# Δ20 IFITM2 differentially restricts X4 and R5 HIV-1

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**CCR5 (R5)-tropic, but not CXCR4 (X4)-tropic, HIV-1 is associated with primary HIV-1 infection and transmission. Recent studies have shown that IFN-induced transmembrane (IFITM) proteins, including IFITM1, IFITM2, and IFITM3, restrict a broad range of viruses. Here, we demonstrate that an IFITM2 isoform (Δ20 IFITM2) lacking 20 amino acids at the N terminus differentially restricts X4 and R5 HIV-1. Δ20 IFITM2 suppresses replication of X4 HIV-1 strains by inhibiting their entry. High levels of Δ20 IFITM2 expression could be detected in $CD4^+$ T cells and in monocytes. Infection of X4 viruses in monocyte-derived macrophages and dendritic cells is enhanced upon depletion of IFITM2 isoforms. Furthermore, we also show that coreceptor use is the determining factor for differential HIV-1 restriction of Δ20 IFITM2. When we replace the C terminus of CCR5 with the C terminus of CXCR4, R5 viruses become more susceptible to Δ20 IFITM2-mediated restriction. In contrast to previous studies, our research reveals that neither X4 nor R5 HIV-1 is suppressed by IFITM2 and IFITM3. The multifactor gatekeeping model has been proposed to explain restriction of X4 viruses in the early stage of HIV-1 diseases. Our findings indicate that Δ20 IFITM2 may serve as a major contributor to this gatekeeping mechanism.**

Δ20 IFITM2 | gatekeeping | HIV | interferon-inducible transmembrane protein | viral entry

IFN-induced transmembrane (IFITM) proteins, including IFITM1, IFITM2, and IFITM3, restrict a broad range of highly pathogenic human and animal viruses (1–3). As their names indicate, expression of IFITM proteins is regulated by IFNs and several proinflammatory cytokines. IFITM3 has been shown to play a central role in suppressing influenza A virus (IAV) entry and replication in vitro and in vivo (4, 5). Association of a single-nucleotide polymorphism (SNP) of IFITM3 with the poor clinical prognosis of H1N1 and H7N9 influenza has also been reported (5–7). IFITM1, IFITM2, and IFITM3 mainly localize to late endosomes and lysosomes. Nevertheless, plasma membrane-associated IFITM1 could be detected (8). Increasing evidence has shown that IFITM proteins inhibit viral entry by interfering with cellular lipid metabolism and impeding fusion between virion and cellular membranes (9). The subcellular distribution of IFITM proteins is thought to be critical for their antiviral activities (3, 10).

The role of IFITM proteins in regulating HIV-1 infection is controversial. Initial characterizations showed that IFITM proteins do not interfere with HIV-1 infection (1, 11). Subsequent studies demonstrated that IFITM1 or IFITM3 can suppress entry and replication of HIV-1 (12–14). The role of cellular and virion-associated IFITM3 in inhibiting cell-to-cell transmission of CXCR4 (X4)-tropic viruses and infection of HIV-1 in new target cells has also been described (13). Increased shedding of HIV-1 gp120 from the plasma membrane of viral-producing cells by IFITM3 expression was thought to be the cause of diminished viral fusion and cell-to-cell transmission (15). However, some of these observations could not be reproduced by other groups (8). More importantly, FLAG-tagged IFITM proteins were widely used in most of these studies (12, 13). FLAG tag has been shown to alter the subcellular distribution of IFITM3 from endolysosomal compartments to the plasma membrane (10). Because the subcellular localization of IFITM proteins is pivotal for their antiviral activities, whether epitope tags similarly change their antiviral properties still needs further investigation.

Recently, Foster et al. (8) observed that different strains of HIV-1 have different sensitivities to IFITM1, IFITM2, or IFITM3. IFITM1 partially restricts entry of CCR5 (R5)-tropic viruses, whereas IFITM2 and IFITM3 inhibit infection of X4 viruses. They also found that transmitted/founder viruses are resistant to IFITM-mediated restriction and increase their susceptibilities to the antiviral activity of IFITM proteins, especially IFITM2 and IFITM3, over the initial 6 mo of acute infection. They hypothesized that founder viruses fuse at the plasma membrane. In contrast, entry of X4 and some propagated R5 viruses happens at endolysosomes; therefore, infection of these viruses can be inhibited by IFITM2 and IFITM3 (8). However, the importance of endocytosis-mediated entry in HIV-1 infection is still under debate (8, 16, 17). Evidence supporting transition of fusion sites of R5 viruses during the course of HIV-1 infection is also lacking. Recent studies showed that dynamin, a cellular protein regulating endocytosis, inhibitors suppress infection of both X4 and R5 HIV-1. Single-virion labeling experiments also demonstrated that HIV-1 virions can traffic to late endosomes (16, 18). Nevertheless, HIV-1 entry does not require a low pH environment, and fusion between viral membrane and plasma membrane of $CD4^+$ T cells has been well documented (19, 20). Removing the motifs essential for CCR5 and CXCR4 recycling or expressing dominant negative dynamin also did not affect infection of HIV-1 (17).

Here, we identify an isoform of IFITM2, Δ20 IFITM2, which has a 20-aa truncation at the amino (N) terminus of full-length (FL) IFITM2. Δ20 IFITM2, together with IFITM1, is highly expressed endogenously in $CD4^+$ T and monocytic cells, whereas low expression of FL IFITM2 and IFITM3 could be detected in

## Significance

**Innate immunity plays an important role in control of viral infection. IFN-induced transmembrane (IFITM) proteins have been shown to inhibit several important human viruses. We identified an isoform of IFITM2, Δ20 IFITM2, which is highly expressed in innate and adaptive immune cells. Functionally, Δ20 IFITM2 preferentially suppresses infection of CXCR4 (X4)-tropic, but not CCR5 (R5)-tropic, HIV-1 and may contribute to restricting transmission of X4 viruses in the acute phase of HIV-1 infection. Our studies also revealed a role of CCR5 in HIV-1 infection. The C-terminal region of CCR5 is critical to the resistance of R5 viruses to Δ20 IFITM2-mediated restriction. Understanding the mechanism by which Δ20 IFITM2 restricts X4, but not R5, viruses may inspire novel approaches to treat HIV-1 diseases.**

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The authors declare no conflict of interest.

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these cells. Unlike other IFITM proteins, Δ20 IFITM2 is distributed to both endolysosomes and the plasma membrane. Functionally, Δ20 IFITM2 substantially suppresses entry of X4, but not R5 or founder, viruses. In our studies, we found that both CXCR4 and CCR5 colocalize with Δ20 IFITM2 at the plasma membrane and that coreceptor use is essential for the resistance of R5 viruses to Δ20 IFITM2-mediated restriction. Although G protein- and arrestin-mediated CXCR4/CCR5 signaling does not seem to play a role in HIV-1 infection, the C terminus of CCR5 is critical to the resistance of HIV-1 to the antiviral activity of Δ20 IFITM2. Several mechanisms have been proposed to explain the association of R5 viruses with acute HIV-1 infection. The high expression level of Δ20 IFITM2 in innate and adaptive immune cells and its differential restriction effects on X4 and R5 HIV-1 indicate that Δ20 IFITM2 may be a major contributor to limiting mucosal transmission of X4 viruses. The importance of coreceptor use in differential susceptibilities of HIV-1 to IFITM2-mediated restriction also suggests that CCR5 may not only serve as an anchor for HIV-1 virions but have a direct role in regulating HIV-1 entry.

## Results

**Δ20 IFITM2 Is Highly Expressed in Innate and Adaptive Immune Cells.** The *IFITM3* polymorphism rs12252-C, which has been controversially associated with poor clinical outcomes in patients with H1N1 and H7N9 IAV infections, was reported to encode an IFITM3 isoform (Δ21 IFITM3) that lacks 21 amino acids at the N terminus (5, 6, 10). We analyzed expression of IFITM2 and IFITM3 mRNA transcripts in lymphoblastoid cell lines (LCLs) and observed that cells homozygously carrying the *IFITM3* polymorphism rs12252-C/C expressed the mRNA transcript encoding FL IFITM3, whereas the previously described transcript, ENST00000526811, encoding Δ21 IFITM3 could not be detected (Fig. 1*A*). Because limited cell types were tested, we could not exclude the possibility that Δ21 IFITM3 may be expressed in other cells or tissues. In our studies, we also identified

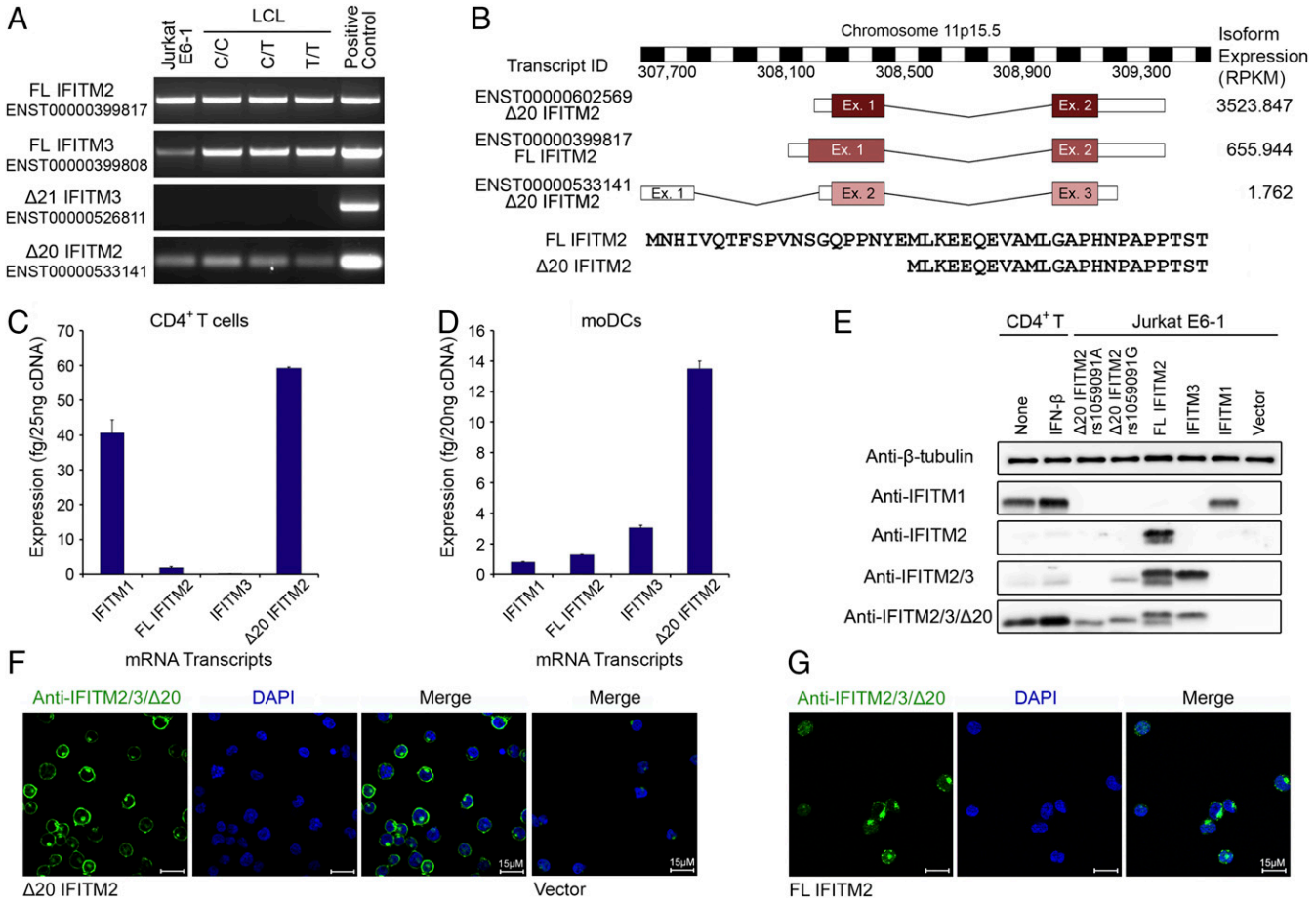


**Fig. 1.** Characterization of Δ20 IFITM2. (*A*) Jurkat E6-1 or LCL cells carrying *IFITM3* polymorphism rs12252-C/C, rs12252-T/C, or rs12252-T/T were treated with 1,000 units (U)/mL IFN-β. Two days later, cells were analyzed by quantitative RT-PCR (qRT-PCR) using specific primers for the indicated IFITM cDNA. DNA electrophoresis was performed to show that a specific Δ20 IFITM2, but not Δ21 IFITM3, transcript could be detected. PCR products of synthetic IFITM cDNA served as positive controls. (*B*) Schematic representation of three major IFITM2 transcripts. RNA expression of these transcripts in whole blood cells analyzed by RNA sequencing was adapted from ref. 21. RPKM, reads per kilobase per million mapped reads. Expression of IFITM mRNA transcripts in anti-CD3 and anti-CD28 antibody-activated $CD4^+$ T cells (*C*) or moDCs (*D*) was analyzed by qRT-PCR. Expression of Δ20 IFITM2 transcript ENST00000602569 is shown. Error bars denote 1 SD ($n = 3$) (IFITM2 primers used for qRT-PCR are shown in Fig. S1*A*). (*E*) Anti-CD3 and anti-CD28 antibody-activated $CD4^+$ T cells were treated with or without 1,000 U/mL IFN-β. Two days later, expression of IFITM proteins was analyzed by Western blotting using the indicated antibodies (details of these antibodies are shown in Fig. S2 *A* and *B* and Table S1). Protein bands from Jurkat E6-1 cells stably expressing the indicated IFITM proteins were used as size markers. Δ20 IFITM2 translates of both *IFITM2* rs1059091-A and rs1059091-G polymorphisms, which have different migration, were included. The rs1059091-G Δ20 IFITM2 was used for our subsequent hyperexpression experiments. Δ20 IFITM2 indicates rs1059091-G Δ20 IFITM2 if there is no additional specification. Vector-transduced Jurkat E6-1 cells or Jurkat E6-1 cells expressing Δ20 IFITM2 (*F*) or FL IFITM2 (*G*) were labeled with an anti-IFITM2/3/Δ20 antibody and 4′,6-diamidino-2-phenylindole (DAPI) and imaged by confocal microscopy.

an alternatively spliced IFITM2 transcript, ENST00000533141, encoding Δ20 IFITM2 (Fig. 1*A*).

Of three major IFITM2 mRNA transcripts, ENST00000399817 encodes FL IFITM2. Transcripts ENST00000602569, a product of multiple transcriptional start sites, and ENST00000533141, an alternatively spliced variant, encode Δ20 IFITM2 (Fig. 1*B*). Previous RNA sequencing analysis has shown that the ENST00000602569 transcript is the predominant IFITM2 transcript, whereas expression of ENST00000533141 is low in human whole blood cells (21). We further investigated expression of IFITM transcripts in $CD4^+$ T and monocytic cells. We observed that the ENST00000602569 transcript encoding Δ20 IFITM2 was highly expressed in unactivated and activated $CD4^+$ T cells, monocyte-derived dendritic cells (moDCs), and macrophages. In contrast, expression of FL IFITM2 and IFITM3 was substantially lower than expression of Δ20 IFITM2 in these cells (Fig. 1 *C* and *D* and Fig. S1). Because protein sequences of IFITM2 and IFITM3 are highly conserved, most commercially available antibodies are cross-reactive (Fig. S2 *A* and *B* and Table S1). Jurkat E6-1 R5 cells stably expressing CCR5 and different IFITM proteins, which were used for our subsequent infection and entry assays, were included as controls for protein expression analyses. Δ20 IFITM2 translates from one of the most prevalent *IFITM2* SNPs, rs1059091-A/G (allele frequency of 0.58/0.42), were also characterized in our studies. We found that expression of Δ20 IFITM2 in activated $CD4^+$ T cells was comparable to or higher than expression of Δ20 IFITM2 in Δ20 IFITM2-expressing Jurkat E6-1 R5 cells (Fig. 1*E* and Fig. S2 *C*–*E*). Although donor variations could be detected, Δ20 IFITM2 expression was substantial in these cells. In addition to $CD4^+$ T cells, expression of Δ20 IFITM2 could be detected in monocytes and moDCs (Fig. S2 *F* and *G*).

Like other IFITM proteins, Δ20 IFITM2 expression was upregulated by IFNs in $CD4^+$ T cells, moDCs, and macrophages, whereas phytohemagglutinin (PHA), which has been widely used for propagating HIV-1, depleted its expression (Fig. 1*E* and Figs. S2*H* and S3). Recent studies have suggested that the N terminus of IFITM proteins interacts with adaptor protein 2 and may regulate their subcellular distribution (8, 10, 14, 22). We observed that Δ20 IFITM2 localized to endolysosomal compartments and to the plasma membrane in A549 cells (Fig. S4 *A* and *B*). The ring distribution of IFITM proteins at the plasma membrane could also be detected in Δ20 IFITM2- or IFITM1-expressing Jurkat E6-1 R5 cells and in unactivated and activated $CD4^+$ T cells (Fig. 1 *F* and *G* and Fig. S4 *C*–*E*). Our data demonstrate that Δ20 IFITM2 is highly expressed in adaptive and innate immune cells. Unlike FL IFITM2 and IFITM3, which mainly localize to endolysosomes, Δ20 IFITM2 is distributed to both endolysosomes and the plasma membrane.

**Δ20 IFITM2 Differentially Restricts X4 and R5 HIV-1.** Recent studies have shown that IFITM proteins restrict a broad range of viruses and that their subcellular distribution is critical to their antiviral activities (1, 2). We sought to examine the effect of Δ20 IFITM2 on HIV-1 replication because cellular entry of HIV-1 happens at the plasma membrane of $CD4^+$ T cells (20, 23–26). Jurkat E6-1 R5 cells stably expressing various IFITM proteins were infected with X4 (strain NL4-3) or with R5 (strain AD8) HIV-1. We observed that Δ20 IFITM2 strongly restricted replication of NL4-3, but not AD8. IFITM1, FL IFITM2, and IFTM3 had marginal effects on HIV-1 replication (Fig. 2 *A* and *B* and Fig. S5*A*). In addition, both rs1059091-A and rs1059091-G Δ20 IFITM2 showed similar suppressive effects on infection of X4 (NL4-3), but not R5 (JRFL), viruses (Fig. S5 *B* and *C*). Δ20 IFITM2-mediated restriction was not caused by receptor or coreceptor down-regulation. Δ20 IFITM2 did not interfere with surface expression of CD4, CCR5, or CXCR4 (Fig. S5*D*). This restriction was also not specific to strain. Replication of different strains of X4 viruses was suppressed in cells expressing Δ20 IFITM2, whereas R5 virus infection, including infection with

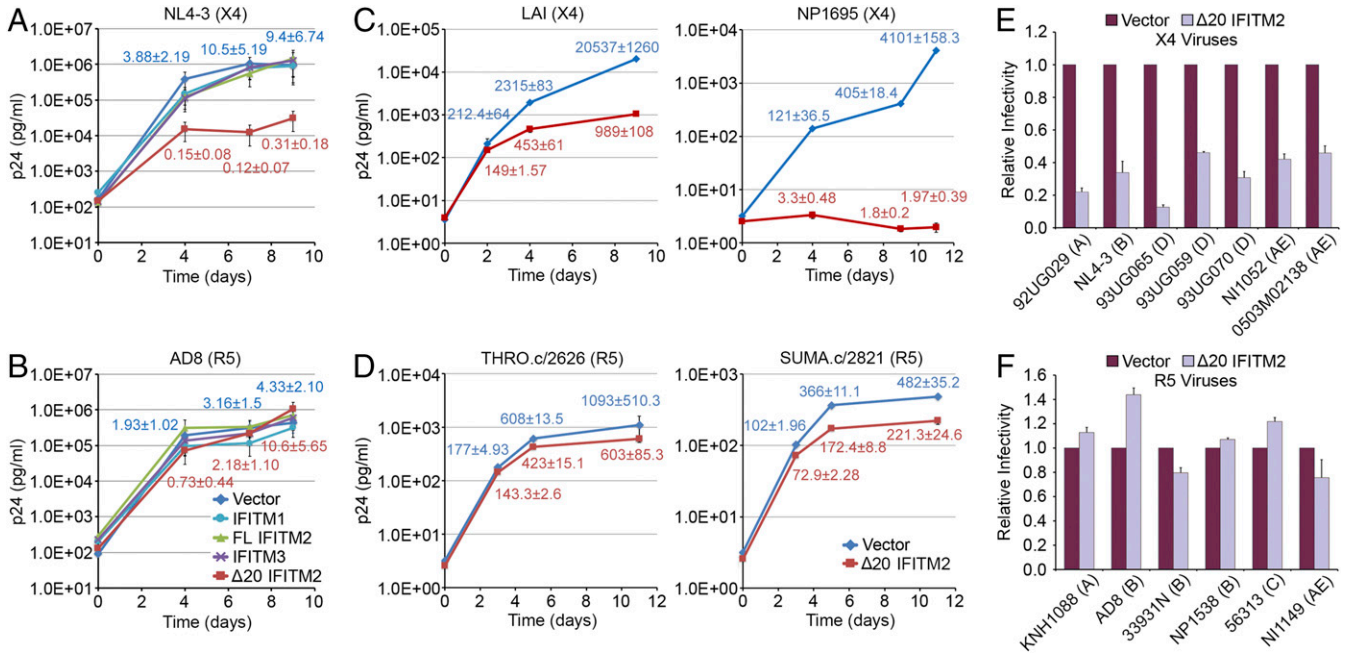


**Fig. 2.** Δ20 IFITM2 differentially restricts replication of X4 and R5 HIV-1. Jurkat E6-1 R5 cells expressing the indicated IFITM proteins were incubated with 100 ng of p24 antigen X4-tropic NL4-3 (*A*) or R5-tropic AD8 (*B*) virus. Supernatants were harvested at the indicated time points, and virus titers were measured by p24 ELISA. Numbers indicate p24 values (mean ± SD × $10^5$; $n = 3$) detected in the supernatants of vector-transduced and Δ20 IFITM2-expressing cells. Experiments similar to the experiments in *A* and *B*, except that the indicated X4 (*C*) or R5 (*D*) viruses were used, were performed. Numbers indicate p24 values (mean ± SD; $n = 3$). Vector-transduced GHOST R5 cells or GHOST R5 cells expressing Δ20 IFITM2 were incubated with the indicated replicating X4 (*E*) or R5 (*F*) virus. Two days later, infected cells were harvested and analyzed by flow cytometry. The relative infectivity was determined as the percentage of $GFP^+$ cells normalized to the percentage of vector-transduced cells. Error bars denote 1 SEM of duplicates.

founder viruses, was unaffected (Fig. 2 *C* and *D*). We further extended our research to include different clades of HIV-1 isolates and found that infection of X4 HIV-1 was substantially inhibited by Δ20 IFITM2. In contrast, infection of R5 viruses was either unaffected or enhanced (Fig. 2 *E* and *F* and Fig. S5*E*).

**Δ20 IFITM2 Inhibits Entry of X4, but Not R5, HIV-1.** To investigate whether Δ20 IFITM2 suppresses infection of X4 viruses by interfering with their entry, viral entry assays using HIV-1 pseudotyped with various viral entry glycoproteins were performed. We found that entry of X4 HIV-1 strains was strongly suppressed by Δ20 IFITM2 in both GHOST R5 and Jurkat E6-1 R5 cells (Fig. 3 *A*–*C* and Fig. S5*F*). Although individual susceptibilities among R5 viruses varied, they were, in general, more resistant than X4 viruses to the antiviral activity of Δ20 IFITM2. Δ20 IFITM2-mediated HIV-1 restriction was correlated to its expression level. Moderate suppression of R5 virus entry could also be detected when Δ20 IFITM2 was highly expressed (Fig. 3*D* and Fig. S5*G*). The importance of Δ20 IFITM2 in regulating X4 virus infection was further demonstrated in our knockdown experiments using human primary cells. (Because the mRNA sequence of the Δ20 IFITM2 ENST00000602569 transcript totally overlaps with the mRNA sequence of the FL IFITM2 ENST00000399817 transcript, siRNA used in our research targeted both IFITM2 isoforms.) When we depleted expression of IFITM2 in moDCs and macrophages, infection of X4 (NL4-3), but not R5 (JRFL), was strongly enhanced (Fig. 3 *E* and *F* and Fig. S6). Our results indicate that Δ20 IFITM2 selectively restricts a broad range of X4 HIV-1 strains by interfering with their entry and that R5 HIV-1 strains are more refractory to Δ20 IFITM2-mediated restriction.

**Epitope Tag Alters Antiviral Properties of IFITM Proteins.** Whether IFITM proteins inhibit HIV-1 entry is under debate (11–13). Suppression of HIV-1 entry and replication by either IFITM1 or IFITM3 has been reported. However, similar phenotypes could not be observed in our studies. Because N-terminal FLAG-tagged IFITM proteins were used in many of these studies (12, 15), we subsequently compared their antiviral properties with the antiviral properties of native IFITM proteins. Compatible with previous work, entry of X4 and, to a lesser extent, R5 HIV-1 could be suppressed by FLAG-tagged IFITM proteins (Fig. S7 *B* and *C*). Among native IFITM proteins, infection of X4 HIV-1 was inhibited only by Δ20 IFITM2 (Fig. S7 *A* and *C*). IAV pseudoviruses were included in our experiments as positive controls. At the expression level tested, native IFITM1, IFITM2, and IFITM3 substantially inhibited IAV entry, whereas infection of X4 viruses was unaffected. In our studies, we also found that IFITM1 and IFITM3 could be packed into HIV-1 virions but that these virion-associated IFITM proteins did not inhibit HIV-1 infection of new target cells (Fig. S8 *A*–*D*). Epitope tags have been shown to alter the subcellular location of IFITM proteins (10). Distribution of FLAG-tagged IFITM proteins to the plasma membrane may result in different phenotypes in previous studies.

A recently published paper described the suppressive effects of IFITM1 on R5 and FL IFITM2 and IFITM3 on X4 HIV-1 infection and emphasized the importance of endocytosis-mediated HIV-1 entry (8). The authors hypothesized that entry of founder viruses and certain strains of R5 HIV-1 happens at the plasma membrane, and therefore is not affected by IFITM2 and IFITM3. U87 glioblastoma cells were extensively used in these studies. Although we observed that IFITM proteins, except IFITM2, strongly suppressed entry of both X4 and R5 HIV-1 in U87 cells (Fig. S8 *E* and *F*), similar inhibitory effects of IFITM1, FL IFITM2, or IFITM3 on HIV-1 infection could not be reproduced in other cells tested in our studies. Because expression of FL IFITM2 and IFITM3 in HIV-1 target cells is relatively lower, Δ20 IFITM2-mediated HIV-1 restriction may reflect a more physiologically relevant condition.

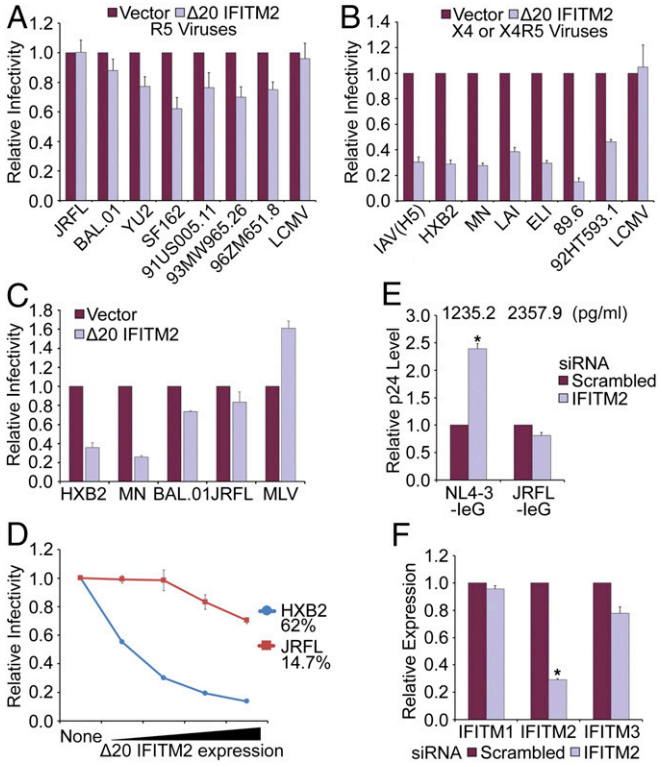


**Fig. 3.** Δ20 IFITM2 differentially restricts entry of X4 and R5 HIV-1. (*A* and *B*) Vector-transduced GHOST R5 cells or GHOST R5 cells expressing Δ20 IFITM2 were incubated with NL4-3-ΔE pseudotyped with the indicated viral entry glycoproteins. Two days later, cells were harvested and analyzed by flow cytometry. The relative infectivity was determined as the percentage of $GFP^+$ cells normalized to the percentage of vector-transduced control cells. LCMV, lymphocytic choriomeningitis virus. (*C*) Vector-transduced Jurkat E6-1 R5 cells or Jurkat E6-1 R5 cells stably expressing Δ20 IFITM2 were incubated with pLenti-based HIV-1–GFP pseudotyped with env proteins from the indicated HIV-1 strains. Two days later, cells were harvested and analyzed by flow cytometry. The relative infectivity was determined as the percentage of $GFP^+$ cells normalized to the percentage of vector-transduced cells. MLV, murine leukemia virus. (*D*) Experiments similar to the experiments in *C*, except that cells expressing different amounts of Δ20 IFITM2, were used. Numbers indicate percentages of infected control cells. (*E*) Primary moDCs were transfected with scrambled siRNA or siRNA targeting IFITM2 transcripts. Two days later, cells were incubated with NL4-3-IeG (X4) or JRFL-IeG (R5). One day later, supernatants were harvested and virus titers were determined by p24 ELISA. Numbers indicate the p24 values detected in the supernatants of scrambled siRNA-transfected cells. Experiments were performed at least three times with similar results. (*F*) Same aliquots of cells used in *E* were analyzed for mRNA expression of the indicated IFITM transcripts using qRT-PCR. Expression of the indicated IFITM mRNA in IFITM2 siRNA-transfected cells relative to expression of the indicated IFITM mRNA in scrambled siRNA transfected controls is shown. Error bars denote 1 SD ($n = 3$). *$P < 0.05$ compared with controls.

**The C Terminus of CCR5 Contributes to the Resistance of HIV-1 to Δ20 IFITM2-Mediated Restriction.** The resistance of R5 viruses raises the possibility that the coreceptor use is the major determinant for Δ20 IFITM2-mediated restriction. We initially examined the distribution of Δ20 IFITM2 and found that it colocalized with both CXCR4 and CCR5 at the plasma membrane (Fig. S9*A*). These findings imply that different sensitivities of HIV-1 strains to Δ20 IFITM2-mediated restriction may not be caused by the uneven distribution of Δ20 IFITM2. Subsequently, we included a CCR5 variant with double mutations in the G protein- and arrestin-binding sites ($CCR5^{DM}$) and two CCR5/CXCR4 chimeras ($CCR5^{CXCR4}$ and $CXCR4^{CCR5}$) by swapping their C termini in our experiments (27) (Fig. 4*A*). These CCR5 or CXCR4 variants did not affect expression of Δ20 IFITM2 (Fig. S9 *B* and *C*).

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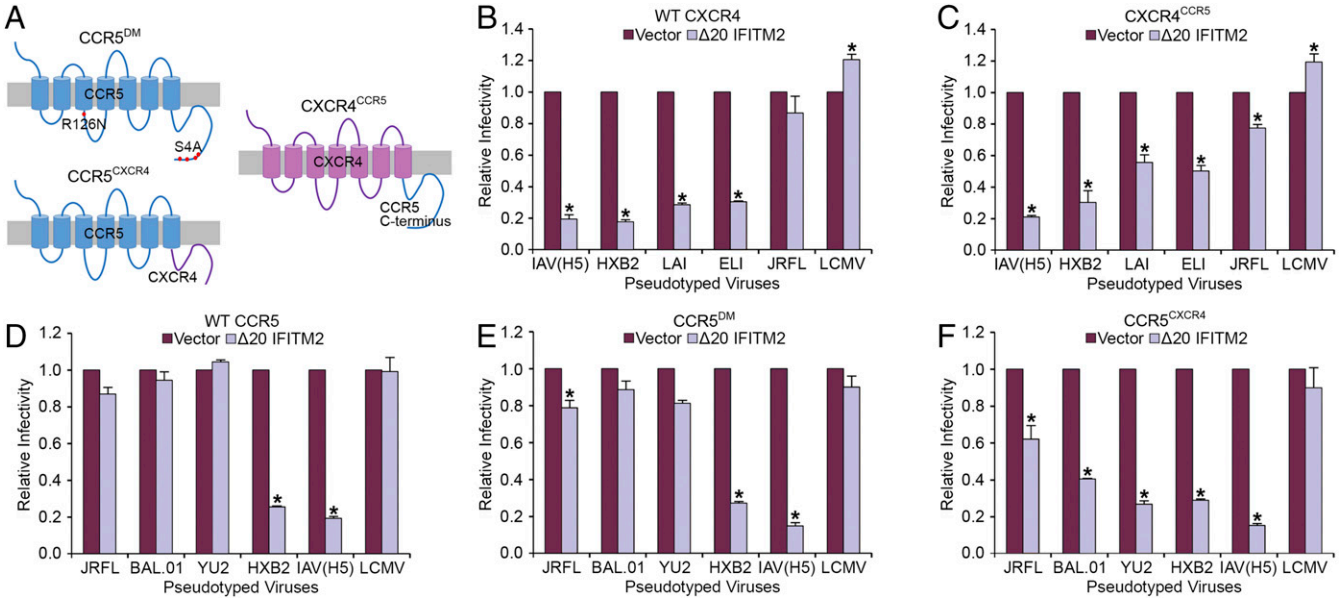


**Fig. 4.** C-terminal region of CCR5 contributes to the resistance of HIV-1 to Δ20 IFITM2-mediated restriction. (*A*) Schematic representation of the CCR5 variant, $CCR5^{DM}$, and CCR5/CXCR4 chimeras, $CCR5^{CXCR4}$ and $CXCR4^{CCR5}$, used in our experiments. Experiments were similar to the experiments in Fig. 3 *A* and *B* except that GHOST R5 cells expressing WT CXCR4 (*B*) or $CXCR4^{CCR5}$ (*C*) were used. Experiments were similar to the experiments in Fig. 3 *A* and *B* except that GHOST X4 cells expressing WT CCR5 (*D*), $CCR5^{DM}$ (*E*), or $CCR5^{CXCR4}$ (*F*) were used. Experiments were performed at least three times with similar results. Error bars denote 1 SD ($n = 3$). $^*P < 0.05$ compared with controls.

However, we observed that entry of R5 viruses became more sensitive to Δ20 IFITM2-mediated restriction in $CCR5^{CXCR4}$-expressing cells, but not in WT CCR5- or $CCR5^{DM}$-expressing cells (Fig. 4 *D*–*F*). In addition, partial restoration of X4 virus entry could be found in cells expressing $CXCR4^{CCR5}$ (Fig. 4 *B* and *C*). These data indicate that the C terminus of CCR5 is critical to the resistance of HIV-1 to Δ20 IFITM2-mediated inhibition. The lack of effect of $CCR5^{DM}$ on R5 virus entry further suggests that CCR5-mediated G protein or arrestin signaling may not interfere with the antiviral activity of Δ20 IFITM2.

## Discussion

Although X4 and R5 HIV-1 could be isolated from body fluids, R5 viruses are exclusively associated with primary HIV-1 infection (28). Several studies have demonstrated that mucin and innate immune peptides preferentially inactivate X4 viruses, that expression of CCR5 in innate immune cells enhances initial infection of R5 viruses, and that macrophages serving as a reservoir provide a steady stream of R5 viruses (29, 30). These mechanisms partially contribute to preferential selection of R5 over X4 viruses but are not sufficient to protect individuals against infection of X4 viruses at the early stage of the HIV-1 disease (31, 32). The multifactor gatekeeping model integrating recent observations has been proposed to explain restriction of mucosal transmission of X4-tropic viruses (31, 32). In our studies, we have demonstrated that Δ20 IFITM2 differentially restricts X4 and R5 HIV-1, indicating that Δ20 IFITM2 may play an important role in this gatekeeping mechanism. Unlike other secreted immune factors, Δ20 IFITM2 is endogenously expressed in HIV-1 target cells and prevents infection of X4 viruses by interfering with their entry. In contrast, the resistance of R5 HIV-1 to Δ20 IFITM2-mediated restriction allows acute-phase infection of R5 viruses. The emergence of X4 viruses could be observed in 50% of clade B HIV-1–infected individuals during their clinical courses (33, 34). Different cytokine expression profiles at the later stages of HIV-1 disease, which may alter expression of Δ20 IFITM2, and *IFITM2* polymorphisms, which may have attenuated antiviral activities, could be potential contributing factors. Subsequent studies are needed to clarify these issues.

CCR5/CXCR4 signaling triggered by association of HIV-1 has been appreciated for decades. Upon association with HIV-1 env proteins, G protein and arrestin are recruited or activated by CCR5/CXCR4 (35). Although the downstream effects of CCR5/CXCR4 signaling on cellular proliferation, migration, and gene expression are well studied, their functions in HIV-1 entry and replication are still being debated (35, 36). Mounting evidence has shown that CCR5/CXCR4 signaling may not play an important role in regulating HIV-1 entry. HIV-1 infection was not affected in cells expressing CCR5 or CXCR4 signaling-deficient variants (35, 36). Comparable to previous work, our research demonstrated that G protein- and arrestin-mediated CCR5 signaling has no effect on entry of R5 viruses in the presence or absence of Δ20 IFITM2. However, the attenuated resistance of R5 viruses to Δ20 IFITM2-mediated restriction in $CCR5^{CXCR4}$-expressing cells indicates that an uncharacterized signaling pathway or cellular mechanism triggered by the C terminus of CCR5 may impede the antiviral activity of Δ20 IFITM2. Furthermore, different sensitivities of R5 HIV-1 strains to Δ20 IFITM2-mediated restriction suggest that variations of interactions between HIV-1 env proteins and CCR5 may modulate the downstream effect of this mechanism. Our studies show that CCR5, in addition to serving as a membrane anchor for HIV-1, regulates HIV-1 entry by interfering with the antiviral activity of Δ20 IFITM2.

A recent report revealed that FL IFITM2 and IFITM3 preferentially restrict X4 and R5 viruses, but not founder viruses, during endosome-mediated HIV-1 entry. The importance of this pathway for HIV-1 infection in human primary cells is still being debated (8, 16, 17, 37). Many studies have demonstrated that membrane fusion of HIV-1 happens at the plasma membrane in $CD4^+$ T cells (20, 24, 25). We have also shown that expression of FL IFITM2 and IFITM3 was substantially lower than expression of Δ20 IFITM2 in HIV-1 target cells. Our findings, together with previous observations, suggest that Δ20 IFITM2 may be an important member of the IFITM family that regulates HIV-1 infection under physiologically relevant conditions. In our studies, we also found that entry of both X4 and R5 viruses could be efficiently suppressed by IFITM1, IFITM3, and Δ20 IFITM2 in U87 cells. However, the same phenotype could not be observed

in other cell types. Because endocytosis-mediated entry of HIV-1 has been documented in U87 cells (8), increased sensitivities of R5 viruses to IFITM-mediated restriction might be a result of the lack of expression of CCR5 cellular cofactors essential for interfering with the antiviral activity of IFITM proteins. Alternatively, the counteracting effect against Δ20 IFITM2 could be strongly attenuated when CCR5 is translocated to the endosomal compartments.

Everitt et al. (5) reported that *IFITM3* polymorphism rs12252-C is associated with the poor clinical outcome of H1N1 influenza. They found that Δ21 IFITM3 encoded by *IFITM3* rs12252-C loses its ability to restrict H1N1 IAV replication in vitro. However, a similar association could not be established in research analyzing more than 5,000 subjects in two separate cohorts (6). In addition, Δ21 IFITM3 has been shown to inhibit infection of IAV efficiently at adequate expression levels (10). Although we have demonstrated that both *IFITM3* rs12252-C and rs12252-T polymorphisms express the RNA transcript encoding FL IFITM3 in LCL cells, we could not exclude the possibility that Δ21 IFITM3 may be expressed in other organs or tissues. In our research, we have also revealed that Δ20 IFITM2 is highly expressed in innate and adaptive immune cells. Recently, the regulatory effects of IFITM3 on cytokine production and memory T-cell survival have been reported (7, 38). Further investigation into the functional role of Δ20 IFITM2 in immune reactions is warranted.

## Materials and Methods

**Viruses and Reagents.** Different strains of replicating HIV-1, molecular HIV-1 clones, and plasmids encoding env proteins from various X4 and R5 viruses were acquired from the NIH AIDS Reagent Program. IFN-β and PHA were obtained from R&D Systems and Sigma–Aldrich, respectively. For RNAi silencing, scrambled siRNA (CGUUAAUCGCGUAUAAUACGCGUAT; Origene) and siRNA targeting IFITM2 (CCAGGCCCAGCGAUAGAUCAGGAGG; Origene) were transfected into cells using RNAiMAX (Invitrogen) according to the manufacturer's instructions.

**HIV-1 Infection.** HIV-1 NL4-3, AD8, NL4-3-IeG, and JRFL-IeG were generated by transfecting 293T cells with HIV molecular clones, pNL4-3, pNL (AD8), pBR-43-IeG (NL4-3-IeG), or pBR-JRFL-IeG (JRFL-IeG), using the calcium phosphate transfection method (39). All viruses were harvested 2 d after transfection. Viral supernatants were filtered through 0.45 μM syringe filters (VWR). For primary cell infection, cells were incubated with 1 mL of viral supernatants and spinoculated at 4 °C, 4,000 × *g* for 30 min. Cells were washed three times with Dulbecco's PBS (DPBS) and then maintained in regular culture media. Viral supernatants or infected cells were collected 24 h after infection. Viral titers of supernatants were determined using a p24 ELISA kit according to the manufacturer's instructions (Advanced BioScience Laboratories). HIV-1 reverse transcripts in infected cells were assayed by real-time PCR. For HIV-1 infection in Jurkat E6-1 R5 cells, cells were inoculated with 100 ng of p24 of HIV-1. Two hours later, cells were washed three times with DPBS and then maintained in regular culture media. Viral supernatants were collected every 2–4 d and titered by p24 ELISA.

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