulation, reservoirs in the HPCs may reseed the HIV in bone marrow after transplantation. This is counterproductive to the HIV reduction achieved by the conditioning regimens. Also, the activation of latent virus during *ex vivo* culture of progenitor cells poises an additional risk (19, 41). All these findings suggest that HPC fraction in the autologous HSPC graft is not suitable for gene editing of HIV-1 gene therapy.

The recent high throughput and non-human primate transplantation studies have demonstrated that long term HSCs are immunophenotypically marked by $CD34^+CD90^+$ and they could be the ideal target cells for gene manipulation (20, 35). Here, we show that $CD34^+CD90^+$ HSCs are limited with HIV receptor/co-receptor, express increased amount of antiviral restriction factors and exhibit resistance to R5-tropic infection, making the $CD34^+CD90^+$ HSCs ideal target cells for CCR5 manipulation. Our HIV-1 infection studies in the HSPC pool, purified progenitors and HSCs, clearly support the predominant infection of R5 tropic virus in the progenitors but not in HSCs. While all our observations are from mobilized healthy donor HSPCs, infected *ex vivo*, reports from the HIV infected patients showed that the G_0 fraction of HSPCs lacked any R5 pro-viral DNA, strengthening the use of our approach (27, 38, 39).

The absence of CCR5 receptor and the increased expression of antiviral restriction factors may play a key role in protecting the $CD34^+CD90^+$ HSCs population from HIV-1 infection during pre and post manipulation. SAMHD1 dependent phenomenon demonstrated to restrict HIV infection in macrophages, resting T cells and dendric cells (43). SAMHD1 over expression in the HSCs points that the HSCs protect themselves by a similar mechanism. The antiviral restriction factors in the $CD34^+CD90^+$ HSCs may also limit the HIV infection post transplantation when it encounters the infected stromal cells (44).

Pre-clinical lentiviral gene therapy studies have reported a low lentiviral transduction in the $CD34^+CD90^+$ HSCs when compared with HPC fraction (45, 46). Our observation of higher expression of antiviral factors in the $CD34^+CD90^+$ HSCs explains the reason behind such a low lentiviral transduction.

The complete elimination of CCR5 expression in the lineages by gene editing the $CD34^+CD90^+$ HSCs will be a safer and long-lasting approach as this provides no choice for gp-120 HIV variants to infect hematopoietic cells. CCR5 Δ32 heterozygous genotype showed delayed but not completely prevented HIV-1 infection underlines the need for biallelic CCR5 editing (47).The mathematical modeling predicted that the autologous HSPC graft with an editing efficiency of 76% or greater is required to control the viral rebound (48). Thus, high frequency bi-allelic CCR5 knockout in HSCs is crucial for clinical success. The high

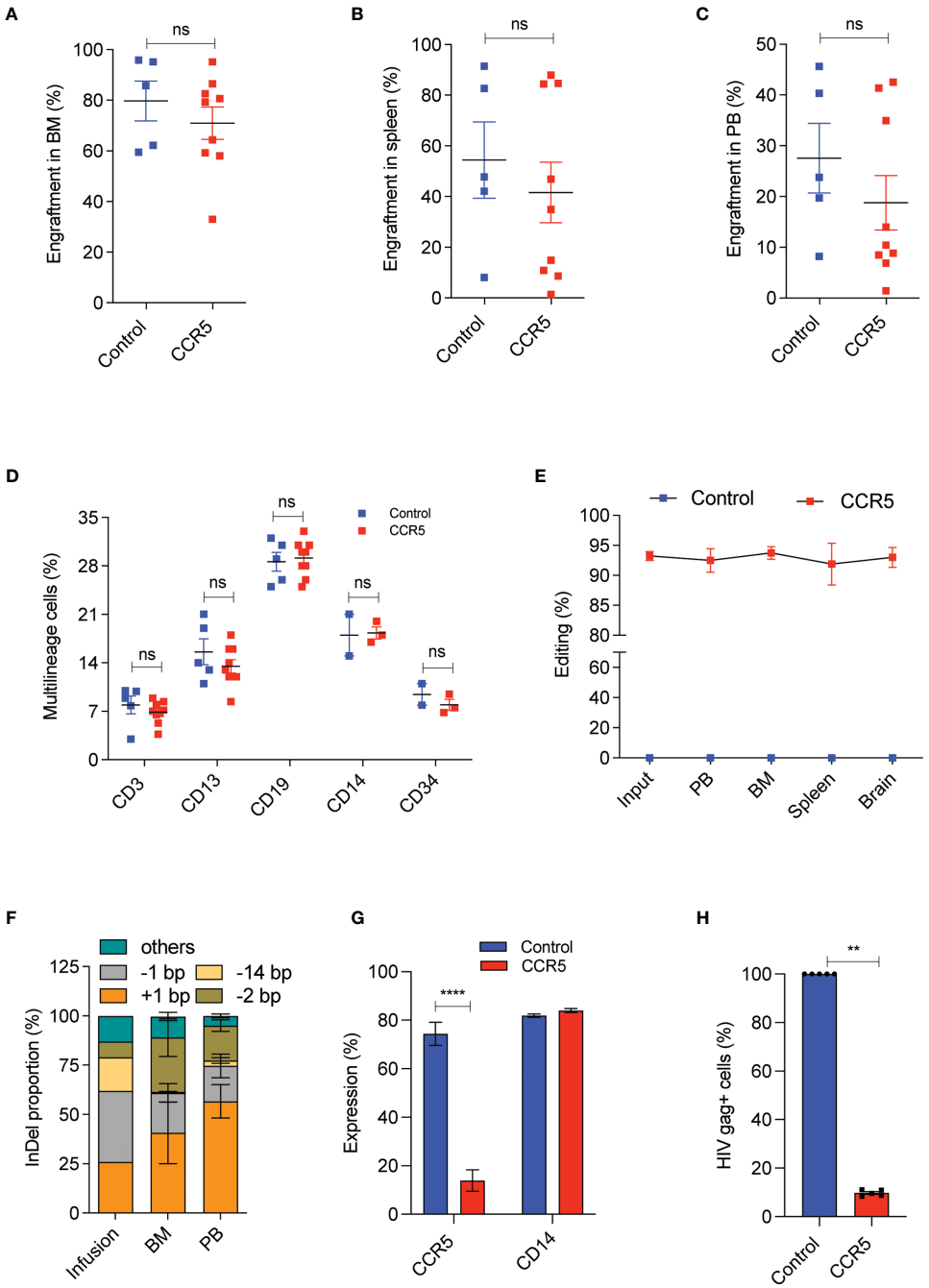

FIGURE 5 | A low dose of CCR5 edited CD34$^+$CD90$^+$ HSCs generates HIV resistant immune system. Experimental Outline: The FACS purified CD34$^+$CD90$^+$ HSCs were cultured with vehicle or RUS for 48 hours, electroporated with tracrRNA and Cas9 (control) or Cas9 RNP targeting *CCR5* and approximately 90,000 cells were transplanted into 7-8 weeks old female NBSGW mice. 16 weeks post transplantation, different tissues of mice were collected and analysed for the engraftment. Each dot indicates a mouse. Error bar denotes mean ± SEM. ns; non-significant. **p ≤ 0.01, ****p ≤ 0.0001(unpaired t test, Two-tailed). **(A)** Percentage of human cell engraftment in bone marrow. **(B)** Percentage of human cell engraftment in spleen. **(C)** Percentage of human cell engraftment in peripheral blood (PB). **(D)** Multilineage (T cells (CD3), Myeloid cells (CD13), B cells (CD19), monocytes (CD14) and HSPCs (CD34)) engraftment in bone marrow. **(E)** Persistence of CCR5 edited cells in different tissues (PB = peripheral blood, BM = Bone marrow). (Genomic DNA were isolated from all mentioned tissues and InDels in CCR5 gene is quantified using human CCR5 specific (E2C5-F, E2C5-R) primers. **(F)** Type and proportion of InDels in the infused product and in engrafted cells (BM = bone marrow, PB = peripheral blood). Others refers to InDels with poor read quality. **(G)** Percentage of macrophages with CCR5 and CD14 expression. The engrafted bone marrow cells were differentiated to macrophages *in vitro* and analysed for the expression of CCR5 and CD14 receptors. **(H)** Percentage of macrophages with HIV infection (Gag$^+$ cells). The engrafted bone marrow cells were differentiated to macrophages *in vitro* and challenged with HIV-1 virus. 6 days post infection, The HIV infection was measured using flowcytometric staining of HIV-gag protein. HIV infection (Gag+ Cells) in control macrophages was normalized to 100.

frequency of bi-allelic editing (>85%), all InDels being functional in disrupting CCR5 expression and the maintenance of indels post transplantation indicate the clinical potential of our approach. Previous works from pre-clinical studies and clinical trial pointed out a reduction in the CCR5 gene edited cell frequency post transplantation (13, 49–51). However, our CCR5-gene edited cell frequency in the infused product and the engrafted cells are comparable. The elimination of HPC fraction during gene editing has likely contributed to the steady level of gene edited cells post transplantation. Notably, The RUS treatment of HSCs facilitated robust and persisted engraftment *in vivo*.

Manipulating large doses of HSPCs for transplantation is challenging and if the HSPCs carry an infectious virus such as HIV it becomes a daunting task. About 2 million HSPCs are being transplanted in the NBSGW mice to achieve >70% human cell chimerism (52, 53). Our observations are in line with the NHP studies that achieved a high level of chimerism with a 10-fold lower infusion product containing only HSCs (35).

The exclusive usage of HSCs for transplantation is associated with its own limitations (54). The dynamics of gene modified stem cell repopulation suggests that, the steady state hematopoiesis is mediated by the HSCs and are stabilized in about 6-12 months post transplantation (55, 56). Thus, it may take up to a period of 1 year for the complete reconstitution of $CCR5^-$ cells in tissues using our approach. Hence, administration of ART for the first year of transplantation is desirable to achieve the full benefit. Studies have also reported a delayed neutrophil reconstitution after transplantation of graft solely containing only the HSCs (35). It will be interesting to see whether the small fraction of HPCs in our graft could potentially provide sufficient numbers of early phase neutrophils. On the other hand, a latest report challenges the bi-phasic hematopoietic reconstitution model and provides evidence that HSCs can also contribute to the early neutrophil recovery (57). Based on this model, CCR5 gene edited $CD34^+CD90^+$HSCs should result in early reconstitution of hematopoiesis with $CCR5^-$ cells.

While, the approach can be used both with allogenic and autologous stem cell transplantation, the factors like graft-versus-HIV reservoir and the extensive conditioning used in allogenic stem cell transplantation may help reduce the amount of viral reservoir, independent of gene editing effect (58). However, GvHD could be a big hindrance in using allogenic graft (4). Our strategy will be limited to R5-tropic but not X4 tropic virus as we see a high expression of CXCR4 receptor in $CD34^+CD90^+$ HSCs and this explain the reason for X4 tropic infection in the HSCs (29). While gene editing of HSPCs is observed to be safe in the ongoing clinical trials, the recent pre-clinical observation of chromothripsis in BCL11A enhancer gene edited HSPCs is a matter of concern (59). Therefore, our future studies will be in the direction of characterization of safety profile of CCR5 edited $CD34^+CD90^+$ HSCs.

In summary, we show that CCR5 gene editing in $CD34^+CD90^+$ HSCs provides uninfected and highly engraftable graft for autologous transplantation and presents a safe and highly efficient gene editing approach for HIV gene therapy. Additionally, culture and gene editing of a low dose of cells would facilitate CCR5 gene editing a simplified and cost-effective gene therapy approach.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institute Review Board (IRB), Christian Medical College, Vellore, Tamil Nadu, India. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by Institute animal ethical committee, Christian Medical College, Vellore, Tamil Nadu, India.

AUTHOR CONTRIBUTIONS

ST designed the study. KVK and ST designed the experiments, analyzed the data, and wrote the manuscript. KVK, JD, VV, AC, PB, MA, AJ, VR, and SR performed the experiments. RK supervised viral studies. AS and GV provided study material and critical inputs. SM and MK provided critical revision of the manuscript. ST acquired the fund. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.792684/full#supplementary-material

Supplementary Figure 1 | HIV receptors in HSPCs, HPCs and HSCs. **(A)** Graphical representation showing sgRNA binding site in CCR5 gene (Nucleotides marked in red showing gRNA targeting region, blue showing Protospacer adjacent motif (PAM) site, shades of blue arrow indicate 246bp deletion when using dual sgRNAs (E2E3) system). **(B)** Representative FACS plot showing the percentage of $CD34^+CD90^+$ cells in unsorted $CD34^+$HSPCs and purity of sorted HPCs ($CD34^+CD90^-$cells) and HSCs ($CD34^+CD90^+$ cells). **(C)** Representative FACS plot showing the percentage of HIV receptors (CD4/CCR5) in $CD34^+$HSPCs, $CD34^+CD90^+$HSCs and $CD34^+CD90^-$ HPCs. The inset in the bottom plot denotes percentage of cells positive for both CD4 and CCR5.

Supplementary Figure 2 | RUS treatment improves the stem cell potential of $CD34^+CD90^+$ HSCs. **(A)** Percentage of highly primitive HSCs ($CD34^+CD133^+CD90^+CD45RA^-CD38^-CD49f^+$) in HSPCs (unsorted), $CD34^+CD90^+$ HSCs and $CD34^+CD90^-$ HPCs. The cells were analysed immediately after purification or sorting. n = 3, Donor:2. **(B)** Representative FACS plot showing percentage cells expressing $CD34^+CD90^+$ in vehicle and RUS treatment. The sorted $CD34^+CD90^+$ HSCs were cultured with vehicle or RUS for 72 hours before FACS analysis. **(C)** Number of GEMM colonies from $CD34^+CD90^+$ HSCs that are cultured with vehicle or RUS for 3 days. **(D)** Percentage of $CD34^+CD90^+CXCR4^+$ cells. The $CD34^+CD90^+$ HSCs were sorted and cultured with vehicle or RUS for 3 days. n = 3, Donors: 2. Error bars denotes mean ± SEM, ns, non-significant. *$p \leq 0.05$, (Unpaired t test, Two tailed).

Supplementary Figure 3 | CCR5 editing in $CD34^+CD90^+$ HSCs generates macrophages with HIV-1 resistance. **(A)** Representative sanger reads from the HSPCs showing intact 32bp sequence which are absent in individuals with delta32 genotype. **(B)** Representative sanger reads from the HSPCs showing intact 32bp sequence which are absent in individuals with delta32 genotype. **(C)** Proliferation kinetics of macrophage progenitor cells from control and *CCR5* edited $CD34^+CD90^+$ HSCs during *in vitro* macrophage differentiation. n = 4, Donors: 2. **(D)** Representative fluorescence micrograph at 10x magnification. The *in vitro* differentiated macrophages were incubated for an hour with fluorescently labelled *E. coli* particle (red). The phagocytosed *E. coli* particle is seen as red colour inside macrophages. (Scale bars were indicated at the right corner of the image. **(E)** Percentage of phagocytosis by control and *CCR5* edited macrophages. Macrophages were incubated with pHrodo Red *E. coli* BioParticles for 1 hr and images were taken with fluorescence microscope and the percentage of phagocytosis was calculated as follows: number cells showing pHrodo Red *E. coli*/ number of total cells*100. n = 2, Donor: 1. **(F)** Representative FACS plot showing the percentage of HIV-1 Gag^+ macrophages from control and *CCR5* edited conditions. Error bars denotes mean ± SEM, ns; non-significant. (Unpaired t test, Two tailed).

Supplementary Figure 4 | Low dose of *CCR5* edited HSCs provides robust engraftment in NBSGW mice with CCR5 null macrophages. **(A)** Representative FACS plot showing the percentage of Human and mice cells in different tissues of NBSGW mice after 16 weeks of infusion. (BM - Bone Marrow, PB - Peripheral Blood and Spleen. Inset values denotes percentage of human cell engraftment calculated using formula (% $hCD45^+$)/(% hCD45 $^+$ +% $mCD45.1^+$) x 100. **(B)** Representative FACS plot showing the percentage of macrophages with CD14 and CCR5. The bone marrow engrafted control and *CCR5* edited cells were *in vitro* differentiated into macrophages and challenged with R5-tropic HIV and analyzed for HIV Gag+ cells as described in *Methods*.

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Biallelic, Selectable, Knock-in Targeting of CCR5 *via* CRISPR-Cas9 Mediated Homology Directed Repair Inhibits HIV-1 Replication

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Transplanting HIV-1 positive patients with hematopoietic stem cells homozygous for a 32 bp deletion in the chemokine receptor type 5 (CCR5) gene resulted in a loss of detectable HIV-1, suggesting genetically disrupting CCR5 is a promising approach for HIV-1 cure. Targeting the CCR5-locus with CRISPR-Cas9 was shown to decrease the amount of CCR5 expression and HIV-1 susceptibility *in vitro* as well as *in vivo*. Still, only the individuals homozygous for the CCR5-Δ32 frameshift mutation confer complete resistance to HIV-1 infection. In this study we introduce a mechanism to target CCR5 and efficiently select for cells with biallelic frameshift insertion, using CRISPR-Cas9 mediated homology directed repair (HDR). We hypothesized that cells harboring two different selectable markers (double positive), each in one allele of the CCR5 locus, would carry a frameshift mutation in both alleles, lack CCR5 expression and resist HIV-1 infection. Inducing double-stranded breaks (DSB) *via* CRISPR-Cas9 leads to HDR and integration of a donor plasmid. Double-positive cells were selected *via* fluorescence-activated cell sorting (FACS), and CCR5 was analyzed genetically, phenotypically, and functionally. Targeted and selected populations showed a very high frequency of mutations and a drastic reduction in CCR5 surface expression. Most importantly, double-positive cells displayed potent inhibition to HIV-1 infection. Taken together, we show that targeting cells *via* CRISPR-Cas9 mediated HDR enables efficient selection of mutant cells that are deficient for CCR5 and highly resistant to HIV-1 infection.

Keywords: CCR5, co-receptor of human immunodeficiency virus type 1 (HIV-1),adipose-derived stem cells (ASCs), CRISPR-Cas9, biallelic mutations, homology directed repair (HDR)

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INTRODUCTION

Since the discovery of the human immunodeficiency virus (HIV) in 1983, the introduction of antiretroviral drug therapy (ART) has turned classically acute HIV infection into a chronic condition. Although ART effectively inhibits HIV replication and disease progression, it does not eliminate the virus (1). Consequently, viral load rebounds when ART is removed and lifelong therapy is required to control viral reactivation and replication (2). Hence, research continues to find a cure for HIV (3, 4).

One potential target is CCR5, a major co-receptor utilized by HIV-1 for cellular entry (5, 6). High levels of CCR5 expression are found in $CD4^+$ T cells and specific myeloid cell types, which become depleted during HIV-1 infection. A small population of individuals are resistant to HIV-1 infection and were found to be homozygous for a naturally occurring 32 bp deletion (CCR5 Δ32) mutation that inhibits CCR5 surface expression and confers resistance to infection by HIV-1 (7–9). So far, population studies were not able to identify deleterious effects of CCR5 Δ32, even in the case of homozygosity, indicating genetic disruption of CCR5 is not associated with major health risks (10). However, subtle changes like increased susceptibility to certain flaviviruses have been reported (11, 12). Based on this natural resistance, cancer patients with an active HIV-1 infection received allogeneic hematopoietic stem cell transplantation (HSCT) from donors homozygous for CCR5 Δ32 (13). In two cases, individuals have been reported with a functional cure from HIV-1 infection (14, 15). Thus, CCR5 Δ32 has been identified as a promising target for curing HIV-1 (16–18). As not all patients have suitable donors, additional approaches are necessary to create a widespread applicable cure for HIV-1 (4). Several approaches for creating a CCR5 deficiency by disrupting its genomic locus have been undertaken, some of them even tested in clinical trials (17–21).

In these successful cases, cord-blood derived hematopoietic stem cells were used as the regenerative cell population (22–24). Besides HSC, other types of human stem cells have been characterized for their hematopoietic potential, such as induced pluripotent stem cells (iPSC), embryonic stem cells (ESC) and mesenchymal stem cells (MSC) (25–30). Adipose tissue derived stem cells (ASCs) are MSC resident within the heterogeneous group of cells within the stromal vascular fraction (SVF), which are more specifically labeled as vascular-associated, pluripotent stem (vaPS) cells (31). They have been shown to be differentiable into cells of all three germ layers including hematopoietic lineage and infectable by HIV-1 *in vitro*, which makes them a potential regenerative source for the blood cell pool depleted during HIV-1 infection (26, 29). In support of this hypothesis transplantation of MSCs isolated from mouse adipose tissue has been shown to efficiently rescue lethally irradiated mice from death as well as resulting in reconstitution of the major hematopoietic lineage (32). In addition, intravenous MSC transfusions in HIV-1 infected nonimmune responder (NIR) individuals showed a significant increase in their naive and central memory $CD4^+$ T-cell counts, but it is unclear if the transplanted MSCs themselves filled up the T-cell compartment (33, 34). MSCs are attracted to latent HIV-1-infected cells and enable virus reactivation from the latent reservoir, so this cell type may play a role in HIV-1 infection beyond the ability to act as progeny for depleted cell pools (35). Because of its abundant availability within the easily accessible white fatty tissue, ASCs have become an attractive source of regenerative cells (31, 36, 37). After isolation from the patient's own adipose tissue, ASCs are suitable for immediate transplantation or expansion, genetic modification, and autologous transplantation. Thus, employing autologous ASCs may bypass bone marrow stromal/stem cells (BMSC) and HSC associated obstacles like complex isolation processes and unsatisfactory cell yields (38, 39). Additionally, autologous transplantation avoids the need for HLA matching and health risks associated with allogeneic stem cell transplantation (40).

Besides many others, CRISPR- Cas9 is a promising gene editing tool enabling the induction of precise changes in the human genome by creating double stranded breaks (DSB) (41–44). The resulting mutations are mediated by two major DNA repair mechanisms: non homologous end joining (NHEJ) and homology directed repair (HDR). While NHEJ mostly creates insertions or deletions (InDels) of smaller size, HDR fixes DSB *via* recombination of homologous sequences. This allows for the integration of foreign sequences into the targeted locus when located within the homology domain (HD) (45–47). Successful gene editing of CCR5 using CRISPR-Cas9 has been reported in a broad variety of studies (23, 27, 48–52). Yet, genomic changes induced by the CRISPR system, especially HDR, exhibit limitations in efficiency and creating predictable genotype outcomes has remained challenging (53–55). Because CCR5 heterozygosity is associated with postponed progression to AIDS in infected patients, only the individuals homozygous for the CCR5-Δ32 frameshift mutation, which lack all CCR5 expression, confer complete resistance to HIV-1 infection (7). Therefore, the efficiency of CRISPR mutations is important for a curative therapy. For inhibition of viral replication in an individual's body, mathematical modeling estimates the fraction of susceptive cells needed to be made refractory to infection lies above 75 - 87.5% (56–58). Consequently, gene edited stem cells used for transplantation should have the highest possible or ideally complete mutational status. By this means it could be possible to provide the patient with a sufficient pool of resistant cells to regenerate the blood system under the selective pressure of HIV-1 infection (59, 60).

In this study, we test an approach for targeting the CCR5 gene and selecting biallelic frameshift mutated cells to create populations consisting of completely CCR5 deficient cells. We hypothesized that integrating two different fluorescent markers using CRISPR-Cas9 mediated HDR induces large frameshift mutations, which subsequently would result in a definite disruption of the CCR5 gene . Cells that constitutively express both fluorescent markers (double positive) could thus be recognized with a bi allelic frameshift mutation and selected rapidly and in large quantities using FACS. We disrupted the CCR5 gene in four different cell types, including human ASCs, and showed loss of CCR5 expression and inhibition of HIV-1 replication.

MATERIALS AND METHODS

Cas9 and gRNA Targeting Plasmids

The pX330-U6-Chimeric_BB-CBh-hSpCas9 expressing a humanized S. pyogenes Cas9 (hSpCas9) from Dr. Feng Zhang (Plasmid #42230, Addgene, Watertown, MA) served as a scaffold. The guide RNAs (gRNAs) were synthesized as single-stranded synthetic oligonucleotides (IDT, Coralville, IA) and the complementary oligonucleotides were annealed to generate double-stranded DNA fragments with 5' ACCG and 5' AAAC overhangs (61). To generate the Cas9-gRNA expressing plasmids (**Figure S1A**), the gRNA linker was ligated into the Cas9 plasmids after BbSI digestion (New England Biolabs (NEB), Ipswich, MA, Cat. # R3539) using T4 DNA Ligase (NEB, Cat. # M0202). Two plasmids, pDONOR-tagBFP-PSM-EGFP (Addgene #100603) and pDONOR-tagBFP-PSM-dTOMATO (Addgene #100604), kindly provided by Jens Schwamborn, served as a template for the donor plasmids. To generate the homology domains (HD), we used a two-step PCR approach to insert the gRNA target sequence at the extremities of the HD into the donor plasmids. Cloning of the final donor plasmids (pDs) (**Figure S1B**) was carried out using Gibson Assembly Cloning Kit (NEB, Cat. # E5510S) in a modified fashion of the protocol published by Jarazo et al. (62). All plasmids were screened for correct formation *via* sanger sequencing.

Cell Culture and Differentiation Assays

TZM-bl cell line (Cat. #ARP5011) and human T-cell Lymphoma Jurkat (E6-1) cell line (Cat. #ARP-177) were obtained through the NIH HIV Reagent Program. TZM-bl and HEK-293FT (Invitrogen, Carlsbad, CA, Cat. #R70007) cell lines and were cultured in low glucose DMEM medium (Invitrogen, Cat. #11885084) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Cat. #26140079) and 1% Penicillin/Streptomycin (Invitrogen, Cat. #15140163) at 37°C and 5% CO_2, while Jurkat cells were cultured in RPMI-1640 (Invitrogen, Cat. #61870036) plus 10% FBS and 1% Penicillin/Streptomycin.

ASCs were isolated from fresh human lipoaspirate samples collected from healthy individuals during surgical procedures. The collection of all human tissue samples was done with the patient's consent in an anonymized fashion and approved by the Institutional Review Board (IRB) of Tulane University, School of Medicine, New Orleans, Louisiana (IRB protocol #168758). Isolation was performed employing a Transpose® RT Tissue Processing Unit (InGeneron, Houston, TX) according to the manufacturer's instructions (36). These cells, designated as a passage 0 (P_0), were then plated at a density of max. 5000/cm^2 in alpha-MEM (Invitrogen, Cat. #12571063), supplemented with 20% FBS in standard cell culture conditions. For the following experiments, only low passage cells (P_{1-3}) were used. To test isolated ASCs for multilineage differentiation potential adipogenic and osteogenic differentiation was performed as previously described (39, 63). For chondrogenic differentiation, cells were plated as a micromass culture using StemPro™ Chondrogenesis Differentiation Kit (Invitrogen, Cat. #A1007101) (**Figure S2**).

Transfection

Since 293FT and TZM-bl are considered easily transfectable cell lines, lipofection was the transfection method of choice. Lipofectamine 2000 (Invitrogen Cat. # 11668500) was used according to the manufacturer's instructions. Jurkat T-cells and especially primary ASCs are considered difficult to transfect. Due to the need of transfecting four different, largely sized (8.4kb/11.7kb) vector plasmids, the Neon Electroporation System (Invitrogen Cat. # MPK5000) was found to be the most promising approach. The system was used according to the manufacturer's instructions. Briefly, 1 μg of each plasmid (4 μg total) and 5 x 10^5 cells were brought into suspension in a 10 μl neon tip. After optimization, ASCs were transfected with 1 pulse at 1500V for 20 ms or 3 pulses at 1400V for 10 ms; while Jurkat cells were transfected with 1 pulse at 1200 to 1300V for 30 ms. The cells were immediately transferred to prewarmed antibiotic-free media. The viability and transfection efficiency was estimated by trypan blue staining and fluorescence microscopy or flow cytometry.

Fluorescent Activated Cell Sorting

In cells with low transfection efficiency, cells underwent sorting for positive transfection 48 hrs post transfection (p.T.). To sort cells for constitutive expression of both fluorescent positive selection marker (PSM), and lacking the negative selection module (NSM) BFP expression ($EGFP^+$,$dTomato^+$,BFP^-) 14 days post transfection, cells were suspended in PBS with 2% fetal bovine serum, 1% Penicillin/streptomycin. Sorting in all cell types was conducted with the same defined gating hierarchy: First FSC-A/SSC-A was used to identify the isoform cell population of interest and exclude debris. Secondly, FSC-W/FSC-H and SSC-W/SSC-H gating helped exclude doublets. Cells were then gated for BFP negativity. In the last gating step, we identified the EGFP and dTomato positive cells. For compensation reasons and identifying positive populations, negative and singly positive control groups were applied. All cell analysis and sorting steps were performed with a BD FACSAria III at the Cell Analysis & Immunology Core Facility at Louisiana Cancer Research Center. EGFP, dTomato and BFP were assessed by using FITC, PE and V450 filter sets respectively. Data was analyzed using FlowJo Software v_10.6.1 (FlowJo LLC, Ashland, Oregon).

Immunophenotyping

ASCs were characterized by immunostaining with differently fluorescent labeled antibodies for mesenchymal and hematopoietic stem cell markers: CD90-APC, CD49b-APC, CD44-FITC, CD105-PE/APC, CCR5-APC, CD4-eFluor, CD34-PE, CD14-PE-Cy5, CD45-PE and CD68-PE (BD Biosciences, Franklin Lakes, NJ) (26). Analogously TZM-bl and Jurkat-T-cells were stained for CCR5 (CD195, BD Bioscience, Cat. #556903) surface expression using standard staining methods. If positive and negative cells were not distinguishable as two separate populations, Overton histogram subtraction technique was utilized for determining the fraction of positive cells (64).

T7EI- Assay

To assess the cleavage efficiency of CRISPR-Cas9 targeted cells, T7 endonuclease I (T7EI) mismatch cleavage assay (IDT, Coralville, IA) was employed. Genomic DNA was isolated using the Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich). A PCR was performed, spanning a segment of 590 bp surrounding the targeted region using T7EI primer pair (**Table S1**). T7EI-Assay was carried out according to manufacturer's instructions and the product was visualized *via* TBE Gel electrophoresis. Cleavage efficiency [Fcut = (b + c)/(a + b + c)] was calculated by measuring the band intensity of the undigested PCR product (a) and each cleavage product (b and c) with ImageJ_1.52a software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA). It should be noted, however, that the mutation rates determined by this strategy underestimate actual mutation frequency since small insertions or deletions (InDels) are not detected.

qPCR for Assessment of Integration and Quantification of the Frequency of Mutation

Targeted and sorted populations were screened for integration of the PSM and disruption of the WT locus by qPCR using four primer pairs (**Figure 1** and **Table S1**). The vector knock-in (VKI) left and right primers span the left and right junction from the PSM across the Homology Arms to Intron 2 or Exon 3 respectively and positive amplification validates VKI of the PSM. The PuroR primer pair span the puromycin resistance gene and detect the either episomal or integrated PSM plasmid present in the population. The DWT (detecting the wildtype) primer pair spans the target site. Integration of the ~4200bp PSM inhibits amplification of the DWT by increasing the distance between the primer pair and therefore indicates integration in the CCR5 locus.

Since this study aims to inhibit the expression of CCR5 by disrupting its genomic locus, the amount of remaining and potentially functional WT Locus is of significant interest. Real-time quantitative PCR is considered a semiquantitative method of measuring a nucleic load, only allowing comparisons within signals detected with the same primer pairs on different genomic samples.

To estimate the percentage of undisrupted CCR5 loci in a targeted population, a standard curve was created from wild type genomic DNA (gDNA) mixed with gDNA derived from a single ASC clone carrying a complete biallelic knock-in of the PSM at different ratios (**Figure S4A**). It was thus possible to infer the percentage of DWT from the ΔCt *via* a linear regression by using the slope of the standard curve. Due to the logarithmic nature of qPCR, a correlation between the Ct value and the amount of wild-type locus exists only in the lower percentage ranges (**Figure S4B**). In a population with low levels of integration, minor ΔCt alterations would reflect increased levels of calculated alleles not carrying a knock-in. Consequently, the percentage of CCR5 alleles not carrying a knock-in (DWT) is only valid for populations in which ΔCt lies within a certain range (<20%), valid for reflecting mutational status. Thus this qPCR approach is rather an approximation of disruption by integration than an

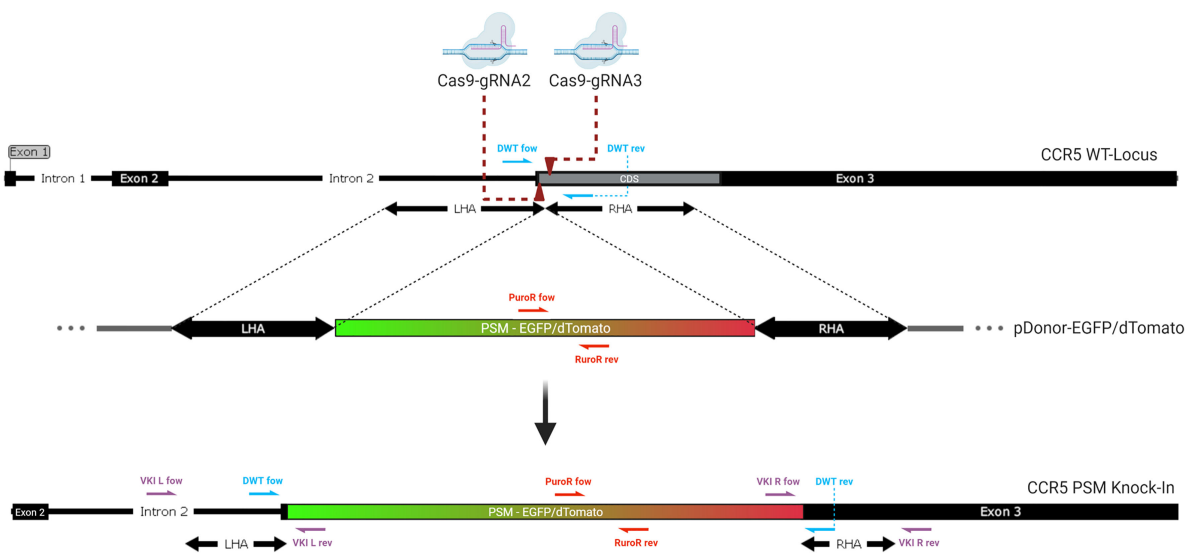


FIGURE 1 | Genomic integration of the donor. Two Cas9-gRNA ribonucleoproteins are directed against closely adjacent sites on the coding sequence (CDS) at the beginning of Exon 3 of the CCR5 gene (dark red arrows). Double stranded breaks lead to integration of a donor template with homologous sequences matching the region surrounding the DSBs. The positive selection module (PSM), a non-homologous graft sequence, which lies between the left (LHA) and right homology arm (RHA) is being integrated into the CCR5 gene as homologous recombination occurs. It contains an expression cassette for either EGFP or dTomato. The knock-in of this long functional sequence creates a massive frameshift mutation, disrupting the CCR5 gene. Half arrows display the location of the primer pairs used for genomic qPCR. (violett: VKI; red: PuroR; blue: DWT).

exact determination. Since CRISPR-Cas9 induced DSB themselves (without the occurrence of HDR) may lead to genetic disruption by InDels, a T7EI assay has been performed on the DWT amplicon. Therefore the mutational frequency is calculated as:

$$[\%\ \text{Mutant Alleles} = \%\ \text{HDR} + (\%\ \text{DWT} \times \%\ \text{InDel})]$$

with [% HDR = 1 – % DWT]. For all populations not targeted with HDR and with DWT ΔC_t signals above ranges considered valid, only the InDel frequency was taken into account for calculating the total remaining WT Alleles.

HIV-1 Infection and Luciferase Reporter Gene Assay

TZM-bl cells express a firefly luciferase (Luc) reporter gene based on HIV-1 infection and HIV-1-Tat expression (50). Viral inhibition assay was performed by infecting 30,000 TZM-bl cells with R5-Tropic HIV-1_{BaL} virus (NIH HIV Reagent Program, Division of AIDS, NIAID, NIH, Manassas, VA Cat. # ARP-510) (titrated to induce >100,000 RLU Luciferase activity) for 3 hours (65). Cells were washed and cultured for 48 hours before lysis with 1x Reporter Lysis Buffer (Promega, Madison, Wisconsin, Cat. # E4030). The cell lysate was centrifuged at 20000 × g for 10 min and 20 µl of the supernatant were mixed with 100 µl of Luciferase Assay Reagent (Promega, Madison, Wisconsin Cat. # E4030) immediately before measuring luminescence with a Lumat LB 9507 (Berthold Technologies GmbH & Co. KG, Bad Wildbad, Germany). The protein concentration, as measured with NanoDrop™ 2000 spectrophotometer (ThermoFisher Cat. # ND-2000), was used to normalize the RLU/ug for each population in quadruplicates.

Statistical Analysis

Results were presented graphically using GraphpadPrism8.1 (GraphPad Software Inc., San Diego, CA) or Excel 14.0.7265.5000 (Microsoft, Redmond, WA). Where meaningful, data is summarized using descriptive statistics such as mean, and standard deviation. Two-tailed student's t-test and Wilcoxon matched-pairs signed-rank test were used as statistical methods and are referred to in combination with the presentation of the data . The study hypotheses were tested at a 5% level of significance throughout the analysis.

RESULTS

Identification of the Most Efficient gRNAs and Their Combinations

Targeting CCR5 *via* CRISPR-Cas9 induced HDR requires three components: the Cas9 nuclease or its encoding sequence, a single guide RNA (gRNA) and a donor which will be integrated into the targeted locus. We hypothesized if cleavage occurs slightly downstream of the beginning of the coding sequence (CDS), mutations may be more likely to inhibit the expression of any functional CCR5 product (**Figure 1**). We used CRISPOR Version 4.99 (http://crispor.tefor.net/) to identify potential gRNAs and CasOFFfinder 2.4 (http://www.rgenome.net/cas-offinder/) to identify potential off-target sites (**Table S2**). gRNAs were excluded if they only displayed two or less mismatches with an off-target site, while three or more mismatches were considered acceptable. The four gRNAs with the highest predicted efficiency and least probability for off target effects were selected for screening (**Table S1**). The aim of this study is to create a selectable, biallelic, frameshift mutation, *via* the integration of two different fluorescent selectable markers, one in each allele. Arias-Fuenzalida et al. published a mechanism for fluorescence guided biallelic HDR targeting selection, using CRISPR-Cas9 and two donor plasmids to induce a single nucleotide change exclusively on one allele, linked to early-onset Parkinson's disease (54). We assumed the integration of fluorescent markers would also act as a large frameshift mutation and therefore efficiently disrupt the CCR5 gene. In our previous study, we showed increased efficiency for biallelic mutations in the CCR5 locus using multiple guide RNAs in ASCs (55). This approach has previously been tested for ZFNs and TALENs and achieved predictable deletions (49). Hence two pCas9-gRNAs were selected and implemented in this study.

To determine whether dual or single targeting is more efficient in this target site and which combination of gRNAs displays the highest cleavage efficiency, four gRNAs (gRNA 1, 2, 3 and 4) as well as their combinations were screened. Transfections and T7EI Assays were performed in triplicates in 293FT Cells (**Figure S3**). gRNA3 showed to induce the highest amount of genomic alterations (32.3 ± 1.7% SD; n=3), followed by gRNA2. gRNA1 displayed only very weak activity and gRNA4 no activity at all. With (44.8 ± 18.0% SD; n=3), the combination of 2 + 3 showed the overall highest cleavage efficiency. Still, in this specific setup, the difference in mutational events by targeting with two gRNAs (2 + 3) rather than one (3) was not found to be statistically significant (p=0.15) as determined by using a two-tailed student's t-test.

Transfection of pDonor-EGFP/dTomato and pCas9-gRNA Together Leads to Constitutive Expression of Both Fluorescent Selectable Markers

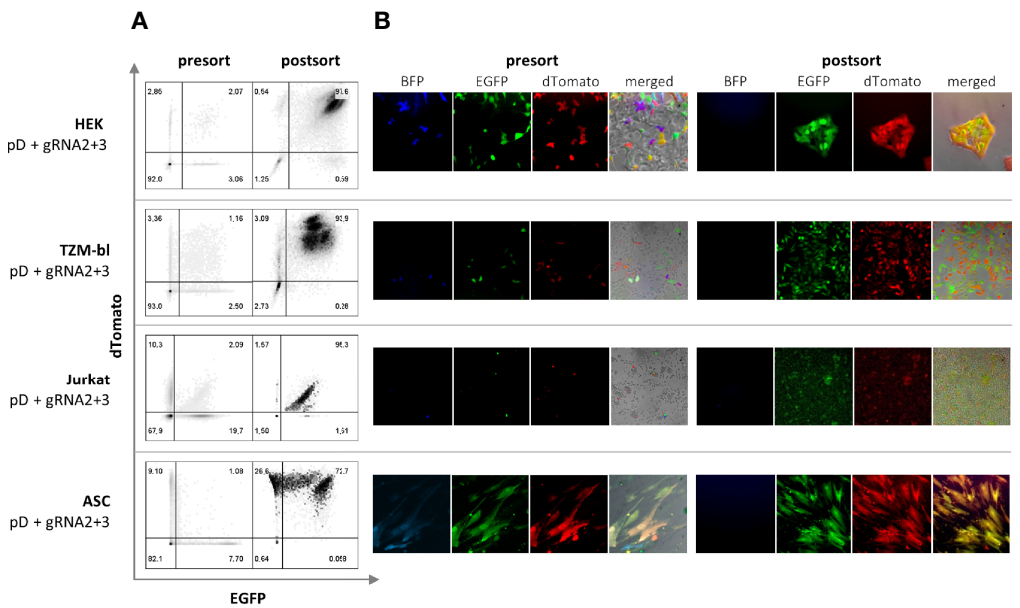
Either an EGFP or dTomato encoding sequence functioned as a positive selection marker (PSM) selectable by flow cytometry for a successful knock-in. DSB induced HDR is often found to be a very inefficient process. Multiple strategies to increase the integration rate have been incorporated into the donor design. First, the length of the homology arms has been shown to have a substantial impact on the integration efficiency (66). Second, linear donors were found to display higher integration rates than circular plasmids. However linear DNA is subject to relatively fast degradation which might limit the amount of donor available for integration. Therefore, gRNAs were integrated into the donor plasmids for linearization in presence of Cas9 activity (66). Third, minimizing the replaced sequence surrounding the DSB (66). Thus, the interior ends of the homology arms include the primary cutting sides of the Cas9. To prevent cleavage activity within the Homology Domain, the protospacer adjacent motif

(PAM), the 5'.NGG.3' sequence of gRNA 2 and 3, was changed to 5'.NCC.3' so it will not be recognized by the Cas9-gRNA complex. All modifications listed above were integrated into the homology arms by designing specific primers (**Table S1**).

In total, four different cell types (293FT, TZM-bl, ASCs and Jurkat T-cells) have been employed for testing the constructs. pDonor and pCas9-gRNA were delivered either by using Lipofectamine 2000 (293FT and TZM-bl) or the Neon Electroporation System (Jurkat T-cells and ASCs). HEK 293FT c ells showed high transfectability (>90% estimated by microscopy, data not shown); however, TZM-bl, Jurkat, and ASCs did not show as high transfection efficiencies (25.0-32.9% TZM-bl; 23.6% Jurkat; 37.9% ASC; assessed by flow cytometry, data not shown). Hence, TZM-bl, Jurkat and ASCs were sorted for positive transfection 48 hr post transfection (p.T.). Additionally, every transfection included the pCas9-gRNA only transfected comparison group, a negative, as well as a pDonor only control. Transfecting the pDonor alone allowed us to estimate the time until the transient expression of fluorescent marker subsides due to plasmid degradation as observed by fluorescent microscopy. After 14 days, control groups in all cell types lost their fluorescent signal (data not shown). Fluorescence displayed beyond this point in time was expected to be subject to constitutive expression due to the integration of the selectable marker into the genome. Consequently, sorting for constitutive expression of both PSMs (dTomato and EGFP) and absence of the NSM (BFP) was carried out on day 14 p.T. (**Figures 2A, B**). Using two sgRNAs, the frequency of double-positive cells was found to be between 1-2% across all cell types. Testing single targeting in TZM-bls induced comparable frequencies (1.3%) with gRNA3, while gRNA2 induced almost no constitutive expression (0.04%) (**Figures 2A, C**). To ensure a correct and stable expression pattern, the sorted population was reanalyzed directly and prior to conducting downstream experiments.

Targeting-Selecting Enables the Selection of Genetically Disrupted Cells

To quantify integration and non-integration of the PSM, real time quantitative PCR was employed (**Figure 3**, row I). In all four cell types, dually targeted and selected cells were compared to WT cells and a dually CRISPR-Cas9 targeted control group (**Figure 3A–D**). WT cells and pCas9-gRNA only transfected



C

%BFP-, EGFP+, dTomato+	HEK pD+gRNA2+3	TZM-bl pD+gRNA2+3	TZM-bl pD+gRNA2	TZM-bl pD+gRNA3	Jurkat pD+gRNA2+3	ASC pD+gRNA2+3
Presort	2.1	1.1	0.04	1.3	2.1	1.1
Postsort	97.6	93.9	Not sorted	95.4	95.3	72.7

FIGURE 2 | Selection of double positive (BFP-, EGFP+, dTomato+) cells *via* FACS. Four different cell types (HEK, TZMbl, Jurkat and ASC) have been transfected with both pDonor and both pgRNA-Cas9 14 days prior to sorting. To compare dual to single targeting, TZMbl have been transfected with both pDonors and single pgRNA-Cas9. **(A)** Cells were sorted for BFP negativity (not shown) and dual positivity for EGFP and dTomato (presort). The sorted population was reanalyzed immediately and 7 days later, prior to conducting the experiments (postsort) **(B)** Flourescent microscopy of cells 14 day post transfection (presort) and 7 days after sorting for double positivity (postsort) **(C)** Quantification of BFP-, EGFP+ and dTomanto+ cells before and 7 days after sorting. To compare dual to single targeting, TZMbl have additionally been transfected with both pDonors and single pCas9-gRNA (pD+gRNA3/pD+gRNA2).

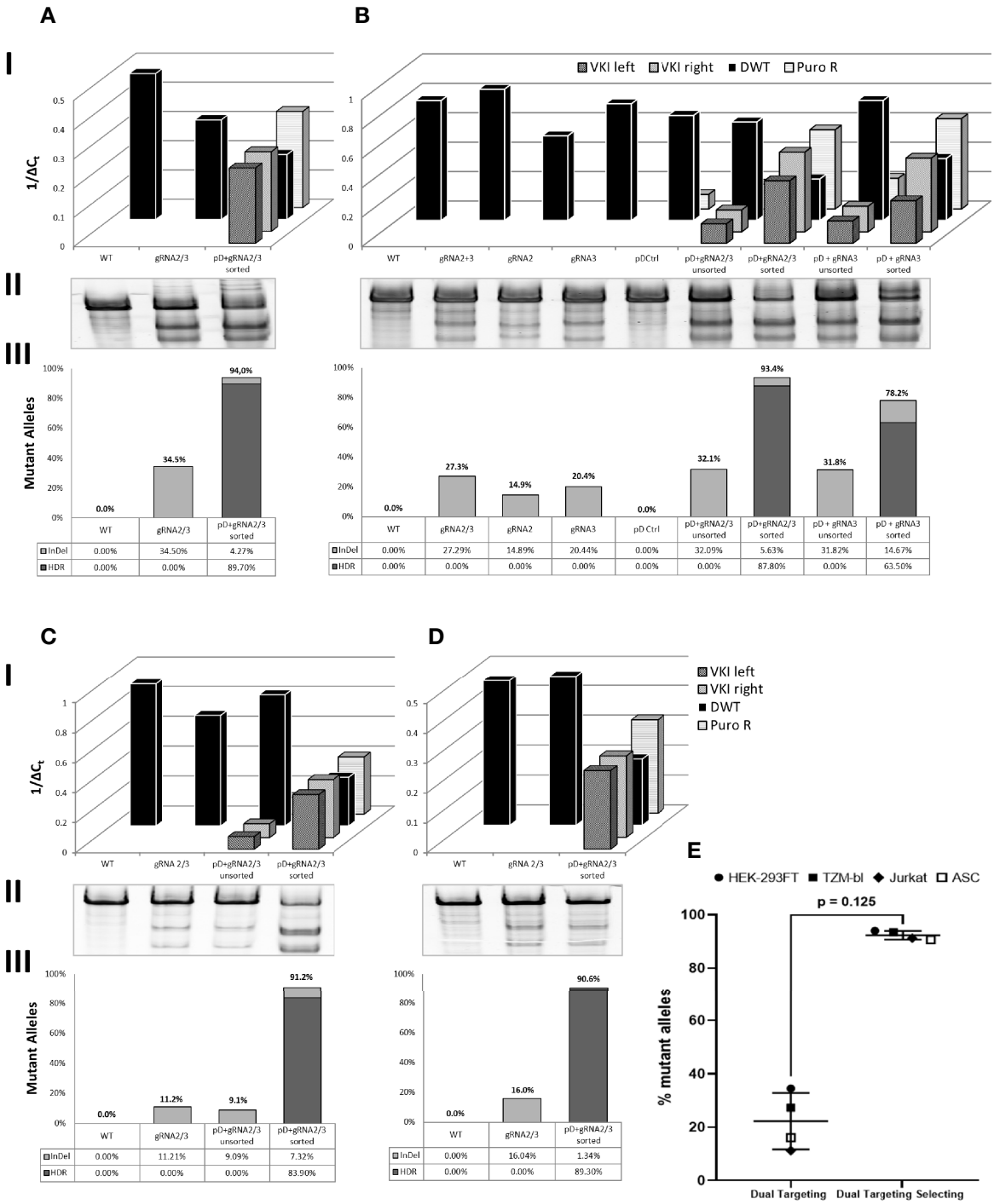


FIGURE 3 | Quantitative analysis of genomic changes in target site. Dually targeted and selected cells (pD + gRNA2/3 sorted) were compared to WT and cells dually targeted with gRNA2/3. Additionally the genomic profile of targeted populations before sorting for dual positivity (pD+gRNA2/3 unsorted) was analyzed in **(B, C)** B also includes comparison groups targeted and target-selected with single gRNAs. I: Real Time quantitative PCR of total PSM (PuroR), the integrated PSM (VKI left/right) and the CCR5 Locus not carrying a knock-in (DWT). qPCR r esults are presented inversely ($1/\Delta C_t$), so a high and a low genomic load are represented by a tall and a low bar respectively. II: T7EI-Assay of the DWT Amplicon. III: Calculation of the Fraction of Mutant Alleles, carrying a knock-in (HDR) or an InDel (InDel): [%Mutant Alleles = HDR + ((1-HDR)*InDel)]. [HDR = 1 - DWT]. DWT, the fraction of alleles not carrying a knock-in, is calculated through linear regression of the ΔCt (DWT) on the standard curve as described above. InDel frequency was calculated as previously mentioned. **(A)** HEK 293FT. **(B)** TZM-bl. **(C)** Jurkat. **(D)** ASC. **(E)** Mean fraction of mutant alleles across all cell types in double positive, dual targeting selecting group (pDonor-EGFP/dTomato + pCas9-gRNA2/3) compared to dual targeted control group (pCas9-gRNA2/3). Error bars show ± SD. Statistical analysis was performed using Wilcoxon matched-pairs signed rank test.

control groups did not show any PuroR or VKI signal. PuroR was detectable in all groups transfected with pDonor, but the transfection of the donor alone (pD Ctrl) did not lead to any integration (VKI). VKI was only detectable in groups generating the DSB (transfected with pDonor and pCas9-gRNA), indicating only simultaneous transfection of all plasmids leads to integration of the Donor. To ensure that both CCR5 alleles are targeted, we included two pDonor plasmids with either EGFP or dTomato fluorescent markers. After selecting the pDonor + pCas9-gRNA transfected groups for double positive cells, the frequency of PuroR and both VKI increased compared to the corresponding unsorted group in TZM-bls and Jurkats (**Figures 3B, C**). More importantly, sorting for double positivity leads to a definite reduction of the CCR5 wild-type allele (DWT). All other control groups (WT, pCas9-gRNA alone, pD Ctrl, unsorted) display almost equal DWT signals, differing by less than one ΔCt within each cell type. Only the targeted and sorted populations had DWT in a range low enough to infer the fraction of alleles not carrying a knock-in (**Figure 3**, row III). T7EI Assay was performed on the DWT amplicon to quantify the InDel frequency within the alleles not carrying a knock-in (**Figure 3**, row II). WT and pD control do not display cleaved fragments. All groups transfected with pCas9-gRNA show cleavage activity with band sizes to the corresponding gRNAs transfected. Targeting with two gRNAs showed a mean mutational activity of 22.3% (± 10.6%; n=4) (**Figure 3E**), which is found to differ between the different cell types (**Figures 3A-D**). In TZM-bls (**Figure 3B**), two gRNAs (27.3%) show slightly higher cleavage efficiency compared to single gRNAs (14.9 – 20.4%) coinciding with the findings in HEK293FT cells (**Figure S3**). Additionally in TZM-bls and Jurkats (**Figures 3B, C**) sorted groups show higher InDel frequencies within the fraction of alleles without recombination (DWT) than their unsorted counterparts as detected by the surveyor Assay (**Figure 3**, row II).

To calculate the total fraction of mutant alleles for the sorted populations, disruption by HDR and InDel frequency were added (**Figure 3**, row III). Assuming all WT, comparison and unsorted groups do not carry any (detectable) KI, indicated by their DWT signal, only the InDel frequency was taken into account for calculating the mutational frequency. Across all cell types (**Figures 3A–D**), dually targeted and selected (double positive) populations showed consistently high mutation rates of averaging 92.4% (± 1.6%; n=4) (**Figure 3E**), of which 87.7% (± 2.6%; n=4) were due to HDR. Double positive TZM-bls targeted with only gRNA3 and two donor-plasmids showed a mutational frequency of 78.2%.

To examine the genomic structure of an individual double-positive cell rather than a whole population, ASC clones were isolated and analyzed for homologous recombination, indicated by detectable VKI and PuroR signals (**Figure 4**). PuroR was positive in all clones. However, VKI signal intensity differed very widely between the different clones and even within one clone comparing VKI left and right. 7/12 clones display no DWT signal, suggesting integration inhibiting DWT amplification on both alleles. In 5/12 clones, DWT did amplify, but with a lower signal than the WT. Still, in these clones at least one allele can be expected to not carry a knock-in. The widely varying signal of the knock-in associated sequences, may be an indication for polyform integration mechanisms.

CCR5 Surface Expression Is Abolished After Selection for Double Positive Cells

The genomic analysis of the targeted selected cells indicated substantial disruption of the CCR5 locus. The CCR5 receptor executes its function by being displayed on the host cell's surface.

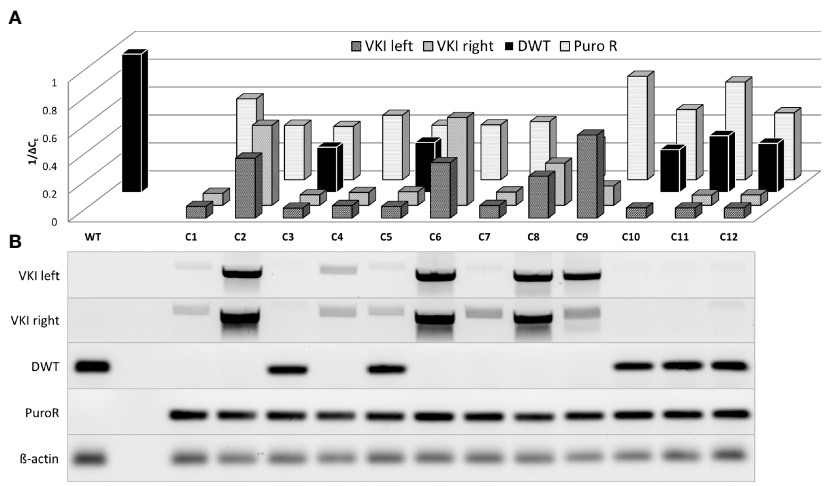


FIGURE 4 | Semiquantitative analysis of genomic changes in dual target-selected, double positive ASC clones. ASCs were transfected with both pDonor and pgRNA-Cas9 (pD+gRNA2/3). 14 days p.T. twelve BFP-, EGFP+, dT+ clones were selected and separately cultured upon reaching enough cells for genomic analysis. **(A)** Real Time Quantitative PCR of sequences associated with HDR was carried out analogously to population analysis. **(B)** Agarose gel electrophoresis of PCR-Products.

To investigate whether the genomic changes lead to a loss in CCR5 surface expression, TZM-bls, Jurkat T cells and ASCs were stained for CCR5 expression (**Figures 5A-C**). Jurkat T cells and ASCs express low levels of CCR5 on their surface. Overton histogram subtraction technique was used to distinguish positive cells (64). However, dual targeting selection was able to reduce the fraction of CCR5 positive cells from 22.4% in WT Jurkat to 6.5% after targeting (**Figure 5A**). Similarly, a reduction of CCR5 positive cells from 60.4% to 25.6% was found in ASCs (**Figure 5B**). In TZM-bl cells, CCR5 is expressed in 97.4% of WT cells (**Figure 5C**). Targeting with CRISPR-Cas9 alone using gRNA2, gRNA3 and dual targeting only lead to minor reduction in CCR5 expression. In contrast, targeting and selection of dual positive cells lead to a significant reduction of detectable surface CCR5 to 9.7% and 2.0% using one and two gRNAs respectively (**Figure 5C**).

Double Positive TZM-bl Cells Show Low Infectability for HIV-1

For successful entry of the host cell, the HIV-1 particle binds first to CD4 and opens the CCR binding domain in the gp120 variable loop; then, binding to CCR5 forms an integration complex, which mediates fusion of the virion into the cell (67). To determine whether the mutations in CCR5 prevented infection, TZM-bls were exposed to HIV-1_{BaL} and infectability was assessed by Luciferase Assay (**Figure 5D**). WT TZM-bls show a significant increase in luciferase activity when challenged with HIV-1 (64.1 ± 8.0 fold increase; n=4) compared to uninfected control. The increase is reduced in cells dually targeted with CRISPR-Cas9 alone (43.5 ± 11.8 fold increase; n=4). Dually targeted and selected TZM-bls show almost no change in luciferase activity, when exposed to HIV-1 (1.2 ± 0.1 fold increase; n=4) compared to uninfected controls (**Figure 5D**). Consequently targeting with two gRNAs leads to a significant reduction in infectability (67.2 ± 17.6%; p=0.03) compared to WT cells but still leaves a high level of infection. In contrast, double positive TZM-bls show only a minor fraction of the infectability of WT Cells (1.9 ± 0.4%; p=0.00043) (**Figure 5D**).

Targeting Selecting ASCs Leaves Regenerative Potential Unaltered

Analogue to testing ASCs prior to conducting experiments, it was investigated whether targeted-selected ASCs keep their multipotency characteristics. Roughly 70% of ASCs appeared to be double positive, leaving the remaining 30% only dTomato positive. Characteristic mesenchymal stem cell marker antigens

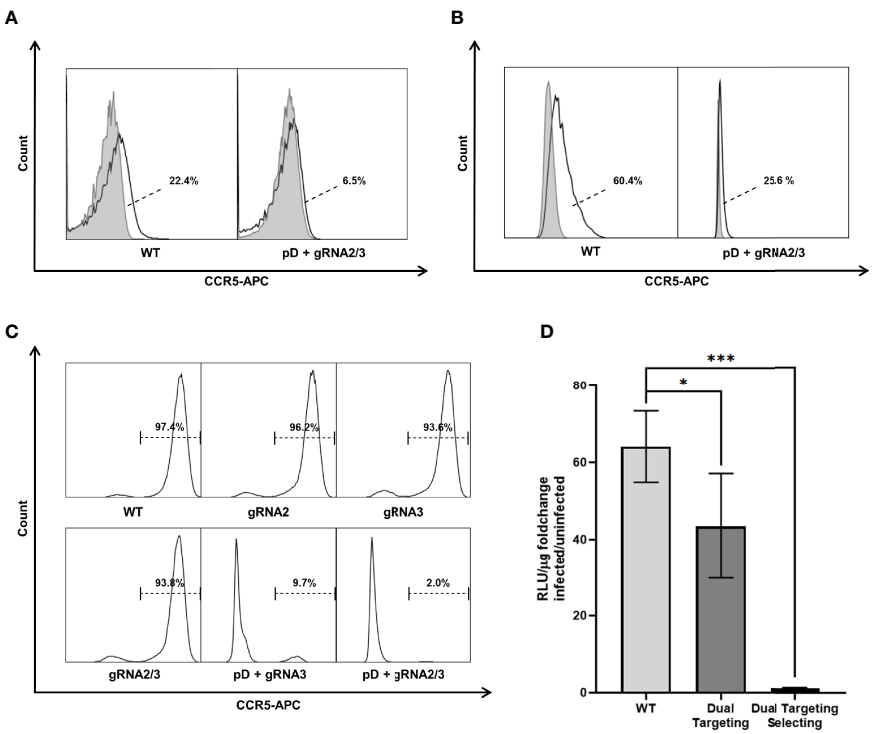


FIGURE 5 | CCR5 surface expression was analyzed by flow cytometry in three different cell types. Untreated (WT) as well as dual targeted-selected (pD+gRNA2/3) populations were stained with an APC labeled Anti-CCR5 Antibody (black lined graph). Unstained negative samples (light gray filled graph) are shown to distinguish the CCR5 positive fraction. **(A)** Jurkat. **(B)** ASC. Fractions are presented next to the Graph. The dashed line points towards the CCR5 positive subset (white area) as calculated by overton subtraction technique. **(C)** TZM-bl includes additional differently targeted populations. Because of the easily distinguishable positive population, only the stained samples are shown. **(D)** HIV-1 infectivity of TZM-bl was measured by Luciferase Assay. Cell lysate was obtained 48h after infection with HIV-1_{BaL}. Uninfected cells were used to measure the background signal. All experiments were carried out in quadruplicates. The results are presented as the fold change in luciferase activity by infection. Error bars show ± SD and significant changes are represented as p-values (*$p < 0.05$, ***$p < 0.0005$).

were displayed on the cell's surface (CD49b, CD105, CD90) (**Figure 6A**). The population was clearly negative for surface CCR5, unlike the WT ASCs which displayed a low but detectable signal when stained for CCR5. Targeted and s elected ASCs were capable of differentiating into adipogenic, osteogenic and chondrogenic lineages (**Figures 6B II-IV**) to the same extent as WT ASCs. Double positive cells consistently expressed EGFP and dTomato throughout differentiation but lost EGFP expression due to fixation and the staining process (**Figure 6B I**).

DISCUSSION

Among many other approaches to create a functional or sterilizing cure for HIV-1, the clinical success in the "Berlin Patient" has made genetically ablating CCR5 a focal point of research in this area (18, 19, 68, 69). New gene editing techniques, in particular CRISPR-Cas9, offer promising possibilities for targeted mutation of the CCR5 gene, but often the mutation rate falls short and not every mutation reliably leads to a functional disruption. Thus, after genetic modification, one obtains a diverse population of cells containing completely disrupted, partially disrupted, or undisrupted cells. Mathematical modeling estimates, for inhibition of viral replication in an individual, the fraction of cells ($CD4^+$ T cells) refractory to infection needs to be above 75-87.5% (56–58). Another study suggests that only 10–20% CCR5 knock- out in $CD34^+$ HSC would maintain $CD4^+$ T cell counts >200 cells/µl after 10 years when the modified cells have a selective advantage (70). To achieve this proportion of modified cells in patients, the generation and transplantation of sufficient amounts of regenerative cells rendered resistant to HIV-1 infection is a prerequisite, especially when there is no previous cytoreduction by ablative chemotherapy. These cells would ideally be autologous, deficient of CCR5, and have complete regenerative potential. Many strategies have been investigated to increase knock- out efficiencies in CCR5 (23, 49, 50, 55, 71, 72). Besides increasing gene editing efficiency, one strategy is to select edited cells prior to transplantation (73). However, stem cell populations such as HSCs, iPSCs, and ASCs, which have been shown to differentiate into CCR5 expressing cells with hematopoietic characteristics, normally show only low to no expression of CCR5 (25–30). Thus, it is not possible to select CCR5 disruption in stem cells based on the absence of the receptor's surface expression, but requires creating the selection based on another phenotype.

In this study we used CRISPR-Cas9 mediated homologous recombination to integrate two different fluorescent markers into the CCR5 gene, functioning as a large frameshift mutation and selectable marker. We hypothesized that this mechanism enables selection of bi allelic frameshift mutated cells based on the genotype which are deficient for CCR5. To completely eliminate CCR5 expression, as in individuals that are homozygous for Δ32, it is expected that both alleles in all cells would need to be edited (55). Instead of increasing the mutation

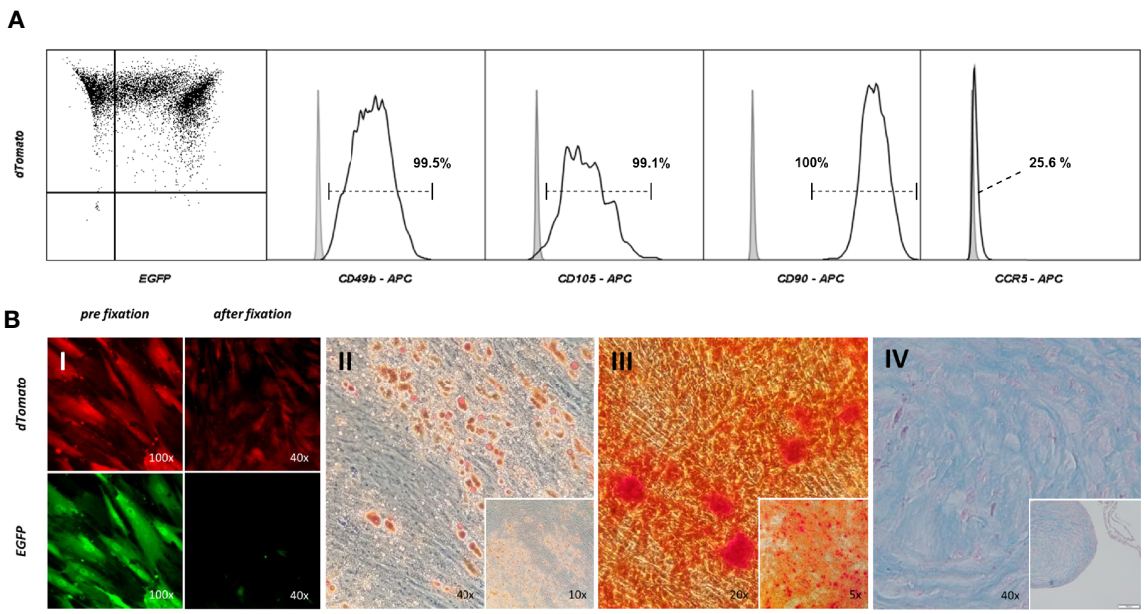


FIGURE 6 | Targeting – selecting ASCs leaves regenerative potential unaltered. **(A)** Immunophenotypic analysis of cell surface profile of double positive ASCs. Cells were stained with fluorescent marker labeled antibodies for CD90, CD49b, CD105 and CCR5. The gray filled graph represents the signal of an unstained control, the black line the population stained with the corresponding antibody. **(B)** Multilineage differentiation Assay. Double positive ASCs were capable of adipo- (II), osteo- (III) and chondrogenic (IV) differentiation. Cells were checked for the correct fluorescent expression pattern. EGFP was washed out during formaldehyde fixation (I).

efficiency or isolating single clones with the desired mutational status, we pursued a strategy enabling a bulk selection of cells with a genomic pattern likely to lead to complete disruption, an approach similar to introducing mono allelic single-nucleotide changes (54, 62). HDR, regardless of zygosity, is known to be a quite inefficient process (42, 53, 74). Therefore, we integrate a strategy of dual targeting to allow for selection of bi allelic mutational events (27, 49, 55). Not all biallelic mutations will be double positive (integration of both fluorescent markers, each on one allele), since 50% of biallelic recombination is expected to be with a single fluorescent marker (either EGFP or mCherry) in both alleles. Sorting for double positive cells ensure that both alleles have been targeted. In order to obtain a sufficient number of successfully edited cells, either a large population or expansion of the autologous regenerative cells would be necessary. Adipose tissue derived stem cells (ASCs) show ideal properties regarding isolation and expansion to support such strategies and represent a potential population for replenishing the immune cell compartment (26, 29, 32, 39). Consequently, we tested applicability in four different cell types, including $CD4^+$ Jurkat T cells and ASCs.

Transfection of Cas9 and gRNA encoding vectors as well as donor plasmids with fluorescent selectable markers lead to integration of the donor and constitutive expression of double-positive fluorescent cells. Coinciding with previous findings (54, 62, 75), we were able to observe 1-2% double positive cells, depending on the cell type and gRNAs used. Performing one sorting step using FACS, we were able to create populations consisting of up to 97.6% constitutively double-positive cells in cell lines and 72.0% in ASCs. Genomic analysis revealed dually targeted and selected double-positive populations carry mutations in 92.3% (± 1.6%; n=4) of all alleles, of which the largest share are disrupted by HDR based on the standard curve. The residual WT CCR5 locus was also significantly reduced. This is a much higher mutational status than what we found or has been previously reported by knock-out studies using single or dual targeting with CRISPR-Cas9 without a selection system (23, 27, 48–51, 72, 76). Coinciding with our previous findings, targeting with two gRNAs displayed higher InDel frequencies than using a single gRNA. For example, single-targeted dual-positive TZM-bls showed a lower mutational frequency than dually targeted cells (78.2% vs. 93.4%). So dual targeting may lead to more thorough DSB formation and homologous recombination than using a single gRNA. Sorting for double-positive cells was shown to select cells the majority of which integrated the PSM (increased VKI) and thus had a disrupted CCR5 gene (decreased DWT). Even though sorting enabled selection of a population heavily disrupted in the CCR5 locus, it did not lead to complete elimination of cells with the wild-type allele. One explanation is the limitation in sorting a population with 100% double-positive cells; however, this cannot be completely responsible for the remaining alleles without integration. In the 12 double-positive clones, genomic DNA analysis shows a DWT signal in 5 of the 12 clones. Although DWT is clearly reduced in these clones compared to the wild type, it indicates at least one CCR5 allele remains unintegrated. Therefore, a certain fraction of double-positive cells do contain CCR5 without a knock-in, and thus the expression of one or both selectable markers does not originate from the CCR5 locus but has been integrated elsewhere. Consequently, double-positive populations cannot be considered as completely biallelic frameshift mutated. However, double positivity is a strong indicator for a high frequency of mutant alleles and disruption of the CCR5 gene. Still, to make precise statements about the extent and properties of bi allelic frameshift mutations in double positive cells, sequencing analysis in a large quantity of clones and/or determining the PSM integration site could be conducted.

More important than achieving complete mutational status was the question of whether targeting and selection is able to create populations deficient for a functional CCR5 receptor and therefore resistant to HIV-1 infection. Natural resistance is conferred by a 32 bp frameshift mutation in CCR5 leading to a premature stop codon after an additional 25 amino acids. Conventional targeting with CRISPR-Cas9 typically induces smaller size InDel mutations that result in minor insertions or deletions, missense, or nonsense mutation not severe enough to prevent expression of a functioning protein. In only a fraction of targeting events are frameshift mutations generated in both CCR5 alleles (55). Previous knock-out studies using conventional CRISPR-Cas9 targeting in TZM-bls were able to reduce the fraction of cells with CCR5 surface expression to as low as 49.2% (48, 50). Using a Lentiviral vector, a 41.2% or 33.3% reduction was possible (50, 72). Single and dual targeting in TZM-bls lead to InDel frequencies between 14.9 and 27.3% as assessed by surveyor assay (**Figure 3B**). However, the reduction in CCR5 surface expression was only as low as 1.2 - 3.8% (**Figure 5C**). This demonstrates that InDels alone do not create mutations severe enough to reliably inhibit CCR5 expression. In contrast, the integration of a large functional sequence including a promoter and terminator into the CCR5 coding sequence can act as a massive frameshift and inhibit proper transcription. We were able to detect a reduction of the measurable surface CCR5 from 97.4% in WT TZM-bl down to 2.0% in double-positive TZM-bls. Therefore in targeted and selected populations the drastic reduction in CCR5 expression correlates with the high frequency of mutation, predominantly caused by HDR. From this it can be concluded that large frameshifts induced by HDR lead to a functional disruption of CCR5 more reliably than it would be the case with InDel mutations alone. Although Jurkat and ASCs express CCR5 at low levels, a similarly significant reduction could be achieved by dual targeting selection in these cell types. Comparable knock-out studies were able to decrease infectability to roughly 40% when using conventional targeting and different transfection techniques in TZM-bls (48, 50). When challenged with HIV-1, double-positive TZM-bls infectability was inhibited 98.1% of WT TZM-bls level, compared to inhibition of 32.7% in the CRISPR-Cas9 dual targeting control. Additionally, the cultivation and modification of stem cells *in vitro*, even if performed carefully, involves a risk of loss in regenerative capacity. When characterized and tested for multilineage differentiation potential, double-positive ASCs showed the same properties as

WT ASCs, suggesting this approach to be successfully applicable in stem cell based therapies. The extent of CCR5 surface expression on MSCs or ASCs in the literature is not clear (26, 29, 77–79), However, we were able to detect slight CCR5 expression on WT ASCs by immunophenotyping, which was eliminated in double-positive ASCs.

Often attempts to create populations with a high rate of a specific mutational pattern are bound to screening and expanding isolated clones. The main benefit associated with the presented strategy is the ability to select highly disrupted cells that are likely to be bi allelic frameshift mutations in a high throughput scale necessary for clinical applications. Although increased, we found the efficiency of HDR-based gene delivery and editing approaches to be a major limitation. We showed that a one-time selection of double-positive transfected cells *via* FACS enriched CCR5 HDR within the population of successfully transfected cells. Implementing alternative gene delivery methods and ways to increase integration could help yield larger quantities of double-positive cells prior to the sorting step. Fluorescent markers like EGFP and dTomato used in this study are beneficial for application in these preclinical proof-of-concept studies; however, they would be less useful in a clinical application creating the need for alternative selectable markers compatible for in-patients use (80). Previous studies have used puromycin selection to provide continuous selection pressure, eliminating cells which were not transfected or l ose the PSM due to plasmid degradation while subculturing (54). A two-drug selection mechanism would also select for bi allelic HDR; or alternatively, a single-drug mechanism would increase the number of edited cells, but not guarantee that both alleles have been targeted. Dual targeting and selection showed consistent outcomes across the tested cell types proving this concept to be reproducible in different scenarios. Our novel approach opens up new therapeutic options to cure patients from HIV-1 infection by using their own pool of regenerative cells. This would not only avoid the risks of lifelong antiretroviral therapy but also those associated with allogeneic transplantation strategies such as myeloablation and the obstacles of HLA matching.

CONCLUSION

Taken together, this study provides proof-of-concept that selection for double-positive cells enriches for the integration of selectable markers into both CCR5 loci. It is thus possible to generate populations highly deficient for CCR5 and resistant to HIV-1 infection, representing an approach to bypass inefficiencies to reliably disrupt the CCR5 gene. The strategy doesn't impair stem cell multilineage differentiation potential, opening up the possibility to be applied in stem cell based therapies. Combined with the application in adipose tissue derived stem cells, this is a novel strategy for the generation of sufficient amounts of HIV-1 resistant autologous regenerative cells. These could partly and repetitively reconstitute the immune system under the selective pressure of an HIV-1 infection and thus represent a possible approach for curing HIV-1.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The collection of all human tissue samples was done with the patient's consent in an anonymized fashion and approved by the Institutional Review Board (IRB) of Tulane University, School of Medicine, New Orleans, Louisiana (IRB protocol #168758).

AUTHOR CONTRIBUTIONS

Conceptualization: SHS, RI, EUA, SEB. Methodology: SHS, DL, RI, EUA, SEB. Investigation: SHS, YR, FMS, KAW, AR. Analysis: SHS, SEB. Writing—Original Draft: SHS, SEB. Writing—Review and Editing: SHS, YR, FMS, KAW, AR, RI, EUA, SEB. Visualization: SHS, SEB. Funding Acquisition: EUA, SEB. Supervision and Administration: SEB. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.821190/full#supplementary-material

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