MAJOR ARTICLE

Interleukin-4–Transgenic hu-PBL-SCID Mice: A Model for the Screening of Antiviral Drugs and Immunotherapeutic Agents against X4 HIV-1 Viruses

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CXCR4-tropic (X4) human immunodeficiency virus type 1 (HIV-1) does not efficiently infect and replicate in severe combined immunodeficiency (SCID) mice reconstituted with human peripheral blood mononuclear cells, termed "hu-PBL-SCID mice," due to, at least in part, relatively low levels of expression of the CXCR4 coreceptor. To overcome this limitation, interleukin (IL)–4–transgenic hu-PBL-SCID mice were derived that spontaneously synthesized human IL-4, which has been shown to enhance CXCR4 expression and promote X4 virus infection in vitro. Experiments reported here show that (1) synthesis of human IL-4 in vivo augmented CXCR4 expression on human CD4$^+$ lymphocytes and importantly led to productive infection of not only X4 HIV-1_{NL4-3} but also multidrug-resistant primary clinical isolates and that (2) the in vivo infection could be significantly blocked by the administration of a CXCR4 antagonist. Altogether, IL-4–transgenic hu-PBL-SCID mice provide a useful model for X4 HIV-1 study and testing/screening of anti-X4 viral drugs.

HIV-1 isolates enter target cells primarily after binding to the CD4 receptor and via the CXCR4 and CCR5 coreceptors [1–5] and are classified into X4 and R5 strains, respectively [6]. The X4 isolates are frequently implicated in the decline of peripheral CD4$^+$ T cell counts characteristic of the late stage of HIV-1 infection proceeding to the development of AIDS [7].

hu-PBL-SCID mice have been extensively used as a small animal model to study HIV-1 pathogenesis [8–14]. Results from a previous study showed that, although infection of human peripheral blood mononuclear cell (PBMC)–reconstituted hu-PBL-SCID mice with a predominantly R5 HIV-1 caused intensive CD4$^+$ T cell depletion, infection of similarly reconstituted mice with the same infectious dose of an X4 HIV-1 resulted in little or no CD4$^+$ T cell depletion [11]. Thereafter, it was noted that this limitation of X4 HIV-1 infection was due, at least in part, to a decrease in the intensity of CXCR4 expression on CD4$^+$ T cells [13]. Thus, it was reasoned that the pathogenic effects of the X4 HIV-1 strains in the hu-PBL-SCID mice might be related to the relative levels of the expression of HIV-1 coreceptor (the state of activation/differentiation) on human CD4$^+$ T cells at the time of infection in these mice. This limitation has to date restricted our ability to use this mouse model for understanding the mechanisms of X4 HIV-1 pathogenesis and for the evaluation of candidate therapeutics against X4 viruses. These findings prompted us to seek alternative strategies for the development of an improved hu-PBL-SCID mouse system that is permissive for infection/replication of X4 isolates.

Human interleukin (IL)–4 has been shown to specifically enhance the cell-surface expression of CXCR4 on

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resting peripheral blood T cells [15]. Furthermore, it has been reported that human IL-4 plays an important role in rendering $CD4^+$ T cells susceptible to X4 HIV-1 infection via enhanced cell-surface expression of the CXCR4 coreceptor in vitro [15–17].

In efforts to overcome the limitation inherent with the use of hu-PBL-SCID mice for the study of X4 HIV-1 as described above, we developed an IL-4–transgenic immunodeficient mouse model that consistently secreted readily detectable serum levels of human IL-4. We show here that X4 isolates readily infect/replicate in this mouse model but not in wild-type (wt) non–IL-4–transgenic mice and that this model can now be exploited for the rapid evaluation of the therapeutic efficacy of new anti-X4 HIV-1 agents in vivo.

METHODS

IL-4–producing mice. Two strains of human IL-4–transgenic immunodeficient mice were bred on the C.B-17-*scid* [18] and BALB/cA-Rag2$^{-/-}\gamma_c^{-/-}$ (dKO) genetic background mice [19, 20] at the Central Institute for Experimental Animals (CIEA) as follows. PBMCs were isolated from a healthy human volunteer and activated in vitro with pokeweed mitogen. RNA was prepared from these PBMCs, and then cDNA was synthesized by reverse-transcriptase polymerase chain reaction (PCR). Human IL-4 cDNA was amplified from the cDNA using one set of primers: 5'-CCCGGGATCGTTAGCTTCTCCTGATAAAA-3' and 5'-GCGGCCGCTATTCAGCTCGAACACTTTGAAT-3'. The product was inserted into the PCR2.1 vector by use of the TA cloning kit (Invitrogen) and the insert sequenced. After confirmation of the sequence, IL-4 cDNA was inserted into pCMVb with a CMV promoter (Invitrogen). To produce transgenic mice, a DNA fragment containing the CMV promoter, IL-4 cDNA, and Poly(A) regions was excised with *Xho*I and *Hind*III sites of pCMVb and microinjected into the pronuclei of fertilized eggs from the 2 strains (C.B-17-*scid* and BALB/cA-dKO) of mice. These eggs were subsequently transplanted into oviducts of pseudopregnant foster recipient mice. The offspring mice were screened to confirm the insertion of the transgene into the genome by PCR, and serum from these mice was screened for levels of human IL-4 by ELISA with a commercial kit (BD). The IL-4 transgene–hemizygous mice were maintained by mating them with wt mice with the same genetic background in the specific-pathogen–free (SPF) facility of the CIEA. The mice were transferred to the SPF and biosafety level 3 facilities of the Institute for Animal Experiments, University of the Ryukyus, and were used for further experiments. The experimental protocols were approved by the Institutional Animal Care and Use Committee on the basis of the Regulation for Animal Experimentation of the CIEA and University of the Ryukyus before the initiation of the study.

Viruses. X4 HIV-1_{NL4-3} was obtained as described elsewhere [14]. Fourteen multidrug-resistant (MDR) HIV-1 clinical isolates were obtained from HIV-1–infected patients who had been treated with highly active antiretroviral therapy (HAART). The viruses were propagated in PBMCs stimulated with phytohemagglutinin (PHA; Sigma), IL-2 (National Institutes of Health AIDS Research and Reference Reagent Program), and IL-4 (Peprotec). Three isolates from these MDR isolates that efficiently grew in the activated cells were selected for further experiments. The titers of virus stocks were determined by end-point titration using a 2-fold limiting dilution of the stock and in vitro PHA-activated human PBMCs, and the infectious units (IU) were calculated.

CXCR4 antagonist. The synthesis and purification of the CXCR4 antagonist KRH-1636 were performed at Kureha Corporation as described elsewhere [21]. As a control, the carrier tartrate was used in parallel.

Transplantation and infection. The control (wt) and the IL-4–transgenic C.B-17-*scid* mice were depleted of NK cells by the intraperitoneal (ip) injection of 0.5–1.0 mg of anti–mouse IL-2R β (TMβ-1) [22] per animal. The IL-4–transgenic and the control BALB/cA-dKO mice do not require TMβ-1 treatment because they lack NK cells [19, 20]. PBMCs were isolated from healthy human donors. Groups of 2–4-month-old IL-4–transgenic mice from each of the 2 background strains and their corresponding non–IL-4–transgenic wt mice were injected ip with PBMCs 3 days later. Groups of mice were challenged 24 h later ip with mock, HIV-1_{NL4-3}, or MDR isolates (2000 IU/500 μL/animal). For the experiments using the CXCR4 antagonist, groups of mice were administered 0.1 mL of 10 mmol/L KRH-1636, the tartrate carrier or saline ip at 1 h before and 1 day after virus infection. At 6–8 days after infection, the mice were killed, their blood was obtained by cardiocentesis, and human lymphocytes were collected from the peritoneal lavage fluids. The serum samples were assayed for levels of human IL-4 by use of an ELISA kit (R&D Systems). The human lymphocytes were analyzed using flow cytometry as described below. The remaining cells were cultured in RPMI 1640 medium (Sigma) supplemented with fetal calf serum and IL-2. The peritoneal lavage fluids, serum samples, and lymphocyte culture supernatants were examined for levels of p24 by use of an ELISA kit (Zepto Metrix).

Flow cytometry analysis. Cell samples to be analyzed by flow cytometry were initially incubated with normal human IgG for blocking of the Fc receptors. For cell-surface staining, aliquots of cells were then stained with Cy5-labeled anti-CD4 (OKT4) and phycoerythrin-labeled anti-CXCR4 (12G5; Dako) or with Cy5-labeled anti-CD3 (OKT3). For intracellular staining, after CD3 staining the aliquots of cells were fixed, permeabilized, and incubated with fluorescein isothiocyanate–labeled anti–HIV-1 Gag p24 (2C2; Y.T. et al., unpublished data). Stained samples were analyzed on a FACSCalibur flow cytometer, using Cell Quest software (BD Pharmingen). Aliquots of cells stained

Table 1. Expression of human CD4, CXCR4, and intracellular HIV-1 p24 in cells from X4 HIV-1–infected hu-PBL-SCID mice.

Category	X4 HIV-1 infection	Mice, no.	$CD4^+$ T cells, %	*P*	$CXCR4^+CD4^+$ T cells, %	*P*	$p24^+$ T cells, %	*P*
C.B-17-*scid* mice								
Control	NL4-3	6	22.1 ± 8.3	<.001	45.2 ± 4.7	<.001	0.1 ± 0.1	NS
IL-4 transgenic	NL4-3	6	66.3 ± 9.0		65.5 ± 6.1		0.1 ± 0.1	
BALB/cA-dKO mice								
Control	NL4-3	5	35.9 ± 5.1	<.01	32.6 ± 1.4	<.001	0.2 ± 0.1	<.05
IL-4 transgenic	NL4-3	3	57.4 ± 8.3		68.2 ± 3.9		3.2 ± 1.2	

NOTE. Cells in peritoneal lavage fluid from control and interleukin (IL)–4–transgenic hu-PBL-SCID mice on either the C.B-17-*scid* or BALB/cA-dKO background were labeled with appropriate monoclonal antibodies and analyzed by flow cytometry, as described in Methods. Analyzed data are shown as mean ± SD values. NS, not significant. The indicated *P* values for the comparison of control vs. transgenic mice for each category are based on Student's *t* test.

with or without each of the antibodies described above were used as controls for the purposes of establishing gates and for the determination of the frequency of positive cells.

Statistical analysis. Data obtained by flow cytometry were analyzed by Student's *t* test with GraphPad Prism (version 4.0c for Mac OS X; GraphPad Software).

RESULTS

Production of human IL-4 in IL-4–transgenic mice. Efforts to construct the IL-4–transgenic mice constitutively synthesizing human IL-4 finally led to the establishment of mice on each of the 2 immunodeficient backgrounds expressing either high or low serum levels of human IL-4 (data not shown). On the basis of preliminary data obtained on the efficiency of virus replication, all subsequent experiments were done using only the 2 strains with high serum IL-4 expression levels. We assayed for levels of human IL-4 in the serum from the $IL\text{-}4^+$ hu-PBL-SCID mice and the wt hu-PBL-SCID mice on either the C.B-17-*scid* or BALB/cA-dKO background after infection with X4 HIV-$1_{NL4\text{-}3}$. Serum from each of the IL-4–transgenic mice on either background contained significant levels of human IL-4 (~800–1800 pg/mL), whereas serum from the control mice on the same background showed nondetectable levels of human IL-4. These data demonstrate that the human IL-4 synthesized by the IL-4–transgenic mice is generated from the transgene but not from the human PBMCs transplanted in these mice.

Effect of human IL-4 on the levels of human CXCR4 and CD4 expression by cells transplanted into mice. Since human IL-4 has been previously documented to enhance the expression of CXCR4 in vitro, experiments were done to examine the expression of human CXCR4 on transplanted $CD4^+$ cells in the peritoneal lavage fluids from HIV-$1_{NL4\text{-}3}$–infected $IL\text{-}4^+$ hu-PBL-SCID mice and control hu-PBL-SCID mice on either the C.B-17-*scid* or BALB/cA-dKO background. There did not appear to be any detectable difference in the absolute amounts of cells recovered from the peritoneal lavage fluids from the IL-4–positive or IL-4–negative hu-PBL-SCID mice on either background (data not shown). Flow cytometry analysis demonstrated that the frequency of human $CD4^+$ cells from the IL-4–transgenic C.B-17-*scid* or BALB/cA-dKO mice was significantly higher than that from the control mice (table 1). As expected, there was a marked increase in the frequency of CXCR4-expressing $CD4^+$ cells from the IL-4–transgenic mice on either genetic background relative to that from the control mice (figure 1*A* and table 1). Thus, these data indicate that human IL-4 produced endogenously is functional in vivo in terms of its ability to enhance human CXCR4 expression on $CD4^+$ cells transplanted into the mice.

Increased frequency of X4 HIV-1–infected cells from IL-4–transgenic hu-PBL-SCID mice. Since the constitutive synthesis of human IL-4 in IL-4–transgenic hu-PBL-SCID mice resulted in the enhanced expression of X4 HIV-1 receptors (human CXCR4/CD4) on the transplanted cells, we reasoned that such cells were likely to be more permissive to the infection and replication of X4 HIV-1. We thus challenged the IL-4–transgenic hu-PBL-SCID mice and control hu-PBL-SCID mice bred on the C.B-17-*scid* or BALB/cA-dKO mice with HIV-$1_{NL4\text{-}3}$. Cells obtained from the peritoneal lavage fluids were analyzed for cell-surface expression of human CD3 (since HIV-1 downmodulates CD4 expression) and the presence of intracellular p24. As seen in figure 1*B* and table 1, although very few if any $CD3^+$ cells from the control or IL-4–transgenic C.B-17-*scid* mice showed p24 expression, there was a >10-fold increase in the frequency of $CD3^+$ T cells that expressed p24 from the IL-4–transgenic BALB/cA-dKO mice relative to the control mice. These data suggest that, while transgene-induced human IL-4 increases the frequency of $CD4^+CXCR4^+$ T cells transplanted into both the C.B-17-*scid* and the BALB/cA-dKO mice, only the latter demonstrates increased sensitivity to X4 HIV-1 infection and replication, at least when this assay is used (see below).

High production of X4 HIV-1 in the culture supernatants of cells from IL-4–transgenic hu-PBL-SCID mice. In an effort

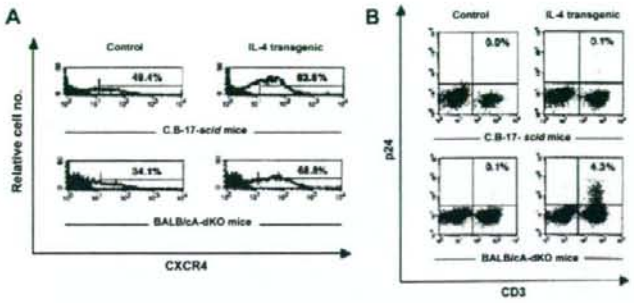

Figure 1. Enhancement of the expression of CXCR4 coreceptor and facilitation of X4 HIV-1 infection and replication in hu-PBL-SCID mice by in vivo production of human interleukin (IL)–4. Groups of hu-PBL-SCID mice, generated from IL-4–transgenic and nontransgenic (control) mice on either the C.B-17-*scid* or BALB/cA-dKO background, were injected intraperitoneally with HIV-1_{NL4-3} at ~24 h after peripheral blood mononuclear cell (PBMC) reconstitution. Six to eight days later, peritoneal lavage fluids were harvested from mice in each group, and cells were collected from the fluids by density-gradient centrifugation. *A*, Cells analyzed for the frequency and mean density of human CXCR4 expression on $CD4^+$ cells by flow cytometry. Data for analyzed cells are depicted by a thick line, and the background control profile is depicted by a thin line and gray shading. The nos. above the bars represent the percentage of positive cells. Data shown are representative of mice in each group from 3 independent experiments. *B*, HIV-1 infectivity. Cells were subjected to flow cytometry after cell-surface CD3 and intracellular p24 staining. Analyzed data are depicted as dot plots. The nos. in the graphs indicate the percentage of $CD3^+p24^+$ cells. Data displayed are representative of mice in each group from 3 independent experiments.

to determine the reason for our failure to detect levels of intracellular p24 in the IL-4–transgenic mice on the C.B-17-*scid* background and to further support the above finding, peritoneal lavage fluids were collected from mock- or HIV-1_{NL4-3}–infected IL-4^+ hu-PBL-SCID mice and, for purposes of control, the HIV-1_{NL4-3}–infected non–IL-4–transgenic mice on the C.B-17-*scid* background. The cells were isolated from the peritoneal lavage fluids, and an aliquot was analyzed for the frequency and the relative density of human CXCR4/CD4; the remaining aliquot was cultured in vitro. In addition, the peritoneal lavage fluids and the culture supernatants of cells at days 1–3 after culture were assayed for levels of p24 production. As displayed in table 2, although the frequency of $CXCR4^+CD4^+$ cells in the IL-4–transgenic mice was significantly higher than that in the nontransgenic mice, the mean fluorescence intensity (MFI) of CXCR4 expressed by the $CD4^+$ T cells from these mice was not increased compared with the control (because of an increase in the frequency of $CXCR4^+CD4^+$ cells with relatively low MFI; see figure 1*A*). Analysis of levels of synthesized p24 demonstrated marked differences, as shown in figure 2. Thus, although the amounts of p24 produced were modest in the peritoneal lavage fluids and the cell-culture supernatants from HIV-1–infected control mice, the levels of p24 produced by those from HIV-1–infected IL-4–transgenic mice were strikingly higher (15,429, 11,844, 1696, and 53 pg/mL in the supernatants on day 3) (mean, 48.9 vs. 7255 pg/mL; >100-fold increase). Although the levels of p24 produced by one of the IL-4–transgenic mice (mouse 12) were similar to those in the control mice, this was likely due to the much lower relative level of human IL-4 (354 pg/mL in serum) produced by mouse 12 than those from the other 3 IL-4–transgenic mice (4227, 6313, and 2356 pg/mL in serum). The present data not only document the fact that the cells from these

Table 2. Effect of the CXCR4 antagonist KRH-1636 on the expression of human CXCR4 by $CD4^+$ cells from X4 HIV-1–infected interleukin (IL)–4–transgenic hu-PBL-SCID mice.

C.B-17-*scid* mice	X4 HIV-1 Infection	CXCR4 antagonist	Mice, no.	$CXCR4^+CD4^+$ T cells, %	*P*	CXCR4 on $CD4^+$ T cells, MFI	*P*
Control	NL4-3	Mock	4	45.5 ± 9.3	<.05[a]	73.7 ± 36.0	NS[a]
IL-4 transgenic	NL4-3	Mock	4	66.7 ± 7.4	NS[b]	73.1 ± 6.2	<.05[b]
IL-4 transgenic	NL4-3	KRH-1636	4	63.0 ± 4.2	...	62.2 ± 4.8	...

NOTE. Control or IL-4–transgenic hu-PBL-SCID mice on the C.B-17-*scid* background infected with X4 HIV-1_{NL4-3} were administered mock KRH-1636 or real KRH-1636. Cells isolated from the peritoneal lavage fluid from the mice in each group were labeled with appropriate monoclonal antibodies and subjected to flow cytometry, as described in Methods. Data analyzed are displayed as mean ± SD values. MFI, mean fluorescence intensity; NS, not significant. The indicated *P* values are based on Student's *t* test.

[a] For the comparison between control mice and IL-4–transgenic mice that received a mock CXCR4 antagonist.

[b] For the comparison between IL-4–transgenic mice that received a mock CXCR4 antagonist and IL-4–transgenic mice that received KRH-1636.

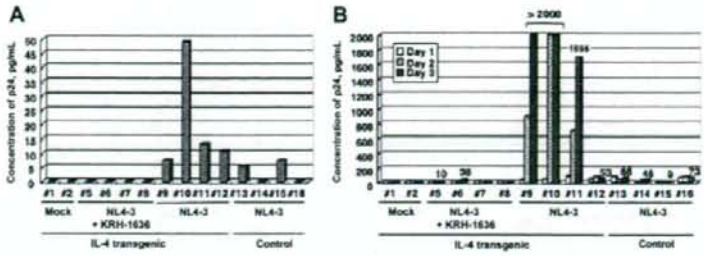

Figure 2. Efficient infection of interleukin (IL)–4–producing hu-PBL-SCID mice with X4 laboratory strain and an inhibitory effect of the CXCR4 antagonist KRH-1636 on infection. Twelve IL-4–transgenic and 4 nontransgenic (control) hu-PBL-SCID mice were generated on the C.B-17-*scid* background. Among them, 8 IL-4–transgenic and 4 control mice were infected intraperitoneally (ip) with the X4 laboratory strain (HIV-1_{NL4-3}) and 4 IL-4–transgenic mice were mock-infected at 1 day after engraftment. To evaluate the effect of KRH-1636, this drug was administered ip twice, at 1 h before and 1 day after infection of 4 IL-4–transgenic mice (NL4-3 + KRH-1636). At 8 days after infection, peritoneal lavage fluids were obtained from the mice in each group. Cells were isolated from the fluids and cultured in IL-2–containing medium. Levels of HIV-1 p24 in the peritoneal lavage fluids *(A)* and culture supernatants at days 1–3 after incubation *(B)* were quantitated for infectivity and replication efficiency by ELISA. With regard to the data on mock-infected mice, only 2 of 4 representative data are presented. The nos. listed above the bars in the graph indicate levels of HIV-1 p24 when it was detectable on day 3 (most of the values were <200 pg/mL, and select samples showed values of >2000 pg/mL). Pound signs (#) indicate mouse nos. Results shown are representative of 3 independent experiments.

IL-4–transgenic C.B-17-*scid* mice are susceptible but also demonstrate that the virus from such cells is replication competent. In addition, these findings suggest that the use of intracellular p24 levels is not a sensitive enough technique and that data using the intracellular p24 assay need to be carefully evaluated. These data also indicate that the IL-4–transgenic hu-PBL-SCID mice provide a powerful model for the study of X4 HIV-1 infection independently of the genetic background of the mice.

Inhibitory effect of the CXCR4 antagonist on infection of IL-4–transgenic hu-PBL-SCID mice with the X4 laboratory strain. In an effort to further validate that the CXCR4 coreceptor was indeed used by the X4 HIV-1 virus in the IL-4–transgenic hu-PBL-SCID mice, we used the X4 virus-entry inhibitor, CXCR4 antagonist KRH-1636. Thus, the IL-4–transgenic hu-PBL-SCID mice on the C.B-17-*scid* background were infected with X4 laboratory strain HIV-1_{NL4-3} and were either mock treated or treated with KRH-1636, and the peritoneal lavage fluids, cells in fluids, and cell-culture supernatants were examined as described above. As shown in table 2, the frequency of $CXCR4^+CD4^+$ cells in KRH-1636–treated IL-4–transgenic mice was marginally lower than that in mock-treated IL-4–transgenic mice. In addition, the MFI of CXCR4 expression by the $CD4^+$ T cells was clearly reduced by KRH-1636 administration. Importantly, treatment with KRH-1636 almost completely blocked X4 HIV-1 infection in these IL-4–transgenic mice (figure 2). These data indicate that X4 HIV-1 infection in transgenic mice is CXCR4 dependent and that our mouse model can be used to develop and test new anti-X4 HIV-1 drugs in vivo.

Therapeutic effect of KRH-1636 on the infection of IL-4–transgenic hu-PBL-SCID mice with MDR clinical isolates. The appearance of MDR HIV-1 clinical isolates has been and continues to be one of the growing problems in a significant number of patients receiving HAART and seriously limits the use of the antiviral drugs that are currently available. Thus, the development of novel adjunct or alternative therapeutics is an urgent need. Since treated patients tend to harbor significantly higher levels of either dual/mixed or X4 viruses [23] and since MDR isolates are not usually refractory to new treatment with drugs from classes that have not been used previously in patients from which the viruses were derived, we finally wanted to examine the effect of KRH-1636 on MDR HIV-1 infection in IL-4–transgenic hu-PBL-SCID mice. For this experiment, we used the IL-4–transgenic BALB/cA-dKO mice instead of the IL-4–transgenic C.B-17-*scid* mice, because the former seems more permissive to X4 HIV-1 infection than the latter, as described above. Before the in vivo study, we confirmed that the in vitro infection by 3 MDR clinical isolates could be inhibited with KRH-1636 (more than ~90% inhibition at the 5-μmol/L level). Thus, groups of IL-4–transgenic hu-PBL-SCID mice were infected with a mixture of these selected MDR isolates containing equal IU of each virus and treated with KRH-1636 or the tartrate carrier control. Thereafter, the cells obtained from the peritoneal lavage fluids were analyzed for the expression of cell-surface human CD4, CD3, and intracellular p24. The serum, peritoneal lavage fluids, and supernatants following in vitro culture of the cells for 24 h were assayed for levels of p24 production. Flow cytometry analysis after CD4 staining demonstrated a significant decline in $CD4^+$ T cells in 2 (mouse 7 and mouse 8) of 4 control-treated mice (figure 3*A*; top profile shows data from 1 of these 2 mice), which was likely due to MDR HIV-1 pathogenesis. However, importantly, no detectable depletion of $CD4^+$ T cells was observed in any of 4 KRH-1636–treated mice (figure 3*A*; bottom profile). As summarized in table 3, the difference in the frequency of $CD4^+$ T cells between the control-treated mice and the

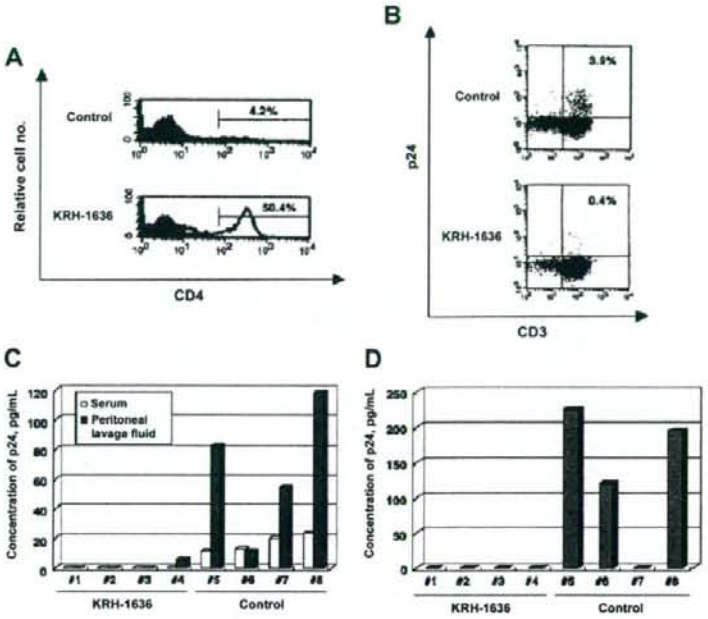

Figure 3. Prophylactic effect of KRH-1636 on infection and pathogenesis by multidrug-resistant (MDR) HIV-1 clinical isolates. Eight interleukin (IL)–4–transgenic hu-PBL-SCID mice (BALB/cA-dKO) were infected intraperitoneally (ip) with a mixture of MDR HIV-1 clinical isolates at 1 day after human peripheral blood mononuclear cell transfer. In an effort to assess the effect of KRH-1636 on HIV-1 infection, this agent or tartrate (control drug) was administered to 4 mice per group ip twice, at 1 h before infection and 1 day after infection. At 7 days after infection, serum and peritoneal lavage fluids were harvested from mice in each group, and cells were collected from the fluids. *A*, Cells examined for human CD4 expression by cell-surface staining and standard flow cytometry. Representative data from a single mouse from the control-treated or the KRH-1636–treated HIV-1–infected mice are shown. The frequency of $CD4^+$ T cells is depicted by a thick line, and the background control is depicted by a thin line with gray shading. The nos. above the bars indicate the percentage of positive cells. *B*, Aliquot of the peritoneal lavage cells analyzed by flow cytometry for the frequency of $CD3^+$ T cells that were positive for the intracellular presence of HIV-1 p24. Representative data of cells from the control-treated and the KRH-1636–treated HIV-1–infected mice are shown. The nos. in the graphs indicate the percentage of $CD3^+p24^+$ cells. *C*, Concentrations of p24 in serum and peritoneal lavage fluid. Concentrations were determined by ELISA to quantify MDR HIV-1 infection and replication efficiency. Pound signs (#) indicate mouse nos. *D*, Levels of in vitro p24 production. The remaining cells were cultured in a microtiter plate containing $IL\text{-}2^+$ medium for ~24 h, and the culture supernatants obtained were assayed for levels of in vitro p24 production by ELISA. Pound signs (#) indicate mouse nos. Results shown are representative of 3 similar independent experiments.

KRH-1636–treated mice was not significant. However, the MFI of CD4 expression was significantly decreased in the control-treated mice (229.3 vs. 296.3; $P < .05$). Results of CD3/p24 staining showed that the frequency of $CD3^+p24^+$ cells was markedly inhibited in the KRH-1636–treated mice, compared with that in the carrier-treated control mice (figure 3*B* and table 3). Furthermore, levels of HIV-1 p24 in the serum samples, peritoneal lavage fluids, and culture supernatants from the KRH-

Table 3. Effect of KRH-1636 on infection and pathogenesis by multidrug-resistant (MDR) HIV-1 clinical isolates in interleukin (IL)–4–transgenic hu-PBL-SCID mice.

BALB/cA-dKO mice	X4 HIV-1 infection	CXCR4 antagonist	Mice, no.	$CD4^+$ T cells, %	*P*	$CD4^+$ T cells, MFI	*P*	$p24^+$ T cells, %	*P*
IL-4 transgenic	MDR	Control	4	14.7 ± 11.9	NS	229.3 ± 33.0	<.05	3.2 ± 0.8	<.01
IL-4 transgenic	MDR	KRH-1636	4	31.3 ± 15.7		296.3 ± 25.2		0.8 ± 0.7	

NOTE. IL-4–transgenic hu-PBL-SCID mice on the BALB/cA-dKO background were infected with MDR HIV-1 clinical isolates and administered tartrate (control) or KRH-1636. Cells in peritoneal lavage fluid from the mice in each group were stained with appropriate monoclonal antibodies and analyzed by flow cytometry, as described in Methods. Data shown here are mean ± SD values. MFI, mean fluorescence intensity; NS, not significant. The indicated *P* values for the comparison between control mice and mice that received KRH-1636 are based on Student's *t* test.

1636–treated HIV-1–infected mice were almost completely reduced relative to those in the control mice (figure 3*C* and 3*D*). Note that the failure to detect the in vitro production of p24 in mouse 7 might result from depletion of $CD4^+$ T cells (figure 3*D*). These data demonstrate that the CXCR4 antagonist KRH-1636 has a marked degree of prophylactic effect on infection with pathogenic MDR clinical isolates in vivo.

DISCUSSION

Humanized mice that have served as valuable small animal models include the SCID-hu Thy/Liv mouse [24–28]. This mouse model, generated by implanting human hematopoietic tissues (human fetal thymus/liver) under the kidney capsule, has been used for the study of HIV-1 and is known for permissiveness to X4 HIV-1 infection [26–28]. However, the use of this model is limited by the fact that the implants are of human fetal organ origins that are not easily available. On the other hand, the hu-PBL-SCID mouse model provides another surrogate in vivo HIV-1 infection assay system. Although this model has led to a number of successful studies of HIV-1 [8–14], there was still a limitation in that it was difficult to demonstrate X4 HIV-1 infection and replication in such mice. Thus, to add extra value to the use of this mouse system for the study of HIV-1, in the present study we developed novel human IL-4–transgenic hu-PBL-SCID mice that enable CXCR4-using HIV-1 strains to efficiently infect and replicate in these mice.

Human IL-4 has low homology with murine IL-4 both at the gene and protein levels, accounting for the lack of cross-reactivity of this cytokine in the 2 species in vitro [29–32]. Results of the experiments reported here indicate that the high efficiency of X4 HIV-1 infection in the IL-4–transgenic hu-PBL-SCID mice was, at least in part, secondary to enhanced expression of viral receptors induced by human IL-4 synthesized endogenously. Interestingly, although there was no apparent increase in the number of cells recovered from the engrafted transgenic mice, there was a significant increase in the number of $CD4^+$ T cells recovered (1.5–3-fold). It is thus possible that the other cell lineages migrate from the peritoneal cavity to other tissues of the mice, resulting in enrichment of the $CD4^+$ T cell lineage. However, further studies of other tissues are needed to clarify this issue. Furthermore, our preliminary experiments indicate that the IL-4–transgenic hu-PBL-SCID mice remain permissive to R5 strain infection (data not shown).

In this report, we created 2 types of novel hu-PBL-SCID mice by transplanting human PBMCs into IL-4–transgenic C.B-17-*scid* and BALB/cA-dKO mice. The data obtained show that hu-PBL-SCID mice using the IL-4–producing BALB/cA-dKO mice appeared more permissive to X4 HIV-1 infection than did those using the IL-4–producing C.B-17-*scid* mice, at least as determined by the presence of intracellular p24. Although the reasons for this difference remain to be determined, it should be noted that, whereas the BALB/cA-dKO mice were derived by double mutation with defects in both the recombinase-activating gene 2 (Rag-2) and the gene encoding the γ_c chain of select cytokine receptors [19, 20], the C.B-17-*scid* mice have only the Rag-2 mutation [18]. Thus, although the Rag-2 mutation prevents the normal maturation of T and B lymphocytes, the γ_c chain mutation abrogates the expression of functional receptors for IL-2 and other cytokines, preventing the expansion of lymphocytes, including NK cells, which play an important role in the innate immune response such as nonspecific rejection of xenogeneic grafts. It is thus possible that the C.B-17-*scid* mice maintain a low but significant residual level of NK cell function, which may play a role in the difference noted above even though they were administered significant levels of anti–IL-2Rβ antibody. Since the BALB/cA-dKO mice are completely deficient in NK cell lineage and function, they are more immunodeficient than the C.B-17-*scid* mice, suggesting that the level and type of immunodeficiency in the BALB/cA-dKO mice may facilitate better engraftment and more efficient viral infection and propagation within these mice. These select defects of the BALB/cA-dKO mice might render the IL-4–transgenic mouse model on this background more valuable and ideal for studies of X4 HIV-1.

Acknowledgments

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SOCS1 is an inducible host factor during HIV-1 infection and regulates the intracellular trafficking and stability of HIV-1 Gag

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Human immunodeficiency virus type 1 (HIV-1) utilizes the macromolecular machinery of the infected host cell to produce progeny virus. The discovery of cellular factors that participate in HIV-1 replication pathways has provided further insight into the molecular basis of virus–host cell interactions. Here, we report that the suppressor of cytokine signaling 1 (SOCS1) is an inducible host factor during HIV-1 infection and regulates the late stages of the HIV-1 replication pathway. SOCS1 can directly bind to the matrix and nucleocapsid regions of the HIV-1 p55 Gag polyprotein and enhance its stability and trafficking, resulting in the efficient production of HIV-1 particles via an IFN signaling-independent mechanism. The depletion of SOCS1 by siRNA reduces both the targeted trafficking and assembly of HIV-1 Gag, resulting in its accumulation as perinuclear solid aggregates that are eventually subjected to lysosomal degradation. These results together indicate that SOCS1 is a crucial host factor that regulates the intracellular dynamism of HIV-1 Gag and could therefore be a potential new therapeutic target for AIDS and its related disorders.

AIDS | pathogenesis | drug target | lysozyme

Human immunodeficiency virus type 1 (HIV-1) infection is a multistep and multifactorial process mediated by a complex series of virus–host cell interactions (1, 2). The molecular interactions between host cell factors and HIV-1 are vital to our understanding of not only the nature of the resulting viral replication, but also the subsequent cytopathogenesis that occurs in the infected cells (3). The characterization of the genes in the host cells that are up- or down-regulated upon HIV-1 infection could therefore provide a further elucidation of virus–host cell interactions and identify putative molecular targets for the HIV-1 replication pathway (4).

The HIV-1 p55 Gag protein consists of four domains that are cleaved by the viral protease concomitantly with virus release. This action generates the mature Gag protein comprising the matrix (MA/p17), capsid (CA/p24), nucleocapsid (NC/p7), and p6 domains, in addition to two small spacer peptides, SP1 and SP2 (5, 6). The N-terminal portion of MA, which is myristoylated, facilitates the targeting of Gag to the plasma membrane (PM), whereas CA and NC promote Gag multimerization. p6 plays a central role in the release of HIV-1 particles from PM by interacting with the vacuolar sorting protein Tsg101 and AIP1/ALIX (7–9). Several recent studies have implicated the presence of host factors in the control of the intracellular trafficking of Gag. AP-3δ is a recently characterized endosomal adaptor protein that binds directly to the MA region of Gag and enhances its targeting to the multivesicular body (MVB) during the early stages of particle assembly (10). The *trans*-Golgi network (TGN)-associated protein hPOSH plays another role in Gag transport by facilitating the egress of Gag cargo vesicles from the TGN, where it assembles with envelope protein (Env) before transport to PM (11). Although the involvement of these host proteins in the regulation of intracellular Gag trafficking has been proposed, the detailed molecular mechanisms underlying this process are still not yet well characterized.

In our current work, we demonstrate that the suppressor of cytokine signaling 1 (SOCS1) directly binds HIV-1 Gag and facilitates the intracellular trafficking and stability of this protein, resulting in the efficient production of HIV-1 particles. These results indicate that SOCS1 is a crucial host factor for efficient HIV-1 production and could be an intriguing molecular target for future treatment of AIDS and related diseases.

Results

SOCS1 Is Induced upon HIV-1 Infection and Facilitates HIV-1 Replication via Posttranscriptional Mechanisms. We and others have shown that HIV-1 infection can alter cellular gene expression patterns, resulting in the modification of viral replication and impaired homeostasis in the host cells (4, 12). Hence, to elucidate further the genes and cellular pathways that participate in HIV-1 replication processes, we performed serial analysis of gene expression (SAGE) using either a HIV-1 or mock-infected human T cell line, MOLT-4 (12). Further detailed analysis of relatively low-abundance SAGE tags identified *SOCS1* as a preferentially up-regulated gene after HIV-1 infection. This finding was validated by both semiquantitative RT-PCR and immunoblotting analysis with anti-SOCS1 anti-

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The authors declare no conflict of interest.

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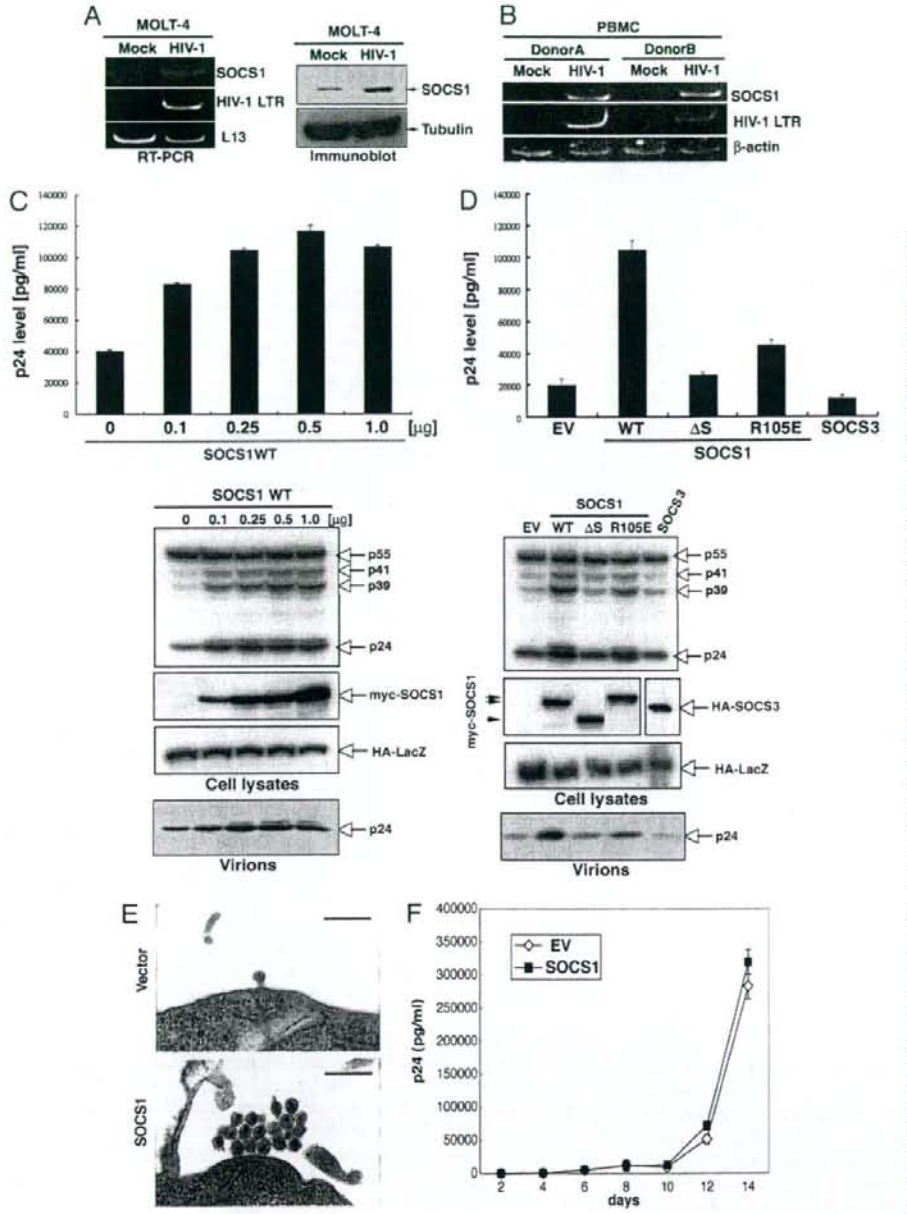

Fig. 1. SOCS1 is induced upon HIV-1 infection and enhances HIV-1 particle production. (*A*) MOLT-4 cells were mock-infected or infected with HIV-1_{NL4-3}, and then total RNA and protein extracts derived from these cells were subjected to semiquantitative RT-PCR (*Left*) and immunoblotting (*Right*), respectively. (*B*) PBMC from two healthy individuals were infected with HIV-1_{NL4-3} or were mock-infected, and SOCS1 expression was examined by semiquantitative RT-PCR. (*C*) 293T cells were transfected with pNL4-3 and cotransfected with various amounts of pcDNA-myc-SOCS1. Forty eight hours after transfection, p24 antigen release into the supernatant in each case was measured by antigen-capture ELISA (*Upper*), and the cell lysates and pelleted viruses were analyzed by immunoblotting (*Lower*). The data shown represent the mean ± SD from three independent experiments. HA-LacZ is a transfection control. (*D*) 293T cells were transfected with pNL4-3 and cotransfected with control vector, SOCS1 (WT), SOCS1ΔS (ΔSOCS box), SOCS1R105E, or SOCS3. Cell lysates and pelleted viruses were then collected after 48 h and subjected to ELISA (*Upper*) or immunoblotting (*Lower*), as described in *C*. (*E*) 293T cells cotransfected with either pNL4-3 plus control vector, or pNL4-3 plus myc-tagged SOCS1 were analyzed by TEM. Note that substantial numbers of mature virus particles can be observed in the myc-SOCS1-transfected cells. (Scale bars: 500 nm.) (*F*) Jurkat cells were infected with virions (adjusted by p24 levels) from either control vector (EV)- or SOCS1-transfected 293T cells. Supernatant p24 levels at the indicated time points were measured by ELISA.

bodies (Fig. 1*A*). In addition, *SOCS1* was found to be up-regulated also in peripheral blood mononuclear cells (PBMC) from two different individuals (following HIV infection, Fig. 1*B*).

Our initial findings that SOCS1 is induced upon HIV-1 infection prompted us to examine whether this gene product affects viral replication. We first cotransfected 293T cells with a HIV-1 infectious molecular clone, pNL4-3 (13), and also pcDNA-myc-SOCS1, and then monitored the virus production levels in the resulting supernatant. We then performed ELISA using an anti-p24 antibody and found that wild-type SOCS1 significantly increases the production of HIV-1 in the cell supernatant in a dose-dependent manner (Fig. 1*C Upper*). In contrast, neither the SH2 domain-defective mutant (R105E) nor the SOCS box deletion mutant (ΔS) of SOCS1 could promote virus production to the same levels as wild type, indicating that both domains are required for this enhancement (Fig. 1*D Upper*). Furthermore, another SOCS box protein, SOCS3, failed to augment HIV-1 replication in a parallel experiment (Fig. 1*D Upper*), indicating that the role of SOCS1 during HIV-1 replication is specific.

We next performed immunoblotting analysis using cell lysates and harvested virus particles in further parallel experiments (Fig. 1 *C* and *D Lower*). Consistent with our ELISA analysis, the expres-

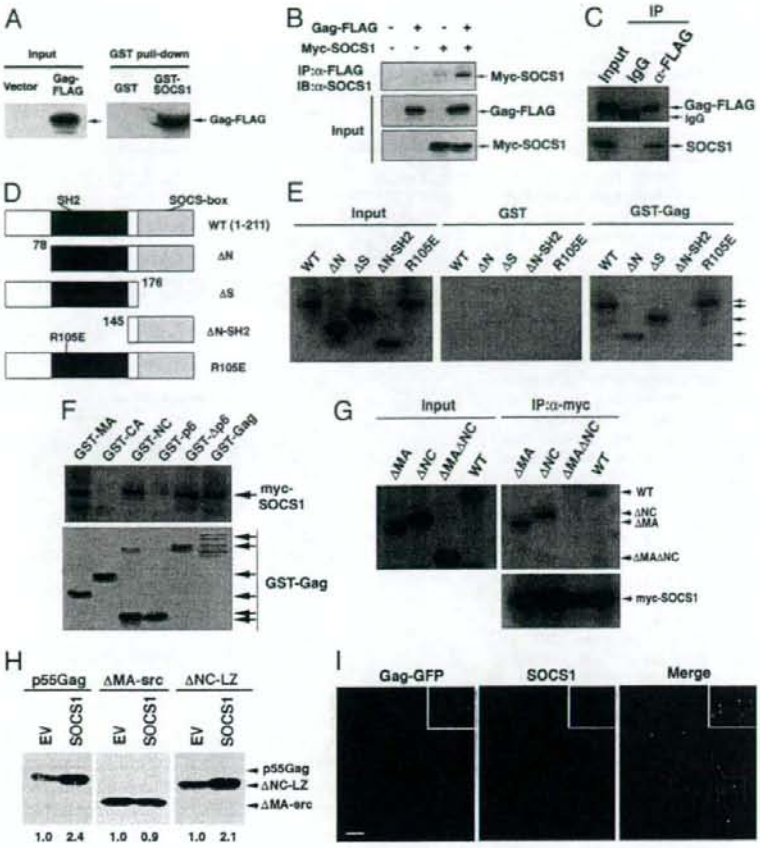

Fig. 2. SOCS1 interacts with HIV-1 Gag. (*A*) Extracts of 293T cells transfected with either empty vector or Gag-FLAG were subjected to pull-down analyses using glutathione–agarose beads with GST-SOCS1 in the presence of 10 ng/ml RNase followed by immunoblotting with anti-FLAG antibodies. (*B*) Extracts of 293T cells transiently expressing myc-SOCS1 and Gag-FLAG were subjected to immunoprecipitation (IP) with anti-FLAG monoclonal antibodies in the presence of 10 ng/ml RNase followed by immunoblotting (IB) analysis with either anti-FLAG or anti-myc polyclonal antibodies. (*C*) 293T cells were transiently transfected with Gag-FLAG, and cell lysates were then subjected to immunoprecipitation with anti-FLAG antibodies followed by immunoblotting with an antibody directed against endogenous SOCS1. (*D* and *E*) 293T cells expressing various myc-tagged SOCS1 mutants (schematically depicted in *D*) were analyzed by GST pull-down analysis with either GST or GST-Gag recombinant protein (*E*). (*F*) GST fusion proteins of the indicated regions of Gag were bound to glutathione beads and incubated with cell lysates from 293T cells expressing myc-SOCS1 in the presence of 10 ng/ml RNase followed by immunoblotting with anti-myc antibodies. (*G*) SOCS1 binds p55 Gag via either its MA or NC domains. 293T cells were transfected with myc-SOCS1 and cotransfected with Gag-FLAG, GagΔMA-FLAG, GagΔNC-FLAG, or GagΔMAΔNC-FLAG. At 24 h after transfection, cell lysates treated with 10 μg/ml RNase were subjected to coimmunoprecipitation with anti-myc monoclonal antibodies followed by immunoblotting with anti-FLAG or anti-myc polyclonal antibodies. (*H*) Functional interaction of SOCS1 with MA but not NC. 293T cells were transfected with wild-type Gag, ΔMA-src, or ΔNC-LZ (Z_{IL}-p6) and cotransfected with either control vector or SOCS1. Supernatant virus particles were then collected after 24 h and subjected to immunoblotting with anti-p24 antibody. Numerical values below the blots indicate fold induction of supernatant p55 signal intensities derived by densitometry. (*I*) Colocalization of SOCS1 with Gag. HeLa cells were transiently transfected with Gag-GFP. After 24 h, the cells were fixed, permeabilized, and immunostained with anti-SOCS1 polyclonal antibody followed by fluorescently labeled secondary antibodies before confocal microscopy. (Scale bar: 10 μm.)

sion of wild-type SOCS1, but neither its SH2 nor SOCS box mutant counterparts, resulted in a marked and dose-dependent increase in the level of intracellular Gag protein, particularly in the case of CA (p24) and intermediate cleavage products corresponding to MA-CA (p41) and CA-NC (p39). This increase was found to be accompanied by an enhanced level of HIV-1 particle production in the supernatant (Fig. 1 *C* and *D Lower*). These results together indicated that SOCS1 facilitates HIV-1 particle production in infected cells and that this role of SOCS1 requires the function of both its SH2 and SOCS box domains. For further details about SOCS1 interaction with MA and NC and SOCS1-enhanced particle production, see supporting information (SI) *Text*.

To examine the morphological aspects of HIV-1 particle production, transmission electron microscopy (TEM) was performed. 293T cells that had been cotransfected with pNL4-3, and either a control vector or a SOCS1 expression construct, were subjected to TEM analysis after fixation in glutaraldehyde. In SOCS1-transfected cells, a significantly increased number of mature virus particles was observed on the surfaces of PM compared with the control vector-transfected cells (Fig. 1*E*). There were also no obvious malformations of the virus particles in SOCS1-expressing cells, such as doublet formation or tethering to PM, which are characteristic of particle budding arrest (14) (Fig. 1*E*). Consistent with this observation, virions from SOCS1-transfected cells were found to be infectious as control viruses in Jurkat cells when the same amounts of virus were infected (Fig. 1*F*). These results together indicate that SOCS1 enhances mature and infectious HIV-1 particle formation.

To elucidate the specific step in HIV-1 production that is enhanced by SOCS1, we next performed gene reporter assays using either luciferase expression constructs under the control of wild-type HIV-LTR (pLTR-luc), or a full-length provirus vector (pNL4-3-luc) (15). Interestingly, SOCS1 overexpression was found not to affect the transcription of these reporter constructs (data not shown), indicating that SOCS1 enhances HIV-1 replication via posttranscriptional mechanisms during virus production.

SOCS1 Interacts with the HIV-1 Gag Protein. The results of our initial experiments indicated that SOCS1 enhances HIV-1 production via a posttranscriptional mechanism. We therefore next tested whether SOCS1 could bind directly to HIV-1 Gag. GST pull-down analysis using C-terminal FLAG-tagged p55 Gag (codon-optimized) and GST-fused SOCS1 revealed that p55 Gag undergoes specific coprecipitation with GST-SOCS1 (Fig. 2*A*). Furthermore, both ectopically expressed myc-tagged SOCS1 and endogenous SOCS1 were found to undergo coimmunoprecipitation with Gag-FLAG in 293T cells (Fig. 2 *B* and *C*). Additionally, GST pull-down analysis with various SOCS1 mutants, as depicted in Fig. 2*D*, further demonstrated that a mutant lacking the both N-terminal and SH2 domain (ΔN-SH2) could not bind

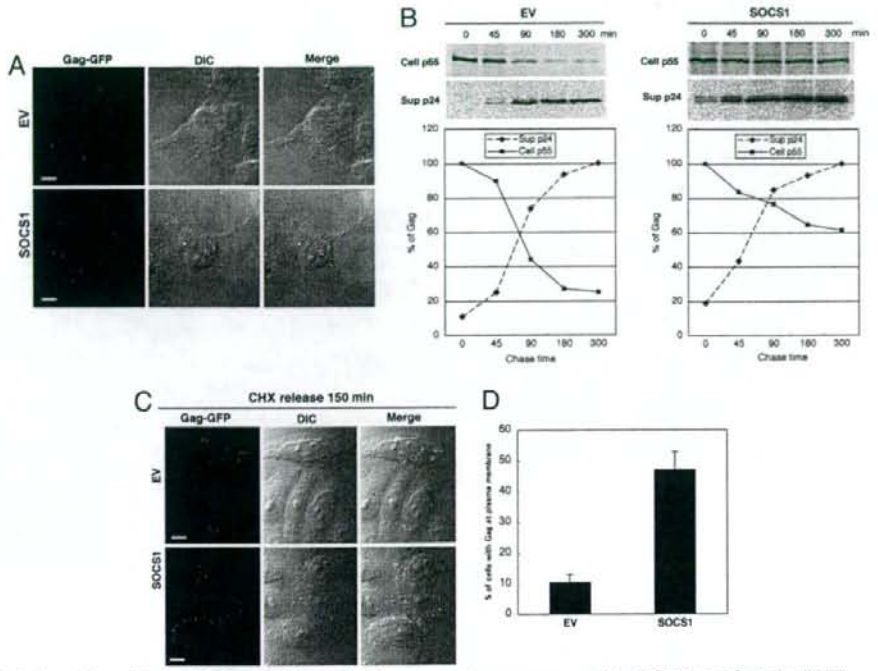

Fig. 3. SOCS1 enhances both the stability and trafficking of HIV-1 Gag. (*A*) HeLa cells cotransfected with pNL4-3 and either control vector (EV) or SOCS1 were immunostained with antibodies targeting anti-p24 (CA). Confocal microscopy with differential interference contrast (DIC) was then performed. (Scale bars: 10 μm.) (*B*) 293T cells were transfected with either a control empty vector (EV) (*Left*) or myc-SOCS1 (*Right*) and cotransfected with pNL4-3. After 48 h, cells were pulse-labeled with [^{35}S]methionine or [^{35}S]cysteine for 15 min and chased for the durations indicated. Cell lysates and pelleted supernatant virions were immunoprecipitated with anti-p24 antibodies followed by autoradiography. (*C* and *D*) HeLa cells seeded on poly-L-lysine-coated cover slides were transfected with either vector control or SOCS1. After 24 h, cells were again transfected with Gag-GFP for 3 h and then treated with 100 μg/ml CHX for 5 h to inhibit protein synthesis. This treatment was followed by incubation with fresh medium; then 150 min after the CHX release, cells were fixed and subjected to confocal microscopy (*C*). (Scale bars: 10 μm.) Cells with Gag protein on the plasma membrane were scored out of 200 transfected cells (*D*).

p55 Gag, whereas an N-terminal or a SOCS box deletion did not affect the binding of SOCS1 to Gag in 293T cells (Fig. 2*E*). This finding indicates that the SH2 domain is important for the interaction of SOCS1 with HIV-1 Gag. Interestingly, the R105E mutant of SOCS1, which disrupts the function of the SH2 domain, still binds Gag (Fig. 2*E*), indicating that the Gag–SOCS1 association is independent of the tyrosine phosphorylation of Gag, as is the case for both HPV-E7 and Vav (16, 17).

To elucidate the SOCS1-binding region of the Gag protein, GST pull-downs with various GST-fused Gag domain constructs were performed. SOCS1 was detected in glutathione bead precipitates with GST-wild-type Gag, GST-Δp6, GST-MA, and GST-NC, but not with other domain constructs (Fig. 2*F*), indicating that SOCS1 interacts with Gag via its MA and NC domains. Consistent with these results, the deletion of both the MA and NC domains of p55 Gag (ΔMAΔNC) completely abolishes its interaction with SOCS1 in coimmunoprecipitation experiments (Fig. 2*G*). Furthermore, *in vitro* analysis with purified proteins also demonstrated that SOCS1 can indeed interact with both the MA and NC regions of HIV-1 Gag in the absence of nucleic acids or other proteins (SI Fig. 5).

We next wished to determine the functional interaction domain in HIV-1 Gag through which SOCS1 functions in terms of virus-like particle production. To this end, we used a MA-deleted Gag mutant with an N-terminal myristoyl tag derived from src (ΔMA-src) (18) and also an NC-deleted Gag mutant with a GCN4 leucine zipper in place of NC, which we herein denote as ΔNC-LZ but which has been described as Z_{IL}-p6 (19). Both of these mutants have been shown still to assemble and bud (18, 19). We found that SOCS1 overexpression can still augment the particle formation of both wild-type Gag and ΔNC-LZ but not ΔMA-src (Fig. 2*H*), indicating that the functional interaction between SOCS1 and HIV-1 Gag is in fact mediated through MA.

To confirm further the direct interaction between SOCS1 and Gag in cells, we examined the intracellular localization of these two proteins. Confocal microscopy revealed that endogenous SOCS1 forms dotted filamentous structures in the cytoplasm and that Gag localizes in a very punctate pattern with SOCS1 from the perinuclear regions to the cell periphery (Fig. 2*I*). These data indicate that SOCS1 interacts with HIV-1 Gag in the cytoplasm during HIV-1 particle production.

SOCS1 Promotes both the Stability of Gag and Its Targeting to the Plasma Membrane. Because we had found from our initial data that SOCS1 increases HIV-1 particle production as a result of its direct interaction with intracellular Gag proteins, we next addressed whether SOCS1 positively regulates Gag stability and subsequent trafficking to PM. Our immunofluorescent analysis with the anti-p24 (CA) antibody initially revealed that SOCS1 overexpression increases the levels of Gag at PM when cotransfected with pNL4-3 at 48 h after transfection, although it was detected at PM in both control and SOCS1-expressing cells (Fig. 3*A*). Furthermore, the levels of cytoplasmic Gag were found to be much lower in the SOCS1-expressing cells compared with the control cells (Fig. 3*A*). These results indicate that SOCS1 enhances Gag trafficking to PM.

To examine next whether SOCS1 affects the stability and trafficking of newly synthesized Gag proteins, we performed pulse–chase analysis. This experiment revealed that SOCS1 significantly increases the stability of the intracellular p55 Gag polyprotein as well as the levels of p24 in the supernatant (Fig. 3*B*). Importantly, p24 was detectable at an earlier time point and reached maximum levels in a shorter period in the cell supernatant of SOCS1-transfected cells compared with control vector-transfected cells (Fig. 3*B*). This finding again suggests that SOCS1 facilitates the intracellular trafficking of newly synthesized Gag proteins to PM.

To confirm this hypothesis further, we performed cycloheximide (CHX) analysis with HeLa cells transfected using either vector control or SOCS1. After 24 h, cells were again transfected with Gag-GFP for 3 h and treated with CHX for 5 h to inhibit protein synthesis. Cells were then cultured in fresh medium without CHX for an additional 150 min and subjected to confocal microscopy. At

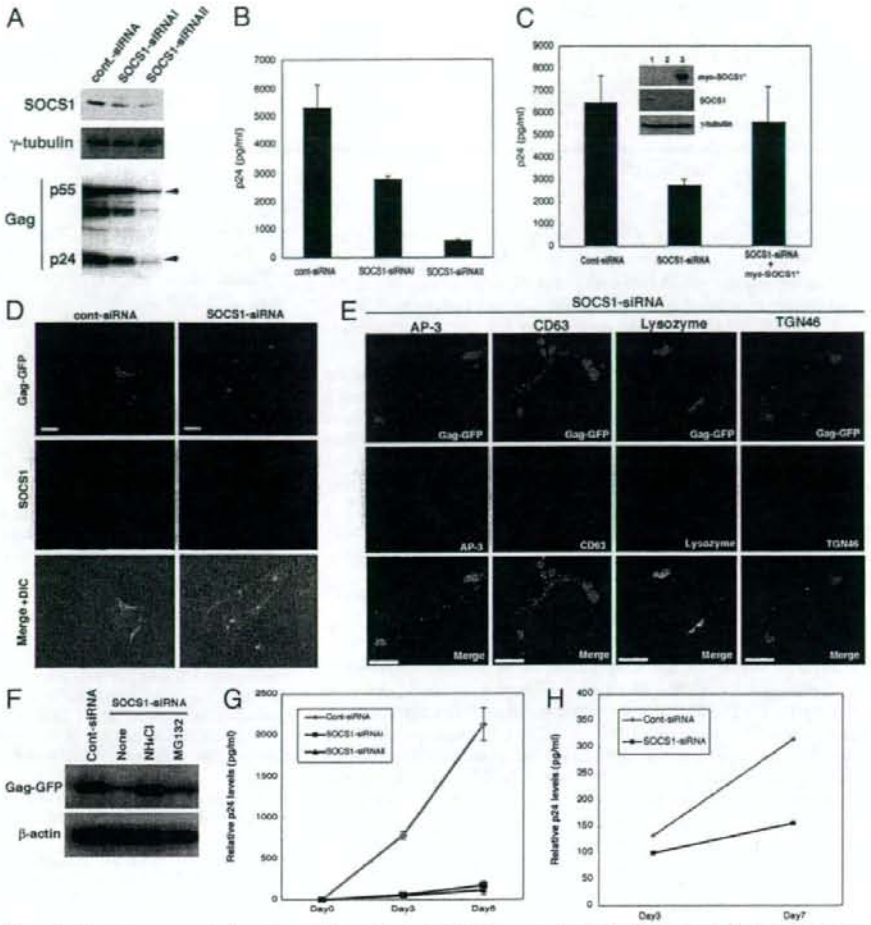

Fig. 4. The targeted inhibition of SOCS1 suppresses Gag trafficking and HIV-1 particle production and enhances Gag degradation in lysosomes. (*A* and *B*) 293T cells were transfected with either control siRNA or two different SOCS1-specific siRNAs (I or II) together with pNL4-3. At 48 h after transfection, cell lysates were subjected to immunoblotting analysis with the indicated antibodies (*A*). Cell supernatants were then subjected to ELISA analysis of p24 levels (*B*). (*C*) 293T cells were transfected with pNL4-3 and cotransfected with control-siRNA, SOCS1-siRNAI alone, or SOCS1-siRNAI plus siRNA-resistant myc-SOCS1 (myc-SOCS1*). After 48 h, cell supernatants were collected and subjected to p24 ELISA. (*Inset*) Immunoblots of the cell lysates. (*D*) HeLa cells were transfected with control or SOCS1-specific siRNA and cotransfected with GFP-Gag. At 48 h after transfection, the cells were subjected to confocal microscopy. (*E*) HeLa cells were transfected with Gag-GFP and SOCS1-siRNA constructs for 48 h. Cells were then fixed and subjected to immunofluorescent analysis with indicated antibodies followed by DAPI staining. (Scale bars: 10 mm.) (*F*) HeLa cells were transfected with Gag-GFP and cotransfected with either control-siRNA or SOCS1-siRNA. After 36 h, the cells were treated with a mock solution, 10 mM NH_4Cl or 10 μM MG132 for another 16 h. Cell were then harvested and subjected to immunoblotting analysis with anti-GFP or anti-β-actin antibodies. (*G*) Jurkat cells were infected with a retroviral vector encoding control (Cont) or two different SOCS1-specific siRNAs (I or II). After selection with puromycin, the cells were then infected with HIV-1_{NL4-3} (multiplicity of infection, 0.1), and p24 antigen levels in cell supernatant were measured by ELISA at the indicated time points. (*H*) Human primary CD4 T cells were separated from healthy donors and infected with lentivirus vectors encoding either control- or SOCS1-siRNAI. The cells were then infected with HIV-1_{NL4-3} (multiplicity of infection, 0.1), and p24 antigen levels in cell supernatant were measured by ELISA at the indicated time points.

this time point, Gag-GFP was found to localize predominantly in a perinuclear region in the control cells (Fig. 3*C*), whereas almost half of the SOCS1-transfected cells exhibited Gag-GFP localization on PM (Fig. 3*D*). These results again indicate that SOCS1 efficiently enhances the trafficking of newly synthesized Gag protein to PM.

The Targeted Disruption of SOCS1 Inhibits Gag Trafficking and HIV-1 Particle Production. To delineate further the role of SOCS1 in the trafficking of Gag and in subsequent HIV-1 particle production, we depleted cellular SOCS1 by siRNA. The significant depletion of SOCS1 expression by two different SOCS1-specific siRNA constructs was confirmed by immunoblotting analysis (Fig. 4 *A* and *B*). Significantly, in cells cotransfected with pNL4-3 and SOCS1-specific siRNAs, both HIV-1 particle release and the levels of intracellular Gag protein are significantly decreased compared with the control cells (Fig. 4 *A* and *B*). Furthermore, the effects of SOCS1-siRNA on the inhibition of HIV-1 particle production was diminished by reexpression with a codon-optimized SOCS1 construct that is resistant to these siRNAs (Fig. 4*C*), indicating that the SOCS1 siRNA suppression of HIV-1 particle production depends on the availability of endogenous SOCS1.

Consistent with these observations, immunofluorescent analysis further revealed that the expression of SOCS1-siRNA dramatically inhibits Gag trafficking such that Gag proteins accumulate in the perinuclear regions as large solid aggregates, as has been reported (20) (Fig. 4*D*). This finding indicates that SOCS1 plays an essential role in the Gag trafficking from perinuclear clusters to PM. Interestingly, these discrete perinuclear clusters of Gag were found to colocalize with lysosome markers, lysozyme, and partly with AP-3, but neither with the late endosome MVB marker CD63 nor the *trans*-Golgi marker TGN46, indicating that Gag is targeted for degradation by lysosomes when the function of SOCS1 is inhibited (Fig. 4*E*). In support of this notion, the levels of intracellular Gag were found to be significantly increased by treatment with a lysosome inhibitor NH_4Cl but not by a proteasome inhibitor MG132 in SOCS1-siRNA cells (Fig. 4*F*), further indicating that the perinuclear clusters of Gag will undergo lysosomal degradation rather than proteasomal degradation when optimal Gag transport to PM is suppressed by the inhibition of SOCS1.

We next addressed whether targeted SOCS1 inhibition would affect HIV-1 particle production in human T cells. The effect of SOCS1 depletion was clearly evident in both HIV-1_{NL4-3}-infected

Jurkat cells and human primary $CD4^+$ T cells, which demonstrated pronounced decreases in virus particle production in SOCS1-siRNA-expressed cells compared with the controls (Fig. 4 *G* and *H*). These results together indicate that the specific inhibition of SOCS1 suppresses the optimal trafficking of Gag to PM, resulting in the degradation of Gag in lysosomes, which in turn leads to the efficient and reproducible inhibition of HIV-1 particle production in various types of human cells.

Discussion

In this work, we report that SOCS1 is an inducible host factor during HIV-1 infection and plays a key role in the late stages of the viral replication pathway via an IFN-independent mechanism (SI Fig. 6). These results represent evidence that SOCS1 is a potent host factor that facilitates HIV-1 particle production via posttranscriptional mechanisms.

SOCS1 has been shown to be a suppressor of several cytokine signaling pathways, and like all SOCS family members it has a central SH2 domain and a conserved C-terminal domain known as the SOCS box (21, 22). Structure–function analyses have further demonstrated that the SOCS1 SH2 domain is required for the efficient binding of its substrates (23, 24). Indeed, our current analyses have also revealed that the SH2 domain of SOCS1 is required for its interaction with the HIV-1 Gag protein. We have shown from our present data that the SOCS box is also required for SOCS1 to function during HIV-1 particle production.

The SOCS box-mediated function of SOCS1 is chiefly exerted via its ubiquitin ligase activity (21, 25). Biochemical binding studies have shown that the SOCS box of SOCS1 interacts with the elongin BC complex, a component of the ubiquitin/proteasome pathway that forms an E3 ligase with Cul2 (or Cul5) and Rbx-1 (21, 26, 27). We show from our current experiments that the SOCS box is required for HIV-1 particle production, indicating the involvement of the ubiquitin/proteasome pathway. However, it is still unknown whether SOCS1 promotes the ubiquitination of Gag and, if so, whether the mono- or poly-ubiquitination of Gag would affect its trafficking and protein stability. Further studies will be necessary to clarify the biological significance of Gag ubiquitination.

Perlman and Resh (20) recently reported that newly synthesized Gag first appears to be diffusely distributed in the cytoplasm, accumulates in perinuclear clusters, passes transiently through a MVB-like compartment, and then traffics to PM. Consistent with these observations, our current work also shows that Gag is accumulated at perinuclear clusters as solid aggregates when its targeting to PM is impaired because of the SOCS1 inhibition.

Another aspect of SOCS1 function during HIV-1 infection was proposed recently. Song *et al.* (28) reported that SOCS1-silenced dendritic cells broadly induce the enhancement of HIV-1 Env-specific $CD8^+$ cytotoxic T lymphocytes and $CD4^+$ T helper cells as well as an antibody response. The induction of the SOCS1 gene in HIV-1 infected cells might therefore disrupt a specific intracellular immune response to HIV-1 in infected host cells.

Based on the strong evidence that we present in our current work that SOCS1 positively regulates the late stages of HIV replication, we conclude that SOCS1 is likely to be a valuable therapeutic target not only for future treatments of AIDS and related diseases, but also for a postexposure prophylaxis against disease in HIV-1-infected individuals.

Materials and Methods

Antibodies and Fluorescent Reagents. Antibodies and fluorescent reagents were obtained from the following sources. Anti-CD63, anti-AP-3, anti-myc (A-14), and anti-SOCS1 (H-93) were from Santa Cruz Biotechnology. Anti-SOCS1 was from Zymed Laboratories. Anti-FLAG (M2) and anti-HA (12CA5) were from Sigma and Roche Diagnostics, respectively. Anti-HIV-p24 (Dako; Cytomation), anti-STAT1, and anti-phospho-STAT1 (Y701) were from BD Transduction Laboratories. Sheep polyclonal anti-TGN46 was from GeneTex.

Plasmid Constructs. Expression constructs for SOCS1 have been described in ref. 29. GST fusion constructs with specific regions derived from the codon-optimized *gag* were generated (MA, CA, NC, p6, Δp6, full-length Gag) by cloning into pGEX-2T (GE Healthcare Bio-Sciences) as described in ref. 30. For retrovirus-mediated siRNA expression, pSUPER.retro.puro vector was digested, as described in ref. 31, with the following sequences: SOCS1-siRNAI, TCGAGCTGCTGGAGCACTA; SOCS1-siRNAII, GGCCAGAACCTTCCTCCTCTT; control siRNA, TCGTATGTTGTGTGGAATT.

Electron Microscopy. Transfected 293T cells were fixed with 2.5% glutaraldehyde and subjected to TEM, as described (14, 32).

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Transmission of Simian Immunodeficiency Virus Carrying Multiple Cytotoxic T-Lymphocyte Escape Mutations with Diminished Replicative Ability Can Result in AIDS Progression in Rhesus Macaques[▽]

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Cytotoxic T-lymphocyte (CTL) responses frequently select for immunodeficiency virus mutations that result in escape from CTL recognition with viral fitness costs. The replication in vivo of such viruses carrying not single but multiple escape mutations in the absence of the CTL pressure has remained undetermined. Here, we have examined the replication of simian immunodeficiency virus (SIV) with five *gag* mutations selected in a macaque possessing the major histocompatibility complex haplotype *90-120-Ia* after its transmission into *90-120-Ia*-negative macaques. Our results showed that even such a "crippled" SIV infection can result in persistent viral replication, multiple reversions, and AIDS progression.

Virus-specific CD8$^+$ cytotoxic T-lymphocyte (CTL) responses exert a suppressive effect on human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV) replication (1, 10, 15, 21, 27). Under the CTL pressure, viral mutations resulting in viral escape from CTL recognition are frequently selected for, with viral fitness costs (2, 5, 8, 9, 12, 16, 19, 20, 24, 25, 26, 28). The transmission of the virus carrying a CTL escape mutation with lower viral fitness between major histocompatibility complex class I (MHC-I)-mismatched individuals can result in reversion of the mutation due to the absence of the CTL pressure (7, 14, 16, 17, 18). Such CTL escape mutations and their reversions have been suggested to be involved in viral evolution (3, 11, 13, 23).

We have developed a prophylactic vaccine using a Sendai virus vector expressing SIVmac239 Gag and shown its protective efficacy against SIVmac239 challenge in a group of Burmese rhesus macaques (*Macaca mulatta*) possessing MHC-I haplotype *90-120-Ia* (20). In these vaccinated macaques that are controlling SIVmac239 replication, $Gag_{206-216}$ epitope-specific CTL responses exerted strong selective pressure on the virus, and rapid selection of a mutant escaping from this CTL was observed at week 5 postchallenge. The virus, SIVmac 239Gag216S, with this CTL escape mutation, GagL216S, leading to a substitution from leucine (L) to serine (S) at amino acid (aa) 216 in Gag showed lower replicative ability than the wild type (14, 20). Two of these vaccinees (macaques V3 and V5) showed an accumulation of additional viral CTL escape mutations in *gag* during the period of viral control and then the reappearance of plasma viremia around week 60 after SIVmac239 challenge (12). The SIV carrying these multiple CTL escape mutations showed lower replicative ability in vitro than the SIV carrying the single GagL216S mutation.

How such viruses with multiple CTL escape mutations replicate and evolve in the absence of the CTL pressure has not yet been well determined, while the reversion of CTL escape mutations has previously been shown by the transmission of viruses with single escape mutations (7, 14, 18). In the present study, we have examined the replication, in the absence of the CTL pressure in *90-120-Ia*-negative macaques, of the SIV with multiple *gag* CTL escape mutations that were accumulated in a *90-120-Ia*-positive macaque.

The induction of $Gag_{206-216}$-specific CTL, $Gag_{241-249}$-specific CTL, and $Gag_{373-380}$-specific CTL responses has previously been observed after SIVmac239 challenge in *90-120-Ia*-positive macaques (12). The *90-120-Ia*-positive vaccinees V5 and V3 showed rapid selection of the GagL216S mutation ($Gag_{206-216}$ CTL escape mutation) and then of an additional two mutations resulting in escape from $Gag_{241-249}$-specific CTL and $Gag_{373-380}$-specific CTL recognition, respectively, during the period of viral control. These were a $Gag_{241-249}$ CTL escape mutation leading to a GagD244E (aspartic acid [D] to glutamic acid [E] at aa 244 in Gag) substitution and a $Gag_{373-380}$ CTL escape mutation leading to GagA373T (alanine [A] to threonine [T] at aa 373) in vaccinee V5 or GagV375A (valine [V] to A at aa 375) or GagP376S (proline [P] to S at aa 376) in vaccinee V3. Viruses at the reappearance of viremia had one or two additional mutations in *gag*, GagI247 L (isoleucine [I] to L at aa 247) and GagA312V (A to V at aa

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312) in vaccinee V5 or GagP172S (P to S at aa 172) or GagV145A (V to A at aa 145) in vaccinee V3. All of these mutations except for the $Gag_{373-380}$ CTL escape mutations resulted in amino acid changes in the Gag CA. We constructed molecular clones of SIVs with these *gag* mutations (12). The SIVs with three CTL escape mutations ($Gag_{206-216}$, $Gag_{241-249}$, and $Gag_{373-380}$ CTL escape mutations) were referred to as group Q SIV mutants, and the SIVs with four or five *gag* mutations selected at the reappearance of viremia as group R SIV mutants. These group Q and R SIV mutants both showed lower replicative ability in vitro than SIVmac239Gag216S, while in the competition assay between groups Q and R, the viral replicative ability was not significantly affected by the GagP172S or GagV145A mutation but was reduced by the addition of the GagI247L and GagA312V mutations (12). These results do not support the possibility of compensation for loss of viral fitness from these mutations (4, 6). In the present study, we have examined the in vivo replication of the SIV carrying five *gag* mutations, GagL216S, GagD244E, GagI247L, GagA312V, and GagA373T, selected in macaque V5 at the reappearance of viremia, which was assumed to show the lowest replicative ability among group Q and R SIV mutants. The macaques were maintained in accordance with the guidelines for animal experiments performed at the National Institute of Infectious Diseases (22).

We first compared the in vivo replication abilities of the SIV with a single GagL216S mutation and the SIVs with multiple CTL escape mutations in *90-120-Ia*-negative macaques (Fig. 1). In the competition between SIVmac239Gag216S and group Q SIV mutants, macaque R02-017 was coinoculated intramuscularly with molecular-clone DNAs of SIVmac239Gag216S and SIVmac239Gag216S244E373T and macaque R05-002 with molecular-clone DNAs of SIVmac239Gag216S and all three group Q SIV mutants. The results of the analysis of plasma viral *gag* genome sequences (Fig. 2) showed selection of SIV mac239Gag216S; i.e., all the mutations other than GagL216S became undetectable in 3 weeks postinoculation, indicating lower replicative abilities in vivo of group Q SIV mutants than of SIVmac239Gag216S, as indicated previously by in vitro competition (12). Further analysis revealed reversion of the selected GagL216S mutation to the wild-type sequence in a few months.

In the competition between SIVmac239Gag216S and group R SIV mutants, macaque R02-023, coinoculated with molecular clone DNAs of SIVmac239Gag216S and SIVmac239Gag 216S244E247L312V373T, showed selection of the former (Fig. 2). This macaque was euthanized at week 6 before exhibiting reversion of the GagL216S mutation. In macaque R02-022, coinoculated with molecular clone DNAs of SIVmac239Gag 216S and all three group R SIV mutants, almost all mutations other than GagL216S became undetectable rapidly but the GagV145A mutation was detected even at week 14. The GagL216S mutation was still dominant without reversion at week 14, and plasma viremia became undetectable after week 14 in this macaque. Both cases indicated a lower replicative ability in vivo of SIVmac239Gag216S244E247L312V373T than of SIVmac239Gag216S.

Additionally, macaque R03-022, coinoculated with the molecular-clone DNAs of SIVmac239Gag216S244E373T and SIV mac239Gag216S244E247L312V373T, showed selection of the

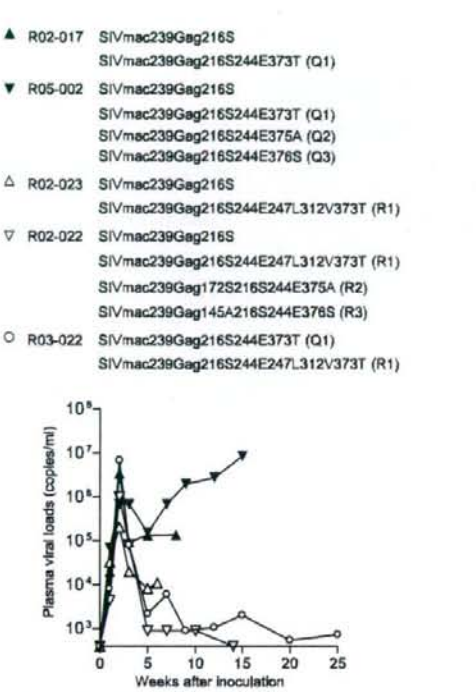

FIG. 1. Plasma viral loads of macaques used for in vivo competition assay (SIV *gag* RNA copies/ml plasma) after inoculation with SIV molecular-clone DNAs. Animals received 10 mg in total of DNAs consisting of an equal amount of each DNA; i.e., macaques R02-017, R02-023, and R03-022 were inoculated with 5 mg of each DNA, and macaques R05-002 and R02-022 with 2.5 mg of each DNA. Plasma viral loads were determined as described previously (20).

former (Fig. 2), indicating a lower replicative ability in vivo of SIVmac239Gag216S244E247L312V373T than of SIVmac239 Gag216S244E373T. In this macaque, reversion of the GagL216S mutation was observed in 6 months, while the GagD244E and GagA373T mutations were still dominant without reversion.

Next, we inoculated *90-120-Ia*-negative macaques with the SIV carrying multiple *gag* CTL escape mutations that was selected in *90-120-Ia*-positive macaque V5 (Fig. 3). The SIV carrying five gag mutations, GagL216S, GagD244E, GagI247L, GagA312V, and GagA373T, that was dominant at the reappearance of viremia in macaque V5, was propagated on rhesus macaque peripheral blood mononuclear cells to prepare the SIVmac239Gag216S244E247L312V373T challenge stock for macaques R05-001 and R06-016. Sequencing analysis confirmed no *gag* mutation except for the five mutations in the challenge virus. These two macaques were challenged intravenously with 1,000 50% tissue culture infective dose of SIVmac 239Gag216S244E247L312V373T. Both of them showed persistent viremia, although the levels of set-point plasma viral loads were low in macaque R06-016. Macaque R05-001, maintaining high viral loads, showed typical signs of AIDS, such as a reduction in peripheral $CD4^+$ T-cell counts, diarrhea, and general weakness, and was euthanized approximately 2 years postchallenge. Autopsy revealed postpersistent generalized

Macaque R02-017 inoculated with molecular clones of
SIVmac239 Gag 216S & SIVmac239 Gag 216S 244E 373T

Wks	aa sequences in Gag		
p-c	**216th**	244th	373rd
1	Mt	Wt(mt)	Wt(mt)
3	Mt	**Wt**	**Wt**
8	**Wt**	**Wt**	**Wt**

Macaque R05-002 inoculated with molecular clones of
SIVmac239 Gag 216S & SIVmac239 Gag 216S 244E 373T
& SIVmac239 Gag 216S 244E 375A & SIVmac239 Gag 216S 244E 376S

Wks	aa sequences in Gag				
p-c	**216th**	244th	373rd	375th	376th
1	Mt	wt/mt	**Wt**(mt)	**Wt**(mt)	**Wt**
3	Mt	**Wt**	**Wt**	**Wt**	**Wt**
12	**Wt**	**Wt**	**Wt**	**Wt**	**Wt**

Macaque R02-023 inoculated with molecular clones of
SIVmac239 Gag 216S & SIVmac239 Gag 216S 244E 247L 312V 373T

Wks	aa sequences in Gag				
p-c	**216th**	244th	247th	312th	373rd
1	Mt	**Wt**	**Wt**	**Wt**	**Wt**(mt)
3	Mt	**Wt**	**Wt**	**Wt**	**Wt**(mt)
6	Mt	**Wt**	**Wt**	**Wt**	**Wt**

Macaque R02-022 inoculated with molecular clones of
SIVmac239 Gag 216S & SIVmac239 Gag 216S 244E 247L 312V 373T
& SIVmac239 Gag 172S 216S 244E 375A & SIVmac239 Gag 145A 216S 244E 376S

Wks	aa sequences in Gag								
p-c	145th	172nd	**216th**	244th	247th	312th	373rd	375th	376th
1	**Wt**(mt)	wt/mt	Mt	wt/mt	**Wt**(mt)	**Wt**(mt)	**Wt**	wt/mt	**Wt**(mt)
3	wt/mt	**Wt**	Mt	wt/mt	**Wt**	**Wt**	**Wt**	**Wt**(mt)	wt/mt
14	wt/mt	**Wt**	Mt	**Wt**	**Wt**	**Wt**	**Wt**	**Wt**	**Wt**

Macaque R03-022 inoculated with molecular clones of
SIVmac239 Gag 216S 244E 373T & SIVmac239 Gag 216S 244E 247L 312V 373T

Wks	aa sequences in Gag				
p-c	**216th**	**244th**	247th	312th	**373rd**
1	Mt	Mt	**Wt**	**Wt**	Mt
3	Mt	Mt	**Wt**	**Wt**	Mt
20	wt/mt	Mt	**Wt**	**Wt**	Mt
25	**Wt**	Mt	**Wt**	**Wt**	Mt

FIG. 2. Dominant viral genome sequences in competition assay. A *gag* DNA fragment was amplified from plasma RNA by reverse transcription and nested PCR and sequenced as described previously (20). The amino acid sequences at the positions where mutations were included in the inoculums are shown. Q and R groups of SIV mutants are described in the text. **Wt**, only the wild-type sequence was detected; **Wt**(mt), the wild-type sequence was dominant but the mutant was detectable (the mutant/wild-type ratio was less than 1/4); wt/mt, the wild type and the mutant were detected equally; Mt(wt), the mutant was dominant but the wild type was detectable (the wild-type/mutant ratio was less than 1/4); Mt, only the mutant was detected. Other than the residues indicated in this figure, no dominant mutation resulting in an amino acid change was detected in the *gag* region in macaque R02-017, R05-002, R02-023, or R02-022, but macaque R03-022 showed one amino acid change resulting in a GagV375M substitution at weeks 20 and 25. p-c, postchallenge.

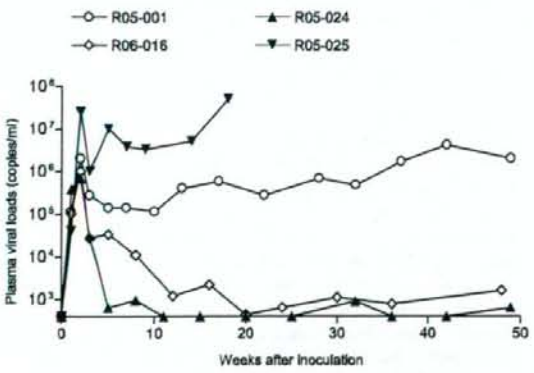

FIG. 3. Plasma viral loads (SIV *gag* RNA copies/ml plasma) in macaques after challenge with SIV carrying five *gag* mutations.

lymphadenopathy conditions and pneumocystis pneumonia. This macaque showed reversion of the GagD244E mutation in a few months, followed by reversion of the GagL216S, GagI247 L, and GagA312V mutations in a year postchallenge, while the GagA373T mutation remained dominant without reversion until euthanasia (Fig. 4). In contrast, macaque R06-016, with lower viral loads, showed no reversion of the five mutations. In the chronic phase, these two macaques showed additional Gag amino acid changes, including GagI140V (I to V at aa 140) and GagV375M (V to methionine [M] at aa 375) that were detected in both. Some of these mutations may contribute to the recovery of viral fitness.

To see the possibility of transmission of the viruses carrying the five gag mutations in the context of the polyclonal, V5-derived SIVs, macaques R05-024 and R05-025 were inoculated with plasma obtained from macaque V5 in the chronic phase of SIVmac239 infection (Fig. 3). For the challenge, plasma was obtained from macaque V5 at weeks 81, 87, 92, 100, and 113

Macaque R05-001 infected with SIVmac239Gag216S244E247L312V373T

Wks	aa sequences in Gag					
p-c	216th	244th	247th	312th	373rd	other residues
1	Mt	Mt	Mt	Mt	Mt	
3	Mt	Mt	Mt	Mt	Mt	
10	Mt	**Wt**	Mt	Mt	Mt	I140V
22	wt/mt	**Wt**	Mt	Mt	Mt	I140V
37	wt/mt	**Wt**	Mt	Mt	Mt	V3A, I140V
42	**Wt**(mt)	**Wt**	Mt	Mt	Mt	V3A, (V68L/M), I140V
49	**Wt**	**Wt**	wt/mt	wt/mt	Mt	V3A, (V68L/M), I140V
55	**Wt**	**Wt**	**Wt**	**Wt**	Mt	V3A, (V68L/M), I140V, (V340M), D429N
86	**Wt**	**Wt**	**Wt**	**Wt**	Mt	V3A, (V68L/M), I140V, D429N
110	**Wt**	**Wt**	**Wt**	**Wt**	Mt	V3A, (V68L/M), (I140V), V375M, (D429E)

Macaque R06-016 infected with SIVmac239Gag216S244E247L312V373T

Wks	aa sequences in Gag					
p-c	216th	244th	247th	312th	373rd	other residues
5	Mt	Mt	Mt	Mt	Mt	
12	Mt	Mt	Mt	Mt	Mt	
30	Mt	Mt	Mt	Mt	Mt	S128P, I140V, V375M
54	Mt	Mt	Mt	Mt	Mt	I140V, V375M

Macaque R05-024 infected with V5-plasma

Wks	aa sequences in Gag										
p-c	3rd	68th	145th	216th	244th	247th	312th	373rd	390th	404th	other residues
1	Mt(wt)	wt/mt	Mt	Mt	Mt	Mt	Mt	Mt	wt/mt	wt/mt	
5	Mt(wt)	wt/mt	Mt	Mt	Mt	Mt	Mt	Mt	wt/mt	wt/mt	I257K
49	Mt	Mt	Mt	Mt	Mt	**Wt**	**Wt**	**Wt**	**Wt**	Mt	A222V, I257K, R485K

Macaque R05-025 infected with V5-plasma

Wks	aa sequences in Gag										
p-c	3rd	68th	145th	216th	244th	247th	312th	373rd	390th	404th	other residues
1	Mt	wt/mt	Mt	Mt	Mt	Mt	Mt	Mt	wt/mt	wt/mt	
5	Mt	wt/mt	Mt	Mt	Mt	**Wt**(mt)	**Wt**(mt)	Mt	wt/mt	wt/mt	
7	Mt	wt/mt	Mt	Mt	Mt	**Wt**	**Wt**	Mt	wt/mt	wt/mt	
14	Mt	wt/mt	Mt	Mt	Mt	**Wt**	**Wt**	Mt	wt/mt	wt/mt	
18	Mt	**Wt**(mt)	Mt	Mt	Mt	**Wt**	**Wt**	Mt	wt/mt	wt/mt	

FIG. 4. Dominant viral genome sequences after challenge with SIV carrying five *gag* mutations. The amino acid sequences at the residues where mutations were included in the inoculums and dominant amino acid changes at other residues in *gag* are shown. In the column of other residues, the predominant mutations with detectable wild-type sequence are shown in parentheses. **Wt**, **Wt**(mt), wt/mt, Mt(wt), Mt, and p-c are defined in the Fig. 2 legend.

post-SIVmac239 challenge and 0.2 ml of each was intravenously inoculated into these two macaques. In the challenge SIV plasma, the five *gag* mutations (GagL216S, GagD244E, GagI247L, GagA312V, and GagA373T) and GagV145A were dominant, and additional *gag* mutations were detected in the MA- and NC-coding regions. In macaque R05-024, exhibiting low viral loads, the SIV GagL216S and GagD244E mutations remained dominant, while reversion of the GagI247L, GagA312V, and GagA373T mutations was observed (Fig. 4). Macaque R05-025, exhibiting high viral loads, developed AIDS and was euthanized at week 18 postchallenge. Autopsy revealed lymphoatrophy and cytomegalovirus infection. This macaque showed rapid reversion of the SIV GagI247L and GagA312V mutations but maintained the GagL216S, GagD 244E, and GagA373T mutations until euthanasia.

In samples from these four macaques challenged with SIV mac239Gag216S244E247L312V373T or V5-derived plasma, we examined the virus-specific $CD8^+$ T-cell responses around 3 months postinfection by flow cytometric analysis of antigen-specific gamma interferon induction (data not shown) as described previously (14, 20). Analyses using vesicular stomatitis virus G-pseudotyped SIV-infected cells as a stimulator revealed SIV-specific $CD8^+$ T-cell responses in macaques R05-001, R06-016, and R05-024, but not in macaque R05-025, which may have contributed to the rapid AIDS progression in this animal. Macaque R05-024, exhibiting lower viral loads and rapid selection of a *gag* mutation resulting in an I257K (I to lysine [K] at aa 257) substitution, showed $CD8^+$ T-cell responses specific for the $Gag_{245-269}$ peptide mixture (a mixture of $Gag_{245-260}$, $Gag_{250-265}$, and $Gag_{255-269}$ peptides), suggesting a possibility of this mutation for viral escape from strong CTL pressure. None of these four macaques showed $CD8^+$ T-cell responses specific for the $Gag_{206-225}$ (a mixture of $Gag_{206-220}$ and $Gag_{210-225}$ peptides), $Gag_{206-225}$.216S ($Gag_{206-220}$.216S and $Gag_{210-225}$.216S), $Gag_{232-255}$ ($Gag_{232-246}$, $Gag_{236-250}$, and $Gag_{240-255}$), $Gag_{232-255}$.244E, $Gag_{236-255}$.244E247L, $Gag_{362-385}$ ($Gag_{362-377}$, $Gag_{367-381}$, and $Gag_{371-385}$), or $Gag_{362-385}$.373T peptide mixture, indicating that CTL responses were not involved in the reversion or nonreversion at residue 216, 244, 247, or 373 in these macaques.

The in vivo competition assay in the present study showed loss of viral fitness from the addition of the GagD244E and GagA373T mutations into SIVmac239Gag216S and further loss of viral fitness from additional GagI247L and GagA312V mutations. The reversion of GagD244E in macaque R05-001, GagA373T in macaque R05-024, and GagI247L and GagA312V in macaques R05-024 and R05-025 (Fig. 4) supports this notion. However, reversion was not observed in all the mutations after challenge with SIV carrying the five *gag* mutations. Challenge with SIVmac239Gag216S carrying the single GagL216S mutation has shown its reversion in 3 months (14), whereas the reversion of the GagL216S mutation was

delayed or not observed after challenge with the SIV carrying five *gag* mutations. This may be due to the predominant selection of the reversion of other mutations or to lower viral replication efficiency in the latter case. Compensatory mutations can also be involved in this delay or nonreversion, but no additional *gag* mutation was observed in the early phase in macaque R06-016. The possibility of a contribution to this delay by GagI140V in macaque R05-001 and GagV145A in macaques R05-024 and R05-025 may be considered, while significant recovery of viral fitness by the latter mutation has not been observed (12).

It has been suggested that a reduction in viral fitness by CTL escape mutations may contribute to HIV/SIV control (19, 20, 28). Pressure by multiple epitope-specific CTLs may result in the selection of HIV/SIV with diminished replicative ability because of accumulating multiple escape mutations. The inefficient viral replication in macaques R02-022 and R03-022 (Fig. 1) and two of four macaques in the second experiment (Fig. 3) may reflect such a lower replicative ability of the mutant SIVs, but conversely, the results of the present study also showed efficient viral replication in macaques R05-001 and R05-025, indicating that the transmission of even such "crippled" HIV/SIV carrying multiple CTL escape mutations can result in persistent viral replication and AIDS progression. It remains unclear what host factors determined the viral replication efficiency in vivo in our study, while macaques with higher viral loads (R02-017, R05-002, R05-001, and R05-025) showed the first reversion earlier than those with lower viral loads (R02-022, R03-022, R06-016, and R05-024), suggesting an association of reversion with viral loads. Earlier reversion may result in the recovery of viral fitness, leading to higher viral loads, or conversely, higher viral loads may accelerate reversion.

Thus, our results suggest that in the transmission of HIV accumulating CTL escape mutations at the cost of viral fitness between MHC-mismatched individuals, even such crippled HIV infection can finally result in AIDS progression. Previous studies on SIVs with single CTL escape mutations showed their rapid reversion, but the present study on SIV with multiple CTL escape mutations indicates that the reversion of all the mutations was not required for the establishment of persistent viral replication or for the onset of disease. Furthermore, it suggests a possibility that CTL escape mutations resulting in viral fitness costs may not always revert rapidly even in the absence of CTL pressure after their transmission into MHC-mismatched hosts and can be transmitted further to other hosts. These results provide an important insight into HIV pathogenicity and evolution in human individuals with divergent MHC polymorphisms.

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