Progress Toward a Human CD4/CCR5 Transgenic Rat Model for De Novo Infection by Human Immunodeficiency Virus Type 1

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Abstract

The development of a permissive small animal model for the study of human immunodeficiency virus type (HIV)-1 pathogenesis and the testing of antiviral strategies has been hampered by the inability of HIV-1 to infect primary rodent cells productively. In this study, we explored transgenic rats expressing the HIV-1 receptor complex as a susceptible host. Rats transgenic for human CD4 (hCD4) and the human chemokine receptor CCR5 (hCCR5) were generated that express the transgenes in $CD4^+$ T lymphocytes, macrophages, and microglia. In ex vivo cultures, $CD4^+$ T lymphocytes, macrophages, and microglia from hCD4/hCCR5 transgenic rats were highly susceptible to infection by HIV-1 R5 viruses leading to expression of abundant levels of early HIV-1 gene products comparable to those found in human reference cultures. Primary rat macrophages and microglia, but not lymphocytes, from double-transgenic rats could be productively infected by various recombinant and primary R5 strains of HIV-1. Moreover, after systemic challenge with HIV-1, lymphatic organs from hCD4/hCCR5 transgenic rats contained episomal 2–long terminal repeat (LTR) circles, integrated provirus, and early viral gene products, demonstrating susceptibility to HIV-1 in vivo. Transgenic rats also displayed a low-level plasma viremia early in infection. Thus, transgenic rats expressing the appropriate human receptor complex are promising candidates for a small animal model of HIV-1 infection.

Key words: HIV-1 • transgenic rats • CD4 • CCR5 • macrophages

Introduction

New small animal models of HIV-1 disease are needed to complement present models in the study of viral pathogenesis, the screening of new drugs, and the testing of vaccine strategies. Limitations of existing animal models include the availability and high cost of nonhuman primates, the ab-

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sence or delayed progression to an acquired immunodeficiency syndrome in some of these models, or permissivity only for related retroviruses (1–3). Current xenotransplant models are informative about select aspects of HIV-1 pathogenesis (4, 5), but present neither a complete range of infected tissues nor the context of an intact immune response.

HIV-1 replication is subject to a variety of potent species-specific restrictions in cells from many nonprimate species (6–10). Over the past years, a number of advances have been made elucidating the molecular bases of such blocks to HIV-1 replication, and these discoveries have recharged efforts to develop transgenic small animal models permissive for HIV-1 infection in the context of an intact immune system. Regarding cellular entry, coexpression of hCD4 and a human chemokine receptor were shown to overcome the entry block in primary T lymphocytes from mice transgenic for either hCD4 and hCCR5 (11) or hCD4 and human CXCR4 (hCXCR4) (12), but these mouse cells exhibited very little or no productive infection. Another restriction to HIV-1 replication in mouse cells is the limited efficiency of the viral regulator, Tat, in activating transcription and transcript elongation from the long terminal repeat (LTR)* of HIV-1, which are normally crucial steps for vigorous viral replication. Recently, a novel Tat-interacting protein, human cyclin T1 (hCycT1) was identified (13). hCycT1 was shown to be crucial to the transcriptional block in mouse cells since expression of hCycT1 drastically enhanced transcriptional activity in mouse NIH 3T3 fibroblasts (13–19) and in primary lymphocytes from transgenic mice (unpublished data). However, 3T3 cells expressing hCD4, hCCR5, and hCycT1 were still unable to support the full HIV-1 replication cycle (16, 17). In this context, a viral assembly block in 3T3 cells was reported (16) that could pose a species-specific, post-transcriptional barrier to HIV-1 replication. Interestingly, this HIV-1 assembly block could be partially complemented by mouse–human heterokaryon fusions (17, 18) suggesting that these mouse fibroblasts lack a specific positive factor required for efficient virus assembly and release.

An alternate approach to small animal model development is the identification of other species that are less restricted for the HIV-1 replication cycle but nonetheless susceptible to transgenic manipulation. In certain rat cell lines, cellular entry constitutes the only absolute block to HIV-1 replication and this restriction can be overcome by coexpression of hCD4 and hCCR5 (19). We and others have identified quantitative and qualitative limitations in various aspects of the HIV-1 replication cycle in rat cell lines (17–19) that appear to be largely cell type–specific, rather than species specific. Importantly, in the context of infections with HIV-1 pseudotypes containing the vesicular stomatitis virus G protein (VSV-G), primary nontransgenic rat cells from the monocyte/macrophage lineage supported all postentry steps in the viral life cycle and secreted substantial levels of infectious virions (19). Furthermore, unlike native mouse and hamster cells, most rat-derived cells supported a robust HIV-1 LTR activity (17–19). This conclusion is also confirmed by a recent report showing that HIV-1 transgenic rats carrying an HIV-1 provirus with functional deletions in *gag* and *pol,* expressed gp120, Tat, and Nef proteins in spleen tissue (20). Although overexpression of hCycT1 thus appears to be nonessential for efficient LTR transactivation in rat cells, our data indicated that the activity of CycT1 may still be a quantitatively limiting factor in this species context (19).

In addition, rats offer several practical advantages for their use as an animal model for HIV-1 infection, including their short gestation time, size, and well-characterized immune system and central nervous system (CNS). In particular, rats have long been considered a valuable model for studying the development and function of the CNS and they are used extensively to study the molecular mechanisms underlying HIV-related CNS pathology (21–23). Rats have also been used frequently as a model organism for basic pharmacological studies, including pharmacokinetic and pharmacodynamic studies on current anti-HIV drugs (24–26). Furthermore, rat transgenesis can be performed with relative ease, thereby enabling the selective expression of human genes that may be essential for full realization of the HIV-1 replication cycle in this species.

In humans, T lymphocytes as well as cells from the monocyte/macrophage lineage, including microglia, are considered to be the most important sites of HIV-1 replication in vivo (27, 28). To mimic the pattern of susceptible tissues and viral pathogenesis in a transgenic rat model, these cell types must express the human transgenes essential for complementation of the HIV-1 replication cycle. Although HIV-1 entry can be mediated by hCD4 acting with one of several members of the human chemokine receptor superfamily or related coreceptors (29), hCCR5 appears to be the critical coreceptor required for transmission and establishment of an infection in humans. Key pieces of evidence in support of this are the almost exclusive presence of CCR5-dependent (R5) viruses in early HIV-1 disease (30) and the high degree of resistance to HIV-1 infection observed in exposed individuals homozygous for a 32-basepair deletion in their CCR5 gene (Δ32 *CCR5*), which prevents the presentation of hCCR5 at the cell surface (31).

Here we report the generation of transgenic rats that coexpress hCD4 and hCCR5 on $CD4^+$ T lymphocytes, macrophages, and microglia. Coexpression of these human transgenes rendered primary rat cells permissive for infection by R5 viruses. In ex vivo cultures, cells of the monocyte/macrophage lineage from these animals could be productively infected by various R5 viruses at levels one to two orders of magnitude higher than those described for comparable transgenic mouse models (11, 12). In vivo, hCD4/hCCR5 transgenic rats challenged systemically

Abbreviations used in this paper: AZT, zidovudine; CNS, central nervous system; CycT1, cyclin T1; Env, envelope; ID, rodent identifier; LTR, long terminal repeat; MIP, macrophage inflammatory protein; MOI, multiplicity of infection; RANTES, regulated upon activation, normal T cell expressed and secreted; RTV, Ritonavir; SIV, simian immunodeficiency virus; VSV-G, vesicular stomatitis virus G protein.

with HIV-1 showed clear evidence of successful infection demonstrated by detection of episomal and integrated HIV-1 cDNA, and early gene expression in cells from spleen, thymus, and blood. Low-level plasma viremia was detectable in transgenic rats up to 7 wk after inoculation. These important steps provide a strong foundation for studies that will address the extent of HIV-1 replication, pathogenesis, and immune responses to HIV-1 in transgenic rats in vivo and elucidate their potential for testing of antiviral strategies.

Materials and Methods

Construction of Transgenic Rats. The transgenic vector for hCD4 has been described previously (32). The vector encoding hCCR5 was prepared to ensure expression in Th cells and in cells from the monocyte/macrophage lineage. For this purpose, an 8.4-kb BstBI-BamHI fragment from intron 1 of the human CD4 gene, shown to contain a monocyte-specific enhancer as well as the CD4 silencer (unpublished data), was inserted as a replacement for the ClaI–HindIII fragment in the middle of intron 1 in the murine construct "b," described previously (33). A 1.15-kb hCCR5 cDNA was inserted into the SalI site in exon 2, replacing the hCD2 cDNA in construct "b," thus yielding the plasmid pMΦE4A.CCR5 (Fig. 1 B). For preparation of transgenic rats, the plasmid vector sequences were excised by digestion with NotI. Rat founders for individual transgenic constructs were generated (Xenogen) by microinjection of the vector DNA into male pronuclei of fertilized oocytes from outbred Sprague-Dawley rats. Founders for the hCD4 transgene were identified by Southern blot analysis of EcoRV-digested tail DNA samples using a HindIII–BamHI fragment from pCD4neo (19) as a probe. Founders for hCCR5 were identified by PCR analysis from tail DNA samples using an internal primer set for hCCR5 cDNA (5′ primer: TCACTATGCTGCCGCCC. 3′ primer: AAACCAAAGTCCCACTGGGCG). Integration-positive founders were mated with nontransgenic Sprague-Dawley rats and F1 progeny were screened by flow cytometry for expression of human proteins in peripheral blood samples.

Antibodies and Flow Cytometry. FACS® analyses were performed as described previously (34), using FITC-, PE-, or APC-conjugated mAbs (BD PharMingen): anti-hCD4 (mAb Leu-3a); anti-hCCR5 (mAb 2D7); anti–rat (r)CD3 (mAb G4.18); anti-rCD4 (mAb OX-35); anti-rCD8a (mAb OX-8); anti–rat macrophage subset marker (ED2-like antigen, mAb HIS36); anti-rCD11b (mAb WT.5); anti-rCD11b/c (mAb OX-42); and anti-rCD45RA (mAb OX-33).

Immunohistochemistry. A standard three-step immunoperoxidase procedure using the Dako LSAB (R) 2 kit (Dako) was performed on formalin-fixed, paraffin-embedded tissues from PBS-perfused rats in principle as described previously (35). Tissues were sectioned onto silanized slides, allowed to dry, deparaffinized, and hydrated through graded ethanols. The tissues were pretreated with 3% H_2O_2 for 10 min to block endogenous peroxidases, heat-treated in citrate buffer, pH 6.0, for 10 min to retrieve antigens and finally buffered in PBS/casein. The slides were incubated with primary antibodies (anti-hCD4 mAb 1F6 [1:10]; Novacastra, or anti-hCCR5 mAb 3A9 [1:10]; BD PharMingen) for 2 h at room temperature, washed, and then stained after the kit protocol with 30-min incubations and final DAB reaction.

Primary Cells and Cultivation. Ex vivo cultures of primary rat lymphocytes, macrophages, or microglia, and cultures of PBMCs or human monocyte–derived macrophages were prepared and propagated as described previously (19).

Chemotaxis Assay. The migration of rCD4$^+$-enriched primary rat splenocytes was determined by using a modification of the method described by Arai et al. (36) with Transwell® plates (pore size: 3 μm; diameter: 6.5 mm, Costar®; Corning, Inc.) and recombinant chemokines (R&D Systems).

Preparation of Viral Stocks. Molecular clones of pYU-2, pJR-CSF, and pNL4–3 were obtained from Beatrice Hahn (University of Alabama at Birmingham, Birmingham, AL), Irvin Chen (UCLA School of Medicine, Los Angeles, CA), and Malcom Martin (National Institutes of Health, Bethesda, MD), respectively, via the National Institutes of Health (NIH) AIDS Research and Reference Reagent Program. pYU-2b, a YU-2/HXB2 recombinant (37), was a gift from Warner Greene (Gladstone Institute of Virology and Immunology, San Francisco, CA). The molecular clone p49–5 (38) was a gift from Bruce Chesebro (Rocky Mountain Laboratories, Hami Hou, MT). pR7/3-YU-2-EGFP (39), which carries an EGFP gene within the *nef* locus driven by the 5′ LTR, was a gift from Mark Muesing (Aaron Diamond AIDS Research Center, New York, NY). Infectious virus stocks were prepared by transfecting 293T cells with proviral DNA as described previously (34). Viral stocks of JR-FL and Ada-M were obtained from Irvin Chen and Howard Gendelman (University of Nebraska Medical Center, Omaha, NE), respectively, via the NIH AIDS Research and Reference Reagent Program. The primary isolates C1 (40) and O3 (41) were gifts from James Mullins (University of Washington, Seattle, WA) and Ruth Connor (Aaron Diamond AIDS Research Center), respectively. Viral stocks were expanded by infection of heterologous human PBMCs. Infectious Ba-L stocks, which had been expanded on human monocyte-derived macrophages, were a gift from Teri Liegler (Gladstone Institute of Virology and Immunology).

For in vivo infections, YU-2 and R7/3-YU-2-EGFP stocks were concentrated using Centricon® Plus-80 columns (Millipore) following the manufacturer's protocol. The infectivity of viral stocks was determined by terminal dilution in quadruplicate on heterologous phytohemagglutinin-activated PBMCs. The titer of R7/3-YU-2-EGFP stocks was determined on HeLa hCD4/hCCR5 cells. The p24 CA concentration was assessed by ELISA (NEN Life Sciences). The molecular clone pNL-4–3 Luc E$^-$R$^-$ (42), a replication-incompetent NL4–3 provirus (along with mutations in *env*, *nef*, and *vpr*), carrying a luciferase gene within the *nef* locus driven by the 5′ LTR, was a gift of Nathaniel Landau (Salk Institute for Biological Studies, La Jolla, CA) via the NIH AIDS Research and Reference Reagent Program. pVSV-G, the mammalian expression vector for VSV-G protein (43), was a gift from Jane Burns (University of California, San Diego, CA). The preparation of NL4–3 Luc E$^-$R$^-$ pseudotype viruses with autologous or heterologous envelopes (Env) has been described previously (44).

Viral Infections. HIV-1 infections of ex vivo cultures were performed in 24-well (lymphocytes, macrophages) or 96-well plates (microglia, macrophages) with the indicated multiplicity of infection (MOI) or p24 CA concentrations. In infection studies on transgenic rat macrophages shown in Figs. 5 and 6, the following reagents were used: anti-hCCR5 mAb 2D7 (BD PharMingen) at 50 μg/ml; zidovudine (AZT) (3′-azido-3′deoxythymidine; Sigma-Aldrich) at 100 μM; Ritonavir (RTV) (Abbott Laboratories, Abbott Park, IL) at 1 μM; LPS (*Escherichia*

coli serotype 0128:B12; Sigma-Aldrich) at 100 ng/ml; or a formalin-fixed *Staphylococcus aureus* crude cell suspension (Sigma-Aldrich) at 0.01%. In vivo infections were performed by trained personnel in accordance with the guidelines and standards for humane animal experimentation set by the UCSF Committee on Research under an approved protocol. All rats were housed under SPF conditions with food and water ad libitum. For intravenous and intraperitoneal injections, rats were deeply anesthetized using isoflurane, and the tail vein or peritoneal cavity was cannulated with a 24G Abbocath®-T catheter (Abbott Laboratories) and YU-2 or R7/3-YU-2-GFP stocks were slowly infused (see specific Figure legends for viral titers). On the indicated days after inoculation, rats were killed with CO_2 and bilateral thoracotomy and organs were removed aseptically. Coded splenocyte, thymocyte, and PBMC samples were analyzed for the presence of HIV-1 cDNA products by PCR as described below. GFP-positive cells were detected by FACS® analyses. Serial blood samples were drawn from the jugular vein. HIV-1 vRNA assays of coded rat EDTA-treated plasma samples were performed by the Gladstone Core Virology Laboratory using the AMPLICOR HIV-1 MONITOR® test version 1.5 (gift from Roche Diagnostics).

Quantification of 2-LTR Circle Junctions. 2-LTR circle junctions were amplified by PCR from extracts of extrachromosomal DNA and quantified by Southern blotting as described previously (45).

Detection of Integrated Proviral DNA. A nested PCR strategy for the specific amplification of HIV-1 proviral DNA that is integrated into the rat genome was developed based on a previously reported method to evaluate HIV-1 integration close to human genomic *Alu* elements (46). We used a rodent identifier (ID) family consensus sequence that is highly abundant in the rat genome (47), to design the oligonucleotide primer BC1-A (5′-GGATTTAGCTCAGTGGTAGA-3′). Genomic DNA was extracted from formaldehyde-fixed cells using the DNAeasy® tissue kit (QIAGEN). During the first PCR reaction, cellular DNA was amplified with the HIV-1 LTR primer A (5′-AGGCAAGCTTTATTGAGGCTTAAGC-3′; reference 46) and primer BC1-A. *Taq* DNA polymerase (0.75 U/25 μl reaction; Stratagene), 200 μM of each dNTP and 500 nM of each primer were used. The reaction was run with the following program: (a) 3 min at 94°C, (b) 30 cycles of 30 s at 94°C, 30 s at 57°C, and 4 min at 72°C, and (c) 10 min at 72°C. A second nested PCR amplification, which allows amplification of a 357-basepair LTR fragment, was performed by using 1 μl of the first reaction with internal HIV-1 LTR primers NI-2 5′ and NI-2 3′ (46). This second PCR was performed using the same PCR conditions as for the first one, except that the annealing temperature was raised to 60°C, the extension time was 1 min, and amplification was run for 25 cycles. PCR products were analyzed by ethidium bromide/agarose gel electrophoresis.

Immunofluorescence Microscopy. Staining for intracellular p24 CA was performed on YU-2–infected rat microglia from hCD4/hCCR5-transgenic or hCD4-transgenic rats. Cells were washed with staining buffer (PBS containing 2% FBS) and then fixed in a 2% paraformaldehyde solution for 1 h on ice. After washing in staining buffer cells were permeabilized in 0.1% saponin for 15 min at room temperature. Cells were again washed in staining buffer and then incubated with a FITC-conjugated anti-p24 mAb (KC57, 1:10; Coulter Immunology) or a FITC-conjugated isotype control mAb. Subsequently, cells were stained with the Alexa Fluor® 488 Signal-Amplification kit (catalog no. A-11053; Molecular Probes) following the manufacturer's instructions. Images were acquired on an inverted Nikon TE-300 light microscope with SPOT RT digital camera using equal exposure times and identical digital filtering for all micrographs.

Results

Construction of Rats Transgenic for Human CD4 or Human CCR5. We developed several independent rat lines transgenic for either hCD4 or hCCR5. In humans, $CD4^+$ T lymphocytes, macrophages, and microglia constitute the

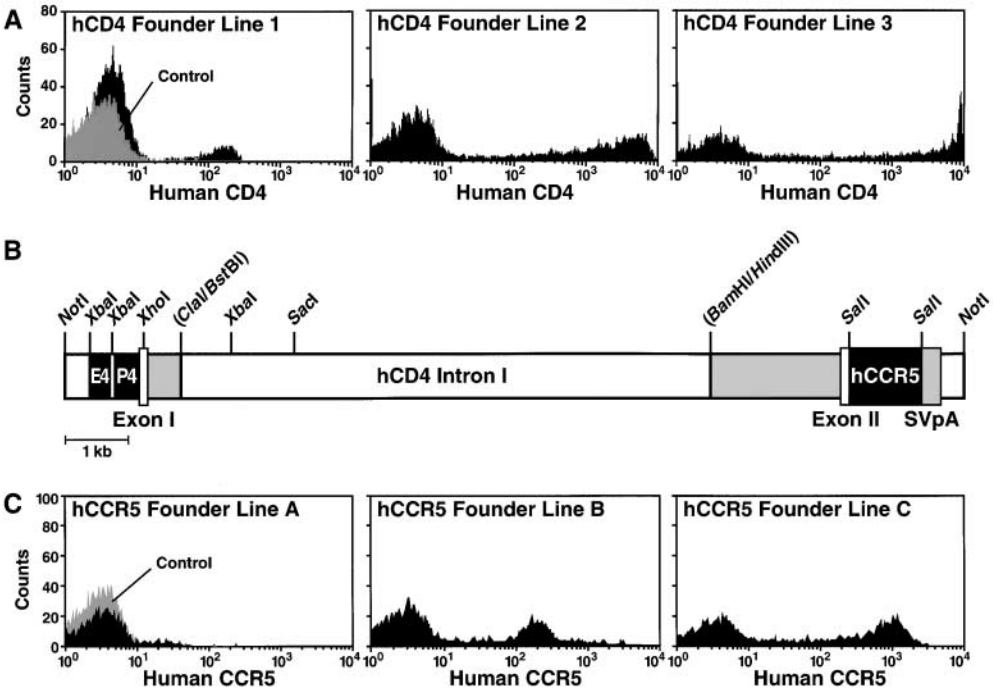

Figure 1. Transgenic rat lines expressing varying levels of hCD4 or hCCR5. Heparinized blood taken from (A) hCD4-transgenic rats, (C) hCCR5-transgenic rats, or nontransgenic littermate controls were stained with anti-hCD4 or anti-hCCR5 antibodies, respectively, and analyzed by flow cytometry. (B) Schematic representation of the transgenic vector for hCCR5 (pMΦE4A.CCR5; for details see Materials and Methods). E4/P4: murine *CD4* enhancer/promoter.

major reservoirs for HIV-1 replication. To mimic this pattern of susceptible tissues as closely as possible, we sought to target expression of the HIV-1 receptor complex to these specific cell types in transgenic rats. For expression of hCD4, we employed a construct, previously studied in transgenic mice (12, 32, 48), that contained the murine *CD4* enhancer linked to a 30-kb human *CD4* minigene that included the promoter and all of the exons (32). For expression of hCCR5, we used a chimeric mouse/human construct that directs expression of cDNA inserts in cells from the monocyte/macrophage lineage and Th cells (Fig. 1 B). Because human, but not murine, CD4 is expressed in the monocyte lineage, this construct was generated with the murine *CD4* enhancer and promoter plus a human *CD4* intron 1 sequence that we found to be required for expression in monocyte lineage cells, including macrophages, dendritic cells, and microglia (unpublished data). The human intronic sequence also contains the transcriptional silencer that restricts expression to $CD4^+$ T cells (33).

Rat founders for individual transgenic constructs were generated by microinjection of the vector DNA into male pronuclei of fertilized oocytes from outbred Sprague-Dawley rats. Five hCD4 integration founders and three hCCR5 integration founders were identified by Southern blot analysis or PCR analysis of tail DNA samples, respectively (data not shown).

As a first assessment of the expression of human transgenes in single-transgenic rats, peripheral blood lymphocytes from transgene-positive F1 progeny were stained with fluorochrome-conjugated antibodies specific for either hCD4 or hCCR5 and analyzed by flow cytometry. Three out of five hCD4 founder lines and all three hCCR5 founder lines expressed detectable levels of the respective human proteins on a subset of peripheral lymphocytes (Fig. 1 A and C). Each transgenic rat line had a unique expression level ranging from significant to very high, and these levels have proven to be stable and heritable. Several reports have demonstrated that surface levels of hCD4 or hCCR5 can be rate-limiting for HIV-1 entry into various cell types (49, 50). Consequently, in this study the transgenic lines with the highest expression levels of human transgenes, namely hCD4 founder line 3 (Fig. 1 A) and hCCR5 founder line C (Fig. 1 C), were interbred and used in subsequent experiments. Transgenic rats coexpressing hCD4 and hCCR5 were healthy and did not reveal any gross pathology compared with nontransgenic littermate controls in analyses of cellular blood composition and a head-to-tail necropsy (data not shown).

To characterize in more detail the cellular expression profiles of human transgenes in hCD4/hCCR5-transgenic rats, we first performed flow cytometry analyses with costaining for human transgenic proteins in conjunction with rat lineage– or lineage subset–specific markers. On peripheral lymphocytes from blood (Fig. 2 A, top panels) or spleen (data not shown), hCD4 and hCCR5 were coexpressed on the rat (r)$CD4^+$ subset, but not expressed on $rCD8^+$ T cells or B cells (data not shown). In thymocytes from transgenic rats both human transgenes were expressed

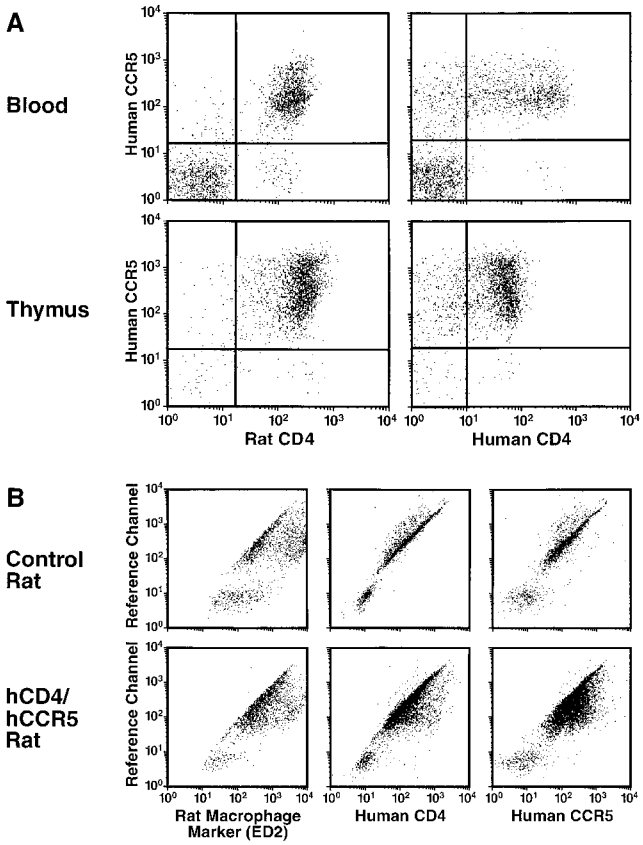

Figure 2. Cell type–restricted expression of hCD4 and hCCR5 in hCD4/hCCR5 transgenic rats. (A) Heparinized blood and thymocytes from a hCD4/hCCR5 transgenic rat were analyzed by flow cytometry for the expression of human transgenes and rCD4. Dot plots shown were gated on the total live lymphocyte population. (B) Subcultured primary rat spleen macrophages were stained for the expression of hCD4, hCCR5, or the rat macrophage activation marker rED2. Nontransgenic rat macrophages served as a control.

on $rCD4^+$ T cells (Fig. 2 A, bottom panels); both the $rCD4^+$ $rCD8^+$ subset, which constitutes the large majority of thymocytes, and the $rCD4^+$ $rCD8^-$ subset, expressed the transgenes (data not shown). Double-negative thymocytes ($rCD4^-$ $rCD8^-$ cells) had no detectable expression of either human transgene (data not shown). Expression analyses were also performed with cells from the monocyte/macrophage lineage isolated from these animals (Fig. 2 B). Since autofluorescence is much higher for these cells compared with lymphocytes, we used dot plots and two-parameter analyses in which unstained cells lie on the diagonal and positively stained cells are apparent due to their shift off the diagonal to the right. Using this approach, we found that macrophages isolated from nontransgenic control rats showed specific staining for the rat macrophage marker rED2, but not for hCD4 or hCCR5 (Fig. 2 B, top row). Importantly, macrophages from hCD4/hCCR5-transgenic rats expressed hCD4 and hCCR5 on the cell surface (Fig. 2 B, bottom row). Similarly, hCD4 and hCCR5 expression was detected on $rCD11b/c^+$ $rCD45^{low}$ rat microglia (51) cultured ex vivo (data not shown).

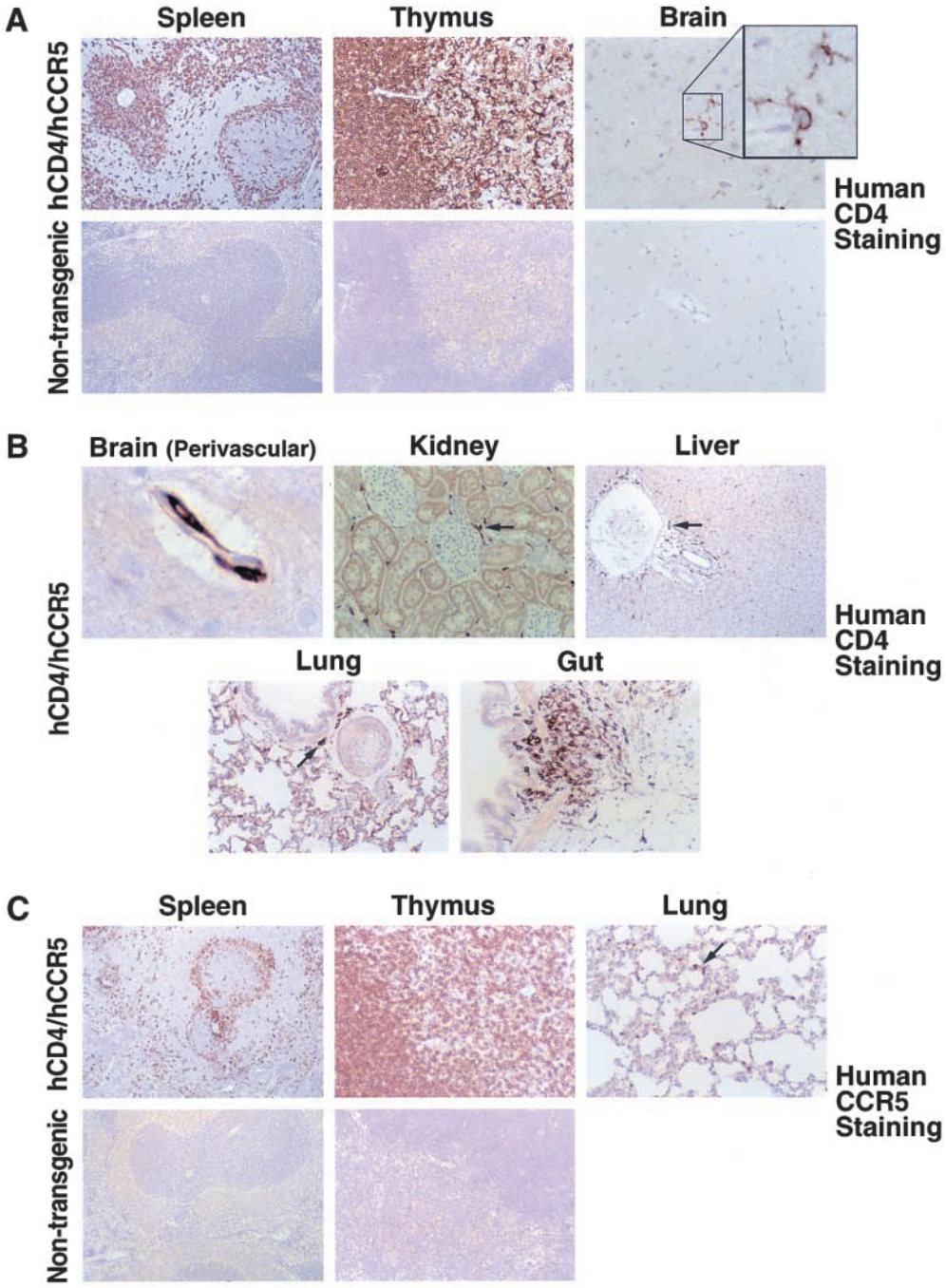

Figure 3. Selective expression of hCD4 and hCCR5 in various tissues from hCD4/hCCR5 transgenic rats. (A–C) Tissues were removed from saline-perfused rats and formalin fixed. Paraffin-embedded sections were stained with either (A and B) an anti-hCD4 antibody or (C) an anti-hCCR5 antibody and secondary step reagents. Equal exposure times were used for comparable micrographs from transgenic and nontransgenic sections. Original magnifications ranged from 40–400×.

Next, immunohistochemical stainings were performed as a complementary approach to assessing the expression of human transgenes, in particular providing insight into the tissue-specific distribution in situ. In transgenic rats, cells with the characteristic morphology and distribution patterns of T lymphocytes and macrophages expressed hCD4. Widespread, intense, and specific cellular hCD4 staining was detected in lymphocyte-rich spleen and thymus sections from a hCD4/hCCR5-transgenic rat (Fig. 3 A). Essentially no staining was detectable in tissues from a nontransgenic control rat with the anti-hCD4 staining (Fig. 3 A), nor did an isotype-matched negative control antibody yield a signal (data not shown). These results validate our immunohistochemistry methodology and confirm the results obtained by flow cytometry. We also conducted the same analyses with a number of other tissues from these animals. In brain sections, throughout the parenchyma we detected positive immunostaining in cells of microglial morphology with highly branched processes (Fig. 3 A). Similarly, larger cells with macrophage-like morphology and granular cytoplasm were frequently found in perivascular zones in the brain, most likely representing perivascu-

lar macrophages (Fig. 3 B). Expression of the hCD4 transgene was also found in tissue-resident macrophages in all other tissues analyzed including kidney, liver, and lung (Fig. 3 B). In the gut, submucosal lymphoid aggregates also showed abundant staining for hCD4. To define the expression pattern of hCCR5 in situ, parallel tissue sections from the same rats were stained with an antibody to this human chemokine receptor. Abundant expression of hCCR5 was found exclusively in the transgenic animal (Fig. 3 C); the expression pattern closely resembled that seen for hCD4, which was expected since both transgenic constructs share key regulatory elements. Collectively, these flow cytometry and immunohistochemistry data indicate that the expression of both human cell surface receptors had been successfully and exclusively targeted to the desired, biologically relevant cell types, that is, $CD4^+$ T cells, macrophages, and microglia in transgenic rats.

Rat Lymphocytes Expressing Human CCR5 Chemotax in Response to Human β–Chemokines. As a first assessment of the functional integrity of hCCR5 expressed on primary rat cells, $rCD4^+$ T lymphocytes from transgenic rats were tested for in vitro migration toward active human chemokines at varying concentrations. Lymphocytes from transgenic rats expressing hCD4 and hCCR5, but not hCD4 alone, chemotaxed in response to the natural hCCR5 β–chemokine ligands human regulated upon activation, normal T cell expressed and secreted (RANTES) and human macrophage inflammatory protein (MIP)-1α (Fig. 4). Similar results were obtained with hMIP-1β (data not shown). The chemotaxis of hCCR5-expressing lymphocytes followed a typical biphasic response to increasing concentrations of human β–chemokines (Fig. 4 A). Similar results were obtained with lymphocytes expressing hCCR5 in the absence of hCD4 (data not shown). Comparable chemotaxis of lymphocyte preparations from transgenic and nontransgenic rats toward the CXCR4 ligand stromal cell–derived factor 1 confirmed cellular viability and the validity of the assay. These results demonstrate that hCCR5 in primary transgenic rat cells retained chemokine-mediated signaling functions linked to the proper biologic responses, suggesting appropriate expression, post-translational modification, and subcellular localization of this human seven transmembrane G-protein–coupled receptor in this species context.

Primary Macrophages and Microglia from Transgenic Rats Can Be Productively Infected by Recombinant and Primary Strains of HIV-1. To assess whether expression of the HIV-1 receptor complex on primary rat cells rendered them permissive for viral infection, transgenic macrophages were first challenged with a set of HIV-1 luciferase reporter viruses. These pseudotypes are based on an Env-deficient HIV-1 proviral backbone containing the firefly luciferase gene within the nef gene locus (pNL4–3 Luc E^-R^-; reference 42), expression of which provides a quantitative marker of successful entry, reverse transcription, integration, and early viral gene expression in a given target cell. Primary macrophages from double-transgenic rats showed coreceptor-specific entry and high-level, early HIV-1 gene expression in ex vivo cultures (Fig. 5 A). Macrophages from hCD4/hCCR5-transgenic rats were highly permissive for infection by the R5 Env pseudotypes JR-FL and Ada-M, but nonpermissive for the hCXCR4-using (X4) Env pseudotype NL4–3. As expected, all macrophage cultures were permissive for the VSV-G pseudotype, which confers entry into a wide range of mammalian cells. As an additional control of specificity, pretreatment of hCD4/hCCR5-expressing rat macrophages with an anti-hCCR5 antibody reduced signals for the HIV-1 JR-FL Env pseudotype by 94%, but did not significantly affect the infection by the VSV-G pseudotype (data not shown). The low-level susceptibility of hCD4 single-transgenic rat macrophages for R5 pseudotypes may indicate the utilization of an endogenous rat cell surface molecule as an inefficient coreceptor for HIV-1 entry.

Next, transgenic rat macrophages were challenged with different, replication-competent strains of HIV-1. Primary macrophages from hCD4/hCCR5-transgenic rats could be productively infected by R5 viruses (Fig. 5 B–D). In particular, double-transgenic rat macrophages challenged with the recombinant R5 strain YU-2b (37) showed increasing p24 CA concentrations over the course of 10 d and reaching up to 18 ng per ml (Fig. 5 B). In contrast, rat macro-

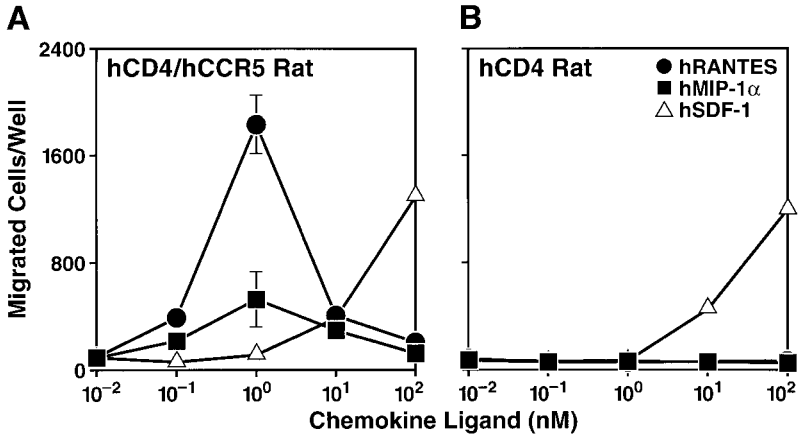

Figure 4. β-chemokine–mediated chemotaxis of hCCR5-expressing, primary rat T lymphocytes. Single cell suspensions of spleen from (A) a hCD4/hCCR5-transgenic rat, and (B) a hCD4-transgenic rat, were enriched for $rCD4^+$ cells by antibody-mediated depletion of $rCD11b^+$, $rCD45R^+$, and $rCD8^+$ mononuclear cells. Subsequently, a transwell chemotaxis assay was performed using human β-chemokines hRANTES and hMIP-1α as specific ligands. human stromal cell–derived factor 1 served as a positive control for viability. The relative number of migrated cells per well was determined and is presented as the arithmetic mean ± SD of triplicates. Results are representative of two independent experiments.

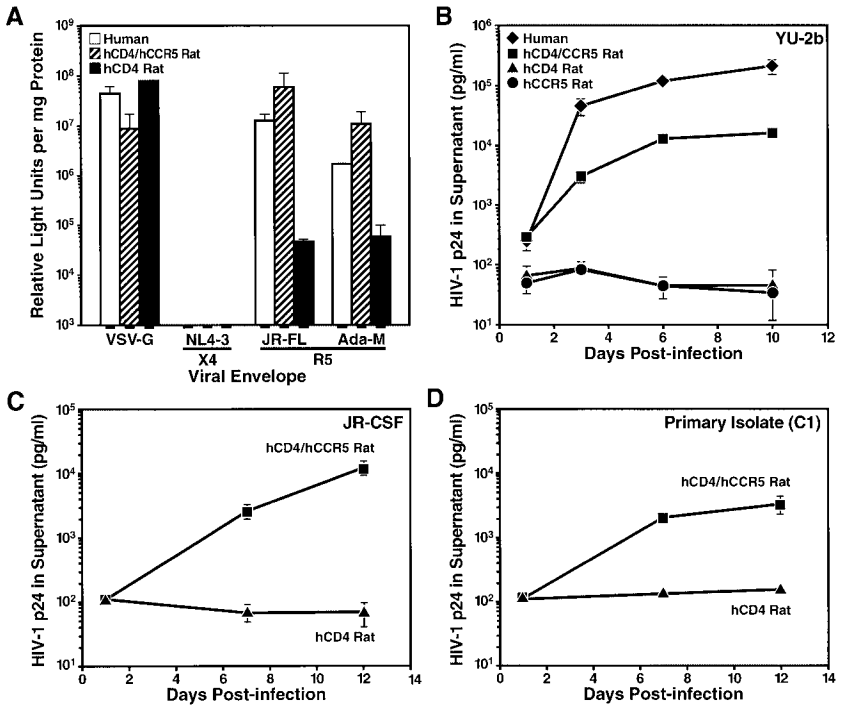

Figure 5. Primary rat macrophages coexpressing hCD4 and hCCR5 are productively infected by recombinant and primary strains of HIV-1. (A) Thymus-derived rat macrophages from a hCD4/hCCR5 transgenic rat, a hCD4 transgenic rat, or $CD14^+$ human monocyte–derived macrophages were cultured in 24-well plates and infected with the indicated HIV-1 luciferase reporter pseudotypes. The luciferase activity in cellular lysates was determined 4 d after infection and is presented here as "relative light units" per milligrams of protein. (B–D) Spleen-derived macrophages from hCD4/hCCR5 transgenic rats or single-transgenic rats (hCD4 or hCCR5 single-positive, as indicated), or human monocyte–derived macrophages, were infected with stocks of replication-competent HIV-1 YU-2b, JR-CSF, or the primary isolate C1 (20 ng p24 CA per well) and p24 CA levels in the supernatant were measured at the indicated time points. All values shown represent arithmetic means of triplicates ± SD.

phages single-transgenic for either hCD4 or hCCR5 were not productively infected, as revealed by p24 CA concentrations at background level. The parallel infection of human monocyte-derived macrophages yielded 9–15-fold higher p24 CA levels. Importantly, double-transgenic rat macrophages could be productively infected by a variety of R5 viruses, including JR-CSF (Fig. 5 C), cloned from the cerebrospinal fluid of an AIDS patient (52), the primary, patient-derived isolates C1 (40) (Fig. 5 D) and O3 (41) (data not shown), as well as the laboratory-adapted, macrophage-tropic strain Ada-M (53) and the NL4–3-based molecular clone 49.5 carrying the V3 loop from Ba-L (38) (data not shown).

Next, to investigate further whether these infections were productive, we studied the effect of a reverse transcriptase or protease inhibitor on HIV-1 infection in macrophages from hCD4/hCCR5-transgenic rats. The reverse transcriptase inhibitor AZT completely abrogated infection by YU-2 (Fig. 6 A), demonstrating that the p24 CA signals depend upon completion of this essential early enzymatic reaction in the viral life cycle and cannot be accounted for by input virus. The processing of the HIV-1 polyproteins p55 Gag and p160 Gag-Pol by the virally encoded protease into functional subunits is an essential step for HIV-1 maturation. Treatment with a protease inhibitor renders newly synthesized virus particles noninfectious (54). In cultures of hCD4/hCCR5-transgenic macrophages infected with YU-2, the presence of the protease inhibitor RTV significantly reduced the secreted p24 CA concentration compared with untreated controls (Fig. 6 A). Based on these considerations, the infection in double-transgenic macrophage cultures likely represents a spreading infection: First, transfer of cell-free supernatant from an infected primary hCD4/hCCR5-transgenic macrophage culture at 5 d after infection onto a second culture from the same rat led to a productive infection in the recipient culture (Fig. 6 C), albeit at low levels. Second, infection of hCD4/hCCR5-transgenic macrophage cultures with YU-2 at a very "low" MOI (0.05) resulted in comparable p24 peak concentrations to that achieved in a "high" MOI (0.5) infection, but with a delay of ~8 d for the cultures infected at the "low" MOI (data not shown).

In addition, certain bacterial compounds, including endotoxins like LPS, have under certain conditions been shown to enhance HIV-1 replication in human cells and macrophages from HIV-1 provirus-transgenic mice ex vivo (55, 56). In hCD4/hCCR5-expressing rat macrophages, both LPS and a formalin-fixed *Staphylococcus aureus* cell suspension measurably enhanced HIV-1 replication (Fig. 6 B). Collectively, our data indicate that a variety of HIV-1 isolates can efficiently and productively infect primary macrophages from hCD4/hCCR5-transgenic rats in a coreceptor-dependent fashion. Furthermore, antiviral drugs that target specific steps in the viral life cycle inhibit HIV-1 replication in primary ex vivo cultures from transgenic rats.

After these provocative results with macrophages, we also investigated the permissivity of brain-derived microglia from transgenic rats. Primary microglia were isolated from neonatal rat brains, enriched by subcultivation, and characterized as described previously (19). Microglial cultures revealed expected morphologic characteristics, including ruffled edges with occasional long, branched processes, small and heterochromatic nuclei, granular vesicles within the cytoplasm, as well as an absence of staining for the astrocyte-specific glial fibrillary acidic protein marker (data not shown). First, to assess the integrity of the early phase of the

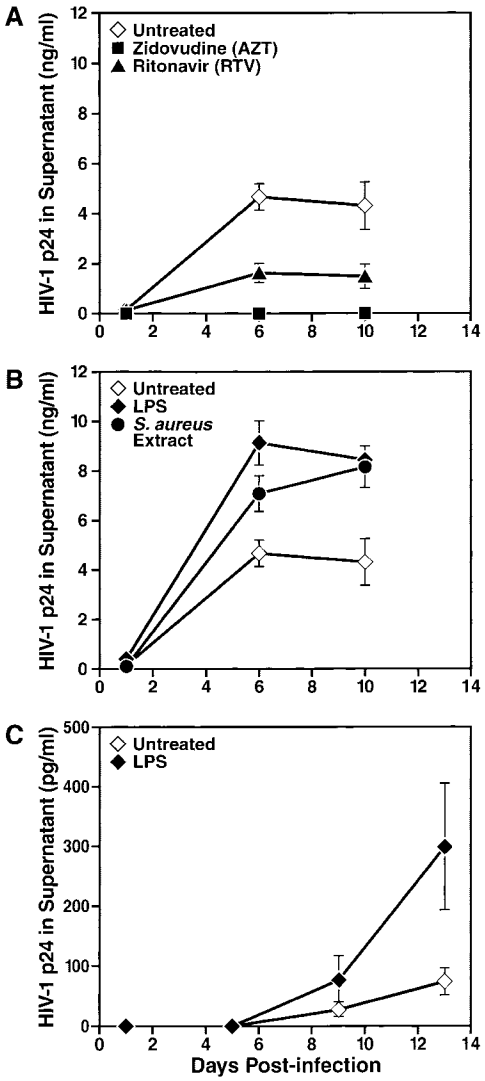

Figure 6. Effect of antiretroviral drugs and bacterial components on HIV-1 replication in transgenic rat macrophages. Spleen-derived macrophage cultures from hCD4/hCCR5 transgenic rats were pretreated with (A) AZT overnight, or RTV, (B) LPS or *Staphylococcus aureus* extract for 5 min before challenge with YU-2 (MOI 0.3). Cells were extensively washed the next day and then continuously cultivated for 10 d in the presence of the respective reagents. (C) Transfer of cell-free supernatants from YU-2-infected macrophages from a hCD4/hCCR5 transgenic rat 5 d after infection onto uninfected cultures from the same rat (p24 CA transferred: 420 pg [untreated], 1160 pg [LPS]). After overnight infection and subsequent washing, the supernatant of secondary cultures was monitored serially for p24 CA concentrations. All p24 CA values shown are the arithmetic means of triplicates ± SD.

HIV-1 replication cycle, microglia were challenged with a series of HIV-1 luciferase reporter viruses. As seen with macrophages from hCD4/hCCR5-transgenic rats, double-transgenic microglia also supported coreceptor-specific entry and robust early HIV-1 gene expression (Fig. 7 A). Comparable results were obtained also for the luciferase reporter pseudotype containing the Env from YU-2 or JR-CSF (data not shown). In contrast, microglia from a non-transgenic control rat were not permissive for either the R5 or X4 pseudotypes, but were readily infected by the VSV-G pseudotype. Second, we sought to determine whether transgenic expression of human entry cofactors on rat microglia would also confer permissivity for the full HIV-1 replication cycle. Microglial cultures from a hCD4/hCCR5-transgenic and a hCD4-transgenic rat were challenged overnight with the R5 virus YU-2 or VSV-G pseudotyped NL4–3 (NL4–3/(VSV-G)). The following day, cultures were extensively washed and 6 d after infection the p24 CA concentration in culture supernatants was determined. In addition, microglia were subsequently fixed and processed for intracellular p24 CA staining. Microglial cultures from double-transgenic rats, but not nontransgenic rats, showed clear evidence for productive infection as indicated both by significant levels of secreted p24 CA (Fig. 7 B) and by the abundant presence of intracellular viral capsid antigen (Fig. 7 C). Interestingly, we occasionally observed large, syncytia-like, multinucleated cellular aggregates in these infected cultures, but not in uninfected controls (data not shown). In contrast, microglia from hCD4 single-transgenic control rats secreted significant p24 CA concentrations after infection with NL4–3/(VSV-G), but not after challenge with the R5 virus (Fig. 7 B). These results demonstrate that coexpression of hCD4 and hCCR5 on microglia from transgenic rats confers coreceptor-specific and substantial infection by R5 strains of HIV-1.

Coexpression of Human CD4 and Human CCR5 Transgenes Overcomes the Entry Block in Primary Rat Lymphocytes, but Does Not Render Them Permissive for Productive HIV-1 Infection. We next sought to determine if coexpression of human transgenes on lymphocytes was also sufficient to overcome the entry block to HIV-1 infection. Mitogen/IL-2–activated primary lymphocytes from hCD4/hCCR5-transgenic rats showed robust signals after infections with the R5 Env pseudotypes Ba-L, Ada-M, or JR-FL (Fig. 8 A). Luciferase levels in transgenic rat lymphocytes were only three to sevenfold lower than those seen in parallel infections of activated primary human T lymphocytes. As a control of specificity, double-transgenic rat lymphocytes were demonstrated to be nonpermissive to infection by the X4 Env pseudotype NL4–3, and nontransgenic control lymphocytes were nonpermissive for either the R5 Env or the X4 Env pseudotypes. These results demonstrate that expression of the HIV-1 receptor complex was sufficient to confer coreceptor-specific entry and robust early, Rev-independent HIV-1 gene expression in primary $CD4^+$ T lymphocytes from transgenic rats.

Challenging the same set of lymphocyte cultures with the replication-competent R5 virus YU-2b revealed that hCD4/hCCR5-transgenic lymphocytes did not secrete significant concentrations of p24 CA (Fig. 8 B), despite clear signs of progression through the first part of the replication cycle, including the abundant expression of a Nef/luciferase reporter (Fig. 8 A). This nonpermissive phenotype was also not overcome by other means of activating rat T lymphocytes, including rCD3/rCD28 costimulation (data not shown). Similar results were obtained with infections by the R5 virus Ba-L (data not shown). In contrast,

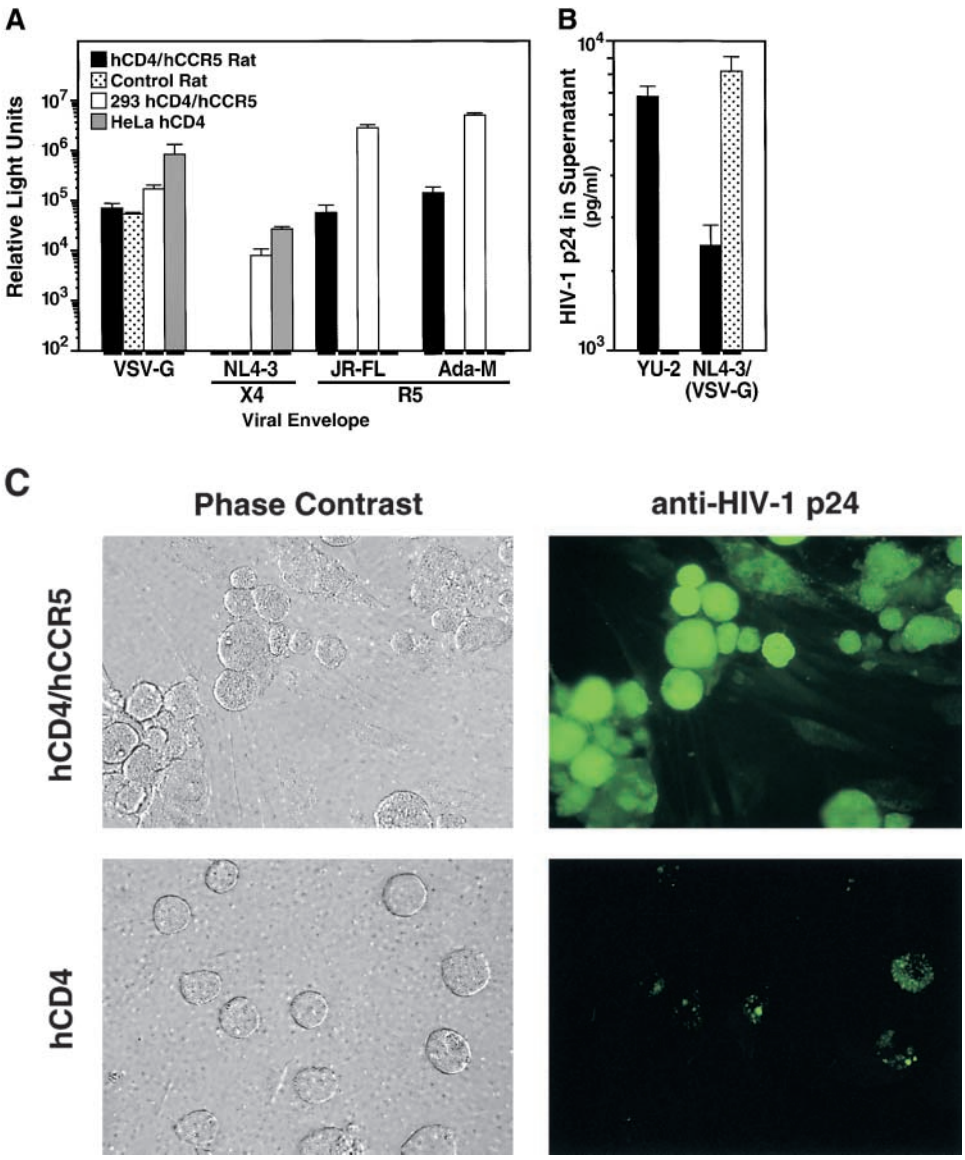

Figure 7. Primary microglia from hCD4/hCCR5 transgenic rats are permissive for productive infection by R5 viruses. (A) Primary microglia from hCD4/hCCR5 transgenic rats or nontransgenic control rats cultured in 96-well plates were challenged with the indicated pseudotyped HIV-1 luciferase reporter viruses and luciferase activity in cellular lysates was determined 2 d after infection. Human 293 hCD4/hCCR5 and HeLa hCD4 cells served as controls. (B) Microglial cultures from hCD4/hCCR5 transgenic or hCD4 transgenic control rats were infected with replication-competent YU-2 (1.5 ng p24 CA per well) or VSV-G pseudotyped NL4–3 (NL4–3/[VSV-G]) (0.7 ng p24 CA per well) overnight and washed extensively the next day, with a postwash p24 CA concentration of <300 pg/ml for all cultures. 6 d after infection the p24 CA concentration in supernatants was quantified and is represented as arithmetic means ± SEM of triplicates. (C) Infected microglia from (B) were fixed, permeabilized, and processed for intracellular p24 CA immunocytochemistry. Phase contrasts are shown on the left, and staining for p24 CA is shown on the right. Staining with an isotype control antibody yielded negligible background signals (data not shown).

human lymphocytes showed a typical infection kinetic over the course of 10 d. Thus, cells from the monocyte-macrophage lineage, but not lymphocytes, from double-transgenic rats could be productively infected by R5 strains of HIV-1.

Transgenic Rats Can Be Infected With HIV-1 In Vivo. Finally, we sought to address whether transgenic rats could be infected with HIV-1 in vivo. In a first experiment, hCD4/hCCR5-transgenic and nontransgenic control rats ($n = 4$ per group) were challenged intravenously with YU-2. At day 3 or 16 after the systemic challenge two rats from each group were killed, and spleens were removed and analyzed for signs of HIV-1 infection. We used a recently described PCR approach to detect circularized HIV-1 cDNA genomes containing two long-terminal repeats (2-LTR circles) (45). 2-LTR circles are formed from linear full-length cDNAs that do not integrate into the host genome, but circularize to form episomes. Their presence in a cell is an established surrogate for successful cellular entry, reverse transcription, and nuclear import of HIV-1. Advantages of this analysis include its unambiguous discrimination from the genomic RNA of the viral inoculum and its quantitative nature. In all transgenic rats, 2-LTR circles were readily detectable in splenocyte samples (Fig. 9), both on day 3 (rat no. 1: 1128 copies per 10^8 splenocytes; rat no. 2: 456 copies per 10^8 splenocytes) and day 16 (rat no. 3: 608 copies per 10^8 splenocytes; rat no. 4: 144 copies per 10^8 splenocytes) after challenge. In contrast, in none of the splenocyte samples from the four nontransgenic rats could 2-LTR circle signals be amplified.

In a second in vivo experiment, we sought to detect proviral DNA that had integrated into the genome of rat cells following systemic viral challenge. Based on a previously reported nested PCR strategy to specifically amplify HIV-1 integrated close to genomic human Alu repeat elements (46) we developed a similar PCR assay using a

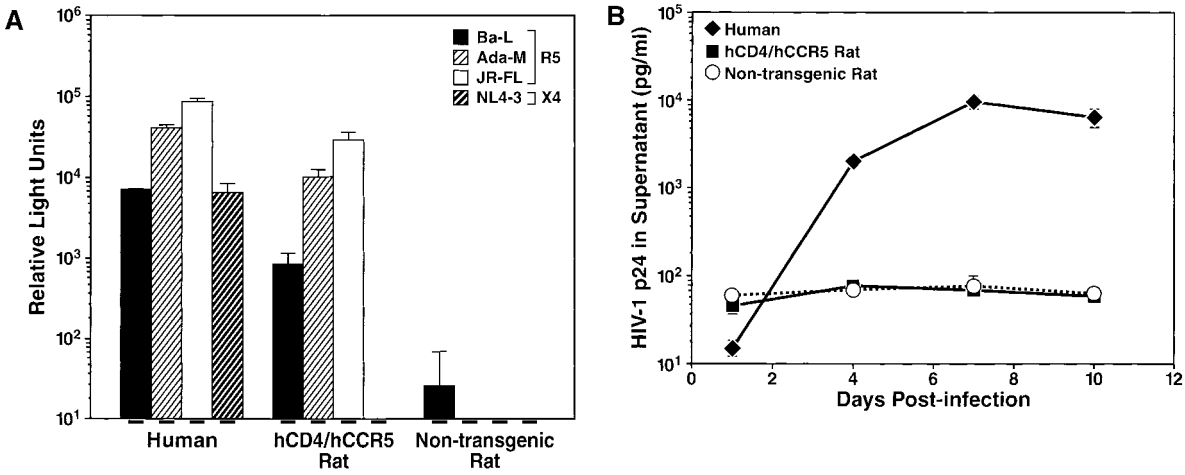

Figure 8. Coexpression of hCD4 and hCCR5 transgenes renders primary rat T lymphocytes permissive for entry and early gene expression of R5 viruses, but does not allow for a productive infection. (A) Mitogen/IL-2–activated, spleen-derived lymphocytes from a hCD4/hCCR5-transgenic rat, a nontransgenic littermate control rat or human lymphocytes were infected with the indicated pseudotyped HIV-1 luciferase reporter viruses. Luciferase activity in cellular lysates was determined 4 d after infection. (B) In parallel, the same lymphocyte cultures were infected with the replication-competent R5 strain YU-2b. The HIV-1 p24 CA concentration in culture supernatants was determined at the indicated time points. All values represent the arithmetic means ± SD of triplicates.

primer specific for a rodent ID family consensus sequence, that is highly redundant in the rat genome (47) (see Materials and Methods for details). Integrated proviral DNA was amplified from genomic DNA samples derived from spleen samples of a hCD4/hCCR5-transgenic rat (Tg #X) infected with YU-2 in vivo (Fig. 9 B, lane 7) or from ex vivo–infected thymocytes as a control (data not shown). The specific 357 bp product was not amplified from uninfected splenocyte samples, splenocyte samples from an infected nontransgenic rat, or thymus samples from Tg #X (Fig. 9 B, lanes 2–6). Similarly, omission of the ID repeat primer from the first PCR for DNA samples from spleen of Tg #X resulted in a loss of the specific product following the second PCR (Fig. 9 B, compare lanes 6 and 7), demonstrating that the exponential amplification of integrated HIV-1 cDNA in the first PCR reaction was necessary for the generation of the LTR-specific product and that extrachromosomal viral DNA cannot account for the band observed.

In a third experiment, we sought to visualize and quantify HIV-1 gene expression in vivo directly. Rats were challenged intravenously with the recombinant R5 virus R7/3-YU-2-EGFP (39) which carries an EGFP gene within the *nef* locus driven by the 5′ LTR. Lymphocytes and macrophages from spleen (Fig. 10) and PBMCs (data not shown) from both hCD4/hCCR5-transgenic rats, but

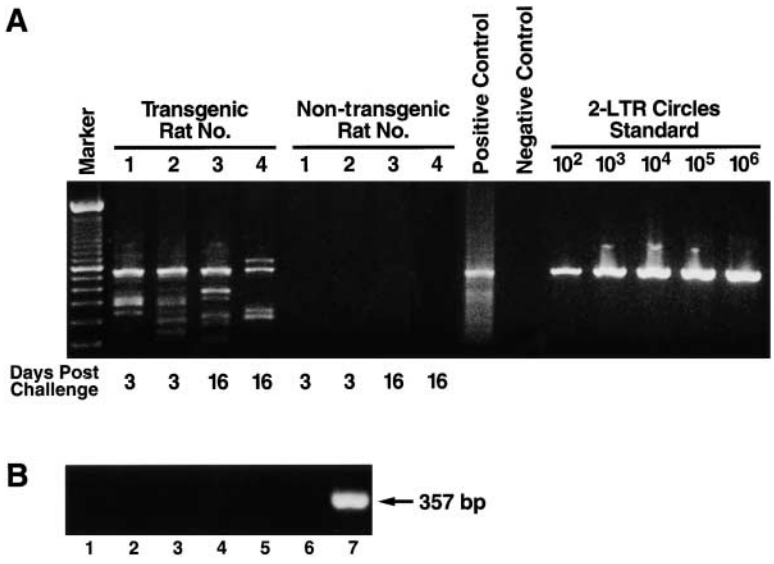

Figure 9. Detection of episomal and integrated HIV-1 cDNA in splenocytes from transgenic, but not nontransgenic rats, challenged with HIV-1 in vivo. (A) hCD4/hCCR5 transgenic and nontransgenic control rats ($n = 4$ per group) were challenged with YU-2 (8×10^5 $TCID_{50}$ per rat), intravenously. On either day 3 or 16 after challenge two rats from each group were killed and spleens removed. Nuclear extrachromosomal DNA was isolated from splenocytes and analyzed for the presence of 2-LTR circles by PCR as described recently (reference 45). Shown is an ethidium bromide-stained gel of PCR products. Positive control: Ex vivo–infected hCD4/hCCR5 transgenic splenocytes; negative control: no DNA. (B) A hCD4/hCCR5-transgenic (Tg #X) and a nontransgenic rat (NTg) were infected with YU-2 (5×10^7 $TCID_{50}$ per rat) intravenously and spleen and thymus were harvested on day 5 after challenge. Integrated proviral DNA in genomic extracts was detected by nested PCR (see Materials and Methods for details). Lanes: (1) no template; (2) NTg spleen; (3) Uninfected Tg #Y spleen; (4) Tg #X thymus, no ID primer; (5) Tg #X thymus; (6) Tg #X spleen, no ID primer; (7) Tg #X spleen. Except for lanes 4 and 6 both ID primer BC1-A and LTR primer A were added to the first PCR reaction. Shown is an ethidium bromide-stained gel of the expected 357 bp PCR product.

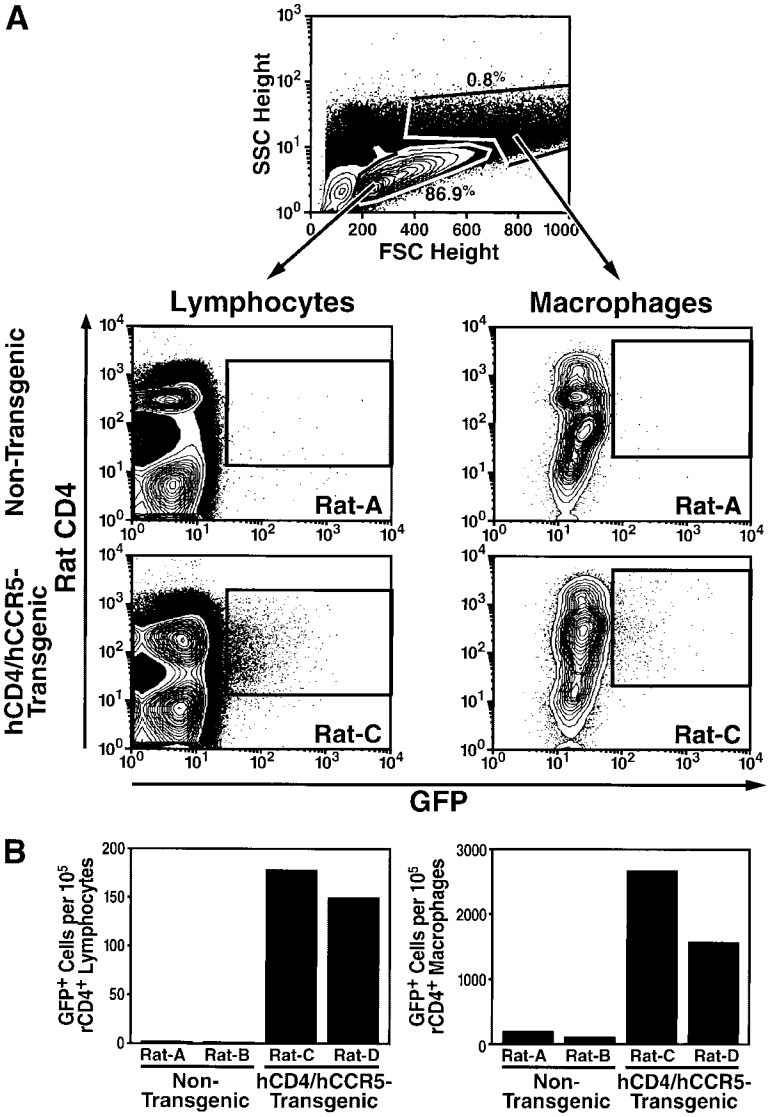

Figure 10. Early HIV-1 gene expression in splenocytes from hCD4/hCCR5 transgenic rats after intravenous infection with R7/3-YU-2-EGFP. Nontransgenic (rat A and B) and double-transgenic rats (rat C and D) were challenged with R7/3-YU-2-EGFP (9×10^6 $TCID_{50}$ per rat). On day 4 after infection, spleen and PBMC samples were harvested and analyzed by multicolor FACS® analysis. (A) Splenocytes were gated on "lymphocyte" and "macrophage" populations based on FSC/SSC profiles previously defined by backgating of $rCD3^+$ and $rCD11b/c^+$ cells, respectively (data not shown). Viable, $7\text{-}AAD^-$ cells in the respective gates are presented as dot plots showing GFP expression relative to rCD4(APC) expression. (B) Quantitative analysis of GFP^+ $rCD4^+$ splenocyte subsets. Data on $\sim 4 \times 10^6$ cells were acquired for each sample.

not from the infected nontransgenic rats or an uninfected transgenic rat, contained a significant population of GFP^+ cells (Fig. 10, and data not shown). A further indication of specificity was that the overwhelming majority of GFP^+ cells were $rCD4^+$ cells (Fig. 10 A), which are the only cells that in these animals express the HIV-1 receptor complex. The frequency of GFP^+rCD4^+ splenocytes was ~0.15% for lymphocytes and 2% for macrophages in transgenic rats (Fig. 10 B). In peripheral blood the frequency of GFP^+rCD4^+ lymphocytes was ~0.01%, with a 10-fold lower background frequency in nontransgenic rats (data not shown).

In a fourth experiment, hCD4/hCCR5-transgenic and nontransgenic control rats ($n = 6$ per group) were challenged with YU-2 by intraperitoneal inoculation. Also, a group of mock-infected double-transgenic control rats was included in this study. Control rats exhibited no gross clinical abnormalities through the postinoculation observation period. Unexpectedly, one HIV-1-inoculated transgenic rat (#521) was observed to have a generalized tonic-clonic seizure 21 d after infection, from which it recovered spontaneously. This transgenic animal was found dead at 43 d after infection, but pathologic assessment was obscured by severe postmortem tissue degradation during the time interval from death to discovery.

Plasma viral loads were monitored in all of these rats weekly for 7 wk after inoculation, and again at three subsequent time points (weeks 11, 13, and 25 after inoculation) (Fig. 11). All samples from the nontransgenic control animals and mock-infected double-transgenic rats (data not shown) were negative (<20 HIV-1 RNA copies per milliliter), indicating the specificity of the experimental system. In contrast, at least one plasma sample each from five of six inoculated hCD4/hCCR5-transgenic rats was positive (range: 20–151 HIV-1 RNA copies per milliliter), while three of them had positive samples at two separate time points. The latest time point for which plasma viremia was detected was 7 wk after inoculation

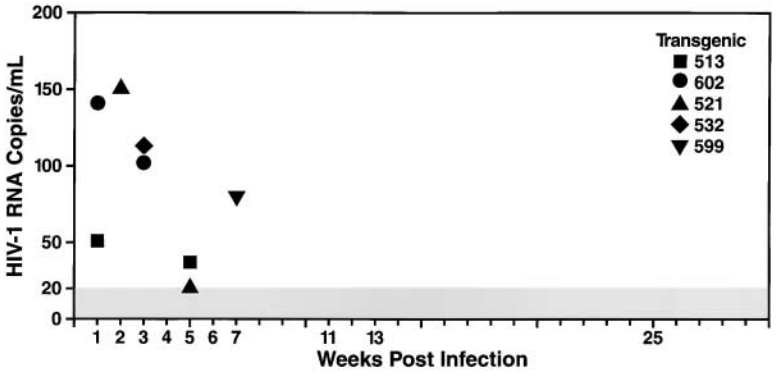

Figure 11. Plasma viremia in hCD4/hCCR5 transgenic rats after intraperitoneal inoculation with YU-2. Six double-transgenic and six nontransgenic rats were inoculated intraperitoneally with YU-2 (10^7 $TCID_{50}$ per rat). Plasma sampling was performed at each of the time points identified with a bold number on the x-axis (weeks 1, 2, 3, 4, 5, 6, 7, 11, 13, and 25 after infection) and HIV-1 vRNA concentrations were determined by the ultrasensitive Roche Amplicor HIV-1 Monitor tests with a limit of detection of 20 HIV-1 RNA copies per milliliter. Shown in this figure are all plasma samples with detectable viremia (transgenic rats 513, 602, 521, 532, and 599). None of the samples from the nontransgenic rats (rats 530, 568, 603, 655, 706, and 523) or one of six transgenic rats (rat 518) scored positive in this analysis.

(#599). At 6 mo after inoculation, two rats from each group were killed (hCD4/hCCR5-transgenic, HIV-1-infected: #513, #532; nontransgenic, HIV-1 infected: #530, #568; hCD4/hCCR5-transgenic, mock-infected: #584, #615) and spleen and thymus samples were analyzed for the presence of 2-LTR circles. Only the tissues from HIV-1 infected, transgenic rats (#513 (spleen) and #532 (spleen and thymus) contained low, but detectable numbers of 2-LTR circles, ranging from 36–108 copies per organ.

Discussion

The development of a transgenic small animal model permissive for HIV-1 infection would aid the study of viral transmission, pathogenesis, and the testing of therapeutic strategies including vaccines. A major obstacle to this end has been the inability of cells from present transgenic rabbit or mouse models to support a robust productive HIV-1 infection (11, 12, 57). We have previously shown that cellular entry constitutes the only absolute block to HIV-1 replication in certain rat cell lines, and that this restriction can be overcome by coexpression of hCD4 and hCCR5 (19). Studies with pseudotyped HIV-1 revealed that primary cells from the monocyte/macrophage lineage from various rat strains supported all steps in the viral replication cycle, including the production of infectious HIV-1. Based on these findings, we hypothesized that reconstitution of the HIV-1 receptor complex in rats would overcome the entry block in primary cells and recapitulate the full viral replication cycle in at least some cell types such as macrophages. Specifically, the mouse CD4 enhancer and the human CD4 intron 1, which contains a monocyte lineage-specific enhancer (unpublished data), were included in both transgenes, as was the human intronic silencer that shuts off CD4 expression in CD8 lineage T cells. A definitive murine promoter element for CCR5 has not yet been identified, and this strategy assured coexpression of hCD4 and hCCR5, which is an absolute requirement for efficient cellular entry of R5 viruses. Several independent, single-transgenic rat lines were generated that displayed distinct expression levels of human transgenes. Detailed expression analyses using flow cytometry and immunohistochemistry indicated that both hCD4 and hCCR5 had been successfully and exclusively targeted to the desired, biologically relevant cell types in transgenic rats.

Although hCCR5-mediated signaling is not required for HIV-1 infection (34, 58, 59), coreceptor-mediated signaling events induced by R5 Env have been suggested to be contributory to HIV-1–related pathology (60, 61). We thus sought to determine whether or not hCCR5 expressed on primary cells from transgenic rats preserved its competence as a signal-transducing chemokine receptor. We found that hCCR5-expressing lymphocytes chemotaxed in response to the natural human ligands for hCCR5, the β-chemokines hRANTES, hMIP-1α, and hMIP-1β, demonstrating that hCCR5 in primary transgenic rat cells retained signaling functions linked to the proper biologic responses. In this context it is also noteworthy that transgenic rats expressing high levels of hCCR5 were healthy and did not reveal any gross abnormalities, particularly within the hematopoietic system. This observation is consistent with the absence of a known physiological phenotype in humans that express markedly different levels of hCCR5 (30).

Coexpression of hCD4 and hCCR5 in transgenic rats indeed rendered primary rat cells susceptible to entry of HIV-1. This process was both hCD4-dependent and coreceptor-specific as demonstrated by several lines of experimental evidence. First, single-transgenic or nontransgenic rat cells were largely nonpermissive. Second, double-transgenic cells were permissive selectively for R5, but not X4, viruses. Third, an anti-hCCR5 antibody specifically blocked infection by an R5 virus. Moreover, entry was not restricted to specific R5 Env, since diverse R5 strains tested were capable of infecting hCD4/hCCR5-expressing primary rat cells. These results suggest that hCD4 and hCCR5 expressed in transgenic rats have the required posttranslational modifications and appropriate subcellular localization to function as an efficient HIV-1 receptor complex in this species context.

Consistent with our earlier studies on nontransgenic rat-derived cells (19), we found that primary cells from the monocyte/macrophage lineage from hCD4/hCCR5-transgenic rats allowed for a productive infection by R5 viruses in ex vivo cultures. Remarkably, levels of productive infection were one to two orders of magnitude higher than those described for comparable transgenic mouse models

(11, 12). R5 primary isolates, molecular clones, and recombinant strains of HIV-1 were all capable of productively infecting hCD4/hCCR5 transgenic rat macrophages. Furthermore, the significant inhibitory effect of a reverse transcriptase and a protease inhibitor further confirmed the productive nature of viral replication in these transgenic rat macrophage cultures. Evidence from low MOI inoculations and from supernatant transfer experiments suggests that the infection in these ex vivo macrophage cultures was spreading. This fact is consistent with our earlier observation that virus released by primary rat macrophages is highly infectious (19). These findings are also important to provide context for the interpretation of viral dynamics in vivo.

In humans, macrophages and microglia are important targets of HIV-1 that support virus replication in vivo (62–66). Several studies have implicated macrophages as a long-term reservoir for HIV-1 in infected individuals (67, 68) and macrophages have been suggested as a source of increasing viremia in later stages of HIV disease, particularly during opportunistic infections (69). Similarly, in a Rhesus macaque study, macrophages were implicated as the principal reservoir for sustained high virus load after the depletion of $CD4^+$ T cells by a simian immunodeficiency virus (SIV)/HIV-1 chimera (70). Regarding the pathophysiology of acute CNS infection by HIV-1, histological studies of specimens from HIV-1–infected humans and SIV-infected rhesus macaques have demonstrated that lymphocytes and monocytes migrate into the brain early in infection (71, 72). A recent study in SIV-infected macaques indicated that perivascular macrophages, which are frequently replenished by peripheral monocytes/macrophages, are the earliest and primary cell type infected in the CNS (73), but the role of lymphocytes in the development of HIV-associated dementia is still unclear. One might speculate that in vivo infections of transgenic rats could provide some insight into this question since productive infection of monocytes/macrophages and T lymphocytes appeared to be naturally uncoupled in these animals (see below). Also, macrophages and microglia are thought to be crucial for the development of HIV-associated dementia because they are the only resident cells that can be productively infected at high levels in the CNS (for a review, see reference 74). Thus, based on the ex vivo data from hCD4/hCCR5 transgenic rats, it is conceivable that certain aspects of HIV-associated CNS pathology can be recapitulated in this small animal model.

Unlike macrophages, primary T lymphocytes from hCD4/hCCR5-transgenic rats were found to harbor a major posttranscriptional block to HIV-1 replication. The early phase of the viral replication cycle was fully intact, since infections by different R5 pseudotypes yielded abundant signals from luciferase reporter viruses. We have previously established that luciferase expression in this cell context is a useful surrogate for HIV-1 Nef protein expression (19). Although the expression level of this reporter gene in rat T cells was only three to sevenfold lower than that found in human lymphocytes, infected transgenic rat lymphocytes failed to secrete significant concentrations of p24 CA. The nonproductive infection of rat lymphocytes was similarly observed previously following infections by HIV-1/(VSV-G), which also demonstrated that there was no significant de novo synthesis of Gag products in rat lymphocytes despite abundant early, Rev-independent gene expression (19). These findings are consistent with an impaired function of Rev in promoting the nuclear export of unspliced viral transcripts in this cell type. This hypothesis is also supported by a recent study on HIV-1 provirus transgenic rats, which revealed the absence of unspliced viral transcripts from the majority of tissues analyzed (20). Future investigation will be needed to define the exact nature of the block in primary rat T cells affecting the transition from the early to the late phase of the viral replication cycle. In the context of an NL4–3 provirus-transgenic mouse model, macrophages, but not lymphocytes, were found to upregulate the expression of p24 CA and infectious virus after polyclonal stimulation (56, 75). In contrast, another study on JR-CSF transgenic mice indicated that comparable, low levels of infectious virus were being released from primary monocytes, as well as T and B lymphocytes (76). It is thus unclear whether mice share this cell type-specific restriction to HIV-1 replication evident in rats. On one hand, the failure of HIV-1 replication to proceed beyond early gene expression in primary rat T lymphocytes may help to dissect mechanisms of $CD4^+$ T cell depletion in an in vivo model. Specifically, transgenic rat T cells should provide a tool to study the individual quantitative and qualitative contributions of hCCR5-mediated signaling to HIV-1 pathology (60, 61, 77, 78) and to study the importance of early HIV-1 gene products for T cell pathology, since infection results in the expression of these products at physiological levels in the absence of structural HIV-1 gene products. For example, the early HIV-1 Nef protein itself has been suggested to harbor a major determinant of pathogenicity for an AIDS-like disease induced by transgenic expression in mice (79). On the other hand, the identification of cellular factors that are essential for HIV-1 replication and that may surmount restrictions in primary rat T lymphocytes would both provide a better understanding of the molecular aspects of HIV-1 replication and increase the range of applications of a transgenic rat model.

In a first set of studies seeking to establish whether or not rat cells in vivo are permissive for HIV-1 infection, systemic challenge with R5 viruses yielded definitive evidence for viral infection in cells from spleen, peripheral blood, and thymus. Cells from hCD4/hCCR5 transgenic, but not nontransgenic rats, contained episomal 2-LTR circles as well as integrated proviral DNA. Early viral gene expression, represented by an EGFP reporter expressed from the nef gene locus, was found in $rCD4^+$ lymphocytes and macrophages in double-transgenic rats. Interestingly, the frequency of infected $CD4^+$ lymphocytes in the spleen of transgenic rats (0.14–0.18%) was in the same range as those described in spleen sections from AIDS patients (0.09–0.64%) in a recent report (80). Our results constitute the

first demonstration of a quantitative HIV-1 infection of lymphoid tissues in a transgenic rodent.

It has been suggested that 2-LTR circles are labile, both in vitro and in vivo, relative to integrated viral genomes, and thus indicative of a recent cellular infection event (45). Further investigations will be required to define the nature of the persistence of 2-LTR circles in transgenic rats, and specifically whether or not this persistence reflects multiple rounds of replication. The detectable, albeit low, levels of 2-LTR circles in spleen and thymus samples from transgenic rats 6 mo after inoculation are encouraging in this respect. In addition, we found that transgenic rats challenged with YU-2 intraperitoneally had detectable plasma viremia up to 7 wk after infection. Although the sample size is small and the levels of viremia were modest, these findings imply that a productive infection may occur in this rodent model. Also, detectable plasma viremia in one transgenic rat (#521) correlated in time with an observed neurological event before an unexplained, spontaneous death.

Taken together, our data demonstrate that hCD4/hCCR5 transgenic rats are promising candidates for a small animal model of HIV-1 infection. Whether the productive infection of cells from the monocyte/macrophage lineage will be sufficient for sustained viral loads in vivo and/or induction of HIV-induced pathogenesis in the present form of the model is currently under investigation. A recent study reported that HIV-1 provirus transgenic rats developed HIV-related pathologies and immunologic dysfunction (20). This indicates that HIV-1 gene expression in this species may be sufficient to recapitulate certain aspects of the disease in humans, and provides an additional basis for optimism that HIV-related pathology will be found in the hCD4/hCCR5-transgenic rat model. Such a system for de novo infection would provide unique experimental opportunities for elucidating aspects of viral transmission, viral dynamics, HIV-1 pathogenesis, immune responses, and efficacy of therapeutic strategies aimed at diverse aspects of the viral life cycle. A systematic assessment of in vivo infections in hCD4/hCCR5-transgenic rats will be needed to define in more detail the features and limitations of this model system, as well as opportunities for enhancement based on further genetic and biological manipulation.

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