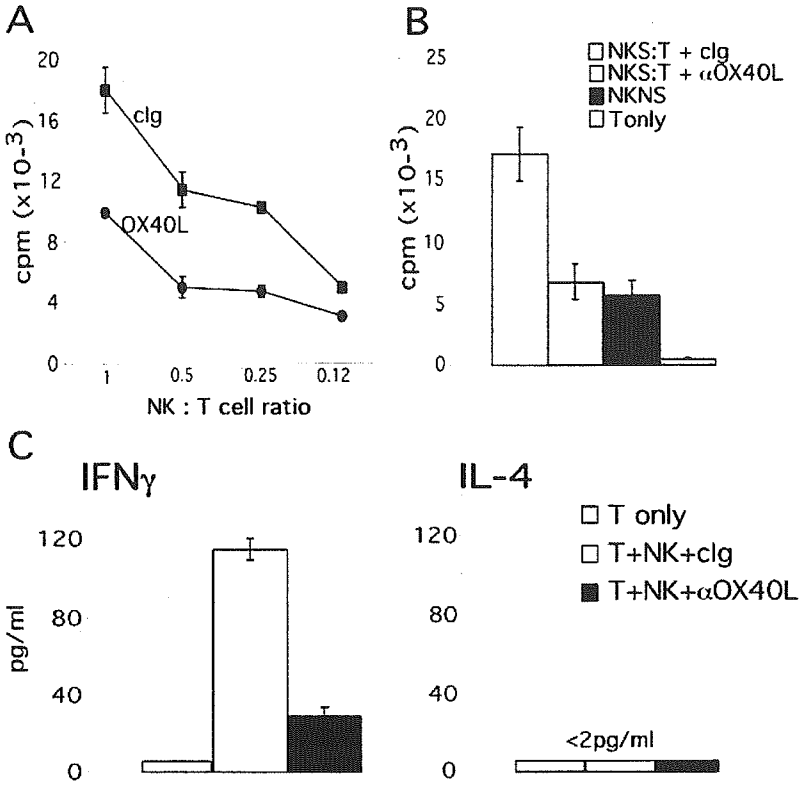

FIGURE 4. Anti-CD3 induced CD4 T cell proliferation and IFN- production costimulated by OX40L on autologous CD16-activated NK cells. *A*, IL-2-activated polyclonal NK cells were stimulated with plate-bound anti-CD16 mAb and fixed with 1% paraformaldehyde. Different numbers of anti-CD16-activated NK cells were plated with 1 10^5 autologous resting CD4 T cells in the presence of soluble anti-CD3 and cultured as described in Fig. 1*B*. Neutralizing anti-OX40L mAb or a clg was added at day 0. A representative experiment of four is shown. Data are represented as the mean of cpm SD (triplicates). *B*, Cocultures of autologous activated NK cells and resting CD4 T cells at a ratio of 1:1 were established as described in Fig. 4*A*, using anti-CD16-stimulated NK cells (NKS) or clg (anti-CD56 mAb)-treated NK cells (NKNS). Neutralizing anti-OX40L mAb or a clg was added to the coculture of NKS and autologous CD4 T cells stimulated with anti-CD3, as indicated. Data are represented as the mean of cpm SE of seven independent experiments. *C*, Activated NK cell-resting CD4 T cell cocultures stimulated with anti-CD3 mAb were established as described in Fig. 4*A*. Neutralizing anti-OX40L mAb or a clg was added to the cocultures, as indicated. Supernatants were collected after 72 h and tested for the presence of IFN- or IL-4. Data are represented as the mean SD (triplicates). A representative experiment of three is shown.

OX40L and B7 contribute to NK cell costimulation of CD4 T cell

We considered that the inability of anti-OX40L mAb to completely block CD4 T cell proliferation induced by activated NK cells may be due to the presence of CD86 (and perhaps CD80) on the activated NK cells (Fig. 2, *C* and *D*). Therefore, additional experiments were performed in which CD16-stimulated NK cells were cocultured with autologous CD4 T cells and anti-CD3 using a mixture of neutralizing mAbs against CD80 and CD86 (38) alone or in combination with anti-OX40L (Fig. 6). Interestingly, while mAbs against CD80 plus CD86 or OX40L individually partially inhibited NK cell-induced T cell proliferation, we observed that the combination of neutralizing mAbs against CD80, CD86, and OX40L completely blocked TCR-dependent CD4 T cell proliferation (results from two different blood donors are shown and are representative of five experiments). Collectively, these data show that CD16-stimulated NK cells efficiently costimulate TCR-dependent CD4 T cell proliferation through the expression of OX40L and B7-family members on the CD16-activated NK cells.

Discussion

Although it has been appreciated that NK cell production of IFN- and possibly other cytokines and chemokines can affect innate and adaptive immune responses, the potential role for direct cell-cell interactions between NK cells and T lymphocytes, in particular CD4 T cells, has not been explored. Roncarolo and colleagues (39) previously reported that human NK cell clones are able to stimulate autologous CD4 T cells, but the molecules involved in this process were not defined. Our unexpected finding that OX40L was up-regulated when NK cell receptors were stimulated on a transformed NK cell line prompted us to re-evaluate how activated NK cells are able to augment the TCR-dependent proliferation of resting autologous peripheral blood CD4 T cells. In this study, we provide evidence that activated human NK cells are able to help TCR-stimulated autologous CD4 T cells by a process that involves both OX40L and B7 costimulation.

Resting peripheral blood NK cells express neither OX40L nor B7, and different stimuli are required to induce these costimulatory molecules. Culture in IL-2 alone was sufficient to induce CD86, but not OX40L. By contrast, stimulation with IL-2 and activation through an NK receptor was required to induce OX40L. In addition to IL-2, IL-12 and IL-15 were also able to prime NK cells such that they up-regulated OX40L when subsequently stimulated via CD16. Because IL-12 and IL-15 are innate cytokines that may be more available at a site of inflammation or an ongoing immune response, these may represent the more physiologically relevant cytokines in vivo.

With respect to the NK receptors that induced OX40L, our first clues were derived from studies of the transformed NKL cell line. Although this cell constitutively expressed OX40L, it can be up-regulated by engaging either the DAP12-associated KIR2DS2 receptor that activates the Syk and ZAP70 tyrosine kinase pathways (40), or by stimulating the DAP10-associated NKG2D receptor that uses a PI3K-dependent activation pathway (33). We do not have Abs that can discriminate between the activating and inhibitory KIR; therefore, in studies of peripheral blood NK cells, we stimulated the NK cells with anti-CD16, which couples to the ITAM-bearing Fc RI and CD3 adapter proteins and activates Syk and ZAP70. When IL-2-preactivated peripheral blood NK cells were stimulated with either anti-CD16 or anti-NKG2D (or exposed to cells expressing the NKG2D ligand, MICA), OX40L was rapidly induced. Interestingly, only a subset comprising 20% of the peripheral blood NK cells expressed OX40L after stimulating NKG2D, despite the fact that essentially all of the NK cells expressed NKG2D. Further studies are needed to determine why expression of OX40L was confined to a subset of the NKG2D-activated NK cells. By contrast, a much larger frequency of NK cells (typically 60% or more) expressed OX40L after CD16

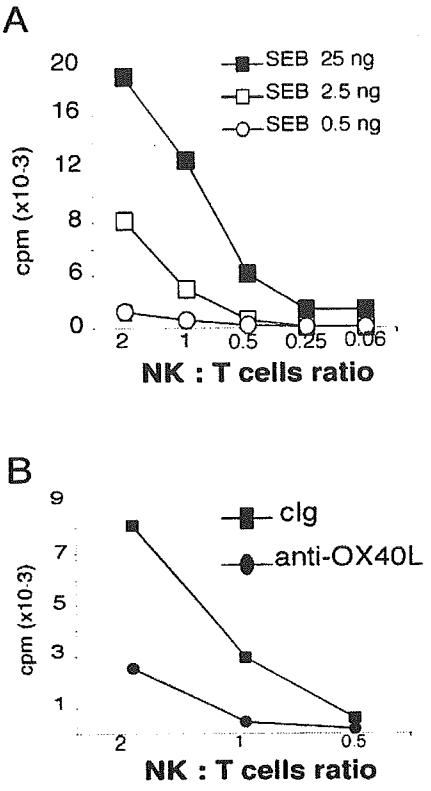

FIGURE 5. OX40L expressed on autologous NK receptor-activated NK cells is involved in SEB-induced proliferation of CD4 T cells. *A*, Anti-CD16-activated NK cells were prepared as described in Fig. 4*A*. Autologous resting CD4 T cells and activated NK cells were cocultured for 5 days in the presence of different concentrations of SEB, as indicated. Data are represented as the mean of cpm SD (triplicates). A representative experiment of two is shown. *B*, Autologous resting CD4 T cells and anti-CD16-activated NK cells at the indicated ratios were cocultured in the presence of 2.5 ng/ml SEB for 5 days. Neutralizing anti-OX40L mAb or cIg was added at day 0. Data are represented as the mean of cpm SD (triplicates). A representative experiment of three is shown.

activation. Many of the NK receptors, e.g., NKp30, NKp44, NKp46, CD16, and the activating KIR (41), use ITAM-based adapter proteins to activate the Syk/ZAP70 tyrosine kinases. Therefore, we suspect that OX40L may be induced when any of these diverse receptors are engaged because they use a common downstream signaling pathway. Together with the ability of IL-2, IL-12, or IL-15 to render the NK cells permissive for NK receptor induction of OX40L, our findings indicate that OX40L may be available in many different physiological situations for potential interactions with T cells bearing OX40.

Where might activated NK cells and CD4 T cells interact? This interaction might happen in peripheral tissues such as the liver in which both NK cells and T cells are resident (42) and accumulate following virus infection (43). Furthermore, a recent report has revealed that NK cells are relatively abundant in the human secondary lymphoid organs (44), and importantly, immunohistochemistry studies have detected NK cells in the parafollicular T cell areas of human lymph nodes (24), providing another possible location in which NK:T cell interactions might occur during an immune response. During a viral or bacterial infection, NK cells in the lymph nodes may be exposed to an environment containing IL-2, IL-12, or IL-15, and potential NKG2D ligands or immune complexes (that engage CD16), thereby providing the stimuli needed for induction of OX40L and allowing them to interact with activated CD4 T cell-expressing OX40.

It should be appreciated that activated human NK cells express high levels of MHC class II (36, 37), which provides them the potential to present Ag to human CD4 T cells. Indeed, in these studies, we have shown that activated NK cells have the capability to directly stimulate CD4 T cell proliferation by presenting SEB to CD4 T cells. Therefore, activated human NK cells possess not only the required costimulatory molecules (e.g., OX40L and B7) for potential interaction with activated CD4 cells, but they also, in theory, have the capacity of present Ags via MHC class II. Collectively, our in vitro experiments provide compelling evidence that human NK cells and autologous CD4 cells can interact and that OX40L is an important participant in this process. It is difficult to provide formal proof of this interaction in vivo in humans. Unfortunately, because activated mouse NK cells (unlike human NK cells) do not express MHC class II, mice do not provide a relevant or appropriate model to examine MHC class II TCR-dependent CD4 cell interactions with NK cells. Although dendritic cells are considered the most potent APCs, the fact that activated NK cells express MHC class II, CD86, and OX40L strongly suggests the possibility that they may also communicate directly with CD4 cells. Otherwise, for what purpose would NK receptor-activated human NK cells express MHC class II, CD86, and OX40L?

Our findings demonstrate that human NK cell costimulation of TCR-induced CD4 T proliferation depends in a large part on OX40–OX40L interactions. Studies conducted using OX40-deficient mice have shown that OX40-deficient CD4 T cells initially become activated to secrete IL-2 (albeit at slightly lower levels than wild-type mice), but they are unable to sustain proliferation (45). Other studies performed on OX40 / mice reported that the impaired in vitro proliferative response to anti-CD3 stimulation

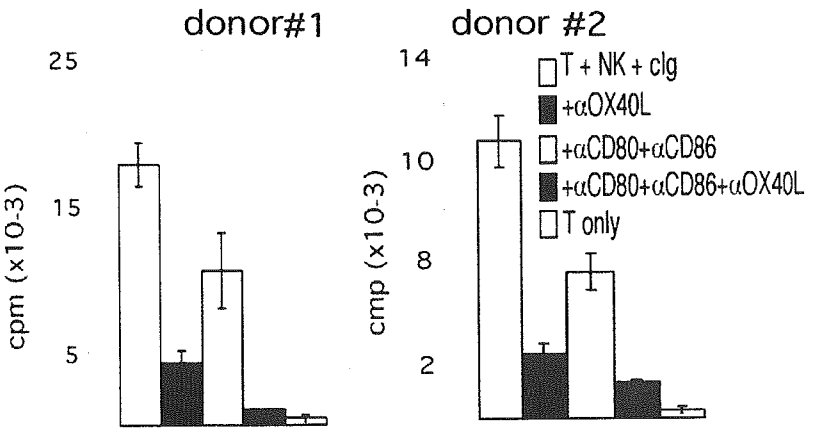

FIGURE 6. NK cell costimulation of TCR-dependent CD4 T cell proliferation involves B7 family members. Cocultures of anti-CD16-stimulated autologous NK and CD4 T cells at a ratio of 1:1 were established and stimulated with anti-CD3 as described in Fig. 4*A*. Neutralizing mAbs against CD80, CD86, and/or OX40L or cIg, as indicated, were added on day 0 at 5 g/ml. Data are represented as the mean SD (triplicates). Two representative experiments of five are shown.

could not be corrected by the addition of exogenous rIL-2 (46). Most significantly, it has been shown that OX40 is a major regulator of anti-apoptotic proteins, such as Bcl-xL and Bcl-2 (45), and strongly promotes the survival of Ag-activated primary CD4 T cells (11). Similarly, the contribution of OX40-OX40L interactions to T cell proliferation that we have observed may favor T cell survival by the induction of Bcl-xL and Bcl-2, although this awaits further evaluation.

Previous studies reported that OX40L expressed on mouse B cells induce a Th2-type response, leading to the expansion of IL-4-producing mouse T effector cells and inhibiting IFN- expression (47, 48). In humans, a role for OX40L in the development of Th2 effector cells has also been reported (49). However, other studies do not support a differential role for OX40L in inducing Th1 vs Th2 differentiation (11, 13, 50, 51). suggesting that it only enhances the pre-existing response. In our studies using activated human NK cells to costimulate autologous CD4 T cells, we observed the production of IFN- , but not IL-4 secretion, by the TCR-activated T cells. These findings suggest that activated, mature human NK cells may preferentially promote T cell IFN- production.

We believe that the induction of OX40L on NK cells by NKG2D ligand-expressing cells might have important implications in the context of tumor surveillance and infectious diseases. It has been shown that the NKG2D ligand MICA is up-regulated on several human tumor cells and, interestingly, soluble MICA has been found in the serum of patients affected by different progressive tumors (52). In addition, several studies have reported that MICA is induced on cells infected with *Mycobacteria tuberculosis* (18), *Escherichia coli* (19), or cytomegalovirus (17). Thus, initial interactions between NK cells and NKG2D ligand-bearing cells or soluble NKG2D ligands may trigger killing and cytokine production and in the presence of IL-2, IL-15, or IL-12 may induce expression of OX40L on the NK cells. Subsequent interactions between OX40L NK cells and OX40 T cells may amplify and sustain an adaptive ongoing immune response. At least under the experimental conditions used, we observed the induction of OX40L only on a subset of activated human peripheral blood NK cells. Further studies are necessary to resolve why some NK cells, but not others, expressed OX40L upon NKG2D stimulation, because all NK cells express NKG2D on the cell surface.

The OX40-OX40L interaction has been shown to induce bidirectional signals. For example, OX40L stimulation by OX40 transduces a signal in dendritic cells, which results in enhanced TNF- and IL-1 production (2). Similarly, triggering of OX40L expressed on activated B cells results in B cell proliferation and Ig secretion (53). Finally, engagement of OX40L on vascular endothelial cells leads to the induction of c-*fos* and c-*jun* mRNA expression and the production of the chemokine RANTES (54, 55). Thus, while our present studies have focused on the potential role of OX40L on NK cell interactions with CD4 T cells, it will also be of interest to examine whether engagement of OX40L on NK cells might regulate their effector functions.

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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

Atsushi Yoshida,[1] Reiko Tanaka,[1] Tsutomu Murakami,[1] Yoshiaki Takahashi,[1] Yoshio Koyanagi,[2] Masataka Nakamura,[3] Mamoru Ito,[4] Naoki Yamamoto,[5] and Yuetsu Tanaka[1]

Department of Immunology, Graduate School and Faculty of Medicine, University of the Ryukyus, Nishihara, Okinawa 903-0215,[1] Department of Virology, Tohoku University Graduate School of Medicine, Sendai, Miyagi 980-8575,[2] Department of Molecular Virology[5] and Human Gene Science Center,[3] Tokyo Medical and Dental University, Chiyoda-ku, Tokyo 113-8519, and Central Institute for Experimental Animals, Kawasaki, Kanagawa 216-0001,[4] Japan

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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

* Corresponding author. Mailing address: Department of Immunology, Faculty of Medicine, University of the Ryukyus, Uehara 207, Nishihara-cho, Okinawa 903-0215, Japan. Phone: 81-98-895-1202. Fax: 81-98-895-1437. E-mail: yuetsu@ma.kcom.ne.jp.

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 3×10^6 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 (5×10^5 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 (3×10^6 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 (5×10^5 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 (2×10^6 cells) collected from the spleens and peritoneal lavage of the immunized mice were cultured for 2 days at 37°C with 2×10^5 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

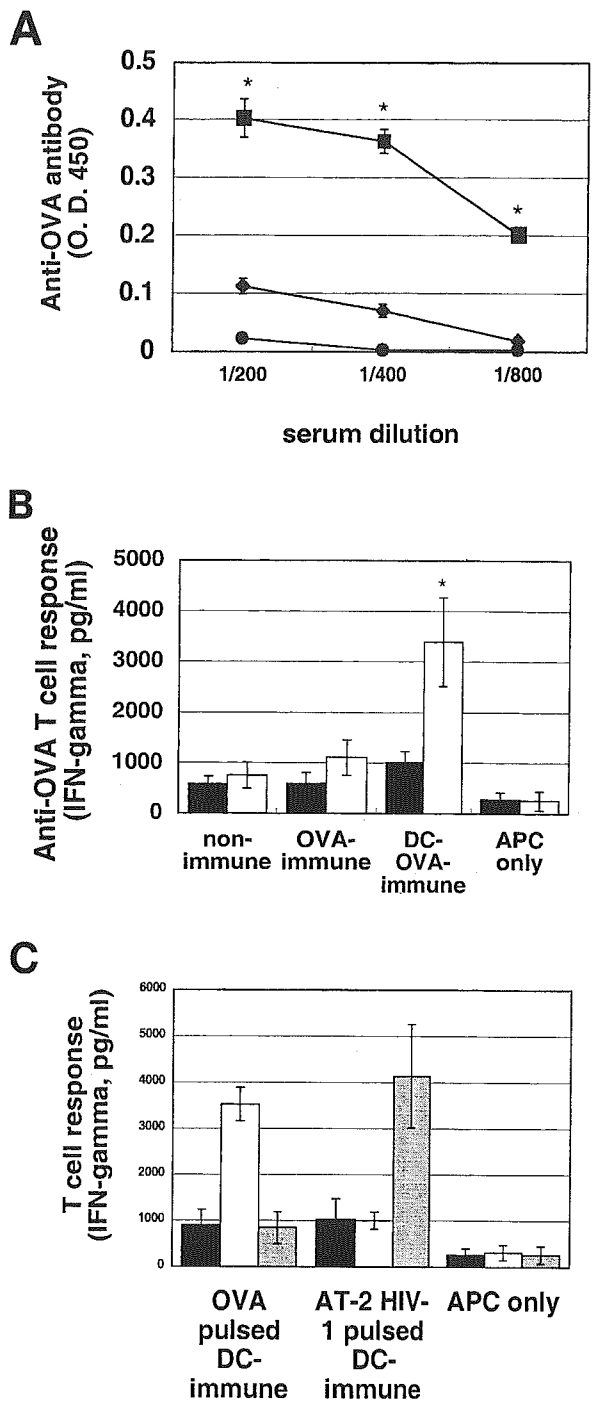

FIG. 1. Induction of antigen-specific human immune responses in hu-PBL-SCID-spl mice by immunization with antigen-pulsed DC. (A) PBMC (3×10^6 cells) alone (nonimmune) (●), with OVA (100 μg) (OVA immune) (◆), or with DC (5×10^5 cells) pulsed with OVA (100 μg) (DC-OVA immune) (■) were engrafted into the 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 (2×10^6 cells) recovered from hu-PBL-SCID-spl mice were cocultured with 2×10^5 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 (2×10^6 cells) recovered from hu-PBL-SCID-spl mice which were immunized with either DC-OVA or DC-AT-2-inactivated HIV-1_{JR-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 2×10^7 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 5×10^6 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 (3×10^6 cells) from normal human donors were transferred into the SCID mouse spleen together with autologous mature DC (5×10^5 cells) pulsed with OVA (100 μg) or AT-2-inactivated HIV-1_{JR-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 (5×10^5 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-

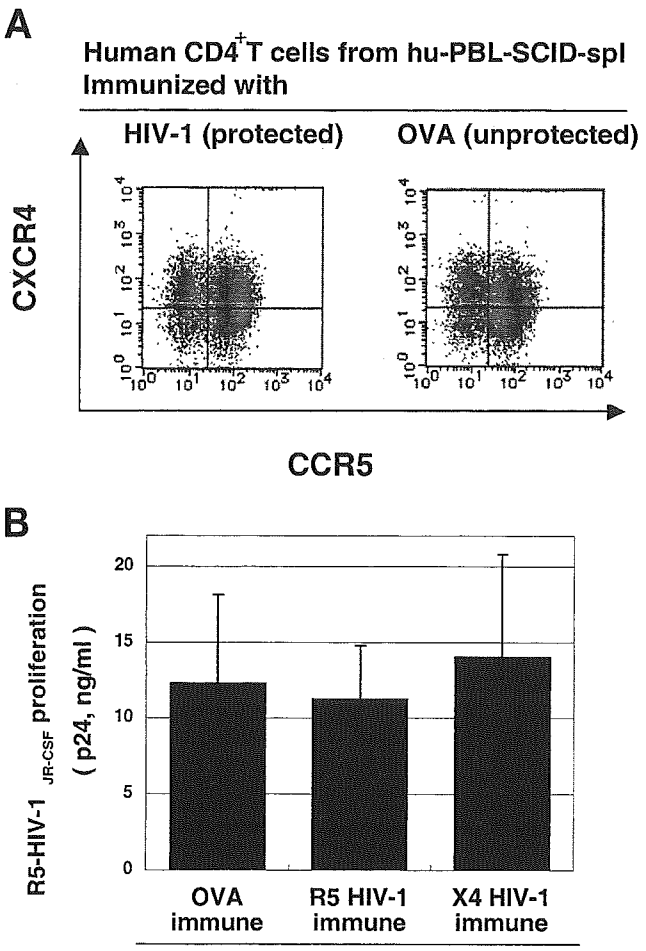

FIG. 2. Human CD4+ T cells from HIV-1-protected hu-PBL-SCID-spl mice express CCR5 and are permissive for R5 HIV-1 infection in vitro. (A) Lymphocytes recovered from hu-PBL-SCID-spl mice immunized with DC-OVA or DC–HIV-1_{JR-CSF} were stained with anti-CD4, anti-CCR5, and anti-CXCR4. The expression profiles 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.

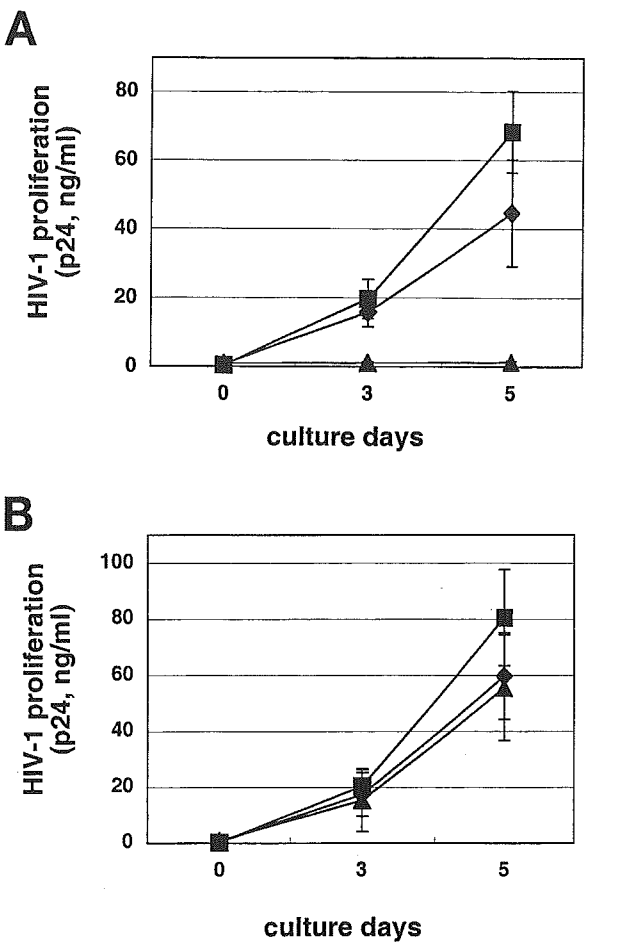

FIG. 3. Inhibition of R5 (A), but not X4 (B), HIV-1 infection by the HIV-1 immune serum. PBMC (5×10^5 cells/well) activated in vitro for 3 days were washed and then incubated in medium (■) or final 20% serum samples obtained from either DC-OVA-immune (◆) or DC–HIV-1_{JR-CSF}-immune (▲) hu-PBL-SCID-spl 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

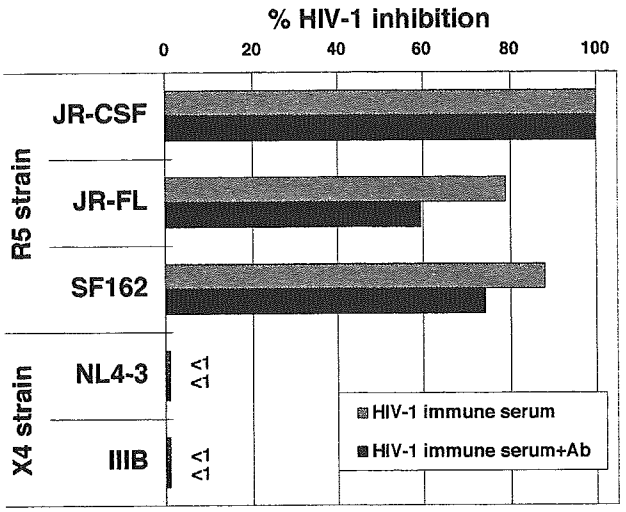

FIG. 4. Inhibition of R5, but not X4, HIV-1 by the HIV-1 immune serum. In vitro-activated PBMC were treated at 37°C for 1 h with 10% pooled serum samples obtained from DC–HIV-1_{JR-CSF}-immune hu-PBL-SCDI-spl mice, followed by the addition of 500 $TCID_{50}$ of HIV-1 (R5 isolates JR-CSF, JR-FL, and SF162 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.

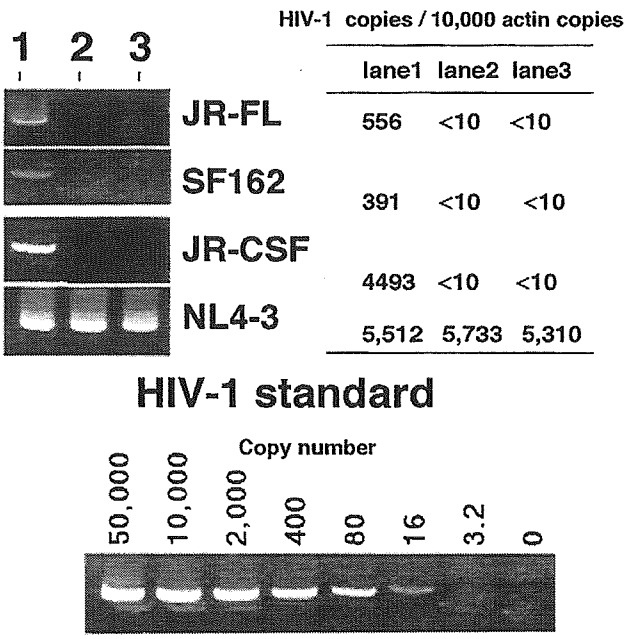

FIG. 5. Blocking of R5 HIV-1 infection in human macrophage cultures. Cultured macrophages or activated PBMC were preincubated in medium alone (lane 1) or with pooled serum samples from DC–HIV-1_{JR-CSF}-immune SCID mice in the absence (lane 2) or presence (lane 3) of a mixture of anti-β chemokines, and 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_{NL4-3}. 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 downregulate 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

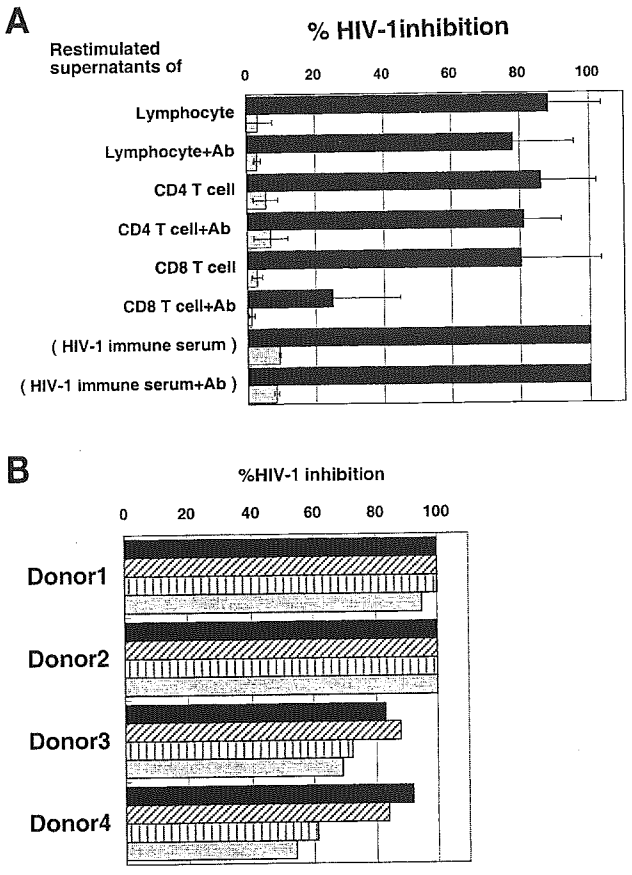

FIG. 6. Identification of the cell population producing the suppressive factor. (A) Lymphocytes from DC–HIV-$1_{JR\text{-}CSF}$-immune hu-PBL-SCID-spl mice were positively selected into human $CD4^+$ and $CD8^+$ T-cell subpopulations by using anti-CD4 and anti-CD8 MAb-conjugated immunobeads. Unfractionated (lymphocytes) or $CD4^+$ or $CD8^+$ T-cell populations (2×10^6 cells) were cocultured with autologous APC (2×10^5 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

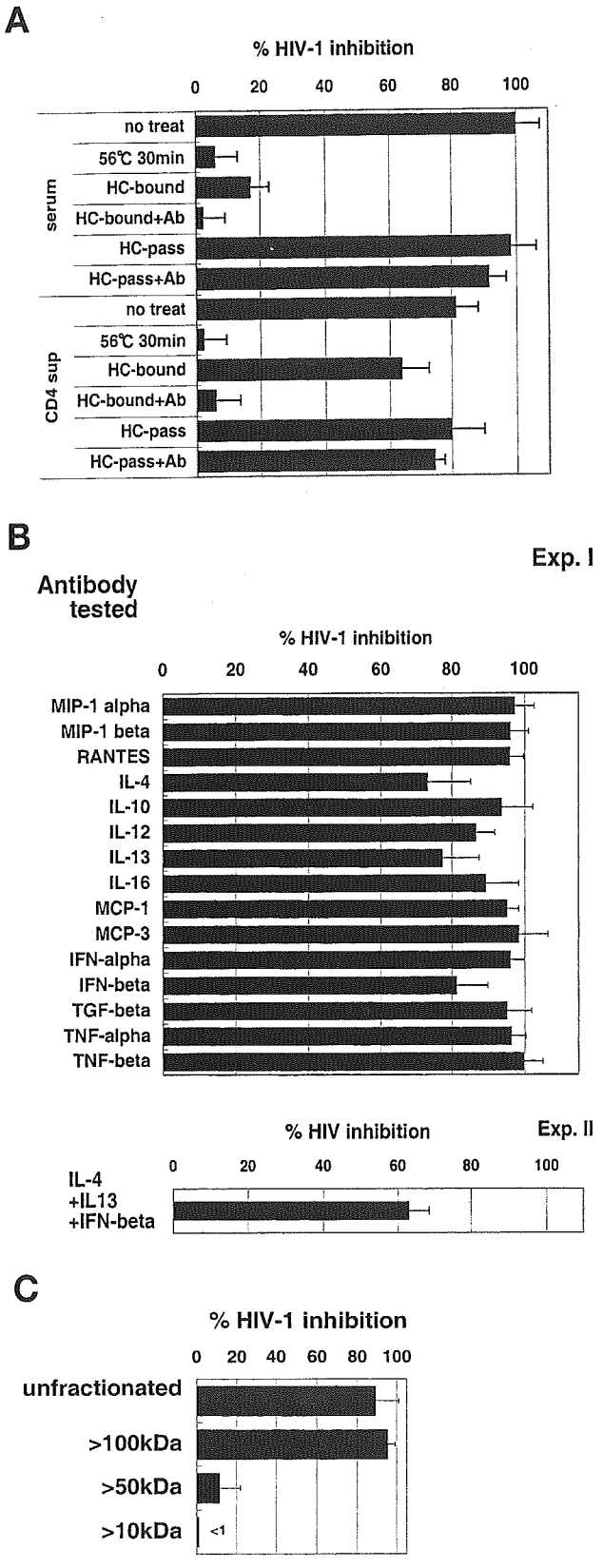

FIG. 7. Partial characterization of the HIV-1 suppressor factor. (A) The HIV-1 immune serum and in vitro-restimulated culture supernatants from DC–HIV-$1_{JR\text{-}CSF}$-immune hu-PBL-SCID-spl mice were heated at 56°C for 30 min or separated into heparin-binding and nonbinding fractions by passage of 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

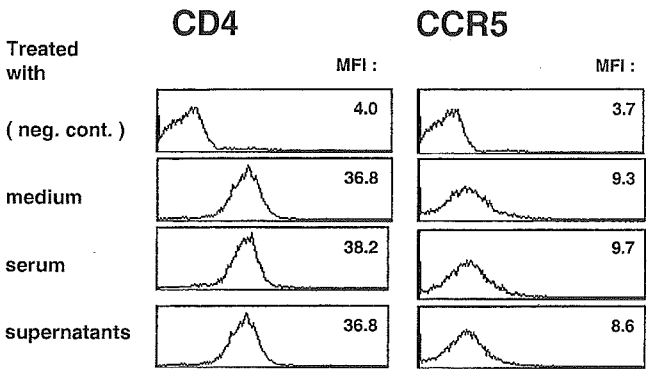

FIG. 8. The suppressor factor has no detectable effect on the levels of CCR5 and CD4 expression by macrophages. Macrophages cultured for 5 days with M-CSF were treated with either the immune serum (10%) or the in vitro-restimulated $CD4^+$ T-cell culture supernatants (50%) generated from the DC–HIV-$1_{JR\text{-}CSF}$-immune 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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HIV-1 Vpr Induces DNA Double-Strand Breaks

Hiroaki Tachiwana,[1,2] Mari Shimura,[2] Chikako Nakai-Murakami,[2] Kenzo Tokunaga,[3] Yoshimasa Takizawa,[1,2] Tetsutaro Sata,[3] Hitoshi Kurumizaka,[1] and Yukihito Ishizaka[2]

[1]Graduate School of Science and Engineering, Waseda University; [2]Department of Intractable Diseases, International Medical Center of Japan; and [3]Department of Pathology, National Institute of Infectious Diseases, Tokyo, Japan

Abstract

Recent observations imply that HIV-1 infection induces chromosomal DNA damage responses. However, the precise molecular mechanism and biological relevance are not fully understood. Here, we report that HIV-1 infection causes double-strand breaks in chromosomal DNA. We further found that Vpr, an accessory gene product of HIV-1, is a major factor responsible for HIV-1–induced double-strand breaks. The purified Vpr protein promotes double-strand breaks when incubated with isolated nuclei, although it does not exhibit endonuclease activity *in vitro*. A carboxyl-terminally truncated Vpr mutant that is defective in DNA-binding activity is less capable of Vpr-dependent double-strand break formation in isolated nuclei. The data suggest that double-strand breaks induced by Vpr depend on its DNA-binding activity and that Vpr may recruit unknown nuclear factor(s) with positive endonuclease activity to chromosomal DNA. This is the first direct evidence that Vpr induces double-strand breaks in HIV-1–infected cells. We discuss the possible roles of Vpr-induced DNA damage in HIV-1 infection and the involvement of Vpr in further acquired immunodeficiency syndrome–related tumor development. (Cancer Res 2006; 66(2): 627-31)

Introduction

A high incidence of malignant tumors, such as non-Hodgkin's lymphoma, Kaposi's sarcoma, and invasive cervical cancer [acquired immunodeficiency syndrome (AIDS)–defining cancers], is epidemiologically associated with HIV-1 infection (1, 2). These neoplasms are attributable mainly to diseases that accompany immunodeficiency, including coinfection with EBV, human herpes virus 8, and human papillomavirus (1, 2). In addition to these AIDS-defining cancers, several non-AIDS-defining cancers also occur with a higher incidence in HIV-infected individuals (3, 4). These reports lead to the assumption that HIV-1 has the potential to induce neoplasms before AIDS develops. Recently, DNA damage responses have been observed in precancerous lesion before inactivation of p53 (5, 6). Interestingly, it has been reported that HIV-1 infection induces DNA damage responses by activating Rad3-related or ataxia-telangiectasia mutated proteins and promoting phosphorylation of their downstream substrates (7, 8). The elucidation of the factor triggering the DNA damage responses to HIV-1 infection is essential to determine the as yet unknown mechanism causing AIDS-related neoplasms. In the present study, we found that HIV-1 infection induces double-strand breaks of chromosomal DNA, as detected using pulsed-field gel electrophoresis (PFGE). We further showed that *vpr*, an accessory gene of HIV-1 encoding a virion-associated nuclear protein, which induces cell cycle accumulation at G_2-M phase and increases ploidy (9), was a factor responsible for double-strand breaks. We discuss the potential ability of Vpr-induced double-strand breaks to develop into neoplasms in HIV-1 infection.

Materials and Methods

Cell culture. MIT-23 and ΔVpr, a mock transfectant, were established from HT1080 (JCRB9113; the Health Science Research Resources Bank) as previously described (9). In MIT-23, Vpr expression is controlled by the rtet promoter on incubation with 3 μg/mL doxycycline (Sigma, St. Louis, MO) for 48 hours.

Virus infection. Vesicular stomatitis virus G protein (VSV-G)–pseudotyped HIV-1 was produced by cotransfection with a plasmid encoding VSV-G (pHIT/G) and the pNL-Luc-E$^-$R$^-$ or pNL-Luc-E$^-$R$^-$ proviral clone (10). (10). The preparation and titration of viruses are described elsewhere (11). Briefly, the concentration of p24 antigen in the culture supernatant was measured using a p24 Gag antigen capture ELISA kit (ZeptoMetrix, Buffalo, NY). The infectivity of the prepared viral stock was examined using MAGIC5 cells. HT1080 cells were infected for 48 hours with viruses that had 200 ng/mL of p24 Gag antigen, giving a multiplicity of infection (MOI) of 0.7.

Immunostaining. Immunostaining was carried out as described (9). A rabbit polyclonal Rad51 antibody raised against the bacterially expressed protein and a mouse monoclonal antibody raised against synthesized peptides of full-length of Vpr (mAb8D1) were used as the primary antibody. Goat anti-rabbit IgG conjugated with Alexa Fluor 488 (Molecular Probes, Inc., Eugene, OR) and goat anti-mouse IgG conjugated with Cy3 (Zymed Laboratories, Inc., San Francisco, CA) were used as the secondary antibodies. Images were captured on a phase contrast microscope, BX50 (Olympus Corp., Tokyo Japan), or a Radiance 2100 laser scanning confocal microscope (Carl Zeiss, Oberkochen, Germany).

Overexpression and purification of Vpr and its mutant. The HIV-1 *vpr* gene was ligated into the *Nde*I and *Bam*HI sites of the pET15b vector (Novagen, Madison, WI). The Vpr protein and VprΔC12 mutant were produced in the *Escherichia coli* BL21 (DE3) Codon(+)RIL strain (Novagen) by induction with isopropyl-β-D-thiogalactopyranoside (IPTG; Nacalai Tesque, Inc., Kyoto, Japan) and were purified as described in Supplementary Method. The concentration of the purified Vpr protein was determined with a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA) using bovine serum albumin (BSA) as the standard.

Isolation of nuclei. Cells scraped from culture dishes were washed once with ice-cold PBS and resuspended in 3 mL of ice-cold 20 mmol/L Tris-HCl buffer (pH 7.6) containing 60 mmol/L KCl, 15 mmol/L NaCl, 5 mmol/L $MgCl_2$, 1 mmol/L DTT, 250 mmol/L sucrose, 0.6% NP40, and

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Requests for reprints: Hitoshi Kurumizaka, Graduate School of Science and Engineering, Waseda University, 3-4-1 Okubo, Shinjuku-ku, 169-8555 Tokyo, Japan. Phone: 81-3-5286-8189; Fax: 81-3-5292-9211; E-mail: kurumizaka@waseda.jp and Yukihito Ishizaka, Department of Intractable Diseases, International Medical Center of Japan, 1-21-1 Toyama, Shinjuku-ku, 162-8655 Tokyo, Japan. Phone: 81-3-5272-7527; E-mail: zakay@ri.imcj.go.jp.

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protease inhibitor mixture (Sigma). The cell suspension was incubated for 10 minutes on ice and the sucrose concentration was adjusted to 1.6 mol/L. Then, the sample was loaded onto a sucrose cushion of 2.3 mol/L sucrose solution and centrifuged at 35,000 × *g* for 30 minutes. The isolated nuclei were obtained in the 2.3 mol/L sucrose fraction. For immunostaining, isolated nuclei were cytocentrifuged to the MAS-coated slide glass (Matsunami Glass IND., LTD., Tokyo, Japan) for 6 minutes at 800 rpm (Thermo Shandon, Chadwick Road, United Kingdom).

PFGE assay. Isolated nuclei were incubated with 10 μmol/L of purified Vpr or VprΔC12 for 15 hours at 30°C. The cells (isolated nuclei) were embedded in agarose plugs at a density of 3×10^5 cells/100 μL. The plugs were treated with proteinase K solution [0.5 mol/L EDTA (pH 8.0), 1% sarcosyl, and 0.5 mg/mL proteinase K] for 38 hours at 50°C. After PFGE was done in a CHEFF Mapper (Bio-Rad Laboratories), the gels were stained with Vistra Green (Amersham Bioscience, Piscataway, NJ).

The DNA-binding assay. The Vpr protein was incubated with ϕX174 single-stranded DNA (ssDNA; 20 μmol/L) or ϕX174 superhelical dsDNA (10 μmol/L) in 10 μL of 8 mmol/L Tris-HCl buffer (pH 8.5) containing 1 mmol/L DTT and 100 μg/mL BSA. The reaction mixtures were incubated for 1 hour at 37°C and were analyzed by electrophoresis on a 0.8% agarose gel in 1× TAE buffer (40 mmol/L Tris acetate and 1 mmol/L EDTA) at 3.3 V/cm for 2 hours. The bands were visualized using ethidium bromide staining.

Nuclease activity. The Vpr protein (18.8 μmol/L) or DNaseI (Invitrogen Corporation, Carlsbad, CA; 0.02 unit/μL) were incubated with ϕX174 superhelical double-stranded DNA (dsDNA; 2.5 μmol/L) in 40 μL of 15 mmol/L Tris-HCl buffer (pH 8.5) containing 1 mmol/L DTT and 100 μg/mL BSA, in the presence of 5 mmol/L $MgCl_2$, $MnCl_2$, $ZnSO_4$, or $CaCl_2$. The reaction mixtures were incubated at 37°C for 30 minutes. After incubation, the samples were treated with proteinase K (0.3 mg/mL) in the presence of 0.1% SDS and the DNA was extracted using phenol-chloroform. The DNA was precipitated by ethanol and was analyzed by electrophoresis on a 0.8% agarose gel in 1 × TAE buffer at 6.6 V/cm for 30 minutes. The bands were visualized with ethidium bromide staining.

The Ni-NTA agarose pull-down assay. Isolated nuclei were disrupted in 20 mmol/L Tris-HCl buffer (pH 8.5) containing 200 mmol/L KCl, 2 mmol/L 2-mercaptoethanol, 0.25 mmol/L EDTA, and 10% glycerol. The extract was incubated with His_6-Vpr (53 μmol/L) for 15 hours at 30°C. After incubation, His_6-Vpr was precipitated with 4 μL of Ni-NTA agarose beads and the beads were washed thrice with 500 μL of 20 mmol/L Tris-HCl buffer (pH 7.6) containing 100 mmol/L NaCl, 5 mmol/L DTT, 10 mmol/L imidazole, 1 mmol/L EDTA, and 0.2% Tween 20. The proteins precipitated with the Ni-NTA beads were analyzed by 16% SDS-PAGE. The bands were visualized by silver staining.

Results

Vpr expression induces chromosomal double-strand breaks. To test whether HIV-1 infection causes double-strand breaks, we used PFGE, which was able to clearly detect the double-strand breaks induced by X-ray irradiation (Fig. 1*A*, *lane 2*; ref. 12). HT1080 cells were infected with HIV-1 that had 200 ng/mL of p24 Gag antigen, giving a MOI of 0.7, and the cellular DNA was fractionated using PFGE. Figure 1*A* (*lane 6*) shows that HIV-1 infection induced double-strand breaks. Interestingly, the amount of HIV-1-dependent double-strand breaks was reduced significantly (Fig. 1*A*, *lane 5*) when the *vpr* gene was deleted from the HIV-1 viral genome (HIV-1ΔVpr). To show that HIV-1-dependent double-strand breaks are attributable to Vpr expression, we examined double-strand break formation in Vpr stable transfectant, MIT-23 (9), in which Vpr expression is controlled by the rtet promoter by doxycycline, and, in ΔVpr, a mock transfectant. As shown in Fig. 1*B*, double-strand breaks were observed in the Vpr-expressing cells (*lane 5, arrow*) but not in the mock transfectants (*lane 4*). Furthermore, Rad51 foci, which are formed

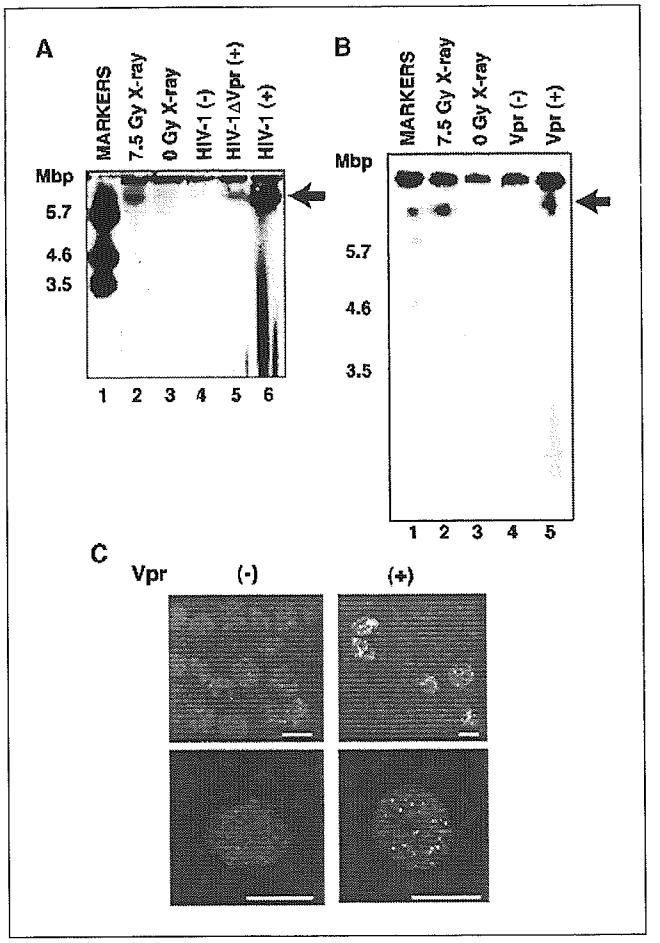

Figure 1. Vpr induces double-strand breaks *in vivo*. *A*, PFGE analysis of double-strand breaks after HIV-1 infection. HT1080 cells were infected with the same amount of HIV-1 or HIV-1ΔVpr (MOI = 0.7) and subjected to PFGE. As a positive control, uninfected cells were analyzed immediately after 7.5 Gy of X-ray irradiation. Molecular mass markers (*lane 1*), control cells (*lanes 3* and *4*), cells subjected to X-ray irradiation (*lane 2*), and cells infected with HIV-1ΔVpr (*lane 5*) or HIV-1 (*lane 6*) are shown. *Arrow*, position corresponding to the double-strand breaks. *B*, PFGE analysis in Vpr-expressing cells. Molecular mass markers (*lane 1*), cells irradiated with 7.5 Gy (*lane 2*), control cells (*lane 3*), mock transfectants (*lane 4*), and cells with Vpr expression (*lane 5*) are shown. *Arrow*, double-strand breaks. *C*, Rad51 focus formation with Vpr expression. An immunohistochemical analysis was used to detect Rad51 in cells with (*right*) or without (*left*) Vpr expression. Bar, 10 μm.

at double-strand break sites (13), were observed with Vpr expression (Fig. 1*C*). These results indicate that Vpr is responsible for double-strand break formation. The double-strand breaks shown in Fig. 1*B* were not the result of an apoptotic process as the DNA ladder typically observed in apoptotic cells (14) was not detected (data not shown).

Vpr has no endonuclease activity. Next, we studied whether Vpr directly induces double-strand breaks. The recombinant Vpr protein was purified to near homogeneity (Fig. 2*A*) and the DNA-binding activity of Vpr was examined. As shown in Fig. 2*B*, purified Vpr bound both ssDNA (*lanes 2-6*) and dsDNA (*lanes 8-12*) in an ATP- and Mg^{2+}-independent manner (15). Then, we examined whether Vpr has nuclease activity. Superhelical dsDNA containing small amounts of nicked circular dsDNA was incubated with Vpr in the presence of various divalent cations. After the incubation, the proteins were removed and the DNA was examined by

electrophoresis. If Vpr induces a double-strand break or nick, the superhelical dsDNA would give rise to linear or nicked circular forms, producing a different electrophoretic pattern. However, the DNA incubated with Vpr in the absence (*lane 2*) or presence of any divalent cation examined (*lanes 4, 6, 8*, and *10*) showed the same migration pattern with control (*lane 1*), indicating that Vpr does not cleave DNA (Fig. 2*C*). Positive control experiments showed that the DNA was digested by DNaseI with $MgCl_2$, $MnCl_2$, or $CaCl_2$ (*lanes 5, 7*, and *11*) but not with $ZnSO_4$ (*lane 9*; Fig. 2*C*). Therefore, these results indicate that Vpr lacks endonuclease or nicking activity.

Vpr induces double-strand breaks *in vitro*. In a second approach, we tested whether purified Vpr induces double-strand breaks in nuclei isolated from HT1080 cells (Fig. 3*A*). First, we confirmed by a laser confocal microscopy that Vpr localizes in nuclei after incubation *in vitro* (Fig. 3*B*). The nuclear DNA was then analyzed for double-strand breaks by using PFGE (Fig. 3*C*). Interestingly, purified Vpr induced double-strand breaks in the DNA of the isolated nuclei (Fig. 3*C, lane 5, arrow*). By contrast, few double-strand breaks were detected without Vpr (Fig. 3*C, lane 4*). Because Vpr alone did not show endonuclease activity (Fig. 2*C*), these results suggest that Vpr interacts with intrinsic nuclear protein(s), which required for double-strand break formation. To identify candidates for the Vpr-interacting nuclear proteins, we did the Ni-NTA pull-down assay. In this assay, recombinant His_6-tagged Vpr was incubated with the extract from isolated nuclei and Ni-NTA beads precipitated proteins bound to His_6-tagged Vpr (Fig. 3*D*). As shown in Fig. 3*D*, His_6-tagged Vpr associated with numerous proteins that were not detected in the control precipitates (*lane 2, asterisks*).

The DNA-binding activity of Vpr is correlated with double-strand break formation. The COOH-terminal region of Vpr is arginine rich and is thought to be an important site for DNA binding to Vpr (15). Nuclear magnetic resonance analysis shows that Vpr has three α-helixes (amino acids 17-33, 38-50, and 56-77) in solution, whereas the COOH-terminal region from amino acid residues 84 to 96 is disordered (16). This suggests that the deletion of the COOH-terminal 12 amino acid residues does not affect the tertiary structure of Vpr. We purified a Vpr mutant protein lacking the COOH-terminal 12-amino-acid residues (VprΔC12; Fig. 4*A*), and examined its DNA-binding activity. Purified VprΔC12 was significantly defective in both ssDNA- and dsDNA-binding activity compared with wild-type Vpr (Fig. 4*B*). Interestingly, VprΔC12 induced double-strand breaks in isolated nuclei but its efficiency was reduced significantly (Fig. 4*C, lane 6*). These results indicate that the DNA-binding ability of Vpr is important for the induction of double-strand breaks by Vpr.

Discussion

Here, we present evidence that HIV-1 Vpr induces double-strand breaks. Our data are consistent with previous observations in Vpr-expressing cells: the up-regulation of gene amplification events that are believed to be introduced by broken DNA strands (17) and the activation of activating Rad3-related/ataxia-telangiectasia mutated, followed by the phosphorylation of their downstream substrate, a histone H2A variant, H2AX, and γ-H2AX and BRCA1 focus formation (8). Biochemical analyses using purified Vpr indicated that Vpr alone has no endonuclease activity (Fig. 2*C*), suggesting that a cellular factor(s), possibly with endonuclease activity, is required for Vpr-dependent double-strand breaks. The factor(s) required for double-strand breaks must preexist in nuclei because double-strand breaks were observed upon incubating a mixture of isolated nuclei and purified Vpr *in vitro* (Fig. 3*C*). As one possible mechanism, Vpr may recruit a nuclease factor to chromosomal DNA, given that the Vpr-dependent double-strand breaks were correlated with the DNA-binding activity (Figs. 4*B* and *C*). Alternatively, Vpr itself may acquire endonuclease activity after modification in the nucleus. Further analyses are necessary to clarify this point.

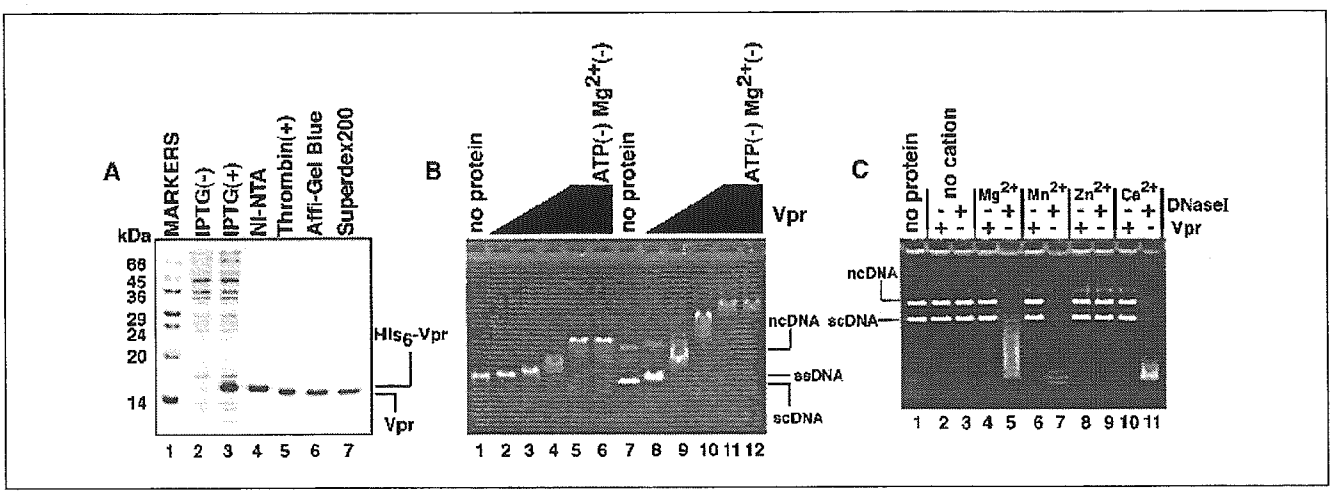

Figure 2. The Vpr-DNA interaction *in vitro*. *A*, purification of recombinant Vpr. Proteins from each purification step were analyzed using 16% SDS-PAGE with Coomassie brilliant blue staining. Molecular mass markers (*lane 1*), whole-cell lysates before (*lane 2*) and after (*lane 3*) induction with IPTG, samples from the Ni-NTA fraction (*lane 4*), the fraction after removing the hexahistidine tag (*lane 5*), the Affi-Gel Blue fraction (*lane 6*), and the Superdex 200 fraction (*lane 7*) are shown. *B*, the DNA-binding activity of Vpr. ϕX174 circular ssDNA (20 μmol/L; *lanes 2-6*) and ϕX174 superhelical dsDNA (*scDNA*; 10 μmol/L; *lanes 8-12*) containing a small amount of nicked circular DNA (*ncDNA*) were incubated with Vpr in the presence of 1 mmol/L ATP and 1 mmol/L $MgCl_2$. Control experiments without ATP and $MgCl_2$ (*lanes 6* and *12*) are included. The Vpr concentrations were 1.25 μmol/L (*lanes 2* and *8*), 2.5 μmol/L (*lanes 3* and *9*), 5 μmol/L (*lanes 4* and *10*), and 10 μmol/L (*lanes 5, 6, 11*, and *12*). *Lanes 1* and *7*, negative controls without protein. *C*, nuclease activity. ϕX174 scDNA (2.5 μmol/L) was incubated with Vpr (18.8 μmol/L; *lanes 2, 4, 6, 8*, and *10*) or DNaseI (*lanes 3, 5, 7, 9*, and *11*) in the absence of divalent cation (*lanes 2* and *3*) or in the presence of 5 mmol/L $MgCl_2$ (*lanes 4* and *5*), 5 mmol/L $MnCl_2$ (*lanes 6* and *7*), 5 mmol/L $ZnSO_4$ (*lanes 8* and *9*), or 5 mmol/L $CaCl_2$ (*lanes 10* and *11*). *Lane 1*, negative control without protein.

In the HIV-1 life cycle, DNA breakage and repair are thought to be essential steps for integrating the double-stranded viral cDNA into the host genome. In this study, we found that Vpr is one molecule responsible for the double-strand breaks that occur upon HIV-1 infection. However, it is also noteworthy that some double-strand breaks were induced in the cells with HIV-1ΔVpr (Fig. 1*A*, *lane 5*), suggesting that other viral factors are also involved. It has been shown that integrase activates the ataxia-telangiectasia mutated–dependent pathway (7) and, thus, the double-strand breaks observed with HIV-1ΔVpr infection are probably owing to integrase. For viral integration to occur, the amount of double-strand breaks induced by HIV-1ΔVpr (Fig. 1*A*, *lane 5*) may be sufficient, because viral production in peripheral blood mononuclear cells was not alleviated by infection with

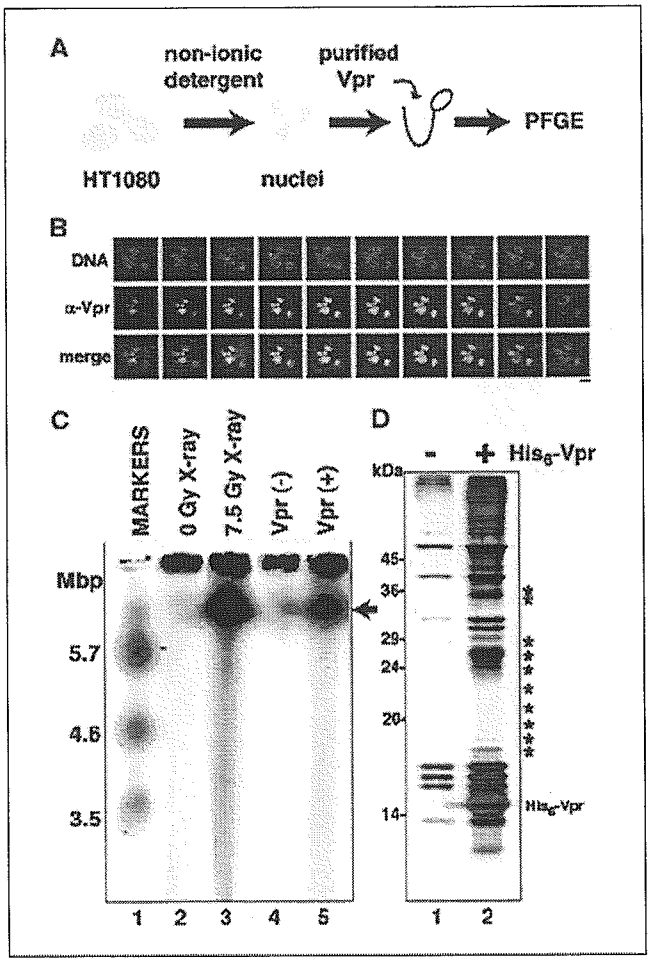

Figure 3. Purified Vpr induces double-strand breaks *in vitro*. *A*, a scheme of the protocol used to detect Vpr-induced double-strand breaks in isolated nuclei. *B*, Vpr localization in isolated nuclei. Isolated nuclei from HT1080 after incubation with Vpr were immunostained by α-Vpr (mAb8D1) and the images were captured by a laser confocal microscopy. The Z-series of optical sections collected at 1 μm steps of the cells were presented. Vpr (*red; middle*), DNA staining by Hoechst (*blue; top*) and their merged images (*bottom*) are shown. Without Vpr incubation, any signals by α-Vpr immunostaining were not detected in isolated nuclei (data not shown). Bar, 10 μm. *C*, PFGE analysis of double-strand breaks in isolated nuclei treated with Vpr. Molecular mass markers (*lane 1*), control cells (*lane 2*), cells subjected to X-ray irradiation (*lane 3*), and isolated nuclei without (*lane 4*) or with 10 μmol/L Vpr (*lane 5*). *Arrow*, double-strand breaks. *D*, Ni-NTA pull-down assay with His_6-tagged Vpr on isolated nuclei. Precipitated proteins bound to His_6-tagged Vpr (*lane 2*) and the control precipitates (*lane 1*) are indicated. *, His_6-Vpr–specific bands.

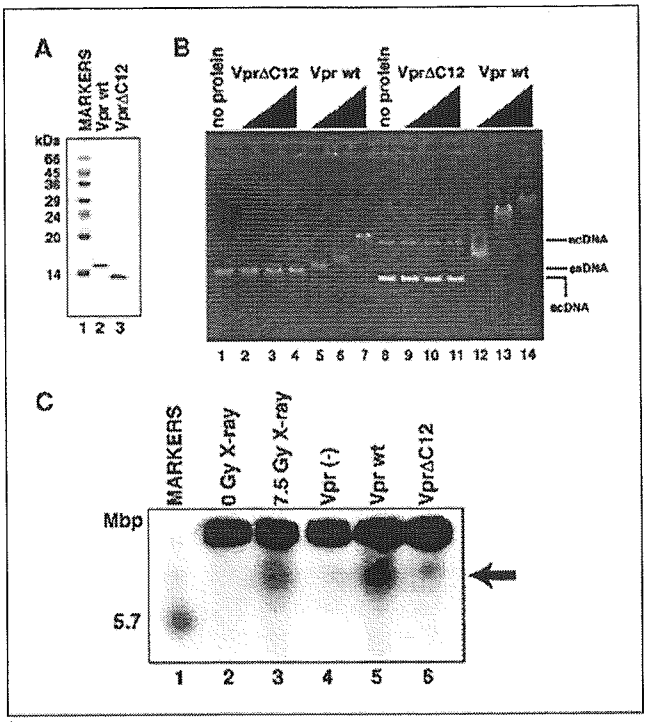

Figure 4. DNA-binding and double-strand break formation by Vpr. *A*, purification of VprΔC12. Purified VprΔC12 was analyzed using 16% SDS-PAGE with Coomassie brilliant blue staining. *Lane 1*, molecular mass markers. *Lanes 2* and *3*, purified wild-type Vpr and VprΔC12 protein, respectively. *B*, the DNA-binding activity of VprΔC12. The DNA-binding experiments were done using the protocol used to obtain Fig. 2*B*. The concentrations of VprΔC12 were 2.5 μmol/L (*lanes 2* and *9*), 5 μmol/L (*lanes 3* and *10*), and 10 μmol/L (*lanes 4* and *11*), and those of the wild-type Vpr were 2.5 μmol/L (*lanes 5* and *12*), 5 μmol/L (*lanes 6* and *13*), and 10 μmol/L (*lanes 7* and *14*). Negative controls without protein (*lanes 1* and *8*) are included. *C*, PFGE analysis of double-strand breaks in isolated nuclei treated with Vpr or VprΔC12. Molecular mass marker (*lane 1*), cells without (*lane 2*) or with (*lane 3*) 7.5 Gy of X-ray irradiation, control nuclei (*lane 4*), nuclei with Vpr (*lane 5*), and nuclei with VprΔC12 (*lane 6*). Vpr was used at 10 μmol/L. *Arrow*, double-strand breaks.

Vpr-deleted HIV-1 (18).[1] Vpr-induced double-strand breaks may be surplus to those required for viral integration (Fig. 1*A*, *lane 6*). The resultant DNA damage may reduce the integrity of the host genome.

Recently, DNA damage signaling was observed at an early stage of tumor development, suggesting that the DNA damage response is a mechanism to prevent the progression of pre-neoplasic lesions (5). If DNA repair is not accomplished correctly or is skipped because of unregulated checkpoint controls, the genomic structure would be altered severely (19). The progression of malignant tumors in AIDS-defining cancers is well documented in oncovirus infections (1, 2). If DNA damage increases the probability of neoplasia, Vpr-induced double-strand breaks with oncovirus infection may accelerate tumor progression during the clinical course of AIDS. In addition to AIDS-defining cancers, non-AIDS-defining cancers also occur at a higher incidence and the factor responsible for such oncogenesis is now a critical issue (3, 4). Vpr-induced DNA damage may result in

[1] M. Shimura, unpublished data.

these AIDS-related malignancies. It is essential to explore the molecular mechanism of Vpr-induced double-strand breaks to clarify their role in HIV-1 infection and their effect on the stability of the host cell genome.

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Improved gene expression in resting macrophages using an oligopeptide derived from Vpr of human immunodeficiency virus type-1

Izuru Mizoguchi [a,c], Yoshihiro Ooe [b], Shigeki Hoshino [a], Mari Shimura [a], Tadashi Kasahara [b], Shigeyuki Kano [a,c], Toshiko Ohta [c], Fumimaro Takaku [d], Yasuhide Nakayama [e], Yukihito Ishizaka [a,*]

[a] *Research Institute, International Medical Center of Japan, 1-21-1 Toyama, Shinjuku-ku, Tokyo 162-8655, Japan*
[b] *Biochemistry Department, Kyoritsu University of Pharmacy, 1-5-30 Shibakoen, Minato-ku 105-8512, Japan*
[c] *Institute of Basic Medical Sciences, University of Tsukuba, 1-1-1 Ten-nodai, Tsukuba 305-8577, Japan*
[d] *Jichi Medical School, 3311-1 Yakushiji, Minamikawachi-machi, Kawachi-gun, Tochigi 329-0498, Japan*
[e] *Department of Bioengineering, Advanced Medical Engineering Center, National Cardiovascular Center Research Institute, 5-7-1 Fujishiro-dai, Suita, Osaka 565-8565, Japan*

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Abstract

Vpr, an accessory gene product of human immunodeficiency virus type-1, is thought to transport a viral DNA from the cytoplasm to the nucleus in resting macrophages. Previously, we reported that a peptide encompassing amino acids 52–78 of Vpr (C45D18) promotes the nuclear trafficking of recombinant proteins that are conjugated with C45D18. Here, we present evidence that C45D18, when conjugated with a six-branched cationic polymer of poly(*N*,*N*-dimethylaminopropylacrylamide)-*block*-oligo(4-aminostyrene) (SV: star vector), facilitates gene expression in resting macrophages. Although there was no difference between SV alone and C45D18-SV with respect to gene transduction into growing cells, C45D18-SV resulted in more than 40-fold greater expression of the exogenous gene upon transduction into chemically differentiated macrophages and human quiescent monocyte-derived macrophages. The data suggest that C45D18 contributes to improving the ability of a non-viral vector to transduce macrophages with exogenous genes and we discuss its further application.

Keywords: HIV-1; Vpr; Nuclear trafficking; Star vector; Gene expression; Resting macrophages

Viral gene transfer systems are commonly used in current gene therapy protocols [1–3], but viral vectors can unfortunately cause side effects, such as severe immunological reactions [4] or leukemogenesis [5]. Thus, it is necessary to develop a non-viral vector system that is both safe and reliable. Various chemical compounds have been synthesized as non-viral vector candidates [6–9], but, in general, they need improvement to allow the expression of the exogenous genes in resting cells [9,10]. Among the obstacles to efficient gene transduction into resting cells is the nuclear membrane, which constitutes a critical barrier that impairs the efficient expression of exogenous genes [9,11]. Although there have been attempts to circumvent this problem, no reliable method has yet been established for effective transduction into resting cells [12–14].

The *vpr* gene, an auxiliary gene of human immunodeficiency virus type 1 (HIV) [15,16], encodes a virion-associated protein [17–19] and is a crucial factor in HIV-1 infection of resting macrophages [20]. It has been proposed that Vpr transports a pre-integration complex containing the viral DNA from the cytoplasm to the nucleus in infected cells [20]. Vpr has three α-helixes (amino acids 17–33, 38–50, and 56–77) [21] but no classical nuclear localization signals [22,23]. It is thought that the nuclear trafficking activity of

* Corresponding author.
E-mail address: zakay@ri.imcj.go.jp (Y. Ishizaka).

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