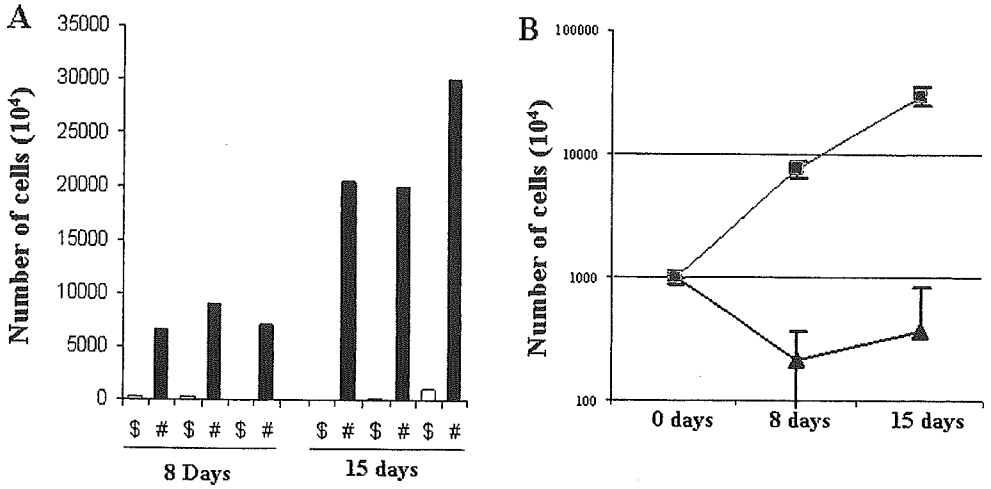


FIG. 2. Comparison of ED-40515(−) cell growth in NOG and NOD-SCID mice. To evaluate the in vivo growth pattern of tumor cells in SCID mice, we inoculated the ED-40515(−) cell line ($10^7$) in both NOD-SCID and NOG mice. (A) Tumor cells obtained from mice on day 8 and 15 were counted by the trypan blue method. Open and black bars represent the number of cells in individual NOD-SCID ($) and NOG (#) mice, respectively. (B) Mean results ± standard error (error bars) from three mice of individual strains on day 8 and 15 (the squares and triangles represent NOG and NOD-SCID mice, respectively).

ED-40515(−) cell line were examined. In vitro cultured ED-40515(−) and SLB-1 cell lines expressed both human CD4 and CD25 abundantly (data not shown). A higher level of IL-2 receptor α (IL-2Rα) (CD25) expression was observed on the surface of the malignant cells associated with all stages of ATL (41–43). Immunohistochemical staining indicated that in vivo tumor ED-40515(−) cells also expressed human CD4 and CD25 (Fig. 1D and E). In addition, we stained in vitro and in vivo samples to evaluate the protein expression of CD8 and CD3. Both in vitro and in vivo samples showed that tumor cells were positive for CD3, but not CD8 (data not shown). We also examined the expression of HTLV-1 viral antigen Tax and Gag (p19) by immunohistochemical staining and Western blot. Cells of the tumor mass formed after inoculation with SLB-1 cell line were apparently positive for the Tax and Gag antigens, whereas those after inoculation of the ED-40515(−) cell line were negative for the Tax and Gag antigens (Fig. 3A and B). These results indicated that there was no obvious change in

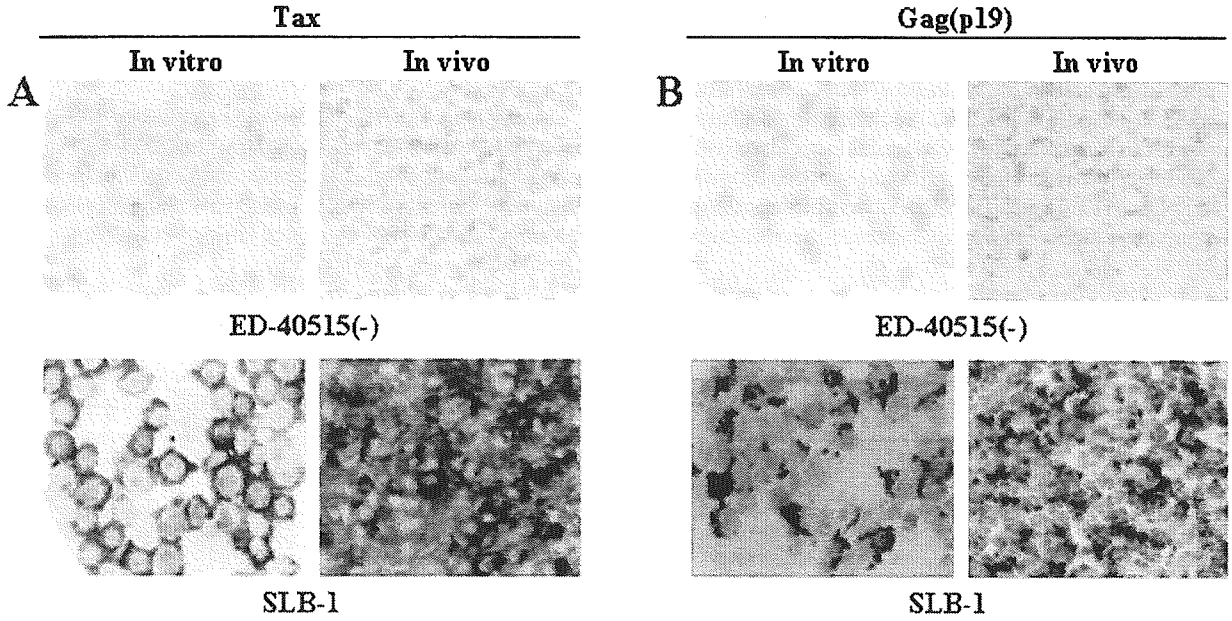


FIG. 3. Viral protein expression in the cell lines inoculated in NOG mice. To investigate viral protein expression in the cell lines inoculated in mice, we performed immunohistochemical analysis. In vitro cytospin sample and in vivo tumor tissue from mice 2 weeks after inoculation with cell lines were stained with anti-Tax (A) and anti-Gag antibody (B). Upper and lower panels of both figures represent the ED-40515(−) and SLB-1 cell lines, and the right and left panels show in vitro and in vivo sample, respectively.

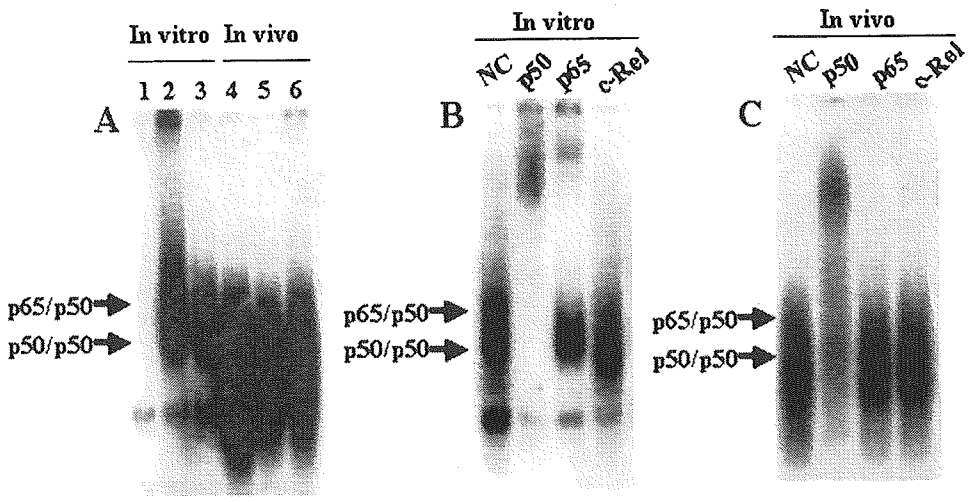


FIG. 4. NF-κB binding activity of ED-40515(−) cells in NOG mice. (A) Analysis of NF-κB binding activity of the ED-40515(−) cell line both in vitro and in vivo by EMSA. Nuclear extracts (5 μg) were treated with $^{32}$P-labeled wild-type NF-κB oligonucleotides. Lane 1 contains the negative control using Jurkat, and lane 2 contains the positive control using the SLB-1 cell line. Lane 3 and lanes 4 to 6 show in vitro and in vivo NF-κB DNA binding of ED-40515(−) cells from three different tumor-bearing mice, respectively. (B and C) Analysis of the NF-κB components of ED-40515(−) cells by supershift assay. Supershift using antibodies specific to p50, p65, and c-Rel subunits of NF-κB were conducted both in vitro (B) and in vivo (C). NC, negative control.

protein expression before and after inoculation of HTLV-1-bearing cells in mice.

**NF-κB binding activity of ED-40515(−) cells inoculated in NOG mice.** High NF-κB binding activity is thought to be crucial for maintaining the characteristics of both ATL/L cells and in vitro transformed cells with HTLV-1. To determine whether or not NF-κB activity is changed in vivo, we performed EMSA. Interestingly, it was shown that in vivo NF-κB DNA binding activity of the tumor cells was even stronger than that of the in vitro sample (Fig. 4A). Furthermore, we investigated whether or not the NF-κB specific bands of tumor tissues originated from the inoculated cells. For this purpose, super-shift assays were performed with nuclear extracts of tumor tissues in the absence or presence of antibodies that specifically recognize the following members of the NF-κB family: p50, p65, and c-Rel. The band was super-shifted by p50 antibody and also by c-Rel antibody, demonstrating that the band contained trans-activating p50 and c-Rel (Fig. 4C). This pattern was apparently different from that of in vitro culture cells of ED-40515(−), where the band was super-shifted by p50 and p65 antibodies (Fig. 4B).

**Antitumor effect of Bay 11-7082 through the inhibition of NF-κB.** To determine the effect of Bay 11-7082 on primary and infiltrative tumor cell growth, we inoculated ED-40515(−) cells ($10^7$) subcutaneously into the postauricular region of NOG mice. Mice were intraperitoneally administered Bay 11-7082 (20 mg/kg of body weight/day) or 1% DMSO together with the inoculation of tumor cells. There was no significant difference in tumor size in any of the mice of both the Bay and DMSO group 20 days after treatment. However, Bay 11-7082-treated mice appeared to be healthy and had no standing of hair, weight loss and cachexia, all of which are signs of near death. In contrast, the control mice showed these clinical signs of near death. Notably, Bay 11-7082 suppressed infiltration of the tumor cells into peripheral blood and other organs while control mice showed infiltration of the tumor cells in peripheral blood as well as other organs of SCID mice (Fig. 5A and B). Accordingly, these results strongly suggest that leukemic infiltration of the tumor cells may contribute to aggravation of the clinical status of the tumor-bearing mice.

We then tested the effects of Bay 11-7082 on the growth of established subcutaneous ED-40515(−) tumors (Fig. 5C and D). Ten days after tumor formation, Bay 11-7082 (20 mg/kg/day) was administered directly into the tumor site daily whereas control mice received 1% DMSO for 15 days. The tumor rapidly grew in control mice, resulting in the development of death signs due to disease progression in all mice. In contrast, the tumors began to regress markedly and later, widespread tumor necrosis occurred in Bay 11-7082-treated mice (Fig. 5C). These data indicate that Bay 11-7082 significantly inhibited the growth of an established tumor.

Collectively, these results suggest that daily administration of Bay 11-7082 could prevent primary tumor growth and disease progression through the inhibition of infiltrative leukemic cell growth in various organs.

## DISCUSSION

ATL is a malignancy of T-lymphocytes etiologically linked to a retrovirus, human T-cell leukemia virus type 1 (HTLV-1) (33, 48). Previous results showed that HTLV-1-infected transformed cell lines do not have sufficient activity to acquire tumorigenic potential in SCID mice, but leukemic cell lines do produce a tumor during a 2- to 4-month follow-up period (10, 16). In the present study using a novel SCID mouse, NOG, successful engraftment of both transformed and leukemic cells was achieved during a 2- to 3-week follow-up period, producing a progressively growing large tumor. This extremely rapid tumor formation is one of the hallmarks of our animal model. Very recently, Liu et al. demonstrated that the efficiency of tumorigenesis by HTLV-1-transformed cell lines is dramatically elevated in NOD-SCID mice when mice are sublethally

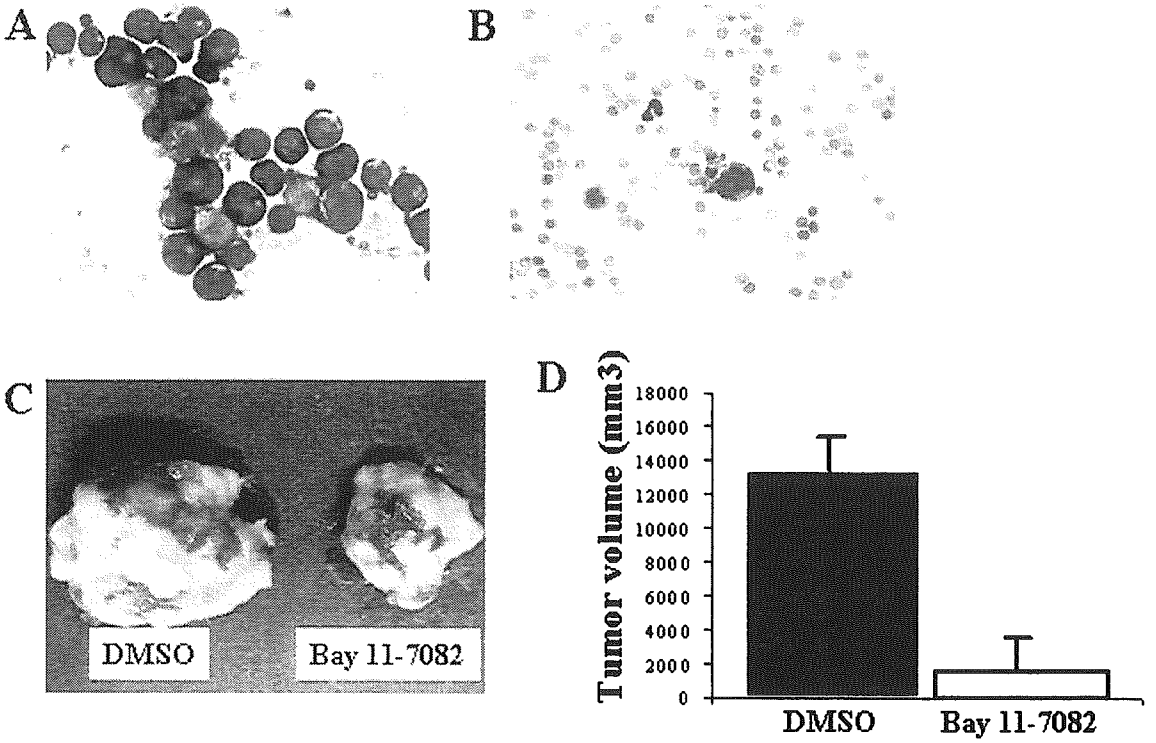


FIG. 5. Effect of Bay 11-7082 on primary and infiltrative tumor cell growth. Mice were injected subcutaneously via the postauricular region with ED-40515(−) cells ($10^7$). At the same time, either 1% DMSO or Bay 11-7082 (20 mg/kg/day) was administered intraperitoneally to the mice every day for 20 days. Another group of experiments were conducted using administration of the same doses of DMSO or Bay 11-7082 at the tumor site 10 days after tumor formation for 15 days. May-Grunwald and Giemsa staining showed infiltration of tumor cells in PBMNCs of mice treated with DMSO (A) or Bay 11-7082 (B) after 3 weeks, respectively. Photographs of subcutaneously formed ED-40515(−) tumors (C) and tumor volume (D) in the mice that had received DMSO or Bay 11-7082 10 days after tumor formation at the tumor site. Black bars represent the DMSO-treated control mice, and open bars represent the Bay 11-7082-treated mice. Each result was obtained from three different mice (means are shown [error bars, standard errors]).

irradiated prior to inoculation (25). Interestingly, it was shown that transformed cell lines produced a hemorrhagic hard tumor and leukemic cell lines produced a soft tumor in NOG mice. The hemorrhagic tumor could be explained by the augmented invasiveness to blood vessels. Further study is needed to determine whether or not this was due to the effect of Tax expressed in transformed cell lines such as SLB-1 and Hut-102.

Infiltrations of HTLV-1-infected leukemic cell lines into various organs are well known in ATL patients (38, 45). We found that SCID mice inoculated with ED-40515(−) cells showed infiltration into peripheral blood, bone marrow, lung, liver, spleen and kidney, and in addition to these organs, cells of the SLB-1 cell line had also infiltrated into the brain and heart (Table 2). These results indicate that HTLV-1-bearing cells in our model could infiltrate in a similar manner as leukemic cells in ATL patients. Leukemic infiltration was more remarkable in Tax-positive transformed cell lines than in leukemic cell lines. Further study is necessary to elucidate whether or not the expression of Tax in transformed cell lines is responsible for this role.

The differing behaviors of HTLV-1-infected cell lines in different types of SCID mice in the formation of tumors are dependent on the host immune system. Natural killer cells in the mice might play an important role in the rejection of implanted tissues or cells in SCID mice (5, 8, 10, 11, 37, 46, 47). In addition, recipient dendritic cells could have a role in transplant rejection (24). In this study, we compared two strains of NOG and NOD-SCID mice to directly assess in vivo HTLV-1-infected cell growth. In addition to the absence of T and B cells, NOG mice have no NK and there are also functional defects in dendritic cells (18). Our data showed that the number of ED-40515(−) cells inoculated in NOG mice was significantly increased about 7.6- and 23.6-fold, whereas the mass of tumor cells inoculated in NOD-SCID mice was reduced about 4.59- and 2.73-fold after 1 and 2 weeks, respectively (Fig. 2). In the present study, HTLV-1-infected cell lines inoculated subcutaneously in the postauricular region over the skeleton of mice permitted us to quickly observe the tumor growth macroscopically and to measure the size of the tumor. By measuring the size of the tumor macroscopically, it was possible to easily compare growth ability between the control mice and mice inoculated with the ED-40515(−) cell line (Fig. 1A). The present animal and inoculation system are applicable to other malignant cells and cell lines of different origins that are unrelated to HTLV-1 (unpublished results), and therefore could be very useful in the study of tumorigenesis of various malignant tissues in general.

Tax is a 40-kDa nuclear oncoprotein, which is responsible for trans-activation of the HTLV-1 long terminal repeat (9, 36), as well as numerous cellular genes involved in T-cell ac-

tivation and growth, such as those encoding IL-2 (26), and the α-chain of IL-2R (CD25, Tac) (3, 7). The malignant cells associated with all phases of ATL express very high levels of IL-2Rα (41–43)without expressing a significant amount of Tax. Our results showed that tumor tissues in NOG mice also expressed both IL-2Rα and T-cell surface molecule CD4 as assessed by the immunohistochemical procedure used in this study, and these levels were reminiscent of those seen in primary ATL cells (Fig. 1D and E).

Many cellular genes are shown to be activating through stimulation of the NF-κB/Rel family of transcription factors when Tax is expressed in the cells. The inactivation of tumor suppressor p53 by Tax in HTLV-1-positive cells is also dependent on activation of the NF-κB pathway (32). Leukemic cells in the ATL patients and leukemic cell lines directly originating from the patients apparently share this character with Tax-expressing cells in the absence of Tax. For this reason, NF-κB, rather than Tax, is considered to play a key role in the pathogenesis of HTLV-1-associated malignancy (15). Recent studies have provided some insights into the multiple aspects of NF-κB involvement in oncogenesis, including the control of apoptosis, the cell cycle, differentiation and migration of the cells (2). In this study, we inoculated a Tax nonexpressing ED-40515(−) cell line in NOG mice to evaluate the role of NF-κB binding activities and Tax expression in tumor cells. As a result, the ED-40515(−) cell line inoculated into SCID mice did not express Tax (data not shown). It was shown that in vivo NF-κB DNA binding activity of the tumor cells was even stronger than those cultured in vitro (Fig. 4A). Moreover, although the NF-κB components of ED-40515(−) cells grown in vitro were p50 and p65 (Fig. 4B), those in tumor cells consisted of p50 and c-Rel (Fig. 2). Such quantitative as well as qualitative alterations in NF-κB activity and the NF-κB components seen in tumor cells in vivo might play an important role in the development of rapid growth of tumors in SCID mice. This NOG mice model we present here would be useful in identifying such in vivo changes of the cells. Further study is necessary to clarify the mechanism underlying the rapid growth of the tumor and in vivo change of NF-κB components.

To develop potential new therapeutic strategies for ATL, Bay 11-7082 was used for the in vivo experiment. Very recently, we showed that Bay 11-7082 induced apoptosis in HTLV-1-infected cell lines and primary ATL cells, whereas the effect against HTLV-1-negative cells was only negligible (29). Bay 11-7082 rapidly and specifically reduced DNA binding of NF-κB in HTLV-1-infected T-cell lines, and down-regulated expression of the antiapoptotic gene, Bcl-XL. Due to its potent and selective inhibition of phosphorylation of IκBα, we predicted that Bay 11-7082 would be useful in the treatment of ATL. Our results clearly indicate that exclusive use of Bay 11-7082 could significantly prevent tumor growth at the inoculated site and infiltrative leukemic cell growth in SCID mice through inhibition of NF-κB activity (Fig. 5). In our NOG mice model, Bay 11-7082 showed no significant and severe adverse effect against the mice during the treatment period. These data strongly suggest that NF-κB might serve as a general therapeutic target of ATL and Bay 11-7082 could be used as a lead compound in the development of anti-ATL drugs.

In summary, our NOG mice model system provides the opportunity to understand and investigate the mechanism of pathogenesis and malignant cell growth of ATL and to develop a novel therapeutic regimen.

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# Induction of Protective Immune Responses against R5 Human Immunodeficiency Virus Type 1 (HIV-1) Infection in hu-PBL-SCID Mice by Intrasplenic Immunization with HIV-1-Pulsed Dendritic Cells: Possible Involvement of a Novel Factor of Human CD4$^+$ T-Cell Origin

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**The potential of a dendritic cell (DC)-based vaccine against human immunodeficiency virus type 1 (HIV-1) infection in humans was explored with SCID mice reconstituted with human peripheral blood mononuclear cells (PBMC). HIV-1-negative normal human PBMC were transplanted directly into the spleens of SCID mice (hu-PBL-SCID-spl mice) together with autologous mature DCs pulsed with either inactivated HIV-1 (strain R5 or X4) or ovalbumin (OVA), followed by a booster injection 5 days later with autologous DCs pulsed with the same respective antigens. Five days later, these mice were challenged intraperitoneally with R5 HIV-$1_{JR-CSF}$. Analysis of infection at 7 days postinfection showed that the DC–HIV-1-immunized hu-PBL-SCID-spl mice, irrespective of the HIV-1 isolate used for immunization, were protected against HIV-1 infection. In contrast, none of the DC-OVA-immunized mice were protected. Sera from the DC–HIV-1- but not the DC-OVA-immunized mice inhibited the in vitro infection of activated PBMC and macrophages with R5, but not X4, HIV-1. Upon restimulation with HIV-1 in vitro, the human CD4$^+$ T cells derived from the DC–HIV-1-immunized mice produced a similar R5 HIV-1 suppressor factor. Neutralizing antibodies against human RANTES, MIP-1α, MIP-1β, alpha interferon (IFN-α), IFN-β, IFN-γ, interleukin-4 (IL-4), IL-10, IL-13, IL-16, MCP-1, MCP-3, tumor necrosis factor alpha (TNF-α), or TNF-β failed to reverse the HIV-1-suppressive activity. These results show that inactivated HIV-1-pulsed autologous DCs can stimulate splenic resident human CD4$^+$ T cells in hu-PBL-SCID-spl mice to produce a yet-to-be-defined, novel soluble factor(s) with protective properties against R5 HIV-1 infection.**

Mice with a genetically inherited severe combined immunodeficiency (SCID mice) develop a surrogate human immune system when injected with human peripheral blood mononuclear cells (PBMC). These mice, termed hu-PBL-SCID mice, have served as a valuable model for the study of human immunodeficiency virus type 1 (HIV-1) pathogenesis (18, 22). It has been shown that the human T cells transplanted into SCID mice are activated (26) and proliferate in response to nominal antigens presented by antigen-presenting cells (APC) of murine origin (34). Thus, experiments have been conducted to induce and study human immune responses in hu-PBL-SCID mice (1, 3, 7, 17). There are, however, two major limitations to the development of strong human immune responses in these hu-PBL-SCID mice. The first is the lack of appropriate human APC, including dendritic cells (DC), while the second is the lack of a suitable microenvironment, such as the presence of normal lymphoid organs and architecture (34). Each of these issues is known to facilitate primary interaction between T cells and APC. To overcome the lack of APC, Delhem et al. (4) have used autologous skin transplants containing tissue DC as a source of APC and have succeeded in demonstrating the induction of primary major histocompatibility complex (MHC)-restricted human T-cell responses against HIV-1 envelope in hu-PBL-SCID mice. Furthermore, Santini et al. (28) have recently reported that HIV-1-pulsed, monocyte-derived human mature DC can stimulate primary human anti-HIV-1 antibody production in the SCID mouse system.

It is reasoned that since hu-PBL-SCID mice are permissive for R5 HIV-1 (23), this animal model should provide us with valuable information for the evaluation of candidate vaccines against HIV-1. Despite the success that has been achieved in the induction of human T- and B-cell immune responses against HIV-1, such HIV-1-immunized hu-PBL-SCID mice have not to date been utilized for the evaluation of protective immunity against HIV-1. In the present study, we found that transfer of human PBMC, together with inactivated HIV-1-pulsed autologous DC, directly into the mouse spleen elicited a protective immune factor against R5 HIV-1 infection. The factor was synthesized predominantly by human CD4$^+$ T cells in response to HIV-1 antigen and appears to be unrelated to the presently identified R5 HIV-1 suppressive cytokines and chemokines. The data presented here not only document the establishment of a novel model to study candidate DC-based

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vaccines against HIV-1 but also provide data to support the existence of a unique factor with R5 HIV-1-suppressive properties that can be potentially exploited as an adjunct to therapy against HIV-1.

## MATERIALS AND METHODS

**Mice.** The SCID mice utilized (C.B-17-*scid*) were purchased from Crea Japan (Kanagawa, Japan). SCID mice lacking natural killer (NK) cells, i.e., NOD/Shi-*scid* $\gamma c^{-/-}$ (8) and BALB/c-rag2$^{-/-}$ $\gamma c^{-/-}$ mice (24), were also used in the present study. The mice were kept in the specific-pathogen-free and P3 animal facilities of the Laboratory Animal Center, University of the Ryukyus. The protocols for the care and use of hu-PBL-SCID mice were approved by the committee on animal research of the University of the Ryukyus prior to initiation of the study. The NK cell lineages in the C.B-17-*scid* mice were depleted by intraperitoneal (i.p.) injection of 1 mg of rat anti-mouse interleukin-2 (IL-2) receptor β (clone TMβ-1) (33) per animal.

**Reagents.** The media used were RPMI 1640 medium (Sigma, St. Louis, Mo.) supplemented with 5% fetal calf serum, 100 U of penicillin per ml, and 100 μg of streptomycin per ml (hereafter called RPMI medium) and Iscove's medium (Lifetechnologies, Grand Island, N.Y.) supplemented with 10% fetal calf serum with the same antibiotics (hereafter called Iscove's medium). Soluble recombinant human IL-4 and granulocyte-macrophage colony-stimulating factor (GM-CSF) were generated from COS cell cultures transfected with the appropriate genes in the expression plasmid DNAs pCMhIL4 and pCMhGM (RIKEN Gene Bank, Ibaraki, Japan), respectively, by the Fugene 6 method (Roche Diagnostics Corporation, Indianapolis, Ind.). The concentrations of human IL-4 and GM-CSF were determined by using commercial enzyme-linked immunosorbent assay (ELISA) kits (BioSource, Camarillo, Calif.). Human recombinant IL-2 and M-CSF were purchased from Shionogi (Osaka, Japan) and Pepro Tech EC Ltd. (London, United Kingdom), respectively.

The anti-human MIP-1α, anti-human MIP-1β, anti-human IL-4, anti-human IL-10, anti-human IL-12, anti-human IL-13, anti-human IL-16, anti-human MCP-1, and anti-human MCP-3 monoclonal antibodies (MAb) were all purchased from R&D Systems (Rockville, Md.). Goat anti-human alpha interferon (IFN-α) and IFN-β were purchased from Pepro Tech. To maintain their neutralizing activity, these antibodies in lyophilized form were reconstituted in accordance with the manufacturer's instructions, and aliquots were kept at −80°C until use.

**Virus.** HIV-$1_{JR\text{-}CSF}$ and HIV-$1_{JR\text{-}FL}$ (9) and HIV-$1_{NL4\text{-}3}$ (2) viral stocks were each produced in the 293T cell line by transfection with the appropriate HIV-1 infectious plasmid DNA, utilizing the calcium phosphate method (31). HIV-$1_{SF162}$ (30) was produced in phytohemagglutinin-stimulated PBMC. HIV-$1_{IIIB}$ was harvested from Molt-4/IIIB cell cultures. The 50% tissue culture infective dose ($TCID_{50}$) was determined by an end point infectious assay with phytohemagglutinin-activated PBMC. For immunization with HIV-1, the viral stocks were prepared in autologous PBMC cultures activated with immobilized anti-CD3 MAb. These HIV-1 preparations were inactivated with aldrithiol-2 (AT-2), as previously described by Rossio et al. (27). AT-2 was removed by three successive ultrafiltration in phosphate-buffered saline (PBS), using 100-kDa-cutoff centrifugal filtration devices (Centriprep 100; Amicon, Beverly, Mass.).

**Generation of monocyte-derived DC.** Fresh PBMC at $3 \times 10^6$ cells/ml in RPMI medium were dispensed into individual wells of 12-well plates (1 ml/well) which had been previously coated with autologous plasma for 30 min at 37°C. The PBMC cultures were allowed to incubate at 37°C for 1 h. After gentle washing with serum-free RPMI 1640 medium, the adherent cells were cultured in Iscove's modification of Dulbecco's modified Eagle's medium (2 ml/well) containing human GM-CSF (500 ng/ml) and IL-4 (200 ng/ml) for 5 days. The resulting immature DC cultures were depleted of contaminating lymphocytes by using a monocyte negative isolation kit (Dynal, Oslo, Norway) and were further cultured with human IFN-β (1,000 U/ml; Toray, Tokyo, Japan) for 1 day to obtain mature DC, essentially as described by Santini et al. (28).

**Transplantation, immunization, and infection.** Groups of SCID mice received mature DC ($5 \times 10^5$ cells) which were pulsed for 2 h at 37°C with either AT-2-inactivated HIV-1 (40 ng of p24) or 100 μg of ovalbumin (OVA) in 100 μl of RPMI medium. These DC were mixed with autologous fresh PBMC ($3 \times 10^6$ cells) in a final volume of 100 μl and then were directly injected into the spleens of SCID mice. Five days later, the same number of DC pulsed with antigen were inoculated into the spleen or peritoneal cavity. Five days later, some mice in each group were sacrificed, blood was collected by cardiocentesis, and human lymphocytes were recovered from the peritoneal cavity by lavage and from the spleen. The remaining mice in each group were challenged i.p. with 1,000 $TCID_{50}$ of HIV-$1_{JR\text{-}CSF}$ (100 μl/animal). After 7 days, the mice were sacrificed, their blood was obtained, and human lymphocytes were collected from the peritoneal cavity by lavage and from the spleen. The peritoneal lavage fluids, sera, and lymphocyte culture supernatants were examined for levels of HIV-1 p24 with an ELISA kit (Zepto Metrix, Buffalo, N.Y.). Fresh lymphocytes were examined for proviral DNA by a quantitative PCR assay (10). Target cells used for in vitro infection assays consisted of normal PBMC activated with magnetic beads conjugated with anti-CD3 and anti-CD28 MAb (Dynal) at a cell-to-bead ratio of 1:1 in RPMI medium containing 20 U of human IL-2 per ml for 3 days. Some experiments utilized cultured macrophages derived from normal human PBMC, which were prepared by culturing adherent PBMC with 20 ng of human M-CSF per ml for 5 to 7 days. These activated PBMC or cultured macrophages ($5 \times 10^5$ cells) were preincubated in 50 μl of medium, diluted serum, and culture supernatant samples at 37°C for 1 h in 96-well U-bottom microtiter plates (BD PharMingen, San Diego, Calif.). Subsequently, 50 μl of the HIV-1 stock containing 500 $TCID_{50}$ of HIV-1 was added to each well. After incubation at 37°C for 3 h, the cells were washed three times and cultured in 200 μl of RPMI medium containing 20 U of IL-2 per ml for 3 to 5 days. HIV-1 replication was monitored by the quantitation of HIV-1 p24 produced in the culture supernatants. In order to determine whether the inhibitor of HIV-1 replication present in the immune sera or culture supernatant fluids consisted of known cytokines, a number of anti-human cytokine neutralizing antibodies at 10 μg/ml were preincubated with the sera or restimulated culture supernatants on ice for 30 min and then analyzed with the infection assay described above. Among the human cytokines tested with the present infection assay, while pretreatment of cultured human macrophages with IL-10, IL-16, IFN-α, IFN-β, tumor necrosis factor alpha (TNF-α), or TNF-β at 50 ng/ml completely inhibited the infection with R5 HIV-$1_{JR\text{-}CSF}$, IL-4, MCP-1, and MCP-3 showed marginal inhibitory effects. We confirmed the biological role of the appropriate inhibitory cytokines with the use of neutralizing antibodies against the respective cytokines, which were shown to completely reverse their HIV-1 inhibition activity at 10 μg/ml (data not shown).

**in vitro restimulation.** For the measurement of antigen-specific human cellular immune responses, lymphoid cells ($2 \times 10^6$ cells) collected from the spleens and peritoneal lavage of the immunized mice were cultured for 2 days at 37°C with $2 \times 10^5$ autologous APC (adherent PBMC) in the presence or absence of either 1 μg of OVA or AT-2-inactivated HIV-1 containing 40 ng of p24 in a volume of 1 ml in individual wells of a 24-well plate (BD PharMingen). The medium consisted of RPMI 1640 supplemented with 20 U of human IL-2 per ml. The concentration of human IFN-γ produced in the culture supernatants was determined with commercial ELISA kits (R&D Systems). Unfractionated or enriched populations of $CD4^+$ and $CD8^+$ T cells purified by the magnetic bead-positive selection method (Dynal) were cultured in 12-well plates (BD PharMingen) in the presence of APC and antigen, as outlined above, for preparation of the HIV-1 suppressive factor and for identification of the potential cell lineage that synthesized such a factor. The purity of the isolated CD4 and CD8 single-positive cells was always > 95% as determined by flow cytometric analysis. Contamination of human B cells within these T-cell fractions was not detected by staining with anti-CD20 MAb (data not shown).

**Assay for human cytokines and antibodies.** Commercial kits for human TNF-α, IFN-α, IFN-γ, IL-4, IL-10, IL-12, IL-13, IL-16, MIP-1α, MIP-1β, RANTES, MCP-1, and MCP-3 (BioSource) TGF-β (R&D Systems), and IFN-β (Fuji Rebio Co. Ltd., Tokyo, Japan) were employed. All assays were performed in accordance with the manufacturer's instructions, and cytokine levels were calculated from values obtained by using standard curves determined with recombinant cytokines. For select experiments depletion of human β-chemokines was achieved with heparin-Sepharose (Pharmacia, Tokyo, Japan). Bound materials were eluted from the column in PBS containing 2 M NaCl. For the measurement of OVA-specific human antibodies, serial dilutions of the serum samples to be tested were added to 96-well ELISA microtiter plates (Nunc, Rochester, N.Y.) which were precoated with 10 μg of OVA per ml at 37°C for 2 h. The bound human antibody was developed with a horseradish peroxidase-conjugated goat anti-human immunoglobulin G (IgG) (American Qualex, San Clemente, Calif.), followed by incubation in a buffer containing tetramethyl benzidine (Sigma) and hydrogen peroxide (Wako Pure Chemical Industries Inc., Osaka, Japan). HIV-1 specific human antibodies were detected by Western blot assay with LAV Blot1 (Fuji Rebio Co.).

**Flow cytometry.** Cell samples were incubated with 0.1 mg of normal human IgG per ml in fluorescence-activated cell sorter buffer (PBS containing 2% fetal calf serum and 0.1% sodium azide) on ice for 15 min and then were stained with fluorescein isothiocyanate- or Cy5-labeled anti-CCR5 (T227) (32), phycoerythrin-labeled anti-CXCR4 (12G5; BD PharMingen), phycoerythrin-labeled anti-CD4 (Beckman Coulter, Fullerton, Calif.), or Cy5-labeled OKT-4 on ice for 30 min. The cells were washed three times in fluorescence-activated cell sorter

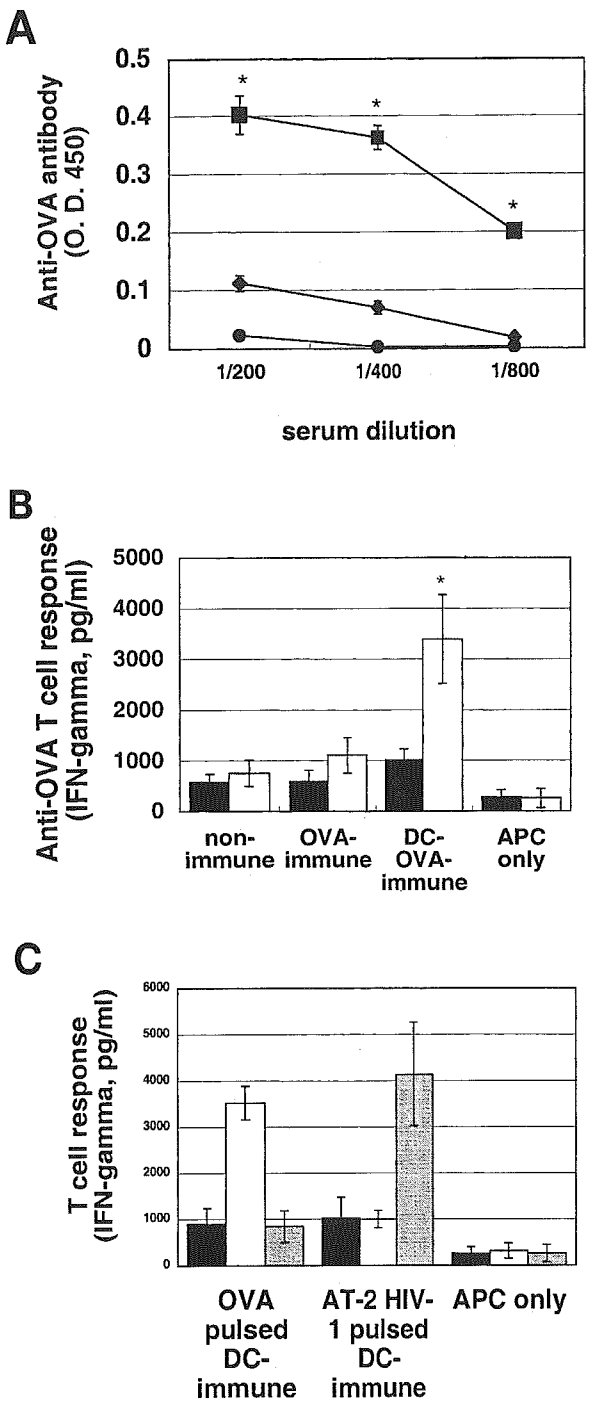


FIG. 1. Induction of antigen-specific human immune responses in hu-PBL-SCID-spl mice by immunization with antigen-pulsed DC. (A) PBMC ($3 \times 10^6$ cells) alone (nonimmune) (●), with OVA (100 μg) (OVA immune) (◆), or with DC ($5 \times 10^5$ cells) pulsed with OVA (100 μg) (DC-OVA immune) (■) were engrafted into the spleens of SCID mice. Five days later, the PBMC-OVA-immunized mice received a booster injection with OVA and the DC-OVA-immunized mice received a booster injection with DC-OVA. Five days later, serum samples were collected and human anti-OVA antibodies were measured by ELISA. Results are expressed as the mean ± standard deviation from six independent experiments. *, $P < 0.05$. (B) Lymphocytes ($2 \times 10^6$ cells) recovered from hu-PBL-SCID-spl mice were cocultured with $2 \times 10^5$ autologous APC (adherent PBMC) in the presence (open bars) or absence (solid bars) of 1 μg of OVA per ml at 37°C for 2 days in 1 ml of RPMI medium containing 20 U of human IL-2 per ml. APC cultured alone served as controls. Results are expressed as the mean ± standard deviation from six independent experiments. *, $P < 0.05$. (C) Lymphocytes ($2 \times 10^6$ cells) recovered from hu-PBL-SCID-spl mice which were immunized with either DC-OVA or DC-AT-2-inactivated HIV-$1_{JR\text{-}CSF}$ were either not restimulated (solid bars) or restimulated as outlined for panel B in the presence of OVA (open bars) or AT-2-inactivated HIV-1 (containing 40 ng of p24) (shaded bars), respectively, for 2 days. Supernatant fluids from such cultures were harvested, and the levels of human IFN-γ were determined by ELISA. All results are expressed as the mean ± standard deviation from six independent experiments. *, $P < 0.05$.

buffer and fixed with 1% paraformaldehyde in PBS. Cells were analyzed on a FACSCalibur flow cytometer, using Cell Quest software (BD PharMingen). Isotype-matched MAb were utilized as controls to stain an aliquot of the cells to be analyzed for purposes of establishing gates and for determination of the frequency of positively stained cells.

**Statistical analysis.** Data were analyzed by Student's *t* test with the Stat View-J 4.02 statistics program (Abacus Concepts, Berkeley, Calif.).

## RESULTS

**Induction of human immune responses in SCID mice.** For the preparation of hu-PBL-SCID mice, SCID mice previously have generally been engrafted with $2 \times 10^7$ fresh human PBMC by i.p. injection. In the studies presented here, we have attempted an intrasplenic (i.s.) transfer of human PBMC and found that this method was superior to the one previously used with regard to both a more efficient engraftment of the human T cells and reduction of mouse death caused by severe graft-versus-host disease (data not shown). By using this i.s. transfer method, the number of PBMC required for initial inoculation could be reduced by approximately 1 log unit for generation of more than $5 \times 10^6$ human $CD3^+$ T cells within 2 weeks (data not shown). In addition, these mice (hu-PBL-SCID-spl mice) produced higher levels of human Ig than those generated by the i.p. transfer. These findings indicate that human T and B lymphocytes directly inoculated into the mouse spleen are more efficiently activated than those inoculated into the peritoneal cavity.

Preliminary studies were carried out to determine the requirements for the generation of antigen-specific human immune responses in these hu-PBL-SCID-spl mice. Subcutaneous immunization of these mice with OVA incorporated into Freund's adjuvant failed to induce detectable anti-OVA-specific human immune responses (data not shown). In the second series of studies, we attempted to immunize the mice with antigen-pulsed autologous mature DC generated from peripheral blood monocytes. Fresh PBMC ($3 \times 10^6$ cells) from normal human donors were transferred into the SCID mouse spleen together with autologous mature DC ($5 \times 10^5$ cells) pulsed with OVA (100 μg) or AT-2-inactivated HIV-$1_{JR\text{-}CSF}$ (containing 40 ng of p24). All of the HIV-1 stocks used were prepared in autologous PBMC cultures in order to avoid contamination with allogeneic antigens. On day 5, the mice received an i.s. booster injection with similarly prepared antigen-pulsed DC ($5 \times 10^5$ cells/animal). After 5 days, the mice were examined for antigen-specific human immune responses (Fig. 1). Sera from the DC-OVA-immunized mice showed a significant human anti-OVA antibody titer (Fig. 1A), and the lymphoid cells from these mice responded to OVA by producing

TABLE 1. Protection of hu-PBL-SCID-spl mice against HIV-1 infection by immunization with HIV-1-pulsed DC[a]

| Donor | Immunization of hu-PBL-SCID-spl mice | *n* | Provirus copy no.[b] ($10^3$) | Culture p24[c] (ng/ml) | Human chemokine (ng/ml)[d] | | |
|---|---|---|---|---|---|---|---|
| | | | | | MIP-1α | MIP-1β | RANTES |
| 1 | OVA | 8 | 10.9 ± 5.2 | 60.3 ± 23.9 | ND[e] | ND | ND |
| | $HIV\text{-}1_{JR\text{-}CSF}$ | 4 | <1 | <0.2 | ND | ND | ND |
| | $HIV\text{-}1_{NL4\text{-}3}$ | 4 | <1 | <0.2 | ND | ND | ND |
| 2 | OVA | 4 | 7.8 ± 4.1 | 9.5 ± 7.6 | 1.65 ± 0.47 | 1.63 ± 0.05 | 0.19 ± 0.05 |
| | $HIV\text{-}1_{JR\text{-}CSF}$ | 4 | <1 | <0.2 | 1.16 ± 0.08 | 1.71 ± 0.14 | 0.11 ± 0.05 |
| | $HIV\text{-}1_{NL4\text{-}3}$ | 4 | <1 | <0.2 | 0.62 ± 0.10 | 0.79 ± 0.29 | 0.12 ± 0.09 |

[a] hu-PBL-SCID-spl mice immunized with either DC-OVA or DC–AT-2-inactivated HIV-1 particles were infected with $HIV\text{-}1_{JR\text{-}CSF}$. After 7 days, sera and lymphocytes recovered from spleens and peritoneal cavities of hu-PBL-SCID-spl mice were examined for HIV-1 infection and serum chemokine levels. Results are expressed as the means ± standard deviations from six independent experiments.
[b] HIV-1 provirus copy number in fresh samples per total recovered cells from individual mice.
[c] HIV-1 p24 concentration in supernatants of lymphocytes ($10^6$ cells/ml) cultured for 5 days.
[d] Human chemokine concentration in serum samples as determined by ELISA.
[e] ND, not determined.

human IFN-γ upon stimulation with OVA-pulsed APC in vitro (Fig. 1B). Importantly, while the DC–HIV-1-immunized mice showed human anti-HIV-1 cellular immune responses (Fig. 1C), the sera from these mice showed very low or no detectable antibody against HIV-1 as determined by Western blot analysis (data not shown).

**Protection against HIV-1 challenge in vivo.** In order to examine whether the induced anti-HIV-1 immune responses are protective, these DC-OVA- and DC–HIV-1-immunized hu-PBL-SCID-spl mice were challenged i.p. with infectious R5 $HIV\text{-}1_{JR\text{-}CSF}$. After 7 days postchallenge, these mice were examined for HIV-1 infection by assaying for provirus in the lymphocytes and levels of p24 in the serum or supernatant fluid of the lymphocyte cultures. As shown in Table 1, all of the mice immunized with DC-OVA were HIV-1 infected. Surprisingly, the mice immunized with DC–$HIV\text{-}1_{JR\text{-}CSF}$ were completely protected against HIV-1 infection. Furthermore, the mice immunized with the inactivated X4 isolate of $HIV\text{-}1_{NL4\text{-}3}$ were also protected against the R5 HIV-1 infection. Similar results were obtained in three other experiments using hu-PBL-SCID-spl mice reconstituted with PBMC from two other donors (Table 1 and data not shown). Based on these results, we reasoned that the protection of the mice against the R5 HIV-1 infection might be mediated by the CCR5-binding human β-chemokines MIP-1α, MIP-1β, and/or RANTES. However, this possibility was found to be unlikely, since the human β-chemokine levels in the immune sera were lower than those required for suppression of the R5 HIV-1 infection in vitro, and the sera from the DC-OVA-immunized mice also contained similar levels of these β-chemokines (Table 1).

**Serum contains a suppression factor.** The levels of CCR5 and CXCR4 expression on the surface of human $CD4^+$ T cells isolated from the DC–HIV-1-immunized mice (protected) were comparable to those from DC-OVA-immunized (unprotected) mice (Fig. 2A). This finding suggests that the $CD4^+$ T cells from the protected groups were just as susceptible to R5 HIV-1 infection in vitro as those from the nonprotected mice (Fig. 2B). These data prompted us to speculate that one potential explanation for these findings could be that the HIV-1-activated human PBMC from the DC-based HIV-1 immunization induce an anti-R5 HIV-1 state in the animals without rendering the human $CD4^+$ T cells intrinsically nonpermissive to R5 HIV-1 infection. Thus, we speculated that some soluble HIV-1 suppressive factors might be involved.

Pretreatment of R5 HIV-1 virus with serum samples from the DC–HIV-1-immunized mice did not inhibit HIV-1 infection (data not shown), suggesting that the factor is not directed against the virus itself. Therefore, target PBMC were pretreated with the immune serum samples and then infected with either R5 or X4 HIV-1, followed by washing and cultivation in IL-2-containing medium. Sera from either DC–R5 HIV-1- or DC–X4 HIV-1-immunized mice, but not those from DC-OVA-immunized mice, markedly inhibited productive infection of the PBMC with R5 HIV-1 (Fig. 3A), but not X4 HIV-1 (Fig. 3B), in vitro. As shown in Fig. 4, the DC–HIV-1-immune serum was also suppressive for infection of the PBMC with the other two R5 HIV-1 isolates but not for infection with the X4 HIV-1 isolates. It is important to note that the R5 HIV-1-suppressive activity was not reversed by the addition of a mixture of antibodies against the three β-chemokines (Fig. 4). The antibody mixture at the concentration utilized (10 μg/ml for each of the three antibodies) was shown to be capable of neutralizing the anti-R5 HIV-1 effect of the cocktail of the three corresponding β-chemokines at 100 ng/ml each (data not shown). In addition, the putative factor also suppressed infection of human macrophage cultures with the three R5 HIV-1 strains, as determined by the absence of proviral DNA (Fig. 5). The HIV-1-suppressive activity neither was associated with cell death nor was MHC restricted (data not shown).

The R5 HIV-1-suppressive activity of the serum was eliminated by heating at 56°C for 30 min, suggesting the unlikelihood of the involvement of HIV-1-neutralizing antibody or IFN-α/β. Removal of serum IgG with protein G-Sepharose did not affect the suppressive activity of the serum (data not shown). The average levels of human cytokines in the sera from the DC–HIV-1-immunized mice were as follows: IFN-α, <10 pg/ml; IFN-β, 20 pg/ml; IL-4, <10 pg/ml; IL-12, <10 pg/ml; IL-13, <10 pg/ml; IL-16, <10 pg/ml; TNF-α, <10 pg/ml; and TGF-β, <10 pg/ml. These data indicate that the serum R5 HIV-1-suppressive activity is mediated by some unknown cytokine(s) of either human or mouse origin.

**Human $CD4^+$ T cells produce the suppression factor.** In order to define the cell lineage origin of the suppressor factor, human lymphocytes from DC–HIV-1- and DC-OVA-immu-

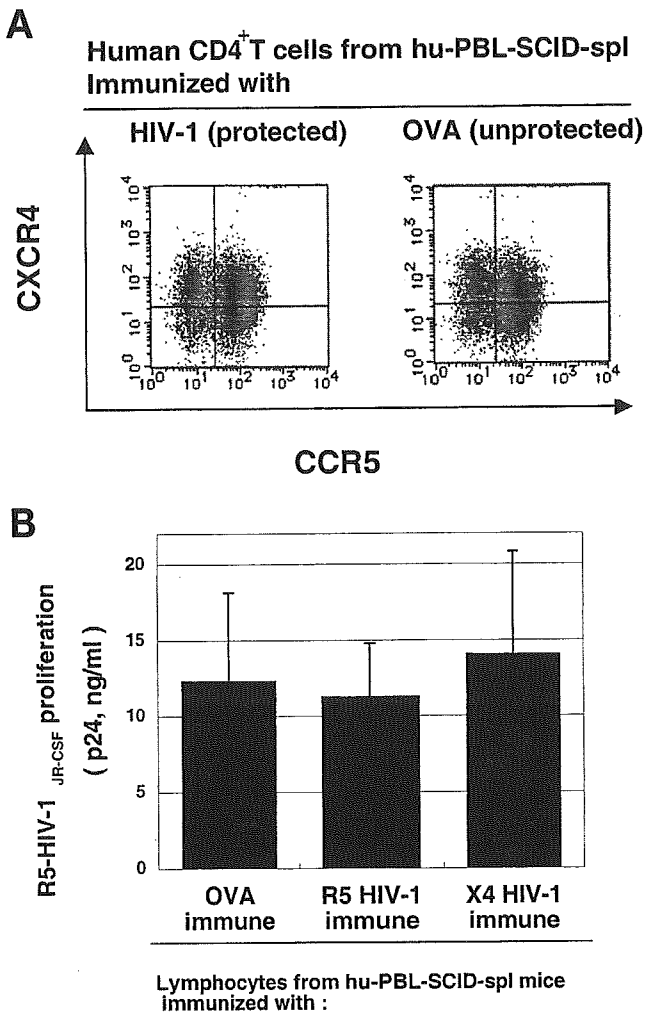


FIG. 2. Human CD4+ T cells from HIV-1-protected hu-PBL-SCID-spl mice express CCR5 and are permissive for R5 HIV-1 infection in vitro. (A) Lymphocytes recovered from hu-PBL-SCID-spl mice immunized with DC-OVA or DC–HIV-$1_{JR-CSF}$ were stained with anti-CD4, anti-CCR5, and anti-CXCR4. The expression profiles of CCR5 and CXCR4 on the CD4+ T cells are shown. (B) Lymphocytes recovered from hu-PBL-SCID-spl mice immunized with DC-OVA, DC–HIV-$1_{JR-CSF}$, or DC–HIV-$1_{NL4-3}$ were washed and infected with 500 $TCID_{50}$ of HIV-$1_{JR-CSF}$ at 37°C for 4 h in vitro. After washing, the cells were cultured for 5 days in RPMI medium containing 20 U of IL-2 per ml. Levels of HIV-1 p24 present in culture supernatants were quantitated by ELISA. All results are expressed as the mean ± standard deviation from six independent experiments.

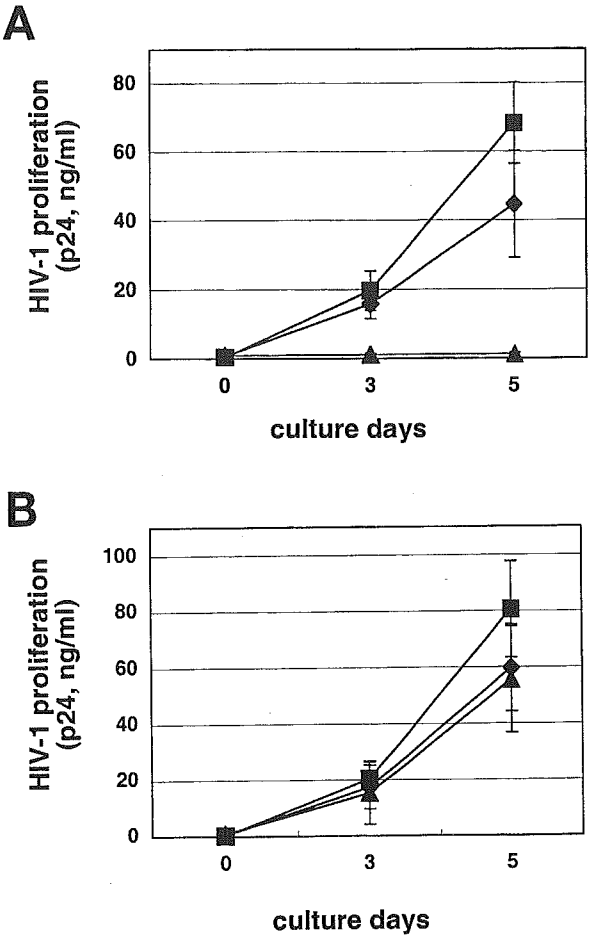


FIG. 3. Inhibition of R5 (A), but not X4 (B), HIV-1 infection by the HIV-1 immune serum. PBMC ($5 \times 10^5$ cells/well) activated in vitro for 3 days were washed and then incubated in medium (■) or final 20% serum samples obtained from either DC-OVA-immune (◆) or DC–HIV-$1_{JR-CSF}$-immune (▲) hu-PBL-SCID-spl mice at 37°C for 1 h, followed by the addition of 500 $TCID_{50}$ of HIV-$1_{JR-CSF}$ or HIV-$1_{NL4-3}$ and further incubation at 37°C for 4 h. After washing, cells were incubated in IL-2-containing medium for 5 days. The level of HIV-1 replication was monitored by quantitating HIV-1 p24 levels in the culture supernatants. Results are expressed as the mean ± standard deviation from six independent experiments.

nized mice were fractionated into human CD4+ and CD8+ T-cell subpopulations by positive selection with antibody-bound magnetic beads. Such enriched populations of CD4+ and CD8+ T cells and the unfractionated PBMC as a control were restimulated in vitro with inactivated HIV-1 and OVA, respectively, in the presence of autologous APC. As shown in Fig. 6A, the human CD4+ T cells from the DC–HIV-1-immunized mice produced a significant amount of the putative HIV-1 suppressor factor(s), which was not reversed by the addition of previously defined neutralizing anti-β-chemokine antibodies. The human CD8+ T cells, on the other hand, also produced R5 HIV-1 suppression factor, but in this case, the suppressive activity was significantly reversed by the addition of the anti-β-chemokine antibodies. Again, none of these samples were suppressive to X4 HIV-1 infection. The finding of suppressor factor synthesis by CD4+ T cells was highly reproducible, since DC–HIV-1-immune CD4+ T cells obtained from the other hu-PBL-SCID-spl mice transplanted with PBMC from four different donors also produced a similarly functioning HIV-1 suppressor factor (Fig. 6B). Since the culture supernatants of OVA-stimulated human CD4+ T cells from the DC-OVA-immunized mice had no or low R5 HIV-1-suppressive activity (data not shown), this suggested that the human CD4+ T cells reactive to HIV-1 antigen are the major producers of the suppressor factor.

**Partial characterization of the suppression factor.** The HIV-1 suppressor factor produced by the DC–HIV-1-immune

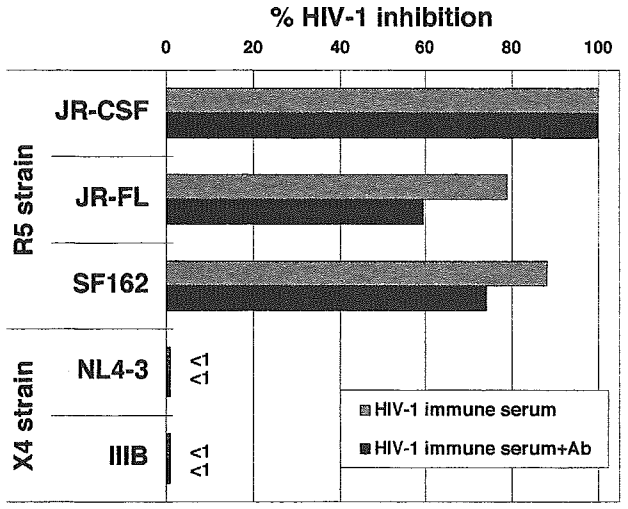


FIG. 4. Inhibition of R5, but not X4, HIV-1 by the HIV-1 immune serum. In vitro-activated PBMC were treated at 37°C for 1 h with 10% pooled serum samples obtained from DC–HIV-$1_{JR\text{-}CSF}$-immune hu-PBL-SCDI-spl mice, followed by the addition of 500 $TCID_{50}$ of HIV-1 (R5 isolates JR-CSF, JR-FL, and SF162 and X4 isolates NL4-3 and IIIB) and further incubation at 37°C for 4 h. After washing, cells were incubated in IL-2-containing medium for 5 days, and levels of HIV-1 p24 in the culture supernatants were quantitated. The HIV-1-suppressive activities of the serum preincubated with the mixture of anti-β-chemokine antibodies (+Ab) were also determined. Percent inhibition was calculated by using values obtained with the medium controls, as follows: JR-CSF, 18.7 ng/ml; JR-FL, 7.6 ng/ml; SF162, 6.6 ng/ml; NL4-3, 18.3 ng/ml; and IIIB, 10.2 ng/ml.

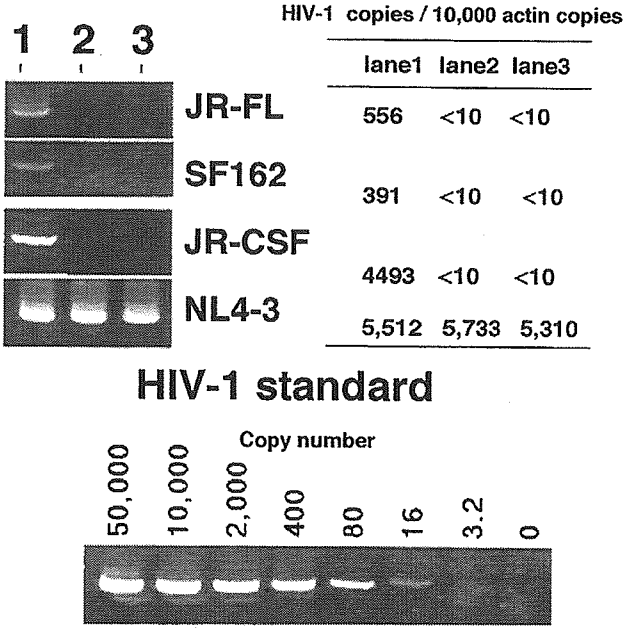


FIG. 5. Blocking of R5 HIV-1 infection in human macrophage cultures. Cultured macrophages or activated PBMC were preincubated in medium alone (lane 1) or with pooled serum samples from DC–HIV-$1_{JR\text{-}CSF}$-immune SCID mice in the absence (lane 2) or presence (lane 3) of a mixture of anti-β chemokines, and then the macrophages were infected with R5 HIV-1 strains (JR-FL, SF-162, and JR-CSF) and the PBMC were infected with X4 HIV-$1_{NL4\text{-}3}$. After washing, the cells were cultured for 2 days and cellular DNA were extracted and analyzed for the number of HIV-1 provirus copies. The signal obtained for actin was utilized as a reference control. The estimated numbers of HIV-1 copies per 10,000 copies of actin are shown in the accompanying table. The data shown are representative of those from three independent experiments.

serum and the restimulated $CD4^+$ T-cell culture supernatants were further characterized. As shown in Fig. 7A, the suppressive activity was eliminated by heating at 56°C for 30 min, was not absorbed by passage through a heparin-Sepharose column, and was not reversed by incubation with the anti-β-chemokine antibodies. It is important to note that the same $CD4^+$ T-cell cultures also did produce heparin-binding R5 HIV-1-suppressive factors. However, these were neutralized by the addition of the β-chemokine antibodies. The antibody neutralization assay results shown in Fig. 7B show that the factor was not likely to be related to the CCR5-binding β-chemokines IL-4, IL-10, IL-12, IL-13, IL-16, MCP-1, MCP-3, IFN-α, IFN-β, TNF-α, and TNF-β. Thus, although the antibodies against IL-4, IL-13, and IFN-β showed marginal neutralizing activity, a mixture of these antibodies did not synergistically block the biological suppressor activity of the factor.

We next examined the molecular size of the factor. The pooled sera from the DC–HIV-1-immunized mice were depleted of β-chemokines by use of heparin-Sepharose and fractionated by serial centrifugation over different molecular sieving filters. Figure 7C shows that the anti-HIV-1 suppressor factor was present in the >100-kDa fraction. Similar results were obtained with the analysis of the in vitro-restimulated DC–HIV-1-immune $CD4^+$ T-cell culture supernatants (data not shown).

The fact that the putative suppressor factor did not down-regulate CCR5 expression on macrophages provides support for the notion that this newly identified HIV-1 suppressor factor does not belong to the CCR5-binding β-chemokine family (Fig. 8). In addition, as mentioned above, the factor did not affect CD4 expression (Fig. 8). These observations suggest that the factor suppresses R5 HIV-1 infection without affecting HIV-1 receptor expression.

## DISCUSSION

In the present study, we showed for the first time that immunization of hu-PBL-SCID mice with HIV-1-pulsed mature DC protected the mice against R5 HIV-1 infection and that the protection was at least partially mediated by a soluble factor(s) present in the serum produced predominantly by human immune $CD4^+$ T cells in response to either R5 or X4 HIV-1 antigen.

It is possible that the DC–HIV-1-immune $CD8^+$ T cells from these mice also produce R5 HIV-1 suppression factors in addition to the previously characterized anti-R5 HIV-1 β-chemokines and CD8 factors known as Cd8 antiviral factors (CAF). However, since the immune $CD4^+$ T cells always produced relatively higher levels of the β-chemokine-independent R5 suppression factor than comparable numbers of immune $CD8^+$ T cells in vitro, we assume that the major producer of the novel factor is the $CD4^+$ T-cell population rather than the $CD8^+$ T-cell population in our hu-PBL-SCID-spl system. While the present in vitro restimulation experiments showed

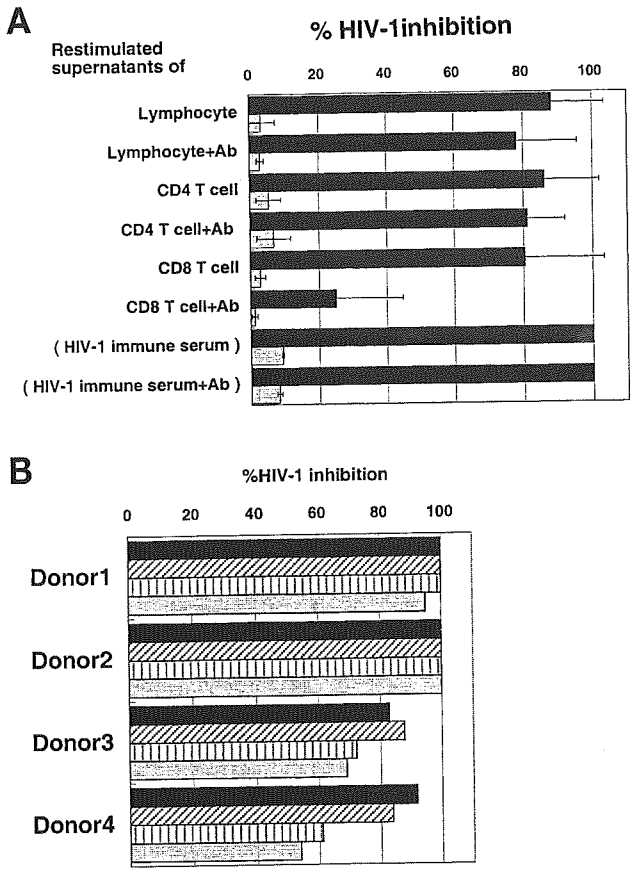


FIG. 6. Identification of the cell population producing the suppressive factor. (A) Lymphocytes from DC–HIV-$1_{JR\text{-}CSF}$-immune hu-PBL-SCID-spl mice were positively selected into human $CD4^+$ and $CD8^+$ T-cell subpopulations by using anti-CD4 and anti-CD8 MAb-conjugated immunobeads. Unfractionated (lymphocytes) or $CD4^+$ or $CD8^+$ T-cell populations ($2 \times 10^6$ cells) were cocultured with autologous APC ($2 \times 10^5$ cells) in the presence of AT-2-inactivated HIV-1 (containing 40 ng of p24) in 1 ml of IL-2-containing medium. After 2 days, culture supernatants were harvested to quantitate the levels of HIV-1 suppressor activity. Activated PBMC (target cells) were pretreated with these culture supernatants (50% final concentration) in the absence or presence (+Ab) of a mixture of anti-β-chemokine neutralizing antibodies and then infected with 500 $TCID_{50}$ of either HIV-$1_{JR\text{-}CSF}$ (solid bars) or HIV-$1_{NL4\text{-}3}$ (shaded bars). After washing, the PBMC were cultured for 5 days, and HIV-1 p24 produced in the culture supernatants was measured. DC–HIV-1-immune serum (20%) was used as a positive control. The percent inhibition was calculated by utilizing the values obtained for the medium controls, which were 19.4 ng/ml for JR-CSF and 25.9 ng/ml for NL4-3. All results are expressed as the mean ± standard deviation from six independent experiments. (B) Immune serum samples (20%) and culture supernatants (50%) of in vitro-restimulated $CD4^+$ T cells, which were prepared as described above from the DC–HIV-$1_{JR\text{-}CSF}$-immune hu-PBL-SCID-spl mice reconstituted with PBMC and DC from four different donors, were examined for suppressive activity against HIV-$1_{JR\text{-}CSF}$ infection of PBMC as described above. p24 values in the infected PBMC cultures were determined on day 5, and the percent HIV-1 inhibition was calculated by utilizing the p24 value obtained with the medium control, which was 23.4 ng/ml. Solid bars, immune serum; diagonally hatched bars, serum pretreated with a mixture of anti-β-chemokine antibodies; vertically hatched bars, restimulated culture supernatants; shaded bars, restimulated culture supernatants pretreated with a mixture of anti-β-chemokine antibodies.

that the DC–HIV-1-immune $CD4^+$ T cells and $CD8^+$ T cells were capable of producing the previously defined β-chemokines in response to HIV-1 antigen, the levels of these chemokines in the immune serum were lower than those required for suppression of R5 HIV-1 infection. In addition, two other facts make it unlikely that the factor described here contains β-chemokines. First, the $CD4^+$ T cells recovered from the protected mice expressed high levels of CCR5, similar to those from the unprotected mice. Second, the immune serum incubated with the macrophages did not induce any detectable down-modulation of CCR5 expression by such macrophages. We submit that such findings support the view that the CCR5-binding β-chemokines are not the major R5 HIV-1-suppressive factor in vivo. However, it is clearly possible that these β-chemokines produced by the $CD4^+$ and $CD8^+$ T-cell subpopulations may work synergistically in vivo with the newly identified antiviral factor described here.

To elicit the anti-R5 HIV-1 status in the hu-PBL-SCID mice, it was necessary to immunize the mice at least twice with HIV-1-pulsed autologous mature DC by i.s. injection. The HIV-1 suppression was mediated by a noncytolytic mechanism. It appears that the production of the suppressor factor is HIV-1 antigen dependent but virus isolate independent and that close contact between naive $CD4^+$ T cells and HIV-1 antigen presented by DC in the secondary lymphoid organs of mice facilitates the primary human anti-HIV-1 T-cell immune responses. The advantage of direct inoculation of simian immunodeficiency virus (SIV) antigen into the lymph nodes, where mature DC reside, to induce a protective immune response has been demonstrated in the simian model (13, 14). Since DC–HIV-1-immunized PBMC specimens from five different healthy individuals produced the HIV-1 suppressor factor, it appears that the factor production is not influenced by MHC background. These observations, together with the finding that the suppressor factor can be induced in a relatively short period (10 days from the initial immunization), indicates that the present DC–HIV-1 immunization protocol may be useful for the potential induction of an immediate protective immune response in HIV-1-infected humans.

The induction of primary HIV-1-specific human immune responses in vitro (37) and in hu-PBL-SCID mice in vivo (9, 10) by DC-based immunization has been achieved. In each case, the key issue is the use of experimentally matured DC. IFN-α/β and CD40L have been demonstrated to be DC maturation factors which induce expression and/or enhanced expression of antigen-presenting MHC class I and II molecules and costimulatory molecules. Unfortunately, it has not been reported so far whether the induced anti-HIV-1 T-cell immune responses in these studies were protective against HIV-1 infection in vivo. In the present study, we have also confirmed that the use of the HIV-1-pulsed mature DC is essential for induction of the anti-HIV-1 status in SCID mice. This view is supported by the finding that human $CD4^+$ T cells in DC-OVA-immunized mice did not produce the suppressor factor in vitro. These findings also suggest that the priming of HIV-1-reactive, naive $CD4^+$ T cells by sufficient numbers of HIV-1-pulsed DC is essential for the production of the putative suppressor factor in vivo.

The precise identity of this newly defined putative suppressor factor remains to be identified, as does the nature of the

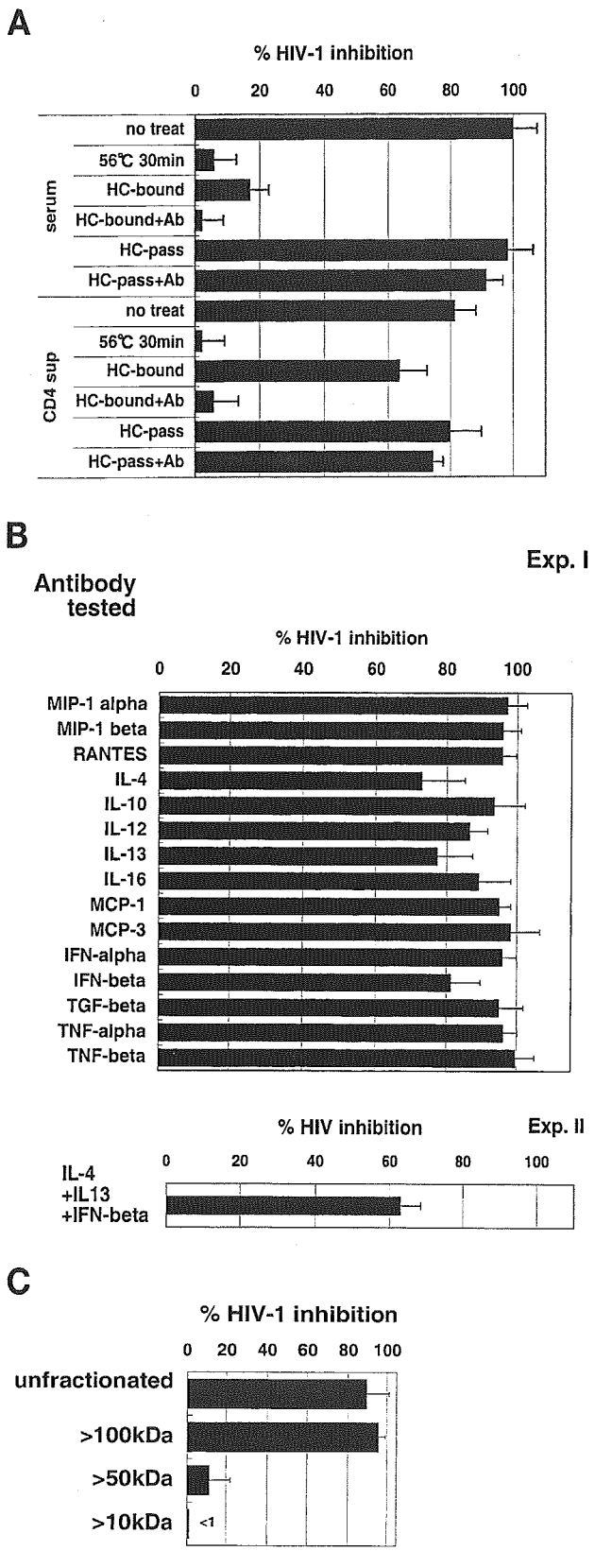


FIG. 7. Partial characterization of the HIV-1 suppressor factor. (A) The HIV-1 immune serum and in vitro-restimulated culture supernatants from DC–HIV-$1_{JR\text{-}CSF}$-immune hu-PBL-SCID-spl mice were heated at 56°C for 30 min or separated into heparin-binding and nonbinding fractions by passage of the serum or supernatant fluid through heparin-Sepharose columns (HC). The heparin-bound fraction was eluted with 2 M NaCl buffer. Thereafter, activated PBMC were pretreated with these samples (at final concentrations of 20% serum and 50% culture supernatants) in the absence or presence (+Ab) of a mixture of anti-β-chemokine antibodies and then infected with 500 $TCID_{50}$ of HIV-$1_{JR\text{-}CSF}$. After 5 days, the p24 level in each culture supernatant was calculated. The percent inhibition was calculated by using the p24 value obtained with the medium control, which was 22.3 ng/ml. (B) Pooled sera (10%) from the DC–HIV-$1_{JR\text{-}CSF}$-immune hu-PBL-SCID-spl mice were preincubated with each anti-human cytokine antibody at 10 μg/ml and then examined for suppressor activity against HIV-$1_{JR\text{-}CSF}$ infection of PBMC. In experiment (Exp.) II, the immune serum was pretreated with a mixture of anti-IL-4, anti-IL-13, and anti-IFN-β for 1 h before its addition to activated PBMC, and the PBMC were then infected with HIV-$1_{JR\text{-}CSF}$ as described above. The p24 value for the medium control was 20.0 ng/ml. (C) Pooled sera from the DC–HIV-$1_{JR\text{-}CSF}$-immune hu-PBL-SCID-spl mice were passed through the HC column, and then aliquots were filtered through 100-, 50-, and 10-kDa-cutoff Centricon filters. The filtrates obtained were examined for suppressive activity against HIV-$1_{JR\text{-}CSF}$ infection of PBMC at a 10% concentration. The data presented are representative of those from four independent experiments. Error bars indicate standard deviations.

HIV-1 antigen which is responsible for the induction of the factor. The approximate equal immunogenicities of R5 and X4 HIV-1 virions in the induction of the HIV-1 suppressor factor suggest that the V3 region of the Env gp120 that defines the selective tropism of the virus for the X4 and R5 receptors and the viral nonstructural proteins are not likely to be involved. Coculture of the HIV-1-immune $CD4^+$ T cells with truncated HIV-1 proteins an/or the use of synthetic overlapping HIV-1 peptides may help in the mapping of the HIV proteins and/or peptides that are potential inducers of this newly defined suppressor factor. From a therapeutic perspective, it will be of interest to determine whether the factor can be produced from $CD4^+$ T cells from HIV-1-infected individuals upon HIV-1 antigen stimulation in vivo and in vitro.

The mechanism for HIV-1 suppression by the present factor appears to be at the level of inhibition of an early stage of virus infection. The preferential suppression of R5 HIV-1 in activated primary PBMC cultures and macrophages in vitro suggests that the factor may belong to the CCR5-binding β-chemokines. However, several findings discount this possibility. These include the findings that (i) the concentrations of MIP-1α, MIP-1β, and RANTES in the DC–HIV-1-immune serum samples were too low to suppress HIV-1 in vitro; (ii) neutralizing antibodies against the three human β-chemokines did not reverse the suppressive activity; and (iii) the suppressor factor was not absorbed by a heparin-Sepharose column. These findings strongly suggest that the factor is not related at least to these groups of β-chemokines. Furthermore, the fact that the levels of expression of CCR5 and CD4 on macrophages after treatment with the factor did not change appreciably diminishes the possible relationship between the factor and the CCR5-binding β-chemokines. The other cytokines known to suppress R5 HIV-1 proliferation in primary macrophages are the Th2 cytokines IL-4 and IL-10 (21) and the proinflammatory cytokines TNF-α (11) and IFN-γ (5, 35). However, the involvement of these cytokines in the present studies of R5 HIV-1 suppression is less likely, since blocking antibodies

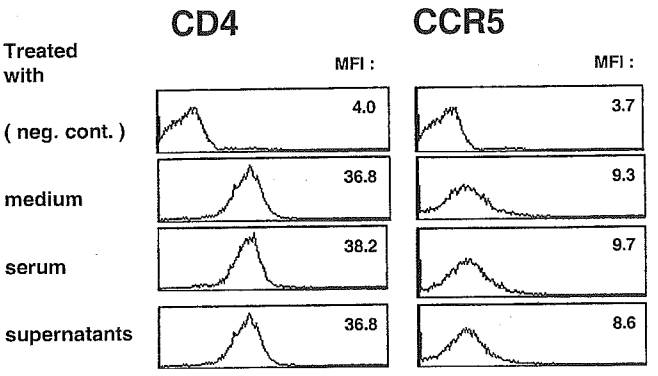


FIG. 8. The suppressor factor has no detectable effect on the levels of CCR5 and CD4 expression by macrophages. Macrophages cultured for 5 days with M-CSF were treated with either the immune serum (10%) or the in vitro-restimulated $CD4^+$ T-cell culture supernatants (50%) generated from the DC–HIV-$1_{JR\text{-}CSF}$-immune hu-PBL-SCID mice at 37°C for 1 h. The cells were FcR blocked and stained with anti-CD4 and -CCR5. The data shown are representative of those from four independent experiments. MFI, mean of fluorescence intensity.

against these cytokines did not interfere significantly with the suppressive activity under the present assay conditions. The involvement of human IFN-α/β, which have been implicated in anti-HIV-1 protective activity in SCID mice (12), is also unlikely, since X4 HIV-1 infection of PBMC was not suppressed by the factor under the same experimental conditions. Since neutralizing antibodies against IL-4, IL-10, and IFN-β alone or in combination showed a marginal blocking effect (20 to 35%) against the present factor, it remains to be resolved whether IL-4, IL-10, and IFN-β, which are known to suppress R5 HIV-1, work synergistically with the present HIV-1 suppressor factor or whether the factor shares epitopes with these molecules and is thus partially cross-reactive with these cytokines. Another possibility is that certain classes of anti-HIV-1 neutralizing antibodies and/or murine serum components may be involved. However, their contribution in vivo is likely to be minimal, since the suppressive activity of the HIV-1-immune serum was heat labile and not absorbed by protein G and since fresh sera from DC-OVA-immune mice had no suppressive activity.

The characteristics of the present HIV-1 suppressor factor are also different from those of other, as-yet-undefined, HIV-1 suppressor factors. First, the present factor is predominantly, but not exclusively, produced by DC–HIV-1-immune $CD4^+$ T cells, while the so-called CAF is produced by $CD8^+$ T cells from HIV-1-infected individuals, and CAF inhibits both R5 and X4 HIV-1 production at the level of viral transcription (15). It is important to note that hu-PBL-SCID mice reconstituted with human PBMC from HIV-1-exposed but uninfected individuals were resistant to both R5 and X4 HIV-1 infection by a $CD8^+$ T-cell-dependent mechanism (36). However, it has not been reported whether the anti-HIV-1 effect in these studies was mediated by the CCR5-binding chemokines or CAF. Although it remains unclear whether the factor exists either as a monomer, as an aggregate, or bound to serum proteins, the high molecular size of the present factor argues against a relationship of this newly identified factor to the human defensins α1, α2, and α3, which have recently been demonstrated to be produced by human $CD8^+$ T cells and to block both R5 and X4 HIV-1 infection of activated PBMC (36). Another CAF candidate is the modified form of bovine anti-thrombin III that is produced by $CD8^+$ T cells from HIV-1-infected individuals (6). This molecule is heat stable, 40 kDa by gel filtration, and suppressive to both X4 and R5 HIV-1 infection of cell lines and is thus clearly different from the present factor. Similarly, differences in molecular size and HIV-1 selectivity in suppression suggest that the present factor is distinct from the secretory leukocyte protease inhibitor that is a potent anti-HIV-1 factor in saliva (19) and from soluble poly anions such as dextran sulfate, heparin, or heparan sulfate, which interfere with CD4- and coreceptor-independent HIV-1 attachment to the cell surface heparan sulfate proteoglycans (29). It has previously been shown that the induction of β-chemokine-independent intrinsic resistance of $CD4^+$ T cells to R5 HIV-1 infection can be achieved in vitro by stimulation of $CD4^+$ T cells with a combination of anti-CD3- and anti-CD28-conjugated immunobeads (25). Furthermore, it has been shown that naive, but not memory, $CD4^+$ T cells from HIV-1-negative donors become resistant to R5 HIV-1 upon dual stimulation with anti-CD3 MAb and either anti-CD28 MAb or CD80 independently of CCR5-binding chemokines (20). Although these $CD4^+$ T cells could suppress R5 HIV-1 replication in activated memory $CD4^+$ T cells, it remains unclear whether these stimulated naive $CD4^+$ T cells secrete HIV-1 suppressor factors identical to the present factor.

In conclusion, the present study has demonstrated for the first time that a DC-based HIV-1 vaccination can induce HIV-1-reactive human $CD4^+$ T cells to produce an as-yet-undefined R5 HIV-1 suppressor factor in hu-PBL-SCID mice. These observations, together with the recent demonstration by Lu et al. (16) that DC pulsed with AT-2-inactivated SIV can stimulate protective anti-SIV-specific T-cell and antibody responses in rhesus monkeys, suggest a rational basis for DC-based immunization against HIV-1 infection in humans.

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# Quantitative Analysis of Human Immunodeficiency Virus Type 1 DNA Dynamics by Real-Time PCR: Integration Efficiency in Stimulated and Unstimulated Peripheral Blood Mononuclear Cells

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**Abstract.** We established a set of real-time PCR assay to accurately quantify human immunodeficiency virus type 1 (HIV-1) DNA in infected cells. Using this assay we were able to measure the strong-stop, full-length/1-LTR circle, 2-LTR circle, and integrated forms of viral DNA, and the data provided was quite consistent with the characteristics of mutant viruses in early phase of infection. Since our assay is particularly applicable to quantify the integrated DNA in small scale of samples, we measured the level of integrated DNA in wild-type virus (WT)- or Vpr-defective virus (ΔVpr)-infected peripheral blood mononuclear cells (PBMC), and examined whether quiescent condition of the PBMC influences integration step of HIV-1. Under stimulating condition approximately 25% of total viral DNA was in integrated form in either WT- or ΔVpr-infected cells. In contrast, under unstimulated condition the level of integration efficiency was not significantly reduced in WT-infected cells, while this efficiency was severely impaired in the absence of *vpr* gene. This result clearly demonstrated a crucial role of the Vpr for nuclear localization and subsequent integration of viral DNA in nondividing cells. Therefore, our assay is useful for analyzing the events in early phase of HIV-1 infection under various conditions.

**Key words:** *Alu*-PCR, HIV-1 DNA, integration, real-time PCR, unstimulated PBMC

## Introduction

Reverse transcription and integration are essential steps in the early phase of retrovirus infection that occurs before subsequent progeny production [1]. Short minus-strand DNA (strong-stop DNA) is an initial product of viral DNA and full-length double-stranded DNA is synthesized. The full-length DNA forms part of a high molecular weight nucleoprotein complex, the preintegration complex (PIC), which is composed of matrix, reverse transcriptase, integrase, and Vpr proteins in case of human immunodeficiency virus type 1 (HIV-1) [2,3]. In addition to the viral components, barrier-to-autointegration factor (BAF) and high mobility group proteins (HMGs) have been implicated as cellular factors in playing a role in integration [4,5]. Although the full-length DNA is integrated into chromosomal DNA to form a provirus in infected cells, a portion of unintegrated full-length DNA becomes circular DNA in nucleus [6]. To elucidate the early steps of HIV-1 infection, it is important to develop an assay for accurately monitoring the efficiency of the steps of DNA conversion from the reverse transcription to the integration under various conditions in infected cells.

Three major forms of HIV-1 DNA can be detected by Southern blot analysis: the full-length, circle, and

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high-molecular-weight integrated forms of viral DNA [7]. In addition, PCR technique also has been successful in quantifying various DNA forms individually, including the strong-stop, which contains R/U5, the full-length, the two-long terminal repeat (2-LTR) circle [8–10], and the integrated forms of DNA [11]. Quantitative PCR has been made possible with the development of real-time PCR [12], and this assay has been used to quantify HIV-1 specific DNA and RNA [6,13–15].

It has been reported that freshly isolated peripheral blood mononuclear cells (PBMC) are resistant to the establishment of productive infection by HIV-1 in unstimulated condition due to labile viral DNA in infected cells and they become susceptible to infection as they differentiate in culture to monocyte-derived macrophages (MDM) [16,17]. Moreover, it has been demonstrated that mutation in *vpr* gene severely impairs the viral replication in terminally differentiated cells such as MDM [18,19]. However, the direct evidence of integration rate in primary cells has not been shown.

In the present study, we established a new real-time PCR assay for accurately measuring the levels of various forms of HIV-1 DNA including the integrated DNA. Using this assay we demonstrate the integration efficiency of HIV-1 in primary cells and significant impairment of the integration in the absence of *vpr* gene in unstimulated PBMC.

## Materials and Methods

### *Cell Culture*

A human T-cell leukemia virus$^+$ and CD4$^+$ cell line, MT-4, was maintained in RPMI 1640 medium containing 10% fetal calf serum (FCS) [20]. Human PBMC were obtained from healthy, HIV-1-seronegative donors. For stimulation of the PBMC, cells were cultured in the presence of 1 μg of phytohaemagglutinin (PHA) (Seikagaku Corp., Tokyo, Japan) per ml for 3 days (PHA-PBMC) and further maintained in RPMI 1,640 containing 10% FCS and IL-2 (100 IU/ml; Shionogi Co., Osaka, Japan). To maintain unstimulated PBMC, the cells were cultured in RPMI 1,640 containing 10% human AB serum (Sigma Chemical Co., St. Louis, MO). 293T cells were maintained in D-MEM containing 10% FCS.

### *Virus Preparation and Infection*

A luciferase-expressing HIV-1 vector DNA containing a deletion of the envelope gene, pNL43luc$\Delta$env (wild-type [WT]), and its mutants were used [21]. The mutants contained a substitution in the integrase catalytic site (D116G) [21], defective Vpr ($\Delta$Vpr) [15], or a deletion of the primer-binding site (PBS) (position 641–651, $\Delta$PBS). Pseudotyped virus was generated by co-transfecting 293T cells with either pNL43luc$\Delta$env or its mutant DNA, and a vesicular stomatitis virus G protein (VSV-G)-expressing plasmid (pHCMVG) [22], using $CaPO_4$. Three days after transfection, the culture supernatants were cleared by filtration and stored at −80°C as virus stock. Virus was treated with DNase I (20 μg/ml) in the presence of 10 mM $MgCl_2$ for 30 min at room temperature. Heat-inactivated (65°C, 30 min) virus in culture medium was used as a negative control for infection. Five hundred thousand cells were exposed to the virus (100 ng of $p24^{gag}$ measured by HIV-1 $p24^{gag}$ enzyme-linked immunosorbent assay [Cellular Products, Buffalo, NY]) at 37°C for 2 h and cultured. Total DNA was isolated 48 h after infection as described [23].

### *Real-time PCR*

For the detection and quantification of the individual forms of HIV-1 DNA, we designed the following primer pairs (Fig. 1) and fluorogenic probes modified with 6-carboxyfluorescein (FAM) reporter dye on the 5′ end and 6-carboxytetramethylrhodamine quencher dye on the 3′ end (the numbering of nucleotide positions corresponds to that for the HIV-1 NL4-3 DNA sequence [24]). We used the following primers and probes to detect the viral DNAs indicated: for R/U5 DNA, sense primer M667 (5′-GGC TAA CTA GGG AAC CCA CTG C-3′, 496–517), antisense primer AA55 (5′-CTG CTA GAG ATT TTC CAC ACT GAC-3′, 612–635), and probe, HIV-FAM (5′-TAG TGT GTG CCC GTC TGT TGT GTG AC-3′, 554–579) [15]; for U5/gag DNA, sense primer LG564 (5′-CCG TCT GTT GTG TGA CTC TGG T-3′, 564–585), antisense primer LG699 (5′-GAG TCC TGC GTC GAG AGA TCT-3′, 679–699), and probe, LG-FAM (5′-CCC GAA CAG GGA CTT GAA AGC GAA-3′, 641–664); for 2-LTR circle, sense primer 2LTR-S (5′-CCC TCA GAC CCT TTT AGT CAG TG-3′, 9,668–9,690), antisense primer

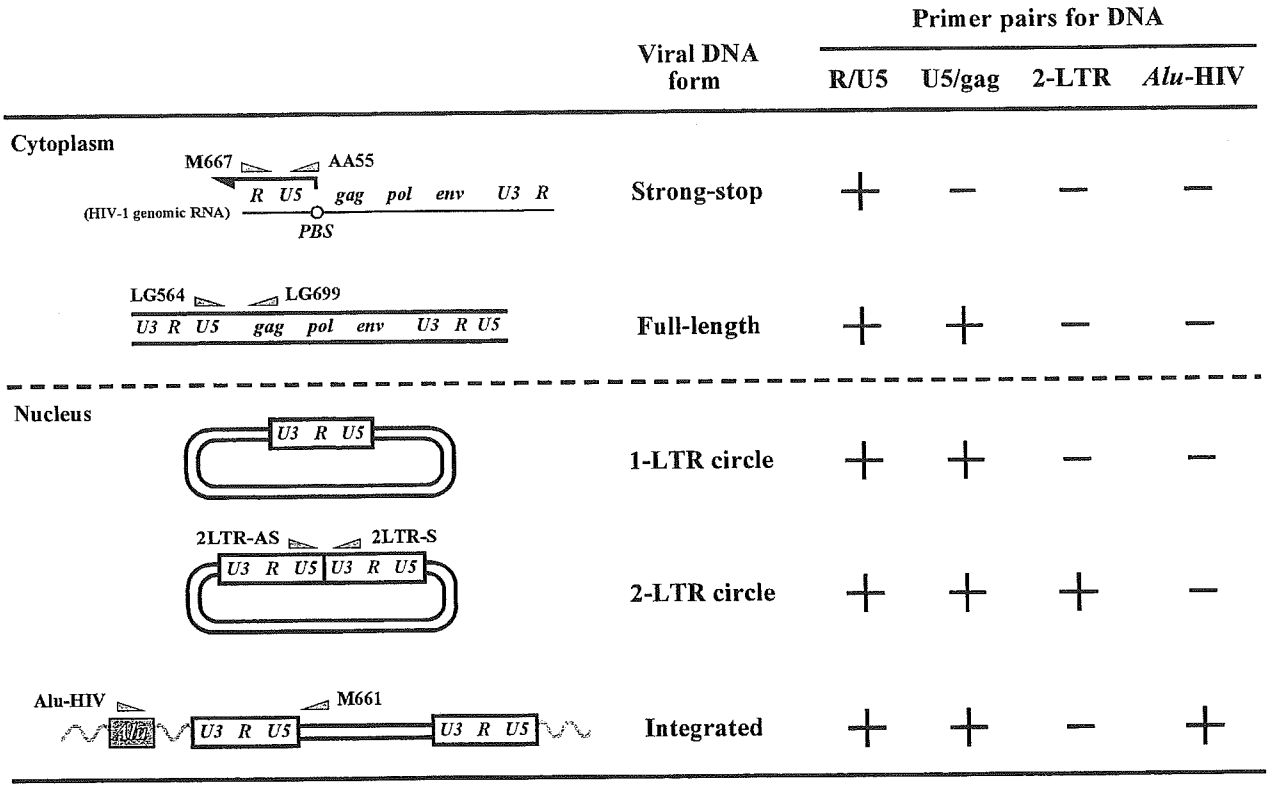


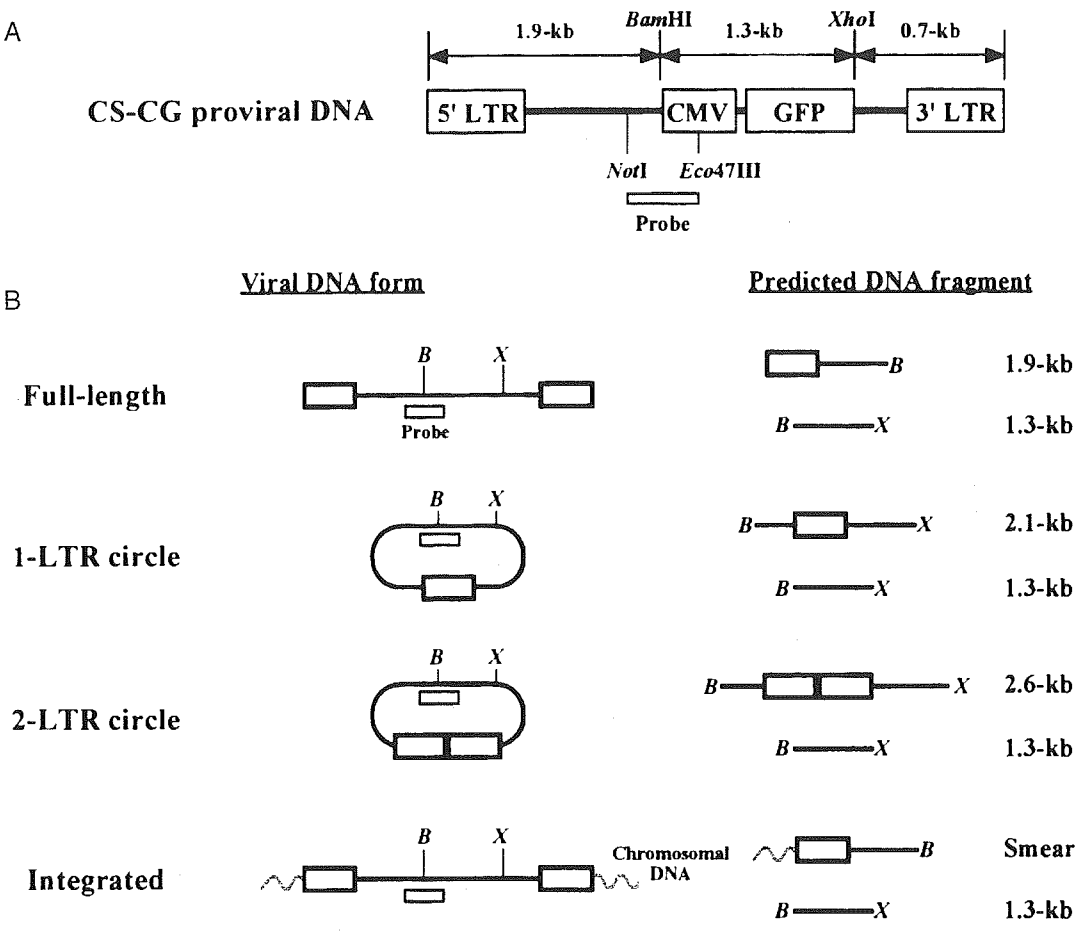

*Fig. 1.* Reactivity of oligonucleotide primers used for the real-time PCR assay. HIV-1 DNA (thick lines) is synthesized from the genomic RNA template (thin line) in the cytoplasm. Reverse transcription is initiated from PBS (open circle) and then, a short minus-stranded DNA is copied up to the 5′ end of the RNA (strong-stop DNA). Synthesis of double-stranded viral DNA (full-length DNA) is completed after the first and second template switches. The full-length DNA enters the nucleus, where it is integrated into the host genome or forms unintegrated circular DNA (1-LTR or 2-LTR circles). The following computations were used to determine the amounts of the individual DNA forms: the copy number of strong-stop DNA = R/U5 DNA copies – U5/gag DNA copies, and the copy number of unintegrated (linear) full-length and 1-LTR circle DNA = U5/gag copies – 2-LTR circle DNA copies – integrated DNA copies.

2LTR-AS (5′-TGG TGT GTA GTT CTG CCA ATC A-3′, 77–98), and probe 2LTR-FAM (5′-TGT GGA TCT ACC ACA CAC AAG GCT TCC-3′, 46–75) (Fig. 1). PCR was performed using an ABI PRISM 7,700 sequence detection system (PE-Applied Biosystems, Foster City, CA) and TaqMan Universal PCR Master Mix (PE-Applied Biosystems). Cycling conditions included a hot start (50°C for 2 min, 95°C for 10 min), followed by 40 cycles of denature (95°C for 15 s) and extension (60°C for 1 min).

For measuring the integrated DNA, a procedure described by Chun et al. [11] was applied with the following modifications. The first PCR was carried out using a GeneAmp XL PCR kit (PE-Applied Biosystems) with an *Alu*-sequence-specific sense primer, *Alu*-HIV (5′-TCC CAG CTA CTC GGG AGG CTG AGG-3′) [11], and an antisense primer made to the HIV sequence, M661 (5′-CCT GCG TCG AGA GAT CTC CTC TG-3′, 673–695) (Fig. 1) [10]. The cycling conditions included a denaturation step (94°C for 3 min), followed by 22 cycles of denaturation (94°C for 30 s), annealing (66°C for 30 s), and extension (70°C for 10 min), and then a final extension (72°C for 10 min). The first PCR product was subsequently diluted 100-, 1,000-, and 10,000-fold, and subjected to a real-time PCR assay for measuring R/U5 DNA using M667, AA55, and HIV-FAM as described above. The level of human β-actin DNA was also measured using the TaqMan PCR Reagent Kit (PE-Applied Biosystems) following the manufacturer's instructions, and the amount of HIV-1-specific DNA per cell was calculated.

### *Establishment of HIV-1-Vector-Transduced Cells*

A green fluorescent protein (GFP)-expressing HIV-1 vector, CS-CG was produced by cotransfecting an


A
CS-CG proviral DNA
1.9-kb
BamHI
1.3-kb
XhoI
0.7-kb
5' LTR
CMV
GFP
3' LTR
NotI
Eco47III
Probe
B
Viral DNA form
Predicted DNA fragment
Full-length
Probe
1.9-kb
1.3-kb
1-LTR circle
2.1-kb
1.3-kb
2-LTR circle
2.6-kb
1.3-kb
Integrated
Chromosomal DNA
Smear
1.3-kb
C
Times after infection
M.W.
4 h
12 h
24 h
48 h
7 w
Higher resolution
6.8-kb
1.3-kb
0.7-kb
2-LTR (2.6-kb)
1-LTR (2.1-kb)
Full-length (1.9-kb)
Total viral DNA (1.3-kb)
1 2 3 4 5 6 7

HIV-1 vector plasmid (pCS-CG) [25], a VSV-G-expressing plasmid (pHCMVG), and a packaging plasmid (pCMVΔ8.2) [26]. After the HIV-1 vector (100 ng of $p24^{gag}$) was treated with DNase I, MT-4 cells were inoculated with the vector, and the cells were cultured for a further 7 weeks. DNA was isolated from the cells, and the amounts of HIV-1 DNA in the R/U5, U5/gag, and 2-LTR circle were measured by real-time PCR as described above.

*Southern Blot Analysis*

MT-4 cells ($5 \times 10^5$ cells) were inoculated with DNase I-treated HIV-1 vector CS-CG (100 ng of $p24^{gag}$) at 37°C for 2 h and subsequently cultured. Total DNA (10 μg) was isolated 4, 12, 24, and 48 h after infection and digested with *Bam*HI and *Xho*I. Purified DNA was treated with RNase and separated with 0.8% agarose gel electrophoresis. After Southern blot transfer, viral DNA was detected by hybridization with a random primed $^{32}$P-labeled probe for CS-CG DNA sequence. Template DNA for virus-specific probe was isolated from pCS-CG as a 1.4-kb *Not*I and *Eco*47III fragment [25].

*Luciferase Assay*

Cells were harvested 72 h after transfection or 48 h after infection, respectively, and subjected to a luciferase assay as described previously [15].

*Cell-cycle Analysis*

Cell-cycle progression was examined by single-color flow cytometric analysis of the DNA content. In brief, after treatment with 70% ethanol, the cell pellet was incubated with 50 μg of RNase A (QIAGEN, Valencia, CA) and propidium iodide (50 μg/ml, Sigma). The DNA content was then analyzed using a FACScan (Becton Dickinson, San Jose, CA).

## Results

*Establishment of a Standard Cell Line for* Alu-*HIV PCR*

To measure the amount of integrated viral DNA, we modified a previously described *Alu*-HIV PCR procedure [11] by applying a real-time PCR system. For preparing a standard DNA that HIV provirus would be present in infected cells, we generated a cell line, MT4/CS-CG that was transduced with a GFP-expressing HIV-1 replication-incompetent vector, CS-CG [25] and monitored viral DNA forms in the transduced cells by Southern blot analysis (Fig. 2). Briefly, DNA from the cells was digested with *Bam*HI and *Xho*I, which divide CS-CG proviral DNA into three fragments and detected with a probe overlapping a 1.9-kb 5′ fragment and a 1.3-kb internal fragment of CS-CG DNA (Fig. 2A). The internal 1.3-kb fragment derived from all viral DNA forms, indicating total amount of viral DNA in infected cells (Fig. 2B). On the other hand, full-length, 1-LTR circle, or 2-LTR circle DNA form can be differentiated from the size of the detected fragment (1.9-, 2.1-, and 2.6-kb, respectively), and integrated DNA can be visualized as a smear more than 1.3-kb band (Fig. 2B). As indicated in Fig. 2C, unintegrated forms of DNA reached a peak within 12 h after infection, then declined (Fig. 2C, lanes 2, 4). In contrast, the formation of integrated DNA gradually increased at later time points after infection (Fig. 2C, lanes 2–5) and, thereafter, more than 80% of the cells were GFP-positive. Seven weeks after

*Fig. 2.* Southern blot analysis of viral DNA forms in CS-CG infected MT-4 cells. (A) Schematic representation of CS-CG proviral DNA. A GFP-expressing HIV-1 replication-incompetent vector, CS-CG was produced from pCS-CG by co-transfecting with VSV-G expressing plasmid and packaging plasmid. The *GFP* gene is expressed under control of CMV promoter [25]. The *Not*I-*Eco*47III restriction fragment was used as a probe for Southern blot analysis. (B) Strategy for detection of viral DNA forms. DNA from CS-CG-infected cells was digested with *Bam*HI (*B*) and *Xho*I(*X*) and detected by Southern blot analysis. The probe overlapping the *Bam*HI site can hybridize with both a 1.9-kb 5′ fragment and a 1.3-kb internal fragment. The internal 1.3-kb fragment derived from all viral DNA forms, indicating total amount of viral DNA. The 1.9-, 2.1-, and 2.6-kb fragment including 5′ LTR correspond respectively to full-length, 1-LTR circle, and 2-LTR circle. However, integrated DNA are visualized as smear band, because those 5′ fragments are joined with chromosomal DNA. (C) Detection of viral DNA forms. DNA were isolated from CS-CG-infected MT-4 cells 4 (lane 2), 12 (lane 3), 24 (lane 4) and 48 h (lane 5) after infection and subjected to Southern blot analysis. Lane 6 shows the DNA from the cells 7 weeks after infection (i.e., standard cell line, MT4/CS-CG). Lane 7 is higher resolution of the lane 5 to distinguish 1-LTR or 2-LTR circles. Lane 1 shows pCS-CG (100 pg) digested with *Not*I, *Bam*HI and *Xho*I as a molecular weight marker (M.W.).