VIROLOGY

JAK/STAT signaling pathway affects CCR5 expression in human CD4$^+$ T cells

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CCR5 serves as R5-tropic HIV co-receptor. Knocking out CCR5 in HIV patients, which has occurred <10 times, is believed important for cure. JAK/STAT inhibitors tofacitinib and ruxolitinib inhibit CCR5 expression in HIV$^+$ viremic patients. We investigated the association of JAK/STAT signaling pathway with CCR5/CCR2 expression in human primary CD4$^+$ T cells and confirmed its importance. Six of nine JAK/STAT inhibitors that reduced CCR5/CCR2 expression were identified. Inhibitor-treated CD4$^+$ T cells were relatively resistant, specifically to R5-tropic HIV infection. Furthermore, single JAK2, STAT3, STAT5A, and STAT5B knockout and different combinations of JAK/STAT knockout significantly reduced CCR2/CCR5 expression of both RNA and protein levels, indicating that CCR5/CCR2 expression was positively regulated by JAK-STAT pathway in CD4$^+$ T cells. Serum and glucocorticoid-regulated kinase 1 (SGK1) knockout affected CCR2/CCR5 gene expression, suggesting that SGK1 is involved in CCR2/CCR5 regulation. If cell surface CCR5 levels can be specifically and markedly down-regulated without adverse effects, that may have a major impact on the HIV cure agenda.

INTRODUCTION

C-C chemokine receptor type 5 (CCR5), which is a G protein (heterotrimeric GTP-binding protein)–coupled receptor (GPCR), plays a key role in immune and inflammatory responses, and is mainly expressed on T cells, macrophages, and dendritic cells. CCR5 also serves as the major co-receptor for R5-tropic human immunodeficiency virus type 1 (HIV1) (*1*). Homozygous CCR5Δ32 individuals are resistant to HIV-1 infection, and those who are heterozygous exhibit reduced viral loads and a slower progression to acquired immunodeficiency syndrome (AIDS) (*2*, *3*). The CCR2 variant 64I was associated with a protective role against HIV infection and a slower disease progression (*4*). Up to this point in time, five of six HIV$^+$ patients who were cured received donor hematopoietic cells, which had the homozygous Δ32/Δ32 mutation. From the start of the HIV epidemic in the 1980s, there have been >80 million people infected with HIV and only 5 of them received CCR5 knockout (KO) cell transplants and were cured, which is a cure rate of 1 of 16 million. Thus, understanding CCR5 gene regulation is critical for the HIV cure effort.

CCR5 is also expressed on tumor cells and plays an important role in tumorigenesis, e.g., the migration and survival of tumor cells (*5*). Moreover, high CCR5 expression can lead to breast and pancreatic cancers (*6*, *7*). Therefore, studying the regulation of CCR5 may be important for malignant pathology as well as HIV.

CCR5 expression level on CD4$^+$ T cells is important for HIV susceptibility. cAMP (adenosine 3′,5′-monophosphate)–responsive element binding protein 1 (CREB-1) is reported as a transcription factor for CCR5. It binds to the CCR5 cAMP response elements (CREs) region and up-regulates CCR5 gene transcription (*8*). Oct 1 and Oct 2 are also designated as CCR5 transcription factors; they inhibit and enhance CCR5 expression in primary human CD4$^+$ T cells, respectively (*9*). There are other factors that are thought to regulate CCR5 expression, such as NF-AT and GATA-1 (*8*). Lower CCR5 expression levels are critical to control R5-tropic HIV/AIDS. Unfortunately, it is difficult to modulate the levels of these transcription factors to reduce CCR5 expression in humans. Thus, finding an effective way to control CCR5 expression in humans would be critical.

The Janus kinase (JAK)–signal transducer and activator of transcription (STAT) pathway plays an important role in cell growth, differentiation, proliferation, and immune response regulation. JAK/STAT genes are involved in HIV persistence, which is related to viral DNA integration in vivo (*10*) and in T cells (*11*). Tofacitinib and ruxolitinib, two U.S. Food and Drug Administration (FDA)–approved JAK inhibitors, reduce CCR5 expression in HIV viremic subjects (*10*). Thus, the JAK/STAT signaling pathway is associated with HIV infection, integration, and the immune response, but its relationship to CCR5 gene regulation in human primary CD4$^+$ T cells is not well established.

Serum and glucocorticoid-regulated kinase 1 (SGK1), which belongs to the AGC kinase family, is a key enzyme in the stress response, cell proliferation, apoptosis, cell survival, and inflammation (*12*, *13*). It also plays an important role in cancer development and is considered to be a target for cancer therapy (*14*). SGK1 is a host factor associated with virus infection. On the one hand, it promoted influenza A virus (IAV) replication (*15*). On the other hand, it inhibited hepatitis C virus (HCV) infection (*16*). Previous studies showed that SGK1 is a target gene of STAT. We hypothesize here that it may be an intermediate gene between the JAK/STAT pathway and CCR5.

To understand the association between JAK/STAT and CCR5, we chose different JAK/STAT inhibitors; each of them has an effect on different JAK/STAT members. IQDMA, an indoloquinoline derivative, down-regulates STAT5 and leads to apoptosis in leukemic K562 cells (*17*). FLLL32, derived from the natural product curcumin, is a dual inhibitor of JAK2/STAT3. It specifically reduced STAT3 and induced apoptosis in a human melanoma cell line (*18*), and it constitutively down-regulated STAT3 in human rhabdomyosarcoma cell lines (i.e., RH28, RH30, and RD2 cells) (*19*). WP1066 is an inhibitor of JAK2 and STAT3 and also inhibits STAT5, without reducing JAK1 and JAK3 levels. Baricitinib is an FDA-approved JAK1/2 inhibitor for rheumatoid arthritis (*20*, *21*) and is also used to treat COVID-19 patients (*22*). An in vitro study demonstrated that

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baricitinib can reduce HIV latency and block HIV reactivation in latently infected cells (*23*).

JAK/STAT signaling pathway is important for the expression of chemokine receptors, such as CCR2 and CCR5. But there is no direct demonstration that CCR5/CCR2 gene expression is associated with JAK/STAT signaling pathway in human primary $CD4^+$ T cells. Thus, in this study, we investigated the regulatory mechanism by using JAK/STAT inhibitors and CRISPR-Cas9 gene KOs. We report that the JAK/STAT inhibitors tofacitinib, ruxolitinib, baricitinib, IQDMA, FLLL32, and WP1066 inhibit CCR2 and CCR5 expression at both the RNA and protein levels in human primary $CD4^+$ T cells. Furthermore, we provide evidence that individual JAK2, STAT3, STAT5A, and STAT5B gene KOs significantly down-regulate CCR5/CCR2 expression ($P < 0.05$). Combined JAK2/STAT or STAT3/STAT5 gene KO shows more impressive reduction of CCR5/CCR2 expression in human $CD4^+$ T cells. Our data reveal that the gene expression of CCR2 and CCR5 is regulated by the JAK/STAT signaling pathway.

RESULTS

JAK/STAT inhibitors reduce CCR5/CCR2 expression in human primary $CD4^+$ T cells

To investigate the association between the JAK/STAT pathway and CCR5/CCR2 expression, we selected nine JAK/STAT inhibitors (Table 1) to treat human primary $CD4^+$ T cells from five different healthy, nonhospitalized donors. Dimethyl sulfoxide (DMSO)–treated cells served as a negative control. CCR2 and CCR5 expression levels were determined by quantitative reverse transcription polymerase chain reaction (qRT-PCR) (Fig. 1, A to E) and flow cytometry (Fig. 1, F to J). Results of baricitinib-treated $CD4^+$ T cells are shown in fig. S1. Six of the nine inhibitors significantly reduced CCR2 and CCR5 RNA expression levels, compared with the DMSO-treated group ($P < 0.01$). No inhibition was consistently observed when P6, AG490, or perficitinib was used (fig. S2, A to C). Perficitinib showed inhibition in one donor at 10 μM (fig. S2D). Moreover, the observed inhibition was dose dependent. Although the reductions in CCR2 and CCR5 gene expression levels were similar at the low doses used, higher doses of IQDMA, FLLL32, and WP1066 showed greater suppression of CCR5 and CCR2 expression, especially of CCR5, up to 10-fold. Reduced CCR5 and CCR2 surface protein levels were also observed by flow cytometry in all treatment groups.

We then investigated whether the reduction of surface protein expression was due to lower CCR2/CCR5 RNA levels at different inhibitor concentrations. We found a positive correlation between CCR5 protein and RNA expression levels in all treated groups (Pearson correlation coefficient $r > 0.9$; $P < 0.05$; fig. S3). Similar results were observed with CCR2 ($r > 0.8$; $P < 0.08$; fig. S3), suggesting that reductions of both CCR5 and CCR2 RNA levels were responsible for lower cell surface protein expression.

To address the toxic effect of these inhibitors on human $CD4^+$ T cells, we determined cell viability by flow cytometry after 48 hours of treatment, with DMSO alone as a control (fig. S4). WP1066, IQDMA, and FLLL32 compounds decreased cell viability at relatively high concentrations, from ~70% viability to 16%, 10%, and 5%, respectively (fig. S4, C to E). All of the other inhibitors used did not decrease cell viability. To confirm whether the reduced CCR2/CCR5 expression is due to decreased cell viability, both CD4 or CXCR4 cell surface expression levels were examined by flow cytometry, after treatment with these inhibitors. There was no decrease observed in either of those proteins on viable $CD4^+$ T cells, after treatment with 10 μM of each of the inhibitors (fig. S5), indicating that CCR5 and CCR2 reduction was due to JAK/STAT inhibition.

We wished to show that JAK/STAT inhibitor–treated $CD4^+$ T cells were specifically resistant to R5-tropic virus infection. Activated $CD4^+$ T cells were treated with JAK/STAT inhibitors for 48 hours and then infected with replication-defective HIV-cycT1-IRES-eYFP (HIV-CIY) pseudotyped with either R5-tropic Env YU2 or VSV

Table 1. JAK/STAT inhibitors used in this study.

Inhibitor	Target protein	IC_{50}	References
WP1066	JAK2 and STAT3, STAT5, ERK1/2	2.30 μM (HEL cells), 2.43 μM (B16 cells), 5.6 μM (U87-MG), 3.7 μM(U373-MG)	(*43*, *60*, *61*)
IQDMA	STAT5	8 μM (A549 cells), 5 μM (HL-60 cells), 8 μM (K562 cells)	(*62*–*64*)
FLLL32	JAK2/STAT3	1.3 μM (A375 cells)	(*65*)
Tofacitinib	JAK3/2/1	Cell-free assays: 1 nM (JAK3), 20 nM (JAK2), 112 nM (JAK1)	(*66*)
Ruxolitinib	JAK1/2	Cell-free assays: 3.3 nM (JAK2), 2.8 nM (JAK1)	(*27*)
Pyridone 6	JAK1,2,3	Cell-free assays: 1 nM (JAK2), 5 nM (JAK3), 15 nM (JAK1)	(*67*)
AG490	STAT3, JAK2/3, STAT5	25 μM (human T cells)	(*68*)
Baricitinib	JAK1/2	Cell-free assays: 5.9 nM (JAK1), 5.1 nM (JAK2)	(*69*)
Peficitinib	JAK1,2,3 and Tyk2	3.9 nM (JAK1), 5.0 nM (JAK2), 0.7 nM (JAK3)	(*70*)

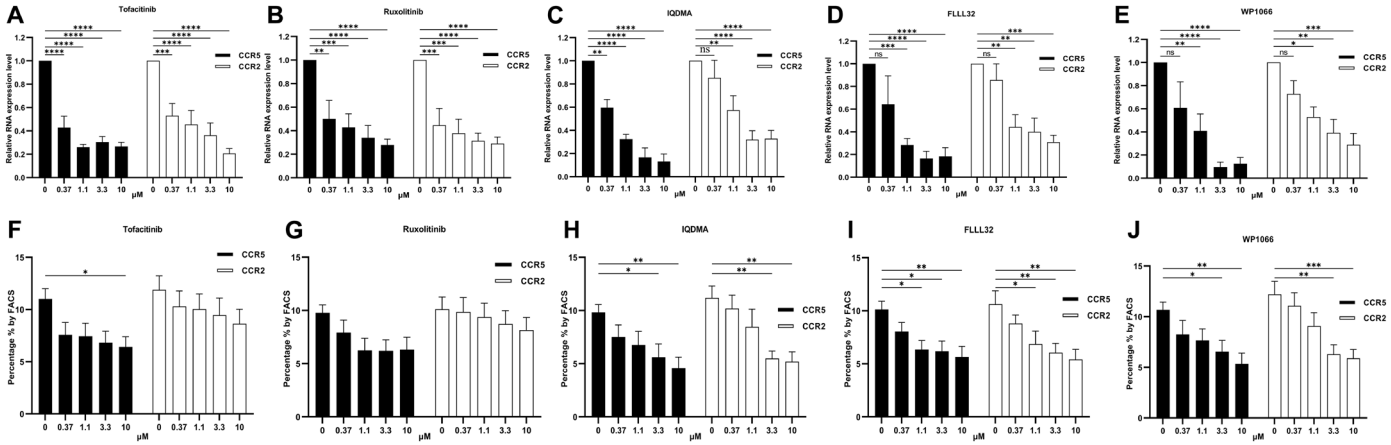

Fig. 1. JAK/STAT inhibitors down-regulate CCR5/CCR2 expression in human CD4$^+$ T cells. Primary CD4$^+$ T cells were isolated and stimulated with anti-CD3/CD28 in the presence of IL-2. Cells were treated with 0, 0.37, 1.1, 3.3, or 10 μM of JAK inhibitors (tofacitinib, ruxolitinib, FLLL32, IQDMA, and WP1066). After 48 hours, CCR2 and CCR5 expression levels were quantified by qRT-PCR [(**A** to **E**); results are normalized by GAPDH] and flow cytometry (**F** to **J**) ($N = 5$). The concentration of 0.0 μM represents DMSO, which was the control. Black rectangles reflect CCR5 levels; white ones are CCR2. Error bars represent SEM, and statistical significance was determined by two-way ANOVA followed by Dunnett's multiple comparison test: ns, not significant; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$.

G. Cells were refed the next day and analyzed for enhanced yellow fluorescent protein (eYFP) expression by flow cytometry after 72 hours. We observed significant reduction in infection of HIV pseudotyped with R5-tropic Env in cells pretreated with the JAK/STAT inhibitors compared to mock or negative control ($P < 0.05$), and no change was observed for VSV G–pseudotyped HIV-CIY infection of CD4$^+$ T cells (fig. S6).

KO of single JAK/STAT member down-regulates CCR2/CCR5 expression

To confirm that CCR5 and CCR2 regulation in CD4$^+$ T cells is driven by the JAK/STAT signaling pathway, we performed CRISPR-Cas9 gene KO in human primary CD4$^+$ T cells. Individual single-guide RNAs (sgRNAs) were designed for each target gene. Activated CD4$^+$ T cells from four healthy donors were separately electroporated with Cas9-GFP (green fluorescent protein) and sgRNA RNP complex. RNA and protein expression levels of CCR2, CCR5, and the KO'd gene in sorted GFP$^+$ cells were determined at day 7 by qRT-PCR (Fig. 2, A to D) and flow cytometry (Fig. 2, E to H), respectively. The jak2 and stat3 RNA expression levels were reduced by 70% after KO ($P < 0.0001$), while stat5A and stat5B RNA expression levels were around 90%, or 10-fold, decreased ($P < 0.0001$). At the protein level, phosphorylated STAT3 (pSTAT3) and Jak2 were reduced by 97%, or 33-fold, whereas phosphorylated STAT5 (pSTAT5) decreased approximately 90%, or 10-fold. This establishes that the CRISPR-sgRNA gene KO works very well.

In the STAT3 KO CD4$^+$ T cells, the RNA levels of ccr2 and ccr5 were significantly decreased, approximately 13- and 2-fold, respectively ($P < 0.0001$; Fig. 2A). A similar reduction was observed in STAT5A, STAT5B, and JAK2 KO cells (Fig. 2, B to D). At the protein level, CCR5 was more than 10-fold reduced in KO groups, except in the STAT5A KO group, in which it was 5-fold reduced; similar results were seen for CCR2. This is consistent with ccr2 RNA expression being very dependent on the JAK/STAT signaling pathway. In all, single JAK/STAT member KO significantly down-regulated CCR2 and CCR5 expression at both RNA and protein levels. Thus, CCR2 and CCR5 are under the regulation of JAK/STAT signaling pathway.

Paired JAK/STAT gene KO impressively reduced CCR2/CCR5 expression

We next determined whether coupled JAK and STAT or combined STAT KO was associated with a greater reduction of CCR2 and CCR5 gene expression and to identify which combination plays a more important role for CCR5 regulation. We performed JAK/STAT double KO using Cas9-GFP and Cas9-RFP (red fluorescent protein), using the related sgRNA. The GFP and RFP double-positive population was then sorted, and the KO'd gene expression levels of CCR2 and CCR5 were quantified by qRT-PCR (Fig. 3, A to E) and flow cytometry (Fig. 3, F to J). All JAK and STAT expression in KO'd cells was greater than 75% reduced at the RNA level, except for stat3 in STAT3/STAT5 KO cells, which was ~60% decreased, whereas protein levels were 90% lower in all KO'd compared to non-KO'd CD4$^+$ T cells, demonstrating that the paired gene KO efficiency is excellent.

We next quantified the reduction of ccr2 and ccr5 RNA expression in KO cells (Fig. 3). Results showed that both ccr2 and ccr5 were very significantly down-regulated in all KO samples (for ccr2, 50- to 100-fold; ccr5, 5- to 10-fold; $P < 0.0001$; Fig. 3, A to E); ccr2 is more sensitive to JAK/STAT KO than ccr5. Although ccr2 and ccr5 expression is different in diverse paired KO cells, we cannot conclude which coupled signaling pathway plays the key role in ccr5 and/or ccr2 gene regulation since the JAK and STAT off-target effects may be different. We also analyzed the changes in CCR2 and CCR5 protein levels after KO (Fig. 3, F and G) and saw that CCR5 was significantly decreased ($P \leq 0.0002$ in all groups). The degree of reduction in JAK2/STAT3, JAK2/STAT5A, JAK2/STAT5B, STAT3/STAT5A and STAT3/STAT5B paired KO cells was 31-, 11-, 18-, 20-, and 11-fold, respectively. CCR2 was also significantly reduced ($P < 0.05$ in all KO groups) and decreased 18-, 7-, 9-, 9-, and 6-fold,

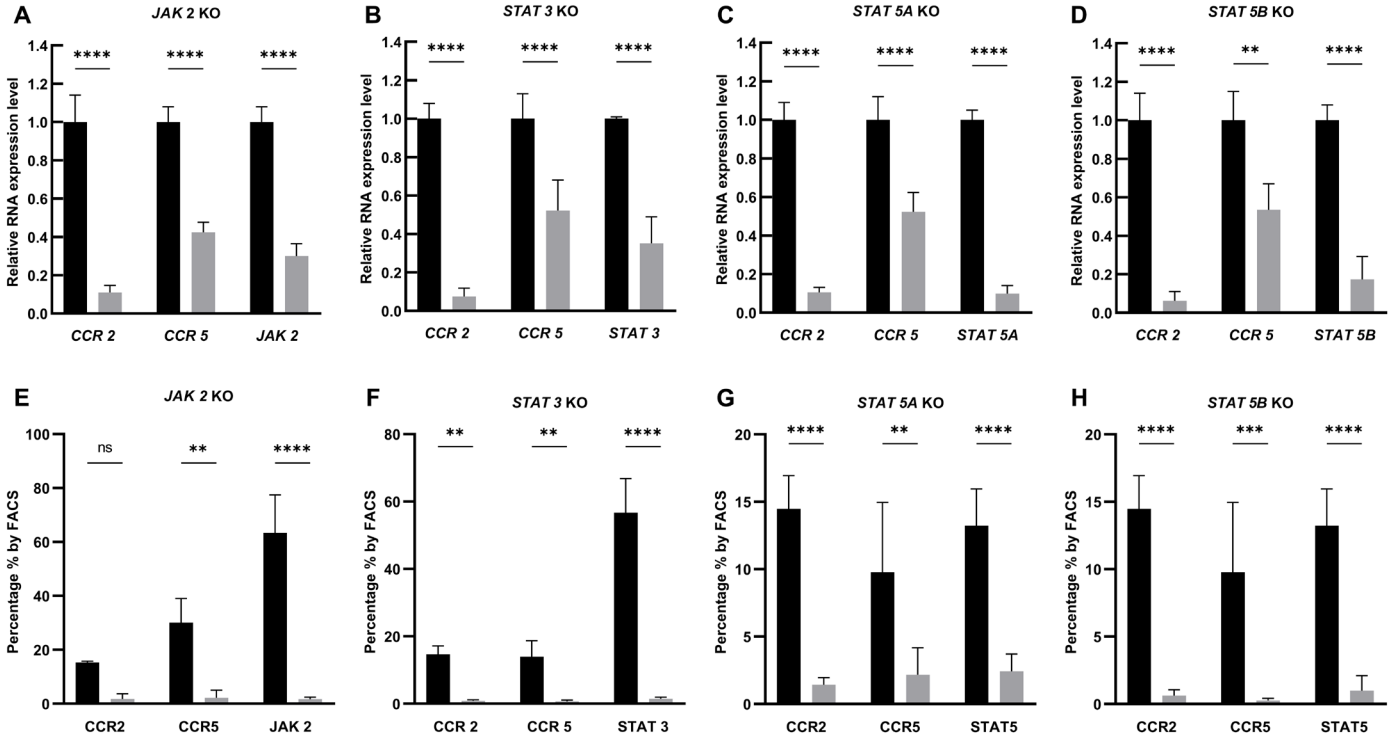

Fig. 2. Single JAK/STAT member KO reduces CCR5/CCR2 expression in primary CD4$^+$ T cells. Activated CD4$^+$ T cells were transfected with Cas9-GFP and gRNA RNPs. GFP$^+$ cell population was positively sorted after 24 hours. Sorted cells were cultured for another 6 days. On day 7, cells were harvested for RNA quantification and staining for flow cytometry. Decreased ccr5/ccr2 RNA levels in activated primary CD4$^+$ T cells after JAK2 (**A**), STAT3 (**B**), STAT5A (**C**), and STAT5B (**D**) gene KO. Mean values from various donors ± SEM ($N = 4$) are shown. Results are normalized using GAPDH. Reduced CCR5/CCR2 cell surface expression level after JAK2 (**E**), STAT3 (**F**), STAT5A (**G**), and STAT5B (**H**) gene KO. Black rectangles are controls, and gray ones are KO. Statistical significance was determined by two-way ANOVA followed by Bonferroni's multiple comparison test: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$.

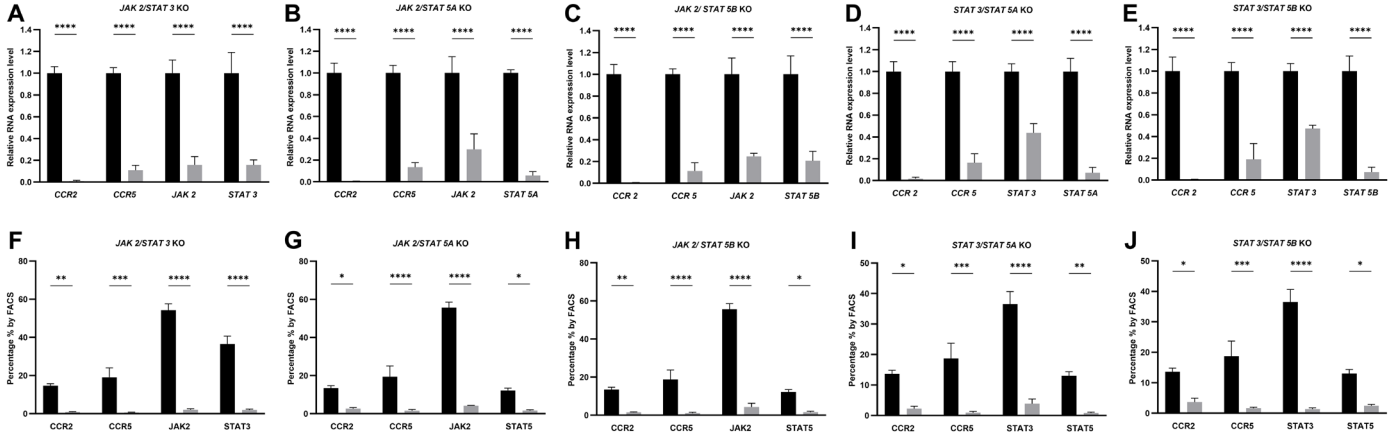

Fig. 3. Combined JAK/STAT member KO reduced CCR5/CCR2 gene expression in primary CD4$^+$ T cells. Anti-CD3/CD28 costimulated CD4$^+$ T cells from four different donors were cotransfected with Cas9-GFP/gRNA1 and Cas9-RFP/gRNA2 complex mix. GFP and RFP double-positive cells were sorted after 24 hours. On day 7, cells were harvested for qRT-PCR [(**A**): JAK2/STAT3, (**B**): JAK2/STAT5A, (**C**): JAK2/STAT5B, (**D**): STAT3/STAT5A, (**E**): STAT3/STAT5B; $N = 3$; results are normalized using GAPDH] or stained for flow cytometry [(**F**): JAK2/STAT3, (**G**): JAK2/STAT5A, (**H**): JAK2/STAT5B, (**I**): STAT3/STAT5A, (**J**): STAT3/STAT5B; $N = 4$]. Black rectangles are controls, and gray ones are KO. Statistical significance was determined by two-way ANOVA followed by Bonferroni's multiple comparison test: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$.

respectively, in the paired KO groups. In some donors, the CCR5 expression was almost completely suppressed, which is consistent with JAK2 and pSTAT both playing very important regulatory roles in CCR5 gene expression.

SGK1 is involved in the regulation of CCR5 and CCR2 gene expression

From the STAT3 and STAT5A transcription factor chromatin immunoprecipitation sequencing (ChIP-seq) datasets (the ENCODE Transcription Factor Targets dataset) and STAT5B interacting protein dataset (the Pathway Commons Protein-Protein Interactions dataset), it is known that CCR5 is one of the target genes for all the three STATs and only STAT3 targets CCR2. Previous investigation showed that there is a STAT binding site in CCR5 promoter region (*24*), but investigation of STAT5A and STAT5B target genes in human T cells observed that STAT5A and STAT5B are not directly binding to either the CCR2 or CCR5 gene (*25*–*27*). Thus, after discovering the clear relationship between JAKs, STATs, and ccr2/ccr5 expression, we attempted to identify possible intermediary genes. The common target genes that have been reported are SGK1, Foxp3, UGCG, and SLC. SGK1 is an important immunomodulation regulator (*28*) and was identified as a regulator of STAT3. It is also important for HIV replication (*29*) and $CD4^+$ T cell and macrophage polarization (*30*, *31*). Thus, we chose to examine SGK1, which has been reported as a potential target gene of STAT3, STAT5A, and STAT5B. We performed CRISPR-Cas9 sgRNA KO of SGK1 and then measured both RNA and protein expression levels of CCR2 and CCR5 (Fig. 4A). We observed that CCR5 and CCR2 RNA levels were both significantly reduced ($P < 0.05$), although SGK1 was only 40% KO'd. Similar results were observed for both protein levels ($P < 0.001$; Fig. 4B). SGK1 is thus involved in the regulation of CCR5 and CCR2; whether it mediates CCR5/CCR2 regulation by JAK/STAT requires further study.

DISCUSSION

CCR5 is the major gene target for the handful of R5-tropic HIV cures in humans (*32*). We and others reported that HIV Elite controllers and Viremic controllers (ECs/VCs) had lower CCR5 expression levels (*33*, *34*). Tofacitinib and ruxolitinib modulate CCR5 but not CXCR4 in HIV viremic donors (*10*), which means that JAK/STAT is involved in CCR5 but not CXCR4 regulation in an HIV viremic patient. CCR2 is not implicated in HIV transmission (*4*), but it is important for HIV infection and AIDS progression (*35*). Here, we demonstrate that the JAK/STAT signaling pathway is directly associated with CCR5 and CCR2 regulation, at both RNA and cell surface protein levels, in human primary $CD4^+$ T cells.

We used JAK and STAT small-molecule inhibitors to screen JAK/STAT members that may regulate CCR5 expression in primary $CD4^+$ T cells. Tofacitinib and ruxolitinib, which are JAK3/2/1 and JAK1/2 inhibitors, respectively, have been studied as potent medications to inhibit HIV in human lymphocyte and macrophages in vitro (*36*). Haile *et al.* (*37*) reported that ruxolitinib showed a dose-dependent reduction in CCR5 expression in primary human macrophages. These studies have shown that JAKs regulate CCR5 expression in primary human macrophages, but no investigations have been performed in primary human $CD4^+$ T cells, which play a key role in HIV transmission and propagation. Here, we report that these two JAK inhibitors significantly inhibited CCR5 expression at the RNA level at a low amount ($P < 0.01$), and protein level at higher concentrations ($P < 0.05$). Baricitinib has been reported to inhibit HIV replication in primary human macrophages and PBMCs at submicromolar level, and it also blocked HIV reactivation in J-Lat T cells and primary human macrophages (*38*). Here, we found that baricitinib also suppressed CCR5/CCR2 expression. Small molecules FLLL32, IQDMA, and WP1066 have been described as JAK/STAT inhibitors in various cancer cell lines (*39*–*43*); no work has been performed using primary $CD4^+$ T cells. Here, we report that these three inhibitors significantly inhibit CCR5/CCR2 expression in primary human $CD4^+$ T cells ($P < 0.01$) at a low concentration.

The "Geneva patient" has been HIV-free for 20 months, off antiretroviral medications after hematopoietic stem cell transplantation (*44*, *45*). The donor had wild-type CCR5, and the patient continued to take ruxolitinib to reduce immunosuppressant use and prevent graft-versus-host disease (GVHD). It is not known whether this "HIV cure" is because ruxolitinib reduced CCR5 expression levels and decreased HIV propagation in $CD4^+$ T cells since the patient's CCR5 levels have not been carefully studied. With respect to the current data, ruxolitinib downregulated CCR5 expression, and recently, Claireaux *et al.* (*34*) reported that HIV-specific $CD4^+$ T cells had lower CCR5 expression in an HIV^+ elite controller, with decreased T cell

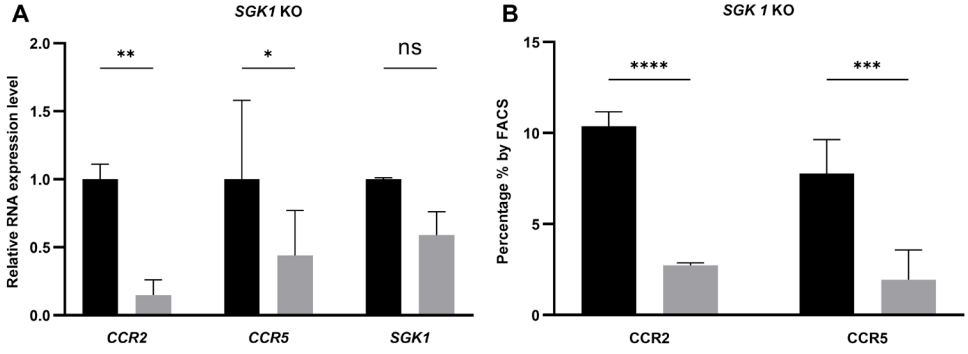

Fig. 4. SGK1 KO decreased CCR2/CCR5 expression in primary $CD4^+$ T cells. CD3/CD28 costimulated $CD4^+$ T cells from four donors were transfected with Cas9-GFP/gRNA against SGK1. CCR2/CCR5 RNA expression levels [(**A**); results are normalized using GAPDH] and protein levels (**B**) were quantified ($N = 4$). Black rectangles are control, and gray ones are KO. Statistical significance was determined by two-way ANOVA followed by Bonferroni's multiple comparison test: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, and $****P < 0.0001$.

susceptibility of R5-tropic HIV entry and reduced virus propagation. Our previous study reported that HIV$^+$ elite and viremic controllers have lower CCR5 and CCR2 expression at both the RNA and protein levels in primary CD4$^+$ T cells (*33*). Maraviroc, which is FDA-approved, binds to CCR5 and prevents the binding of HIV Env to CCR5 (*46*, *47*). Unfortunately, as HIV Env evolves, it can mutate and recognize maraviroc bound to CCR5 and then enter cells (*48*). Here, we showed that JAK/STAT inhibitors reduced CCR5 expression and the treated CD4$^+$ T cells and then became relatively resistant to R5-tropic pseudotyped, single-cycle HIV infection. This is an expected result since it has been known for several decades that if CCR5 expression is reduced or absent, R5-tropic virus cannot enter cells. CCR5 down-regulation is thus important for natural R5-tropic HIV control in some individuals.

To further study CCR5/CCR2 regulation by the JAK/STAT signaling pathway, we used CRISPR/Cas9-gRNA gene editing to KO target genes. Since Cas9 was fused to eGFP, eGFP$^+$ cells were sorted to accurately quantify gene KO. Although all sorted cells had been transfected, the editing efficiency varied because of possible CRISPR off-target effects (*49*). Here, the target gene expression was reduced after KO, but off-target effects and mutations were not evaluated. Single and combination KO of JAK/STAT members significantly reduced the expression of CCR2/CCR5 ($P < 0.01$). On the basis of gene KO efficiency and CCR2/CCR5 reduction levels, JAK2 and STAT3 are more important than other family members for CCR5 transcription. In 2001, Mellado *et al.* (*50*) identified JAK1 (but not JAK2 or JAK3) to be associated with CCR5 expression, while CCR2 promoted JAK2 activation in human embryonic kidney (HEK)–293 cells transfected with CCR5 and CCR2, respectively. Here, we uncovered the importance of JAK2 in regulating CCR5 expression in primary human CD4$^+$ T cells.

SGK1 KO notably reduced CCR5/CCR2 expression. SGK1 is a potential therapeutic target of chronic GVHD and cancers. In the "Geneva patient" case, the patient took ruxolitinib, which is FDA approved and is also an inhibitor for JAKs. Here, we found that both ruxolitinib and SGK1 affect CCR2/CCR5 expression. We hypothesize that SGK1 mediated CCR2/CCR5 regulation by JAK/STAT, but it was not further investigated. Future work will probe the role of SGK1 in this regulatory pathway.

Although the CCR5 and JAK/STAT relationship has been reported in a number of studies involving HIV and cancer (*10*, *37*, *51*, *52*), this study is important because there has been no investigation of the JAK/STAT pathway in regulating CCR5 gene expression in human primary CD4$^+$ T cells, a major cellular target of HIV in humans. In addition, no study has reported that SGK1 is involved in CCR2/CCR5 expression.

JAK/STAT signaling pathway regulates CCR5 expression and function, thereby affecting CCR5-mediated signaling. On the other hand, CCR5 signaling can activate the JAK/STAT signaling pathway. It does so by mediating the cell binding of CCL5 and other chemokines such as CCL3, RANTES, and MIP-1 (*53*–*55*), activating JAK kinases, and then phosphorylating and activating STAT proteins. This interaction may involve complex signaling cross-regulation and regulatory mechanisms in primary human CD4$^+$ T cells. Certainly, if cell surface CCR5 levels can be specifically and markedly down-regulated in HIV$^+$ individuals without adverse effects, that could have a major impact on the cure agenda.

MATERIALS AND METHODS

Inhibitors

P6, AG490, peficitinib, tofacitinib, ruxolitinib, FLLL32, IQDMA, baricitinib, and WP1066 were obtained from MedChemExpress (CA, USA; Table 1). All inhibitors were dissolved in an appropriate volume of DMSO to make a 10 mM stock concentration. These stocks were then diluted 1000-fold in complete RPMI 1640 to prepare 10 μM working solutions of each. Those solutions were then diluted threefold serially in RPMI 1640 to prepare 3.3, 1.1, and 0.37 μM working solutions.

Peripheral blood mononuclear cell collection and CD4$^+$ T cell isolation

Deidentified human peripheral blood mononuclear cell (PBMC) samples were obtained from the New York Blood Center. PBMCs were isolated using SepMate Tube combined with Lymphoprep (STEMCELL Technologies, Vancouver, BC, Canada) according to protocol. CD4$^+$ T cells were sorted by negative selection, using a EasySep Human CD4+ T Cell Isolation Kit (STEMCELL, Vancouver, BC, Canada) (*56*). Expression of cell surface CD4 on the isolated T cells was confirmed by flow cytometry via cell surface staining using phycoerythrin (PE)–conjugated anti-human CD3 antibody (clone OKT3; eBioscience, San Diego, CA) and fluorescein isothiocyanate (FITC)–conjugated anti-human CD4 antibody (clone SK3; BioLegend, San Diego, CA). Briefly, purified T cells were resuspended in staining buffer [phosphate-buffered saline (PBS) with 2% fetal bovine serum (FBS)] in the presence of antibody on ice for 30 min, washed with 2 mM EDTA in PBS, and then fixed on ice for 20 min using IC fixation buffer (eBioscience). Fixed cells were resuspended in staining buffer and analyzed by flow cytometry (LSRII, BD, Franklin Lakes, NJ) and FlowJo software (version 10.9, Ashland, OR). The percentage of CD4$^+$ T cells was >93% after purification.

Primary CD4$^+$ T cell stimulation, transfection, and intracellular staining

Purified CD4$^+$ T cells were stimulated for 72 hours using anti-CD3 antibody (1 μg/ml; clone OKT3, BioLegend) on precoated plates, in the presence of anti-CD28 (2 μg/ml; clone 28.2, BioLegend) and recombinant interleukin-2 (rIL-2) (100 IU/ml; STEMCELL), and then cultivated in complete medium (RPMI 1640 with 10% FBS), supplemented with rIL-2 (100 IU/ml). CD4$^+$ T cells were transfected using the Neon Electroporation Transfection System according to protocol. sgRNA, which was synthesized by Integrated DNA Technologies (IDT) (Table 2), and Cas9-GFP (or RFP) (IDT) were mixed (1:1.2 ratio) and incubated at room temperature for 20 min to prepare the ribonucleoprotein (RNP) complex. Activated CD4$^+$ T cells were harvested, washed with PBS, and resuspended in T buffer (from Neon Transfection System, Invitrogen). Cell suspension (0.01 ml; equivalent to 5×10^5 cells) was gently mixed with sgRNA-Cas9-GFP RNP complexes and electroporated at 2100 V per 10 ms per three pulses. Cells were immediately transferred into 0.5 ml of prewarmed 10% FBS in RPMI 1640 medium and incubated overnight at 37°C in 5% CO_2. The next day, GFP$^+$ or GFP$^+$/RFP$^+$ cells were positively sorted using FACSAria (BD Biosciences) or Bigfoot (Thermo Fisher Scientific) and cultivated for another 6 days before further analysis (*57*, *58*).

Transfected CD4$^+$ T cells were collected, washed, and stained for surface markers in the dark, followed by fixation and permeabilization.

Table 2. sgRNA sequences used for human gene knockout.

Target gene	sgRNA sequence	Location
JAK2	GTAACTATCCATAAGCAAGA	Exon 8, chr9:5055756-5055775
STAT3	GAGACCGAGGTGTATCACCA	Exon15, chr17:42326138-42326157
STAT5A	ACATTCTGTACAATGAACAG	Exon 4, chr17:42292018-42292037
STAT5B	TAAGAGGTCAGACCGTCGTG	Exon 11, chr17:42217249-42217268
SGK1	TTCAAGATGGACTGAACTTC	Exon 5, chr6:134174057-134174076

Assessment of CCR2 and CCR5 cell surface levels was conducted according to previously published procedures (*33*). After permeabilization, cells were intracellularly stained for JAK and/or STAT expression with anti-JAK and/or anti-STAT antibodies for 1 hour in the dark at 4°C, followed by IC fixation buffer fix for 20 min at 4°C in the dark, according to previously described procedures (*10*, *59*). The following antibodies were used in this study: anti-CD195–PE (CCR5; clone HEK/1/85a; BioLegend, San Diego, CA) or CD195-APC (allophycocyanin) (clone J418F1; BioLegend), anti-CD192–APC or BV605 (CCR2; clone K036C2; BioLegend), anti-JAK2–Alexa Fluor 647 polyclonal (Thermo Fisher Scientific), anti-pSTAT3–Alexa Fluor 647 or PE/CY7 (clone A160898, Thermo Fisher Scientific), and anti-pSTAT5–PE-Cy7 (clone A17016B.Rec, Thermo Fisher Scientific), anti-CD4–APC (clone: RPA-T4, BioLegend), and anti-human CXCR4–PE (clone:12C5, BioLegend). A viability dye (VIVID, LIVE/DEAD kit, Invitrogen) or DAPI (4′,6-diamidino-2-phenylindole) was added to the antibody cocktail to exclude dead cells. Acquisition of cells was conducted by LSRII flow cytometry using FACSDiva software. The data were analyzed using FlowJo version 10.9.0 software (Tree Star, Ashland, OR).

Inhibitor treatment, RNA extraction, and qRT-PCR

Activated $CD4^+$ T cells were treated with serial diluted inhibitors (0.00, 0.37, 1.10, 3.33, or 10.00 μM) for 48 hours. Cells were then collected and divided into two parts: RNA was extracted from one aliquot using RNeasy Kit (Qiagen), and the other aliquot was stained to assess CCR2 and CCR5 surface expression levels.

For qRT-PCR, gene expression levels were normalized using glyceraldehyde-3-phosphate dehydrogenase (GAPDH). All qRT-PCR assays were run using Bio-Rad CFX 96 Real Time System using Superscript III Platinum SYBR Green One-Step qRT-PCR Kit (Invitrogen). Gene expression data were analyzed by the $2^{-\Delta\Delta Ct}$ method. Results were analyzed by GraphPad Prism 9 (GraphPad Software Inc.). All qRT-PCR primers used are listed in table S1.

Single-cycle HIV production and T cell transduction

293T cells at ~70% confluence were transfected with plasmids HIV-cycT1-IRES-YFP (HIV-CIY) and either pSRα-YU2 Env or pME-VSV G (pan-tropic control) using the calcium phosphate method, and the pseudotyped lentiviral particles were collected after ~72 hours. Virus transduction efficiency was measured using as targets GHOST-HI5 cells (HOS cells stably expressing both CD4 and CCR5) by flow cytometry 72 hours after infection. The same International Units (IU) of pseudotyped HIV was then used to transduce inhibitor-treated $CD4^+$ T cells, and the percentage of $eYFP^+$ cells was determined by flow cytometry after 72 hours.

Statistics

Statistical significance was determined by two-way analysis of variance (ANOVA) followed by Dunnett's or Bonferroni's multiple comparison test for inhibitor-treated samples or determined by similar, followed by Bonferroni multiple comparison posttest. $P < 0.05$ was set as the threshold for statistical significance. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$. Error bars represent SD.

Supplementary Materials

This PDF file includes:

Table S1
Figs. S1 to S6

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