scientific reports

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Assembly of CCR5 with gp120 inhibits the HIV infectivity specifically in T cells

Joyce Appiah-Kubi[1,2], Yuan Yuzhe[3], Takeo Kuwata[4], Hiromi Terasawa[1], Perpetual Nyame[1], Wright Ofotsu Amesimeku[1], Md. Jakir Hossain[1], Shuzo Matsushita[4], Tomohiro Sawa[1], Yosuke Maeda[1,5], Shinji Harada[6], Keisuke Yusa[3] & Kazuaki Monde[1,7]✉

For the entry of R5 human immunodeficiency virus type 1 (HIV-1) into target cells, CCR5 functions as a coreceptor following the binding of the envelope glycoprotein gp120 to the receptor CD4. While CD4 is known to be strongly downregulated after infection, the fate of CCR5 post-infection remains unclear. We investigated the surface expression of CCR5 on PM1/CCR5 cells following infection with HIV-1_{JR-FL}. Flow cytometry using anti-CCR5 MAb (T21/8 and 2D7) revealed that CCR5 was not downregulated on the surface of infected cells. Notably, CCR5 was found to be coassembled with HIV-1 Gag at the viral budding sites. A specific subset of CCR5, designated as $CCR5^A$, which is recognized by T21/8 but not 2D7, was significantly colocalized with gp120 on the cell surface. Virions incorporating CCR5 were immunoprecipitated using T21/8 MAb but not with 2D7 Mab, indicating that $CCR5^A$ was incorporated into progeny virions. The efficiency of $CCR5^A$ incorporation into virions was positively correlated with the level of CCR5 expression on the cell surface. Moreover, incorporation efficiency was approximately 83-fold higher in $CD4^+$ T cells than in adherent $CCR5^+$ cells. Furthermore, the incorporation of $CCR5^A$ into HIV-1_{JR-FL} virions resulted in a 25% decrease in viral infectivity. These findings suggest that $CCR5^A$ may have a detrimental effect during the late stage of the HIV-1 replication cycle.

Keywords HIV-1, Assembly, CCR5 incorporation, CCR5 epitope mask

C–C chemokine receptor type 5 (CCR5) is a member of the G-protein-coupled receptor (GPCR) superfamily and is expressed on memory T lymphocytes and macrophage lineages[1]. CCR5 functions as a coreceptor in conjunction with CD4, facilitating the entry of R5 human immunodeficiency virus type 1 (HIV-1) into a target cell[2–4]. In contrast, X4-tropic HIV-1 strains utilize CXCR4 (CD184) as a coreceptor, which is more broadly expressed than other chemokine receptors[5]. Following HIV-1 entry, the CD4 receptor on the cell surface is robustly downregulated by viral accessory proteins such as Nef, Vpu, or Env[6–10]. Nef promotes CD4 internalization and directs it toward lysosomal degradation[11–16]while Env and Vpu inhibit the trafficking of newly synthesized CD4 to the plasma membrane[17,18]. Retention of CD4 on the cell surface impairs HIV-1 replication by trapping and aggregating nascent virions due to the high affinity of gp120 for CD4[19]. This retention also reduces viral infectivity by limiting the recruitment of gp120 or gp120–CD4 complexes to the budding virion surface[7,9]. Therefore, efficient downregulation of CD4 is crucial for HIV-1 replication in infected cells.

Similarly to CD4, CCR5 is also downregulated during HIV-1 infection, although the downregulation is only partial and not as pronounced as that of CD4[20,22]. This modest decrease in CCR5 expression is thought to protect infected cells from HIV-1 superinfection[21]. These observations were based on detection using a 2D7 monoclonal antibody (MAb), which recognizes an epitope located within the second extracellular loop of CCR5, specifically around residues Lys^{171}-Glu^{172} [23]. In previous work, we reported that CCR5 is incorporated into the progeny viral envelope from the infected cell[24]. However, 2D7 MAb could not detect CCR5 on virion, whereas another

[1]Department of Microbiology, Faculty of Life Sciences, Kumamoto University, Kumamoto 860-8556, Japan. [2]Department of Virology, Noguchi Memorial Institute for Medical Research, University of Ghana, Accra, Ghana. [3]Graduate School of Science, Technology and Innovation, Kobe University, Hyogo 650-0047, Japan. [4]Collaborative Research Program with the Chemo-Sero-Therapeutic Research Institute for Anti-viral Agents and Hematological Diseases, Joint Research Center for Human Retrovirus Infection, Kumamoto 860-0811, Japan. [5]Department of Nursing, Kibi International University, Takahashi 716-8508, Japan. [6]Department of Medical Virology, Faculty of Life Sciences, Kumamoto University, Kumamoto 860-8556, Japan. [7]Collaboration Unit for Infection, Joint Research Center for Human Retrovirus Infection, Kumamoto University, Kumamoto 860-0811, Japan. ✉email: monde@kumamoto-u.ac.jp

antibody, T21/8 MAb, successfully captured CCR5-containing virion in a virus precipitation assay. T21/8 MAb recognizes the N-terminus region of CCR5 [25], suggesting that CCR5 may adopt an alternative conformation - termed $CCR5^{A}$ -, in which the second extracellular loop is masked, while the N-terminus remains accessible. It remains unclear whether the observed downregulation of CCR5 in infected cells reflects the actual loss of the protein or a conformational change to $CCR5^{A}$ that renders the 2D7 epitope inaccessible. In this study, we demonstrate that a portion of cell surface CCR5 exists in the $CCR5^{A}$ form and is incorporated into progeny the viral infectivity. Thus, CCR5 has a negative effect on HIV-1 replication at the late stage of infection.

Results

Expression of both $CCR5^{A}$ and $CCR5^{N}$ is not downregulated in HIV-1-infected PM1/CCR5 cells

In this study, we designated the alternative conformational form of CCR5 as $CCR5^{A}$, which is recognized by the T21/8 MAb but not recognized by 2D7 (Fig. 1A). In contrast, the normal form of CCR5, referred to as $CCR5^{N}$, is recognized by both T21/8 and 2D7 (Fig. 1A). To assess potential functional differences between $CCR5^{A}$ and $CCR5^{N}$, we treated cells with the natural ligand of CCR5, CCL5 (RANTES) (Fig. 1B and C). Binding of RANTES induced marked internalization of both $CCR5^{A}$ and $CCR5^{N}$. This result suggests that $CCR5^{A}$ retains the ability to undergo ligand-induced internalization or that $CCR5^{A}$ represents a minor population of CCR5 on the cell surface of uninfected cells. To determine whether surface CCR5 expression is downregulated by HIV-1 infection, we compared CCR5 expression levels between p24 Gag-positive and -negative PM1/CCR5 cells (Fig. 1D and E). Cells were infected with HIV-1_{JR-FL}, HIV-$1_{JR-FLDNef}$, HIV-1_{NL4-3}, or HIV-$1_{NL4-3DNef}$. Flow cytometry using T21/8 and 2D7 MAbs showed no significant difference in CCR5 expression between p24 Gag-positive and -negative populations, indicating that CCR5 is not downregulated upon infection. Although a previous study reported that HIV-1_{SF2} Nef can downregulate CCR5 surface expression[26]our data showed that HIV-1_{JR-FL} and HIV-1_{NL4-3} Nef do not have this effect (Fig. 1D and E). To further validate these findings, we infected peripheral blood mononuclear cells (PBMCs) with HIV-1_{JR-FL} and HIV-$1_{JR-FLDNef}$ (Fig. 1F). In cells infected with HIV-1_{JR-FL}, CCR5 expression appeared to be elevated, likely because HIV-1_{JR-FL} preferentially targets cells with high CCR5 expression. However, a similar increase in CCR5 expression was also observed in cells infected with HIV-1_{NL4-3} (data not shown), and no effect of Nef deficiency was detected (Fig. 1F). These results indicate that CCR5 expression is not downregulated by HIV-1_{JR-FL} infection. Notably, the amount of CCR5 recognized by the T21/8 antibody was significantly increased following infection (Fig. 1E and F). Collectively, these results suggest that Nef-mediated CCR5 downregulation is strain-dependent and that the $CCR5^{A}$ isoform may be selectively expressed or preferentially stabilized on the cell surface following infection.

$CCR5^{A}$ coassembled with HIV-1_{JR-FL} Env at the budding platform in infected PM1/CCR5 cells

In HIV-1_{JR-FL}-infected PM1/CCR5 cells, Gag was found to colocalize with Env at the plasma membrane (Fig. 2A and B). CCR5 also coassembled with HIV-1 Gag at the viral assembly sites (Fig. 2C and D). We previously reported that the V3 domain of HIV-1 gp120 plays a role in the incorporation of CCR5 into viral particles[24]. Based on this, we examined the distribution of CCR5 relative to Env in HIV-1-infected PM1/CCR5 and MAGIC5 cells. MAGIC5 cells are HeLa-derived adherent cells that express CD4 and CCR5[27]. In PM1/CCR5 cells, CCR5 showed strong colocalization with HIV-1_{JR-FL} Env at the Gag-enriched regions of the plasma membrane but not with HIV-1_{NL4-3} Env (Fig. 3A, B and E). Notably, $CCR5^{A}$, as detected by T21/8 MAb, colocalized more significantly with HIV-1_{JR-FL} Env than CCR5 detected by the 2D7 MAb (Fig. 3A and E). These results suggest that while both $CCR5^{A}$ and $CCR5^{N}$ may localize at the budding platform, $CCR5^{A}$ is recruited more efficiently. Currently, no antibody is commercially available that specifically distinguishes $CCR5^{A}$ from $CCR5^{N}$. Therefore, direct evidence of $CCR5^{A}$ localization at the virus budding site remains elusive. However, our previously findings demonstrated that only $CCR5^{A}$, but not $CCR5^{N}$, is incorporated into the progeny virions[24]. This strongly suggests that the CCR5 colocalizing with HIV-1_{JR-FL} Env is predominantly $CCR5^{A}$. In contrast, in MAGIC5 cells, there were no significant differences in CCR5 colocalization at the virus budding platform between HIV-1_{JR-FL} and HIV-1_{NL4-3}-infected cells (Figs. 2E and F and 3C and D, and 3E). Altogether, these results indicate that $CCR5^{A}$ preferentially coassembles with HIV-1_{JR-FL} Env in the $CD4^{+}$ T cell-specific manner.

Env enhances the incorporation of CCR5 into virions

The incorporation of host membrane proteins into HIV-1 particles can be assessed by immunoprecipitation using specific antibodies[28,29]. As a control, we first confirmed that HLA-DR was incorporated into HIV-1 particles derived from PM1 or PM1/CCR5 cells. Virions bearing HLA-DR on their envelope were successfully precipitated with anti-HLA-DR antibodies, and the captured particles were quantified by p24 ELISA (Fig. 4A and B). Next, CCR5 incorporation was assessed using the T21/8 and 2D7 MAbs. HIV-1 particles produced from PM1 or PM1/CCR5 cells were precipitated with high efficiency (73% or 84%, respectively) by T21/8. In contrast, 2D7 MAb could not capture CCR5-positive viruses, indicating that $CCR5^{A}$ but not $CCR5^{N}$ was incorporated into virus particles, consistent with our previous findings[24]. To investigate the role of Env in $CCR5^{A}$ incorporation, we compared virions produced with or without Env expression. HIV-$1_{JR-FLΔEnv}$ particles showed reduced immunoprecipitation efficiency with T21/8, dropping from 77 to 48% (Fig. 4B). This result indicates that Env enhances the incorporation of $CCR5^{A}$ into viruses. A similar trend was observed in virus preparations from PM1/CCR5 cells (Fig. 4C). Interestingly, the presence of Env had the opposite effect on HLA-DR, reducing its incorporation into viruses (Fig. 4B and C).

We then examined whether $CCR5^{A}$ incorporation varies depending on viral coreceptor usage (Fig. 4D and E). Using R5-, R5/X4-, and X4-tropic HIV-1 strains, we measured T21/8-mediated capture of virions produced from PM1 and PM/CCR5 cells. $CCR5^{A}$ incorporation into HIV-1_{JR-FL} was comparable to that observed with other R5 strains, such as HIV-1_{Ba-L} or HIV-1_{YU-2}. Dual-tropic HIV-$1_{89.6}$ and X4-tropic HIV-1_{NL4-3} and HIV-1_{IIIB} were also captured by T21/8, albeit with lower efficiency. In all cases, virions produced from PM1/CCR5

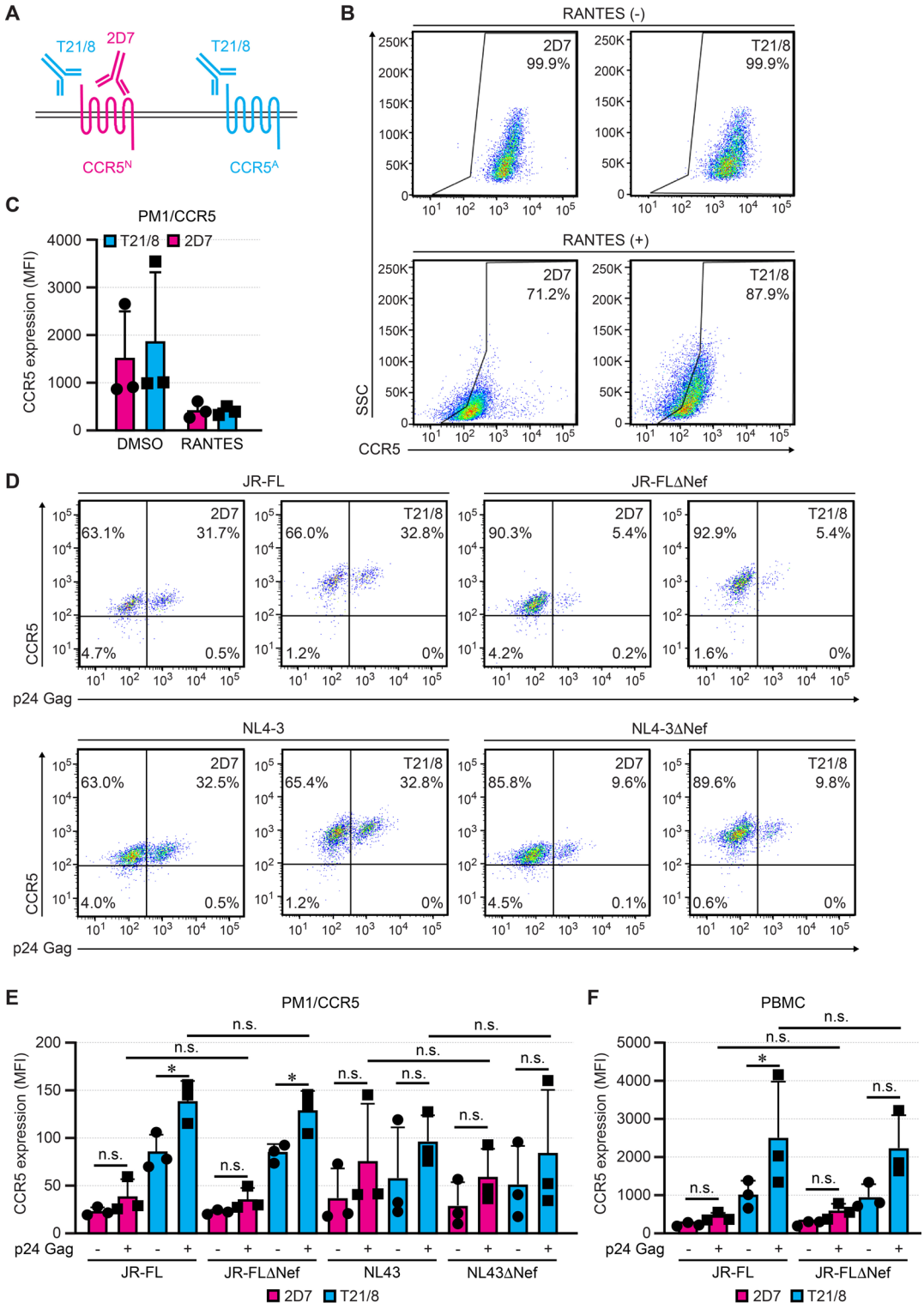


Fig. 1. CCR5 expression is not down-regulated by HIV-1 infection. (**A**) T21/8 MAb recognizes $CCR5^A$ but not 2D7 MAb. (**B** and **C**) The number of CCR5-positive population (**B**) and the MFI of CCR5 (**C**) in RANTES-treated PM1/CCR5 cells were analyzed by T21/8 or 2D7 MAb. (**D**) Cell surface expression of CCR5 in HIV-1_{JR-FL}, HIV-$1_{JR-FLDNef}$, HIV-1_{NL4-3}, or HIV-$1_{NL4-3DNef}$ -infected PM1/CCR5 cells was analyzed by T21/8 or 2D7 MAb. (E and F) The MFI of CCR5 in the p24-negative and positive cell population was measured in PM1/CCR5 cells (**E**) and PBMC (**F**). For statistically significant analysis, the data from three independent experiments are shown as mean ± standard deviations. The P values of the results were determined by the student's t-test. *, $P < 0.01$; n.s., not significant.

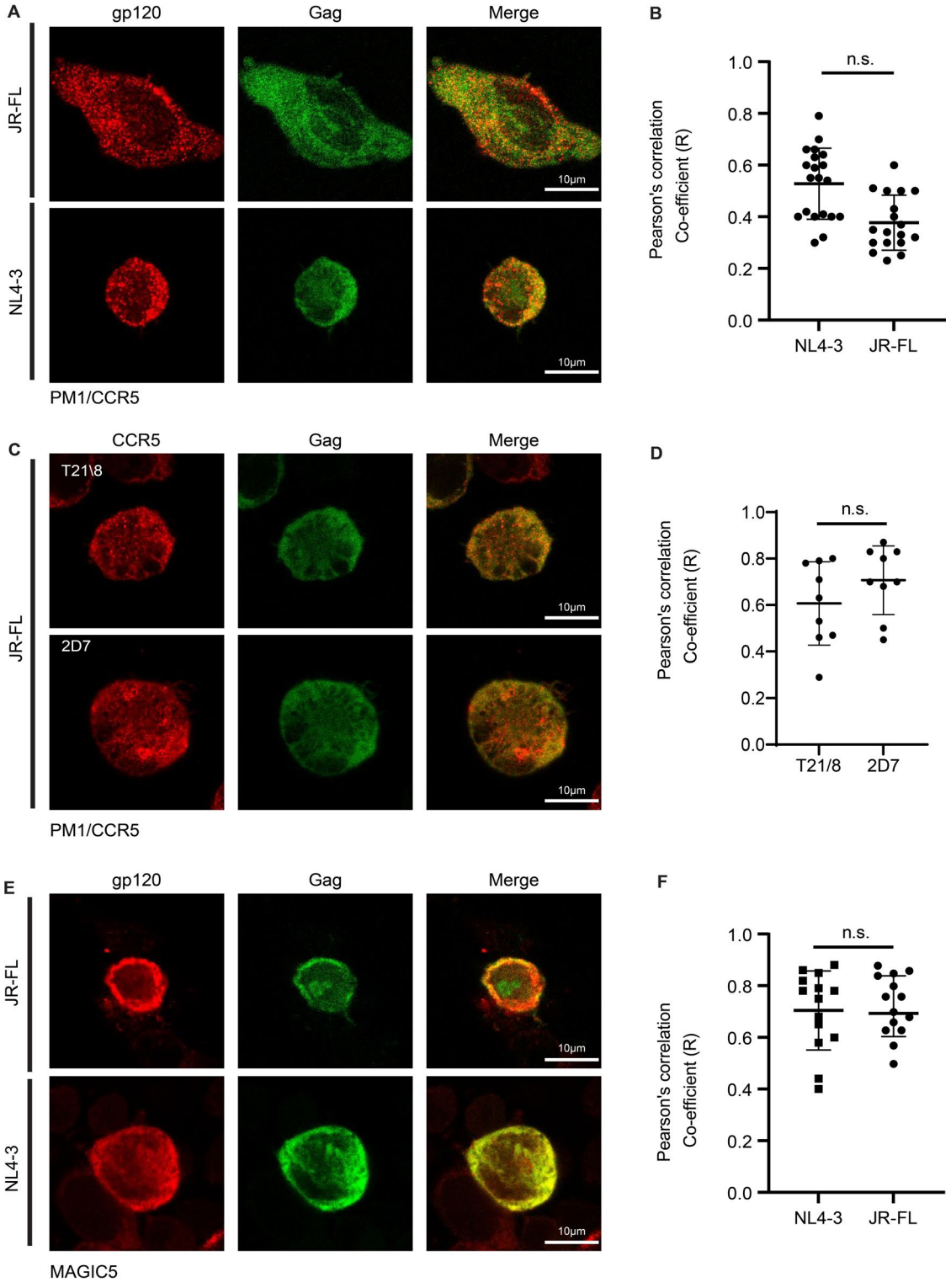


Fig. 2. CCR5 colocalized with HIV-1 Gag at the plasma membrane. Colocalization of Gag with gp120 in PM1/CCR5 cells (**A**) and MAGIC5 cells (**E**) infected with HIV-1_{JR-FL} or HIV-1_{NL4-3} was observed by confocal microscopy. (**C**) Colocalization of Gag with CCR5 in PM1/CCR5 cells infected with HIV-1_{JR-FL} was observed by confocal microscopy. (**B**, **D**, and **F**) Error bars show the means ± standard deviation of repeated tests. The correlation (r) between the MFI of Gag and gp120 (B and F), CCR5, and Gag (**D**) was calculated using Pearson's correlation test using ZEN software. Each dot denotes an individual cell, $n > 15$. *, $p < 0.05$; *n.s.*, not significant. Significant differences were calculated by one-way ANOVA.

cells were captured more efficiently than those from PM1 cells, indicating that CCR5 surface levels influence $CCR5^A$ incorporation.

$CCR5^A$ incorporation into virions depends on CCR5 expression levels in various $CD4^+$ T cell lines

Next, we examined the incorporation of CCR5 into HIV-1 particles produced from various $CD4^+$ T cells exhibiting different surface expression levels of CCR5. Jurkat cells, which naturally express very low levels of CCR5 (mean fluorescence intensity [MFI] < 10; Fig. 5A), produced virions that were captured by T21/8 at an efficiency of 22%, but not by 2D7 (Fig. 5B). Similarly, viruses derived from MOLT-4/CCR5 cells (MFI < 10) were captured by T21/8 at 33%, while virions from PM1, PM1/CCR5, Jurkat/CCR5, or CEM/CCR5 cells (MFI > 10) were precipitated with efficiencies ranging from 65 to 88%. These results demonstrate a positive correlation between CCR5 expression levels on producer cells and the efficiency of virion capture by T21/8 MAb. Notably, viral particles produced from Jurkat/CCR5 cells, which expressed high levels of CCR5 (MFI > 400), were also exceptionally captured by 2D7, with capture efficiencies of 91% for T21/8 and 72% for 2D7. These findings suggest that $CCR5^A$ is preferentially incorporated into virions at the budding sites. However, when CCR5 expression is extremely high, $CCR5^N$ may also be incorporated into virions, likely due to its increased surface availability during viral assembly.

$CCR5^A$ incorporation into virions produced by $CCR5^+$ adherent cells is inefficient

To investigate whether CCR5A incorporation into HIV-1 virions is cell-type dependence, we examined virus particles generated from various $CCR5^+$ adherent cell lines (Fig. 5C). Virions produced by HeLa/CCR5, MAGI/CCR5, and MAGIC5 cells (MFI < 100) were barely captured by the T21/8 MAb, indicating minimal CCR5A incorporation (Fig. 5D). By contrast, 293T/CCR5, GHOST/CCR5, and NP2/CCR5 cells expressed higher levels of CCR5 than the HeLa-derived $CCR5^+$ cell lines. Among these, virions from GHOST/CCR5 cells were captured by T21/8 at 28%. $CCR5^A$ incorporation was detectable in viruses produced from 293T/CCR5 and NP2/CCR5 cells with high CCR5 surface expression (MFI > 400). However, none of the virions from $CCR5^+$ adherent cells were captured by the 2D7 MAb, indicating the absence of $CCR5^N$ incorporation. To compare CCR5A incorporation efficiency quantitatively, we plotted the CCR5 MFI values against the CCR5 incorporation index for both $CD4^+$ T cells and $CCR5^+$ adherent cells (Fig. 5E). The two groups showed clearly distinct incorporation profiles. A non-linear regression model was applied using the equation $y = 1 - e^{a(x-b)}$ to estimate the 50% incorporation point. The best-fit curve for $CD4^+$ T cells was $y = 1 - e^{-1.31(x-0.35)}$, while that for $CCR5^+$ adherent cells was $y = 1 - e^{-0.44(x-1.20)}$. These curves yielded 50% incorporation thresholds of MFI of $10^{0.88}$ for $CD4^+$ T cells and $10^{2.8}$ for $CCR5^+$ adherent cells. This difference corresponds to an 83-fold higher incorporation efficiency in CD4 + T cells compared to $CCR5^+$ adherent cells. Importantly, the data suggest a distinct threshold behavior: $CCR5^A$ incorporation into virions from CD4 + T cells required CCR5 expression exceeding MFI $\geq 10^{0.36}$, whereas, in adherent cells, incorporation was only detectable when MFI exceeded $\geq 10^{1.2}$. These findings strongly suggest that the mechanism of $CCR5^A$ incorporation in $CD4^+$ T cells differs fundamentally from that in adherent cells.

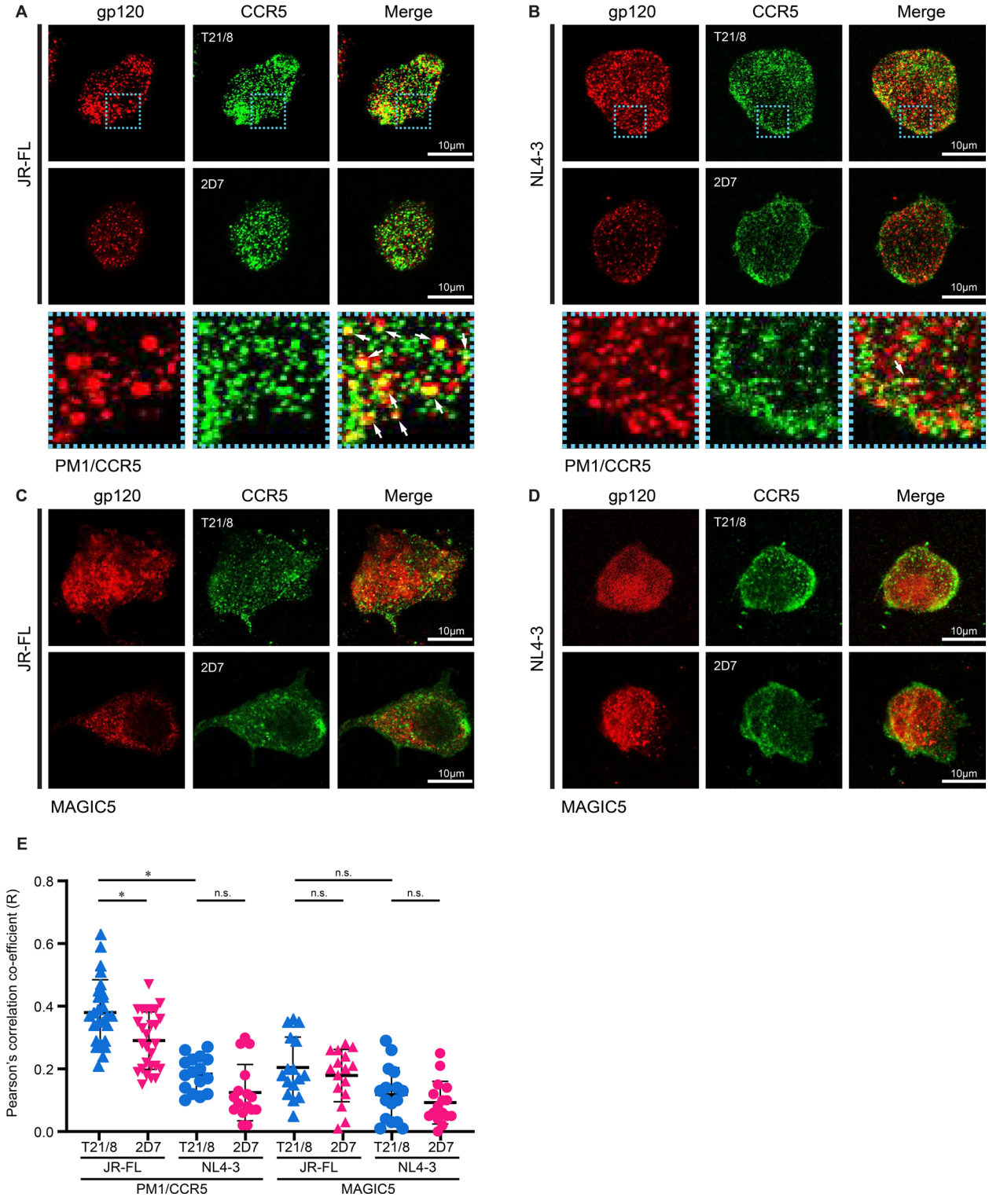
Given that macrophages are a physiologically relevant CCR5 + adherent cell type, we further examined $CCR5^A$ incorporation into virions produced by PBMCs and monocyte-derived macrophages (Fig. 5F). PBMCs from three healthy donors ($n = 3$) were infected and analyzed. Virions released from PBMCs were captured by T21/8 at 24–25% efficiency but not by 2D7, indicating the presence of $CCR5^A$ but not $CCR5^N$. In contrast, virions produced by macrophages were not captured by either antibody, suggesting that $CCR5^A$ is not incorporated into progeny virions in macrophages (Fig. 5F).

$CCR5^A$ incorporation reduces viral infectivity

Finally, we investigated whether the incorporated $CCR5^A$ into virions affects viral infectivity. A fraction of HIV-1_{JR-FL} produced from PM1 cells incorporates $CCR5^A$ on the viral envelope. To examine its effect, $CCR5^A$-positive virions were selectively removed from the virus population by immunoprecipitation using the T21/8 MAb (Fig. 6A). The remaining virions, which lacked surface $CCR5^A$, were then analyzed for infectivity (Fig. 6A). We compared the infectivity of T21/8-depleted viruses to those treated with control antibodies (anti-p24 or 2D7) using viruses produced from either 293 T or PM1 cells. As expected, no significant difference in infectivity was observed between T21/8- and 2D7-treated virus preparations derived from 293 T cells, which lack CCR5 expression (Fig. 6B). In contrast, the infectivity of HIV-1_{JR-FL} produced by PM1 cells significantly increased by approximately 25% following the removal of $CCR5^A$-positive virions via T21/8 immunoprecipitation. No such increase was observed in samples treated with 2D7, which does not recognize $CCR5^A$, and the infectivity was comparable to that of viruses treated with anti-p24 antibody (Fig. 6B). We were unable to analyze virions from PM1/CCR5 cells using T21/8 because nearly all virions were precipitated from the supernatant due to the high levels of $CCR5^A$ incorporation (data not shown). These results indicate that the presence of $CCR5^A$ on virions negatively impacts HIV-1_{JR-FL} infectivity. Taken together, our data suggest that $CCR5^A$ exerts an inhibitory effect during the late stage of the HIV-1 replication cycle.

Discussion

This study provides a comprehensive characterization of CCR5 behavior from the early to late stages of HIV-1 replication, as summarized in Fig. 7. (i) Constitutive expression and viral entry: $CCR5^A$ appears to be constitutively expressed on the surface of $CD4^+$ T cells. Upon initial binding of HIV-1 to cell-surface CD4, a conformational change in gp120 facilitates subsequent interaction with either $CCR5^N$ or both $CCR5^N$ and $CCR5^A$. This interaction enables membrane fusion and the entry of the viral core into the cytoplasm. (ii) Post-entry Regulation: At the post-entry step, CCR5 is not significantly downregulated on the surface of cells infected with either HIV-1_{JR-FL} or HIV-1_{NL4-3}. (iii) Assemble at the budding platform: During virion assembly, CCR5 accumulates at



the budding platform. HIV-1 Env either selectively interacts with $CCR5^{A}$ or induces a conformational shift in $CCR5^{N}$, converting it to $CCR5^{A}$. Although specific detection of $CCR5^{A}$ at the budding platform is limited due to the lack of a $CCR5^{A}$-specific antibody, the dominant incorporation of $CCR5^{A}$ suggests that it aggregates at the budding site. (iv) Preferential incorporation into virions. $CCR5^{A}$ is preferentially incorporated into budding virions. $CCR5^{N}$ can also be incorporated into virions, but only when CCR5 surface expression is exceptionally high, as seen in Jurkat/CCR5 cells (MFI > 400; see Fig. 5). Env enhances this incorporation, with efficiency being approximately 83-fold higher in $CD4^{+}$ T cells than in $CCR5^{+}$ adherent cells. Additionally, $CCR5^{A}$ incorporation occurs in both R5- and X4-tropic viruses, although it is more strongly promoted by R5 HIV-1 Env (Fig. 4). (v) Impact on infectivity: The incorporation of $CCR5^{A}$ into the viral envelope reduces HIV-1_{JR-FL} infectivity by approximately 25% in viruses released from PM1 cells.

◀**Fig. 3**. CCR5^{A} predominantly colocalizes with HIV-1$_{JR-FL}$ Env. Colocalization of Env with CCR5 in PM1/CCR5 cells infected with HIV-1$_{JR-FL}$ (**A**) or HIV-1$_{NL4-3}$ (**B**) was observed by confocal microscopy. Colocalization of Env with CCR5 in MAGIC5 cells infected with HIV-1$_{JR-FL}$ (**C**) or HIV-1$_{NL4-3}$ (**D**) was observed by confocal microscopy. CCR5 is stained using T21/8 MAb or 2D7 MAb. The bar indicates 10 μm. (**E**) Error bars show the means ± standard deviation of repeated tests. The correlation (r) between the MFI of CCR5 and gp120 in PM1/CCR5 and MAGIC5 was calculated by Pearson's correlation test using ZEN software. Each dot denotes an individual cell, $n > 15$. *, $p < 0.05$; *n.s.*, not significant. Significant differences were calculated by one-way ANOVA. (**F**) Colocalization of gp120 and Gag in MAGIC5 cells infected with HIV-1$_{JR-FL}$ or HIV-1$_{NL4-3}$ was observed by confocal microscopy. The bar indicates 10 μm. (**G**) Error bars show the means ± standard deviation of repeated tests. The correlation (r) between the MFI of gp120 and Gag in MAGIC5 was calculated by Pearson's correlation test using ZEN software. Each dot denotes an individual cell, $n > 15$. *, $p < 0.05$; *n.s.*, not significant. Significant differences were calculated by one-way ANOVA.

Prior to infection, the R5 HIV-1 receptors CD4 and CCR5 are co-localized within lipid rafts, which are specialized lipid microdomains, on the surface of the CD4^{+} T cell line, such as the PM1 cell line[30]. Following viral entry, which is mediated by interactions between Env, CD4, and CCR5, CD4 is strictly downregulated by viral proteins, including gp120, Vpu, and Nef[6,10,17,18]due to its detrimental impact on the infectivity of progeny virions[7,31]. Additionally, partial downregulation of CCR5 has been reported as a mechanism to prevent superinfection, likely through competition for the small residual amounts of CD4 remaining on the cell surface[21]. However, in our study, we observed no reduction in surface CCR5 expression on infected CD4^{+} T cells. This apparent discrepancy may be explained by strain-specific differences among HIV-1 isolates. For example, sequence variation in the Nef protein affects its functional activities, such as HLA class I downregulation, which is known to differ by strain[32]. Therefore, it is possible that Nef from HIV-1$_{JR-FL}$ and HIV-1$_{NL4-3}$ lacks the ability to downregulate CCR5 expression.

In this study, we did not observe substantial down-regulation of CCR5 on the surface of PM1/CCR5 cells and PBMCs when analyzed using T21/8 and 2D7 MAbs (Fig. 1). Notably, the MFI of CCR5 detected by T21/8 MAb was consistently higher than that detected by 2D7 MAb (Fig. 1D). Assuming that the detection sensitivity of both antibodies is equivalent, this suggests that a greater proportion of CCR5 is recognized by T21/8 than 2D7. Since T21/8 recognizes both CCR5^{N} and the alternative form CCR5^{A}, whereas 2D7 recognizes only CCR5^{N}, this finding implies that CCR5^{A} is constitutively expressed on CD4^{+} T cells. We further observed that treatment with the natural ligand RANTES reduced CCR5 surface levels similarly when measured by either 2D7 or T21/8, indicating that CCR5A likely retains its functionality as a chemokine receptor. Importantly, the epitope recognized by 2D7 has been mapped to the second extracellular loop of CCR5, specifically between residues Lys^{171}-Glu^{172} [23]. The 2D7 MAb has been shown to efficiently block the infectivity of various M-tropic and dual-tropic HIV-1 strains in vitro[33]suggesting that CCR5^{A}, which lacks the 2D7 epitope, may not function effectively as a co-receptor for viral entry. Further studies examining CCR5^{A} expression and its role in HIV-1 Env binding in CD4^{+} T cells will be important to clarify the mechanistic relevance of this alternative CCR5 form.

CCR5^{A} was colocalized with Env at the virus-budding sites, and R5-tropic HIV-1 Env more efficiently promoted the incorporation of CCR5^{A} into viral particles, suggesting a possible interaction between Env and CCR5^{A}. Moreover, endogenous levels of CCR5^{A} in PM1 cells exerted a modest inhibitory effect on the late stage of HIV-1$_{JR-FL}$ replication. This suggests that CCR5^{A} may interfere with receptor engagement during viral attachment or disrupt the conformational changes in gp120 required for membrane fusion. However, the inhibitory effect was moderate rather than lethal. As a result, HIV-1 may not have been subjected to strong evolutionary pressure to downregulate CCR5 expression, unlike the strict downregulation of CD4 by the viral proteins Nef, Vpu, and Env[6–10].

Numerous host-derived molecules have been reported to be incorporated into HIV-1 particles[34–42]. For example, P-selectin glycoprotein ligand-1 (PSGL-1), has been shown to be incorporated into virions, but its presence on the viral surface inhibits infectivity[43–45]. In contrast, the incorporation of ICAM-1 enhances HIV-1 attachment to the target cells[46,47]. In this study, we found that the antibody reactivity against CCR5 incorporated into virions was markedly altered. If this phenomenon is not unique to CCR5, and similar changes in antibody reactivity occur for other incorporated host proteins, it is possible that additional, previously undetected host molecules are also incorporated into virions. This suggests that when analyzing target molecules incorporated into virions via methods such as viral immunocapture, fluorescence microscopy, or flow virometry, it may be necessary to use multiple antibodies targeting different epitopes to ensure accurate detection.

Interestingly, CCR5 has been reported to form an intracellular oligomer[48]. The loss of the 2D7 epitope in CCR5^{A} may be associated with CCR5 oligomerization on the cell surface. We previously reported that HIV-1 preferentially utilizes monomeric CCR5 for entry, suggesting that CCR5 oligomerization reduces viral entry efficiency[49]. Based on this report, it is conceivable that, following entry, HIV-1 may induce the conversion of monomeric CCR5 into an oligomeric form to prevent superinfection, thereby optimizing the production of progeny virions. Notably, in conditions of CCR5 overexpression, such as in Jurkat/CCR5, CCR5^{N} may be coassembled with CCR5^{A} and incorporated into virions. Whether oligomerized CCR5 leads to conformational changes that result in the formation of distinct CCR5 isoforms, and whether such forms are selectively incorporated into the virions, remains to be determined.

In our previous study[49]we also demonstrated that maraviroc (MVC), a clinically approved CCR5 antagonist, induces the CCR5 oligomerization. If oligomerized CCR5 is indeed incorporated into virions, MVC might exert a dual inhibitory effect on HIV-1 replication: by promoting the incorporation of less functional CCR5^{A} into budding virions, and by blocking CCR5-mediated viral entry into target cells. Further investigation into the

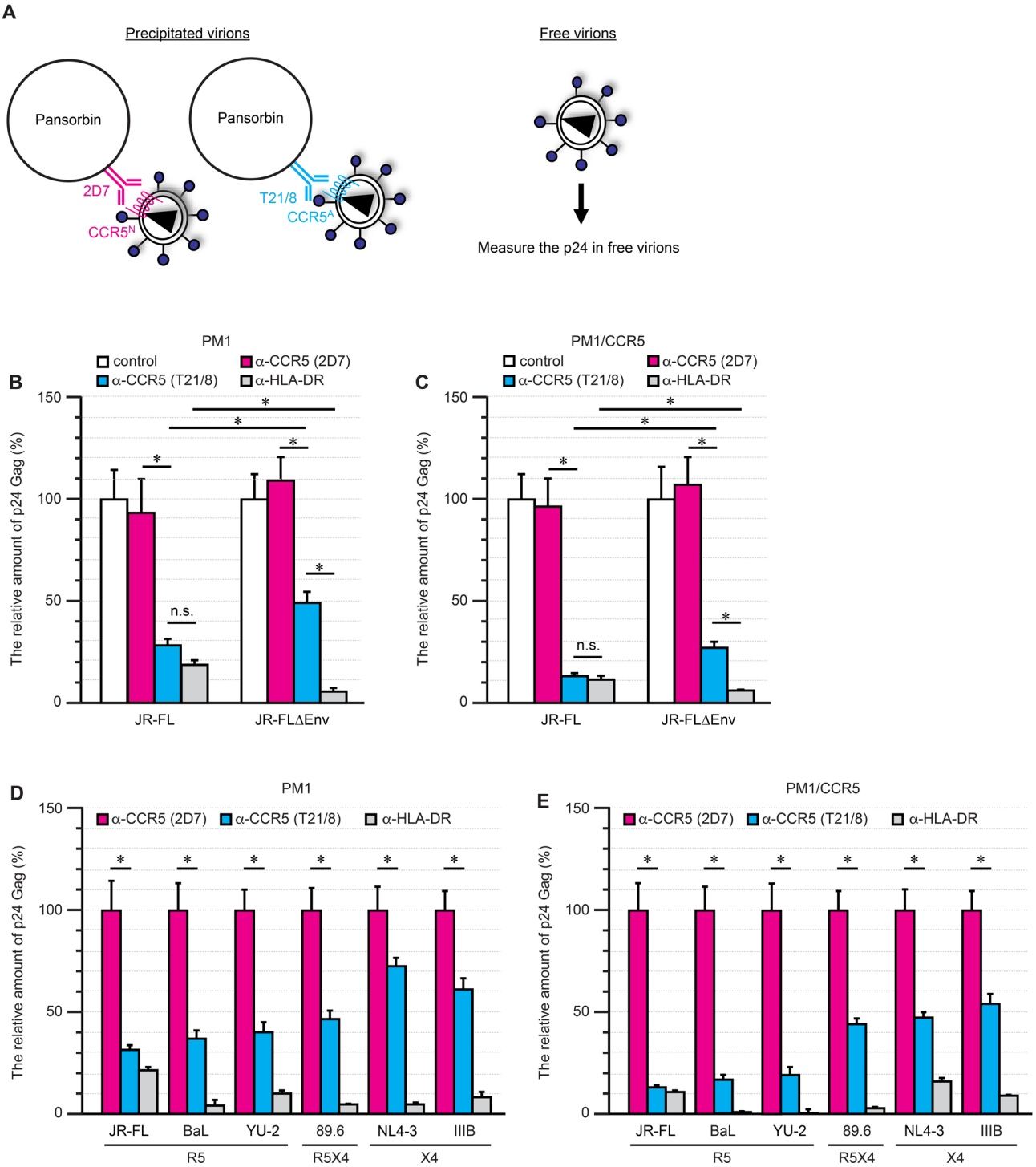


Fig. 4. R5 HIV-1 Env accelerates the CCR5^{A} incorporation into virions. (**A**) The incorporation of CCR5 into HIV-1_{JR-FL} produced from PM1 (**B**) or PM1/CCR5 (**C**) cells was measured by immunoprecipitation assay. The incorporation of CCR5 into R5, R5/X4, or X4 HIV-1 produced from PM1 (**D**) or PM1/CCR5 (**E**) cells was measured by immunoprecipitation assay.The immunoprecipitation assay was performed using T21/8, 2D7, or anti-HLA-DR MAb (L247). Cell-free virus (5 ng), produced from each of the T cell lines, was incubated with 1 μg of antibody for 8 h. Virus–antibody complexes were precipitated by adding Pansorbin cells. After precipitation of virus–antibody complexes, the amount of p24 Gag in supernatants was determined. (B-E) The analysis was repeated three times; error bars represent the S.D. of three replicates. *, $p < 0.01$. Statistically significant differences were calculated by the *t-test*.

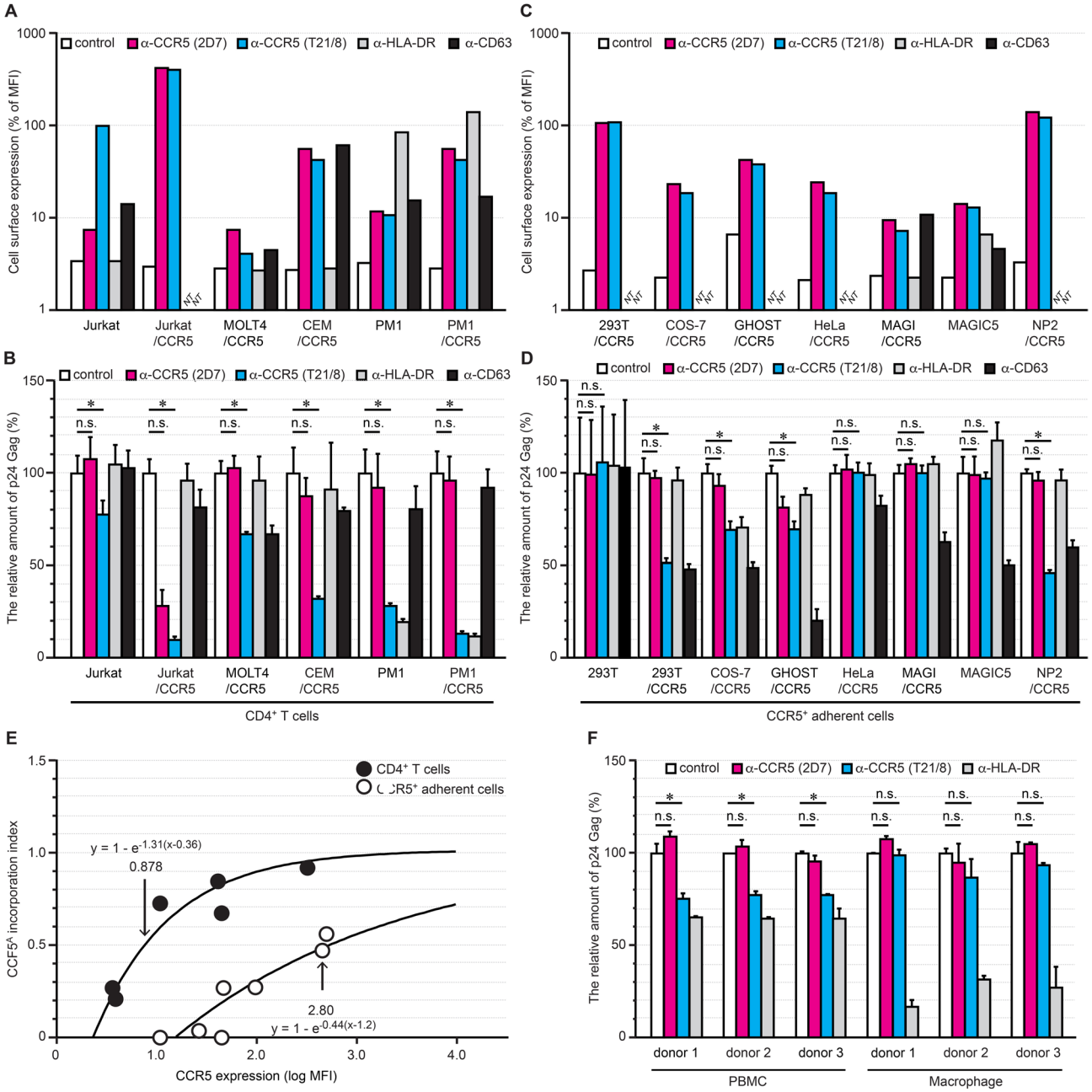


Fig. 5. CCR5 incorporation into the virions is in a cell-type-specific manner. (**A**) The expression of CCR5 in $CD4^+$ T cell lines (**A**) and $CCR5^+$ adherent cell lines (**C**) was analyzed by flow cytometry with an antibody directed against CCR5 (2D7 or T21/8), HLA-DR (L243), or CD63 (MX-49.129.5). (**B**) The incorporation of CCR5 into HIV-1_{JR-FL} generated from $CD4^+$ T cell lines (**B**), $CCR5^+$ adherent cell lines (**D**), PBMCs, or macrophages (**F**) was measured by immunoprecipitation assay. (B, D, and F) For statistically significant analysis, the data from three independent experiments are shown as mean ± standard deviations. The P values of the results were determined by the student's t-test. *, $P < 0.01$; n.s., not significant. (E) The ratio of CCR5 incorporation into virions (x-axis) to CCR5 expression in producer cells (y-axis) was compared between $CD4^+$ T cells (black circles) and $CCR5^+$ adherent cells (white circles). The ratio was plotted using panels B and D.

effects of MVC on CCR5 incorporation could provide valuable insight for the development of next-generation coreceptor-targeting antiviral therapies.

Methods

Cells and viruses

GHOST/CCR5 cells, human $CD4^+$ T-cell lines PM1[50], and Jurkat[51] cells were provided by the NIH AIDS Research and Preference Reagent Program, Division of AIDS at the National Institute of Allergy and Infectious Diseases.

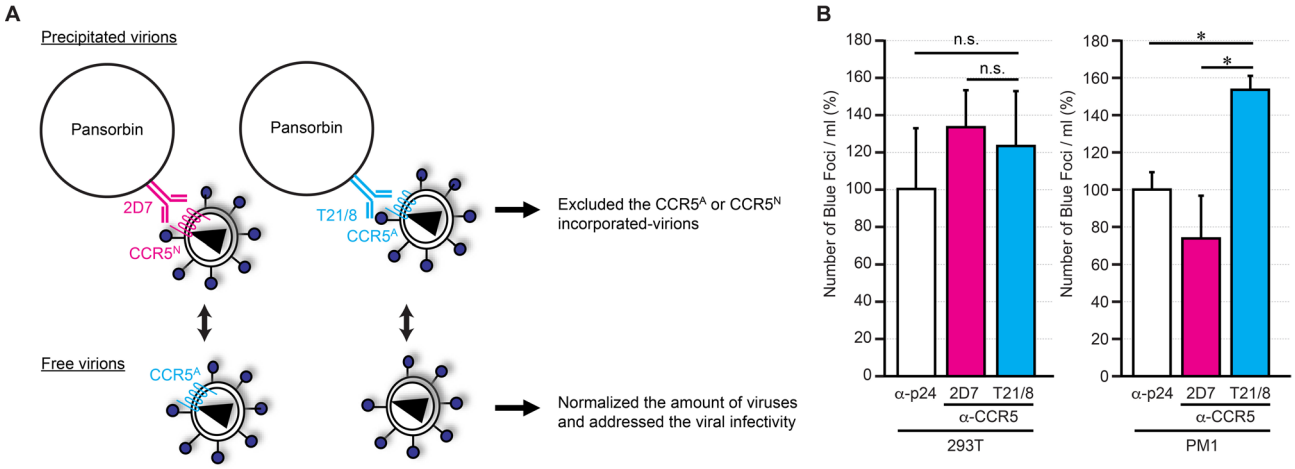


Fig. 6. CCR5^{A} incorporation reduces the viral infectivity of HIV-1$_{JR-FL}$. (**A**) HIV-1$_{JR-FL}$ generated from PM1 was precipitated by anti-p24 or anti-CCR5 (T21/8 or 2D7) MAb, and the infectivity was determined in MAGIC5 cells. Since the p24 antigen is not expressed on the virus surface and is not affected by immunoprecipitation, the anti-p24 Mab was used as a negative control. (**B**) The viruses were harvested from 293 T and PM1/CCR5. MAGIC5 cells were infected with each antibody-purified virus. At 48 h post-infection, cells were fixed and stained with 5-bromo-4-chloro-3-indolyl-β-d-galactoside. MAGIC5 is a HeLa-derived cell line that expresses CD4 and CCR5 and has a ß-galactosidase gene driven by HIV-1 L. For statistically significant analysis, the data from three independent experiments are shown as mean ± standard deviations. The P values of the results were determined by the student's t-test. *, $P < 0.01$; n.s., not significant.

PM1/CCR5[52], Jurkat/CCR5, MOLT-4/CCR5, and CEM/CCR5 cells were generated by standard retrovirus-mediated transduction. The CD4^{+} T cells were maintained in RPMI1640 (Invitrogen) supplemented with 10% heat-inactivated fetal calf serum (FCS; Vitromex). MAGIC5 cells[27] were kindly provided by Dr. Tatsumi at the National Institute of Infectious Disease, Japan. NP2/CCR5 cells were kindly provided by Prof. Hoshino at Gunma University. 293T/CCR5, HeLa/CCR5, and COS7/CCR5 cells were generated by retrovirus-mediated transduction. MAGI/CCR5 cells were similarly generated from MAGI cells[53]. The CCR5^{+} adherent cells were maintained in Dulbecco's modified Eagle's medium (ICN Biomedicals) supplemented with 10% heat-inactivated FCS. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque gradient centrifugation and activated by 20 U/ml interleukin-2 (IL-2) (Roche Molecular Biochemicals, Indianapolis, Ind.) and 10 µg of phytohemagglutinin (PHA) (Difco Laboratories, Detroit, Mich.). After an overnight incubation with PHA, cells were washed, and the culture was continued with IL-2 for 5 days. Monocyte-derived macrophages (MDMs) were purified from PBMCs by plastic adherence and cultured for 5 days in RPMI 1640 medium supplemented with 10% human AB^{+} serum (Nabi, Boca Raton, Fla.), 100 µg of penicillin, and 100 µg of streptomycin per ml, and 12.5 ng of macrophage colony-stimulating factor (M-CSF) per ml. pJR-FL was kindly provided by Prof. Y. Koyanagi at Kyoto University. For the HIV-1$_{JR-FL}$ preparation, 293 T cells (1×10^{6}) were transfected with 10 µg of pJR-FL DNA using ProFection Mammalian Transfection System (Promega). The supernatant was collected at 28 h post-transfection, filtered through a 0.22 µm filter unit (Millipore), and stored at − 80 °C until use.

Flow cytometry

PM1/CCR5 cells were treated with RANTES (50 nM) for 2 days. PM1 or PM1/CCR5 cells were infected with HIV-1$_{JR-FL}$ at 50 TCID$_{50}$ for 2 h and incubated at 37 °C and 5% CO_2. Forty-eight hours post-infection, the cells were fixed by 4% paraformaldehyde (4% PFA), and incubated at 4 ºC for 1 h. The cells were permeabilized with 0.1% saponin and incubated in a blocking buffer (10% FBS in PBS) for 30 min. Then, the cells were incubated in the staining solution with anti-CCR5 antibody (2D7, NIH Reagent Program) or CCR5 (T21/8, BioLegend) at 4 °C for 30 min, then incubated with the AlexaFluor647-conjugated anti-mouse antibody (Thermo Fisher Scientific). After washing, the cells were incubated with fluorescein isothiocyanate (FITC)-conjugated anti-p24 antibody (KC57, Bechman Coulter) for 1 h. To determine the MFI value for CD4^{+} or CCR5^{+} adherent cells, cells were incubated in the staining solution (3% FBS plus 0.05% sodium azide in PBS) with anti-human CCR5 (2D7 or T21/8), anti-human CD63 (MX-49.129.5, Santa Cruz Biotechnology), or anti-HLA-DR (L243, BD Biosciences Pharmingen) antibody at 4 °C for 30 min. The cells were washed twice using 10% FCS in PBS and fixed using 4% PFA, and stained with anti-human CD4 (SK3, BD Biosciences Pharmingen), anti-human CCR5 (2D7 or T21/8), anti-human CD63 (MX-49.129.5, Santa Cruz Biotechnology), or anti-HLA-DR (L243, BD Biosciences Pharmingen) antibody at 4 °C for 30 min. Cells were washed with PBS, and FITC-conjugated goat anti-mouse IgG antibody was used for antibody staining. Flow cytometry was performed using a FACSVerse and FACSCalibur flow cytometer (Becton Dickinson), and the results were analyzed using FlowJo ver. 10.5.3 and BD Cell Quest ver. 3.1 software (BD Biosciences Pharmingen).

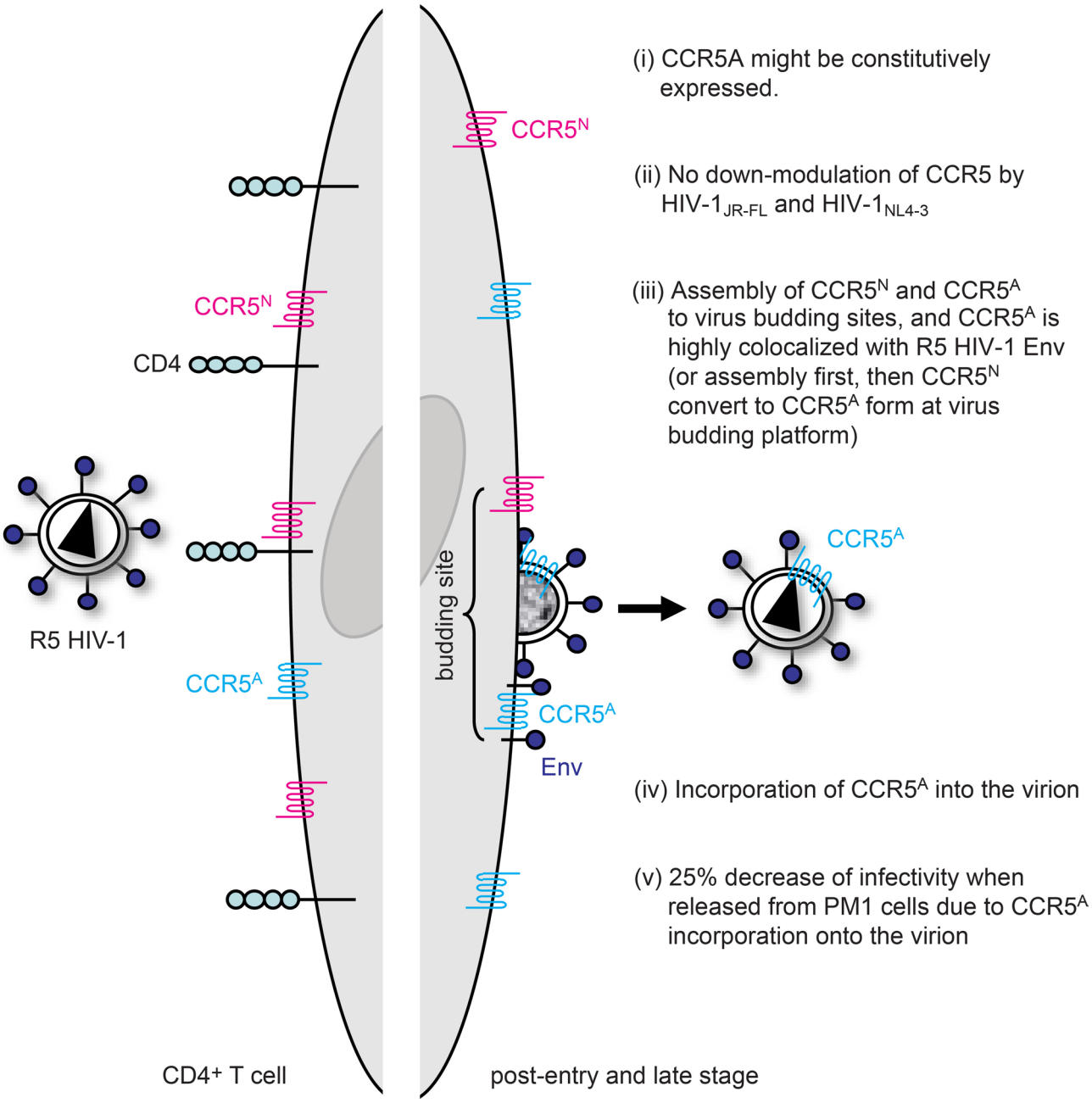


Fig. 7. Model of CCR5 behavior post-infection.

Immunofluorescence microscopy

PM1/CCR5 infected cells were washed twice with 10% FBS in PBS, stabilized using 4% PFA, and incubated at 4 °C for 1 h. The cells were permeabilized with 0.1% saponin and incubated in a blocking buffer (10% FBS in PBS) for 30 min. The cells were incubated in the staining solution (10% FBS in PBS) with the following MAbs: anti-gp120 (49G2)[54]anti-p24 (#24 – 4; NIH reagent program), FITC-conjugated-anti-p24 (KC57), anti-CCR5 (T21/8) (BioLegend), and anti-CCR5 (2D7) (BD Biosciences Pharmingen) antibodies for 1 h at 4°C. The cells were washed twice in a blocking buffer, and secondary antibodies (anti-mouse IgG AlexaFluor 488, and anti-mouse/anti-human IgG AlexaFluor 546) were added to the cells respectively and incubated for 1 h at 4 °C. Confocal laser scanning microscopic analysis was performed on a Zeiss LSM700. Using the Zeiss LSM700, the top section of the cell was taken. Colocalization between CCR5 and gp120 was quantified using the ZEN software (Zeiss), with which we calculated the Pearson correlation coefficient (R-value). We set the entire cell body of each CCR5 and gp120-coexpressing cell as the region of interest for this analysis. $R = 1$ represents perfect co-localization, and $R = 0$ represents random distributions of fluorescence intensities.

Virus precipitation assay

A virus immunoprecipitation assay was performed as previously described[35] with anti-HLA-DR (L243), anti-CD63 (ab8219), anti-CCR5 (T21/8) (BioLegend), anti-CCR5 (2D7) (BD Biosciences Pharmingen), or anti-CXCR4 (12G5) (BD Biosciences Pharmingen) MAbs. Virus (5 ng p24 Gag) in PBS containing 3% bovine serum albumin (BSA) was mixed with the MAb at a concentration of 10 µg/ml in a final volume of 100 µl and incubated for 12 h at 4 °C. Then, 10 µl of Pansorbin (Calbiochem), a suspension of heat-killed Staphylococcus aureus cells pretreated for 1 h with 3% BSA, was added to the virus/MAb mixture. After incubation for 30 min at room temperature, captured viruses were removed by centrifugation (350 × g for 30 min). p24 Gag in the supernatant was quantified by a p24 Gag enzyme-linked immunosorbent assay (ELISA).

Virus infectivity assay after purification with 2D7 or T21/8 MAb—HIV-1_{JR-FL} generated from PM1 was precipitated by anti-p24 or anti-CCR5 (T21/8 or 3A9) MAb, and the infectivity was determined using MAGIC5 cells. Briefly, 5×10^3 of MAGIC5 cells were plated into 48-well tissue culture plates 1 day before infection. After absorption of the virus for 2 h at 37 °C, cells were washed and further incubated at 37 °C in 5% CO_2. At 48 h post-infection, cells were stained with 5-bromo-4-chloro-3-indolyl-β-d-galactoside, and the number of blue foci in each well was counted[53].

Data availability

All data analyzed during this study can be obtained from the corresponding author upon reasonable request.

Received: 21 January 2025; Accepted: 18 July 2025
Published online: 21 July 2025

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Acknowledgements

We thank Dr. Masashi Tatsumi and Dr. Hiroo Hoshino for providing MAGIC5 and NP2/CCR5 cells. Also we would like to thank Dr. Yoshio Koyanagi for providing pJR-FL. The following reagents were obtained through BEI Resources, NIAID, NIH: GHOST CCR3+ CXCR4+ CCR5+ Cells, ARP-3944 contributed by Dr. Vineet N KewalRamani and Dr. Dan R. Littman, PM1 Cells, ARP-3038, contributed by Dr. Paulo Lusso and Dr. Robert Gallo, Jurkat (E6-1) Cells, ARP-177.

Author contributions

KM, YM, TS, SH, and KY conceived and coordinated this study. KM, JAK, HT, YY, PN, WOA, and MJH performed experiments. TK prepared the anti-gp120 antibody. All authors have read and approved the final manuscript.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

Correspondence and requests for materials should be addressed to K.M.

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