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Macrophage-Tropic Strains of Human Immunodeficiency Virus Type 1 Utilize the CD4 Receptor

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To characterize the role of CD4 in human immunodeficiency virus type 1 (HIV-1) infection of macrophages, we examined the expression of CD4 by primary human monocyte-derived macrophages and studied the effect of recombinant soluble CD4 and anti-CD4 monoclonal antibodies on HIV-1 infection of these cells. Immunofluorescence and Western blot (immunoblot) studies demonstrated that both monocytes and macrophages display low levels of surface CD4, which is identical in mobility to CD4 in lymphocytes. Recombinant soluble CD4 and the anti-CD4 monoclonal antibody Leu3a blocked infection of macrophages by three different macrophage-tropic HIV isolates, and the cytopathic effects of HIV-1 infection were similarly prevented. Dose-response experiments using a prototype isolate which replicates in both macrophages and T lymphocytes showed that recombinant soluble CD4 inhibited infection of macrophages more efficiently than in lymphocytes. These results indicate that CD4 is the dominant entry pathway for HIV-1 infection of macrophages. In addition, recombinant soluble CD4 effectively blocks HIV-1 infection by a variety of macrophage-tropic strains and thus has the potential for therapeutic use in macrophage-dependent pathogenesis in HIV disease.

Human immunodeficiency virus (HIV) infects monocytes, macrophages, and other cells in addition to lymphocytes. HIV infection of macrophages has received special attention, since, as in other lentivirus infections, these cells are believed to play a central role in pathogenesis, functioning as a reservoir for viral persistence and dissemination (18, 20, 36, 37). Furthermore, infected macrophages are readily detected in the brains (17, 30, 47), lungs (3, 17), and other tissues (41, 52) of patients with acquired immunodeficiency syndrome (AIDS) and may be particularly important in the genesis of the neurologic (5, 31) and pulmonary (40) sequelae of AIDS.

HIV infects T lymphocytes through the association of its surface glycoprotein (gp120) with CD4 (12, 34), a 55-kilodalton glycoprotein expressed on the surface of the helper-inducer lymphocyte subset that interacts with class II major histocompatibility complex antigen (14). CD4-gp120 binding and lymphocyte infection can be blocked by certain monoclonal antibodies (MAbs) directed against CD4 (29, 35, 43) and by recombinant soluble CD4 (rsCD4) molecules (13, 16, 24, 45, 50). Human monocytes and macrophages also express CD4 (11, 28, 54; H. Teppler, S. H. Lee, E. P. Rieber, and S. Gordon, AIDS, in press), although its normal function on these cells is not known.

Previous reports examining the role of CD4 in macrophage infection have yielded conflicting results (19, 21, 23, 28). Since a number of CD4-negative cells have recently been shown to be infected in vitro (6, 22, 49), it is possible that alternative receptors for HIV exist. Furthermore, HIV type 1 (HIV-1) isolates vary in their ability to replicate in macrophages (5, 8, 17, 19, 31), and alternative cellular receptors may play a role in this tropism.

This study was designed to address two questions. (i) Do primary human monocyte-derived macrophages (MDM) express membrane-associated CD4? (ii) Do HIV-1 strains tropic for macrophages utilize CD4 as their receptor? Our results demonstrate that macrophages express surface CD4 and that selected anti-CD4 antibodies block infection, indicating that HIV-1 utilizes the CD4 receptor for infection of macrophages. In addition, we found that rsCD4 is an effective inhibitor of HIV-1 infection of macrophages.

MATERIALS AND METHODS

Cell isolation and culture. Monocyte-enriched populations were prepared from peripheral blood mononuclear cells (PBMC) of seronegative volunteers by selective adherence to gelatin followed by plastic adherence as previously described (8). Monocytes were seeded at 2×10^5 to 2.5×10^5 cells per well in 48-well plastic tissue culture plates (GIBCO Laboratories, Grand Island, N.Y.) and maintained with Dulbecco modified Eagle medium (GIBCO Laboratories) containing 10% fetal calf serum (Hyclone Laboratories, Inc., Logan, Utah), 10% horse serum (GIBCO Laboratories), glutamine (600 μg/ml), penicillin (100 U/ml), streptomycin (100 U/ml), granulocyte-macrophage colony-stimulating factor (50 U/ml; Genetics Institute, Cambridge, Mass.), and macrophage colony-stimulating factor (100 U/ml; Genetics Institute). Every 3 to 7 days, the cultures were refed with fresh medium (50 to 100% exchange), and approximately every 10 days, they were washed with phosphate-buffered saline (PBS) to remove nonadherent cells. After initial purification, ≥90% of the cells were monocytes, as determined by surface markers, nonspecific esterase, and latex phagocytosis. After 7 days in culture, ≥97% of the cells were MDM by the same criteria, and T lymphocytes were undetectable.

MDM morphology was examined in cells cultured in plastic tissue culture plates, which were washed with PBS, allowed to air dry, fixed with methanol, and stained by a

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modified Wright-Giemsa method (Dif-Quik; American Scientific Products, McGaw Park, Ill.).

Peripheral blood lymphocyte (PBL) cultures were prepared from PBMC by serial depletion of adherent cells as described previously (8) and maintained at 5×10^5 to 10×10^5 cells per ml in RPMI 1640 containing 15% fetal calf serum (GIBCO Laboratories), glutamine (300 μg/ml), penicillin (100 U/ml), streptomycin (100 U/ml), and nonessential amino acids (1%; Sigma Chemical Co., St. Louis, Mo.). PBL were supplemented with phytohemagglutinin (PHA-L; 5 μg/ml; Sigma) for the first 3 to 4 days in culture and thereafter with interleukin 2 (10%, vol/vol; Electro-Nucleonics, Inc., Silver Spring, Md.). Every 3 to 7 days, 50 to 80% of the culture was removed and replaced with fresh medium. After 4 days in culture, ≥75% of these cells were T lymphocytes, as determined by surface markers, and ≤2% reacted with surface markers for monocyte/macrophages.

Detection of CD4. Immunofluorescent detection of surface markers on PBL and freshly isolated monocytes was carried out with the anti-CD4 MAb OKT4 (Ortho Diagnostic Systems Inc., Westwood, Mass.), anti-T-cell MAb OKT3 (Ortho), anti-monocyte/macrophage MAb Leu-M3 (Becton Dickinson and Co., Mountain View, Calif.), and isotype-matched control antibodies. PBL and freshly isolated monocytes in suspension were stained as previously described (8), with the addition of 15% AB-positive human serum to all incubation buffers to block MAb binding to Fc receptors, and 5,000 cells were analyzed by flow cytometry (Facscan; Becton Dickinson).

For immunofluorescent detection of CD4 on adherent MDM, we used pooled hyperimmune rabbit serum raised against purified recombinant CD4 (Smith Kline & French Laboratories, King of Prussia, Pa. [13]), with rabbit serum directed against an irrelevant viral antigen (15) as a control. MDM were cultured on plastic tissue culture chamber slides (Lab-Tek; Nunc Inc., Naperville, Ill.), and PBL were spotted onto polylysine-coated glass slides and allowed to air dry. Slides were fixed with either methanol or 2% paraformaldehyde in PBS for 10 min, washed twice with PBS, and overlaid with 20% goat serum (Organon Teknika Corp., Durham, N.C.) and 20% AB-positive human serum for 20 min to block Fc receptors and nonspecific binding. Cells were then incubated for 45 min with the rabbit antiserum diluted 1:60 in PBS containing 5% goat serum and 5% AB-positive human serum, washed three times with PBS, and incubated for 45 min with fluorescein-conjugated goat $F(ab')_2$ anti-rabbit immunoglobulin G (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) diluted 1:100 in PBS containing 50% fetal calf serum, 5% goat serum, 5% AB-positive human serum, and 0.15% Evans blue as a counterstain to minimize autofluorescence. Slides were then washed, mounted with aqueous medium (Citifluor Ltd., London, England), and examined by fluorescent microscopy.

Western blot (immunoblot) analyses were performed on PBL, freshly isolated monocytes, MDM, and the CD4-negative cell line U373-MG (22) with the same polyclonal anti-CD4 or irrelevant rabbit sera. Cells (10×10^6 to 20×10^6) were washed with PBS and lysed with 0.5 to 1 ml of lysis buffer (20 mM Tris [pH 8.0], 120 mM NaCl, 2 mM phenylmethylsulfonyl fluoride, 2 mM EDTA, 2 mM NaF, 0.2% sodium deoxycholate, 0.2% Nonidet P-40) containing 4 μg of aprotinin (Sigma) per ml, 1 μg of leupeptin (Sigma) per ml, 2 μg of pepstatin A (Sigma) per ml, and 0.2 μg of soybean trypsin inhibitor (Sigma) per ml. Lysates were clarified by centrifugation, and protein concentration was determined by Lowry assay (kit 5656; Sigma). Equal amounts of protein (300 μg) of each lysate were mixed 1:1 with 2× Laemmli sample buffer (32), heated at 65°C for 30 min, and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12.5% resolving gel). Samples were then electroblotted onto nitrocellulose paper (BA85; Schleicher & Schuell, Inc., Keene, N.H.), which was air dried and incubated in blocking buffer (PBS containing 5% nonfat milk, 10% human serum, and 0.3% Tween). Strips of the nitrocellulose were then incubated with rabbit antiserum diluted 1:500 in blocking buffer for 1 h at 37°C, washed four times with PBS containing 0.3% Tween 20, and incubated with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (Sigma) diluted 1:75 in buffer for 1 h at 37°C. After being washed, the strips were incubated with substrate (0.05% chloronaphthol) for 15 min at 37°C, rinsed with distilled water, and air dried.

Viruses. HIV-1 strain SF162 (4) was isolated from cerebrospinal fluid, and HIV-1 strain DV (11) was isolated from PBMC. HIV-1 strain 89.6 is a fresh isolate (second passage) obtained in our laboratory from an individual with AIDS by cocultivation of whole PBMC with PBMC from seronegative donors (25). Virus stocks were grown in phytohemagglutinin-interleukin 2-stimulated lymphocytes (strains SF162 and 89.6) or SUP-T1 cells (strain DV) (46) and titered by 50% tissue culture infectious dose ($TCID_{50}$) endpoint dilution on PBL or SUP-T1 cells. The multiplicity of infection (MOI) was calculated as the $TCID_{50}$ per cell. For PBL, the MOI was based on the total number of cells counted at the time of infection, of which approximately 50% were CD4-positive T lymphocytes. For MDM, the MOI was based on the number of cells initially plated and therefore represented an underestimate of the actual MOI, since, by the time of infection, a proportion of the cells was lost from the MDM cultures.

rsCD4 inhibition of HIV-1 infection. Monocyte/macrophages were infected as MDM after 7 days in culture, and PBL were infected after 4 days in culture. Cell-free virus was incubated for 30 min at 37°C with rsCD4 (Smith Kline & French [13]) and added to 2×10^5 to 2.5×10^5 cells for 90 min at 37°C. The cells were then washed four times with PBS and refed with 0.5 ml of culture medium containing rsCD4 at the same concentration. Every 3 to 7 days, MDM wells were refed and PBL were split and resuspended in 0.5 ml of culture medium with rsCD4. rsCD4 was maintained in the culture medium throughout the experiment and replaced each time the cultures were fed. The rsCD4 used in these experiments was pharmaceutical-grade purified protein which was tested and shown to be free of endotoxin and other contaminants.

MAb inhibition of HIV-1 infection. Anti-CD4 MAb Leu3a (Becton Dickinson) was purchased as purified immunoglobulin G at 1 mg/ml and washed four times with PBS in a 30,000-molecular-weight microcentrifuge exclusion filter (Ultrafree-MC; Millipore Corp., Bedford, Mass.) to remove azide. B33.1, used as a control, is a MAb directed against a nonpolymorphic domain of HLA-DR (provided by B. Perussia, The Wistar Institute, Philadelphia, Pa. [39]) and was purified from mouse ascitic fluid (Affi-gel; Bio-Rad Laboratories, Richmond, Calif.). Seven-day-old MDM cultures and 4-day-old PBL cultures were incubated with 10 μg of MAb per ml for 30 min at 37°C. Virus was then added, along with additional MAb to maintain a constant concentration, and incubated for 90 min at 37°C. Cells were washed four times with PBS and refed with 0.5 ml of medium containing MAb. MAb was maintained in the culture medium throughout the experiment and replaced each time the cultures were fed.

HIV-1 detection. $p24^{gag}$ antigen production by PBL was

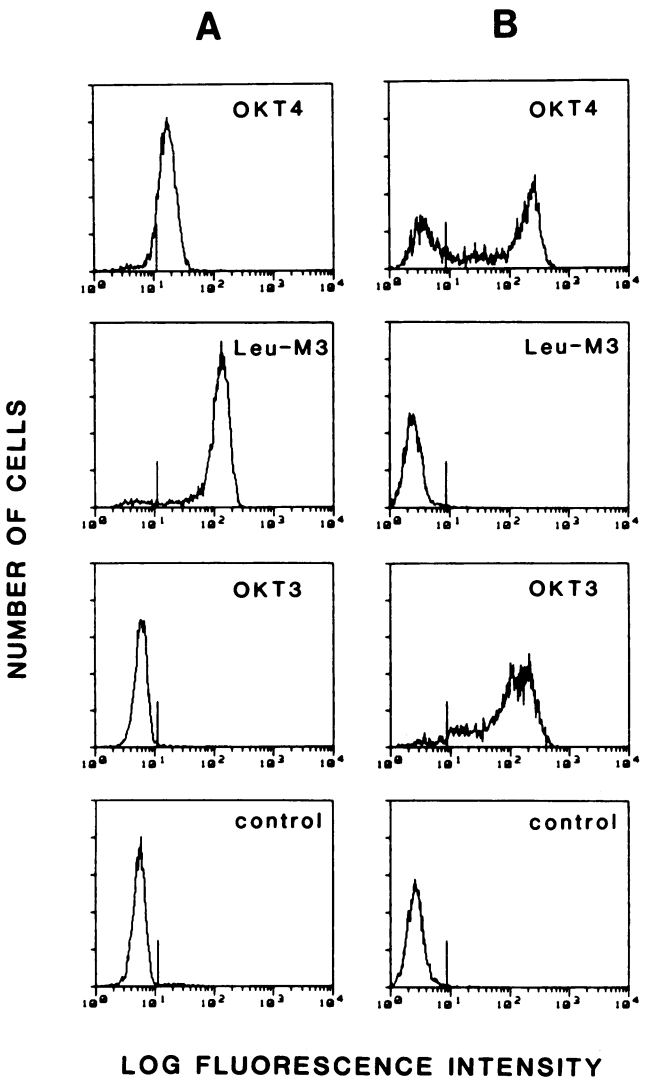

FIG. 1. CD4 expression of monocytes compared with PBL. Monocytes immediately after isolation (A) and PBL (B) were stained with MAb OKT4 as well as markers for monocytes (LeuM3) and T cells (OKT3) and control antibody and evaluated by flow cytometry. The scale for fluorescence intensity is the same for all profiles, and the vertical line in each panel indicates the threshold for positive staining based on control antibody (immunoglobulin G1 control shown).

determined on whole cultures lysed with 1% Triton X-100 (8). Antigen production by MDM in culture supernatant and, at selected time points, in cell lysates as well, was determined. p24 antigen was measured by an antigen capture enzyme-linked immunosorbent assay kit (Coulter Electronics, Inc., Hialeah, Fla.).

RESULTS

CD4 expression by MDM. To compare the levels of CD4 expression in PBL and freshly isolated monocytes, we stained both cell types with the anti-CD4 MAb OKT4 and analyzed them by flow cytometry (Fig. 1). The monocyte population was homogeneous and expressed low levels of CD4. The proportion of cells staining positive for CD4, when compared with those positive for an isotype-matched control

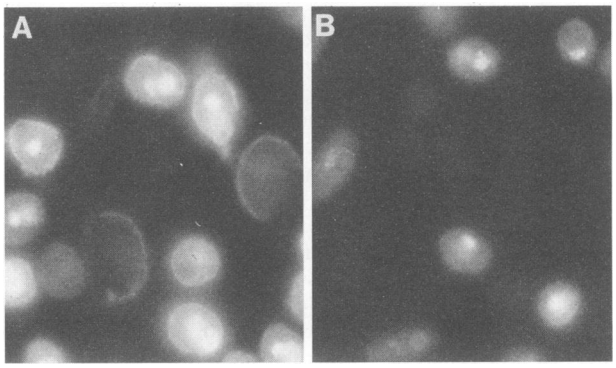

FIG. 2. CD4 expression of MDM. Seven-day-old MDM cultured on plastic tissue culture chamber slides were fixed with 2% paraformaldehyde, stained for CD4, and examined by immunofluorescence. Shown are MDM stained with pooled hyperimmune anti-CD4 rabbit serum (A) and control rabbit serum (B). The cytoplasmic signal is autofluorescence. Magnification, ca. ×270.

antibody, ranged from 35 to 65% in different experiments. It is important to note that a positive monocyte signal represented low-level CD4-positive monocytes and not contaminating T cells, since the population was homogeneous in its staining, and ≥90% of the cells stained with the antimacrophage MAb Leu-M3 while ≤2% reacted with the T-lymphocyte MAb OKT3. In contrast, mitogen-stimulated PBL showed two distinct populations, one CD4 negative and one CD4 positive, and the level of expression in the positive population was much greater than in monocytes (approximately 10-fold).

Because we infected MDM with virus after 7 days of culture, we asked whether CD4 could be detected at that point and, if so, where in the cell it was distributed. We were unable to detach adherent MDM in a form suitable for flow-cytometric analysis, and CD4 could not be detected by fluorescence microscopy using MAbs (8). Therefore, we used a polyclonal serum prepared against recombinant CD4 to examine the cells by both fluorescent microscopy and Western blotting. After 7 to 10 days in culture, approximately 30% of the MDM showed CD4 staining associated with the plasma membrane (Fig. 2). Many cells also had focal internal autofluorescence, and in order to look for cytoplasmic CD4 we permeabilized MDM with methanol and counterstained them with Evans blue, which screens intracellular autofluorescence (8). These permeabilized cells showed focal intracellular anti-CD4 staining, indicating cytoplasmic CD4 localization as well (results not shown). In contrast, approximately 50% of the PBL showed very intense staining which was distributed exclusively at the cell membrane (not shown). To demonstrate that this pattern was specific for CD4, we stained HeLa cells and HeLa cells expressing a transfected CD4 gene (HeLa/T4; courtesy of R. Axel) and found that only the HeLa/T4 cells gave a positive signal (not shown).

To compare the relative levels and molecular sizes of CD4 in MDM and PBL, we performed Western blot analyses of whole-cell lysates with the polyclonal anti-CD4 antiserum (Fig. 3). The levels of CD4 (normalized by total protein content) in fresh monocytes and 10-day-old MDM were similar and were considerably lower than in PBL. Comparable results were observed at 5, 7, and 14 days of culture (results not shown). CD4 migrated at the same molecular weight in all of the lysates, indicating that the receptors

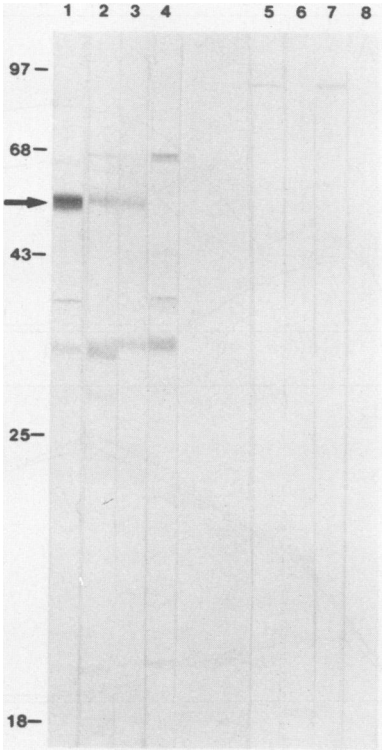

FIG. 3. Western blot detection of CD4. Lysates were prepared from PBL (lanes 1 and 5), monocytes immediately after isolation (lanes 2 and 6), MDM at day 10 in culture (lanes 3 and 7), and the CD4-negative cell line U373 (lanes 4 and 8). Equal amounts of protein were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting using pooled anti-CD4 hyperimmune rabbit serum (lane 1 to 4) or control rabbit serum (lanes 5 to 8). The mobility of molecular weight markers (in thousands) is shown on the left, and the CD4 signal is indicated by the arrow.

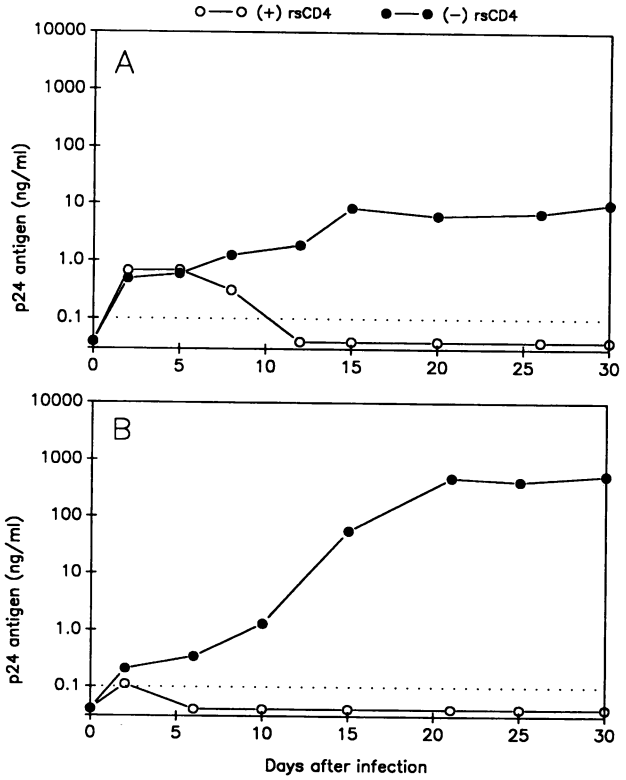

FIG. 4. Effect of rsCD4 on HIV-1 infection of MDM (A) and PBL (B). HIV-1 strain SF162 (approximately 10^3 $TCID_{50}$s) was incubated with 20 μg of rsCD4 per ml for 30 min at 37°C and then incubated for 90 min with 7-day-old MDM cultures or 4-day-old PBL cultures (MOI = 0.005 $TCID_{50}$ per cell). After the cultures were washed and refed, rsCD4 was replaced at the same concentration and the cultures were maintained as described in Materials and Methods. The cultures were tested periodically for p24 antigen production, and MDM values represent the means of results from duplicate wells.

expressed on MDM and PBL are similar. Occasionally, bands of 22 and 24 kilodaltons were also seen in MDM but not in monocytes or PBL. These bands were markedly diminished or eliminated by the addition of protease inhibitors to the cell lysate and probably represent postlysis cleavage products.

rsCD4 inhibition of HIV-1 infection of MDM. We selected as a prototype macrophage-tropic isolate HIV-1 strain SF162, which replicates in both MDM and PBL (8). MDM were cultured for 7 days before infection to allow the differentiation of monocytes into macrophages and to further remove the few residual, poorly adherent contaminating lymphocytes by serial washing. PBL were infected after 4 days in culture, following mitogen stimulation and serial depletion of adherent cells. MDM and PBL were infected with equal amounts of virus which had been preincubated with rsCD4 for 30 min. rsCD4 was kept in the culture medium throughout the experiment in order to maintain its effect on subsequent rounds of infection, and the cultures were monitored by measuring p24 antigen production.

Infection of both MDM and PBL by HIV-1 strain SF162 was markedly inhibited by 20 μg of rsCD4 per ml (Fig. 4). At the MOI used in this experiment (MOI = 0.005 $TCID_{50}$ per cell), infection was prevented in both MDM and PBL. This effect was sustained over 4 weeks in culture. To determine whether these effects were due to intracellular sequestration or blocking of viral release, we measured p24 antigen levels in selected MDM cell lysates. Cell-associated antigen levels were similar to levels in the supernatant (data not shown). Low levels of antigen detected in some cultures infected in the presence of rsCD4 during the first week after infection most likely reflected residual inoculum, although a low-level abortive infection cannot be excluded.

To compare the efficiency of rsCD4 in inhibiting MDM and PBL infection, cells were infected with a higher titer of HIV-1 SF162 than that used in the earlier experiment (MOI = 0.1 $TCID_{50}$ per cell) which was preincubated with increasing concentrations of rsCD4 (Fig. 5). All of the concentrations tested inhibited MDM infection with a striking dose-response effect, although none blocked infection completely. At this higher MOI, 20 μg of rsCD4 per ml resulted in inhibition of infection in MDM by approximately 2 orders of magnitude (Fig. 5A), rather than the complete inhibition seen with the lower MOI (Fig. 4A). In contrast, in PBL infected with this higher MOI, 40 μg of rsCD4 per ml resulted in only modest inhibition (Fig. 5B), and although rsCD4 was maintained in the culture, the inhibitory effect was gradually overcome. This reduction in the efficiency of rsCD4 blocking at higher virus input was seen reproducibly with both MDM and PBL.

MAb inhibition of HIV-1 infection of MDM. We then

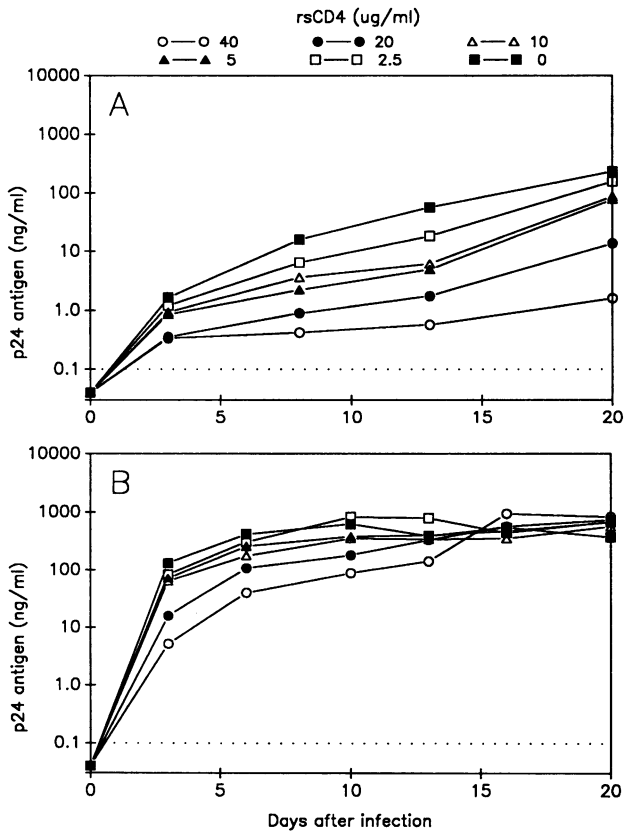

FIG. 5. Dose-response effect of rsCD4 on HIV-1 infection of MDM (A) and PBL (B). HIV-1 strain SF162 (approximately 2×10^4 $TCID_{50}$s) was incubated for 30 min at 37°C with various concentrations of rsCD4 and then incubated for 90 min with 7-day-old MDM cultures or 4-day-old PBL cultures (MOI = 0.1 $TCID_{50}$ per cell). After the cultures were washed and refed, rsCD4 was replaced at the same concentration and the cultures were maintained as described in Materials and Methods. The cultures were tested periodically for p24 antigen production, and MDM values represent the means of results from duplicate wells.

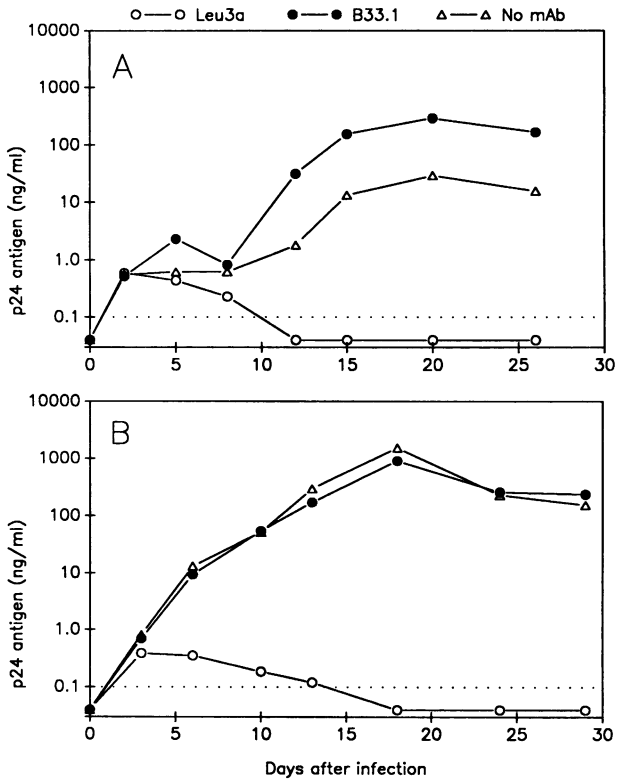

FIG. 6. Effect of MAbs on HIV-1 infection of MDM (A) and PBL (B). MDM cultured for 7 days and PBL cultured for 4 days were incubated with MAb Leu3a or B33.1 at 10 μg/ml or without antibody for 30 min at 37°C and then incubated with HIV-1 strain SF162 (approximately 10^3 $TCID_{50}$s) for 90 min (MOI = 0.005 $TCID_{50}$ per cell). After the cultures were washed and refed, MAb was replaced and the cultures were maintained as described in Materials and Methods. The cultures were tested periodically for p24 antigen, and MDM values represent the means of results from triplicate wells.

examined the effect of the anti-CD4 MAb Leu3a, which is known to block HIV-1 infection of T lymphocytes. As a control we used MAb B33.1, which is directed against a nonpolymorphic determinant of HLA class II (DR) that is expressed on both macrophages and stimulated T lymphocytes (results not shown). Leu3a at 10 μg/ml completely prevented infection of both MDM and PBL by HIV-1 strain SF162 (Fig. 6), while MAb B33.1 used at the same concentration did not block infection. Similar blocking was seen with the anti-CD4 MAb OKT4A, while various other non-CD4 MAbs failed to inhibit infection (data not shown). p24 antigen levels in MDM cell lysates showed a pattern similar to that of levels in supernatants (data not shown).

Inhibition of other HIV-1 isolates. To determine whether these effects of rsCD4 and anti-CD4 MAbs apply in general to macrophage-tropic strains, we examined the effect of rsCD4 and MAbs on MDM infection with two other HIV-1 isolates. HIV-1 89.6 is a low-passage strain isolated in our laboratory which replicates well in MDM and was used at the same MOI as SF162 (0.005 $TCID_{50}$ per cell). HIV-1 DV has intermediate macrophage-tropic characteristics, requiring a higher inoculum to achieve production macrophage infection (8, 11), and was therefore used at a higher MOI (0.1 $TCID_{50}$ per cell). Twenty micrograms of rsCD4 per milliliter inhibited infection of MDM by both strains, although strain DV, inoculated at a 20-fold higher MOI, was blocked less completely (Fig. 7). In addition, MAb Leu3a used at 10 μg/ml completely blocked MDM infection with both isolates (data not shown).

Effect of rsCD4 and MAbs on cytopathic changes of HIV-1 infection. When infected with macrophage-tropic strains of HIV-1 in vitro, MDM undergo varying degrees of cell fusion resulting in the development of large syncytia (8). Consistent with the effects on viral replication, both rsCD4 and MAb Leu3a prevented the cytopathic effects of all three HIV-1 strains (Fig. 8).

DISCUSSION

We have shown that MDM express membrane-associated CD4 and that both rsCD4 and anti-CD4 MAbs block infection of MDM by three macrophage-tropic HIV-1 strains. These results demonstrate the importance of CD4 as the entry pathway for macrophage infection. Furthermore, our results confirm the potential usefulness of soluble CD4 (6, 13, 16, 24, 27, 44, 45, 50, 53) or CD4-derived molecules (2, 51) as a therapeutic strategy for AIDS, particularly in aspects of disease pathogenesis in which the macrophage is thought to play an important role.

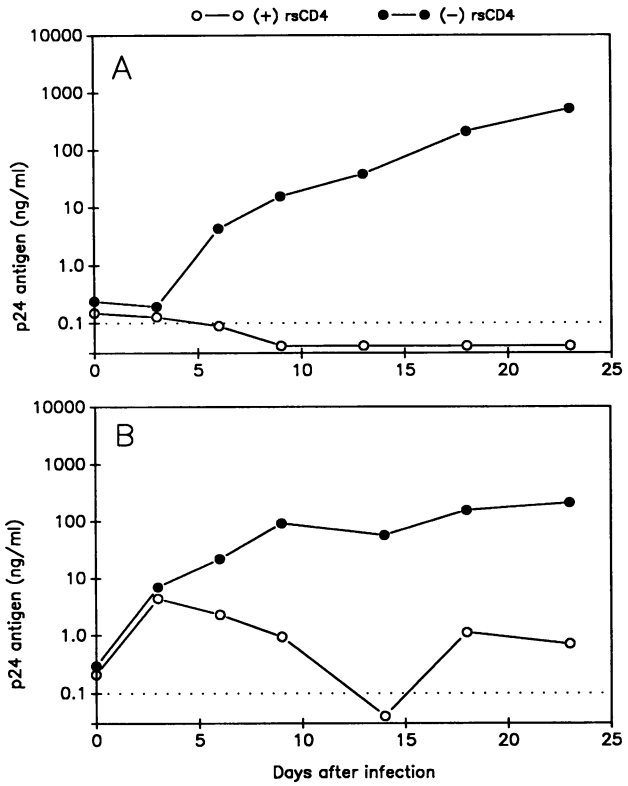

FIG. 7. Effect of rsCD4 on MDM infection by HIV-1 strains 89.6 (A) and DV (B). Strain 89.6 (approximately 1×10^3 $TCID_{50}$s) and strain DV (approximately 2×10^4 $TCID_{50}$s) were incubated for 30 min at 37°C with 20 μg of rsCD4 per ml. They were then incubated for 90 min with 7-day-old cultures of MDM (strain 89.6 MOI = 0.005 $TCID_{50}$ per cell; strain DV MOI = 0.1 $TCID_{50}$ per cell). After the cultures were washed and refed, rsCD4 was replaced at the same concentration and the cultures were maintained as described in Materials and Methods. Cultures were tested periodically for p24 antigen production, and values represent the means of results from triplicate wells.

We found that cultured MDM contain CD4 in quantities that are readily detected by Western blotting and by fluorescence microscopy. Our findings confirm prior studies which have demonstrated CD4 expression by monocytes or MDM (10, 11, 28, 54) and show that the protein both is present at the cell membrane and is the same size as CD4 in PBL. Our results differ from those of studies which have failed to find surface CD4 expression on MDM at the time of infection with HIV (19, 28). This is probably because our approach of microscopic immunofluorescence, unlike flow cytometry, can detect surface receptor expression even with the high background of autofluorescence which cultured macrophages develop (8, 28) and because our polyclonal anti-CD4 antiserum is more sensitive than MAb.

Previous studies have reported that CD4 is the main pathway for HIV infection of the promonocytoid U937 cell line (1, 6, 7, 21, 38), which has been used as a surrogate for primary monocytes and macrophages in studies of tropism. However, in contrast to primary cells, U937 cells express very high levels of CD4, and they do not resemble primary macrophages in their pattern of permissiveness for HIV-1 isolates (8). In recent studies which have examined the role of CD4 in macrophage infection, Homsy et al. (23) reported that MAb Leu3a blocked MDM infection, whereas Kazazi et al. (28) found that Leu3a inhibited infection of monocytes but not of 5-day-old MDM. Capon et al. (2) reported that rsCD4 and CD4 immunoadhesions inhibited infection of fresh monocytes with one macrophage-tropic HIV-1 isolate. Using macrophages treated with DEAE-dextran to enhance virus uptake, Harbison et al. (21) showed that rsCD4 inhibited infection by HIV-1 IIIB, a strain which replicates poorly in macrophages (8). We have extended these studies by using both rsCD4 and monoclonal antibodies, examining several HIV-1 isolates, infecting cells in the absence of polycation enhancers such as Polybrene or DEAE-dextran (33), varying the experimental conditions of blocking, and comparing the efficiencies of rsCD4 blocking in MDM and PBL. Our results demonstrate the importance of CD4 in primary macrophage infection by macrophage-tropic HIV-1 isolates.

In our experiments, lower concentrations of rsCD4 inhibited macrophage infection than were needed to block infection of lymphocytes. This could be due to the lower level of CD4 expression on macrophages, or it could reflect the production of lower levels of infectious virus by macrophages (8). We also found that the concentration of rsCD4 required to block infection of both MDM and PBL depended greatly on the amount of the virus challenge (MOI). Previous studies have shown inhibition of T-cell infection with lower concentrations of rsCD4 than we required. This may be because those studies used lower inocula (16, 45), HIV isolates which might be more sensitive to rsCD4 inhibition (6), or vesicular stomatitis virus pseudotypes, which do not lead to reinfection and amplification in culture (6). It is also possible that virus stocks contain different ratios of particles to infectious units or different amounts of free gp120, which could affect the efficiency of rsCD4 blocking. The levels of rsCD4 used in our blocking experiments are higher than those achieved in vivo in preliminary pharmacokinetic studies (27, 44). However, it is difficult to extrapolate the experimental conditions of infection (an inoculum of 1×10^3 to 2×10^4 $TCID_{50}$s) to the in vivo situation.

Previously, we and others have shown that HIV-1 strains vary in their tropism for macrophages (5, 8, 17, 19, 31), and the determinants responsible for this tropism are unknown. These results suggest that the ability of certain HIV-1 strains to infect macrophages does not result from the use of another receptor instead of CD4, since all three macrophage-tropic strains tested exhibited similar dependence on CD4. However, it is still possible that macrophage tropism results from the use of an accessory macrophage-specific receptor in addition to CD4. Recently Cordonnier et al. (9) showed that infection of U937 cells may require gp120-cell interactions beyond those needed for gp120-CD4 binding, since mutations in gp120 could eliminate infectivity for U937 cells without abolishing CD4 binding or infectivity for lymphoid cell lines. Whether macrophage tropism is determined by similar binding or entry effects, other early postentry events, or at the level of virus expression is as yet unknown.

It is important to note that our results do not exclude the possibility that under special circumstances macrophages could be infected by other pathways as well, such as antibody- or antibody- and complement-mediated entry via Fc or complement receptors, either in conjunction with or independent from CD4 (23, 26, 42, 48).

Because HIV strains vary widely in their genetic and biologic characteristics, it is necessary to confirm that these observations can be generalized to a variety of isolates. These results apply to three macrophage-tropic strains of

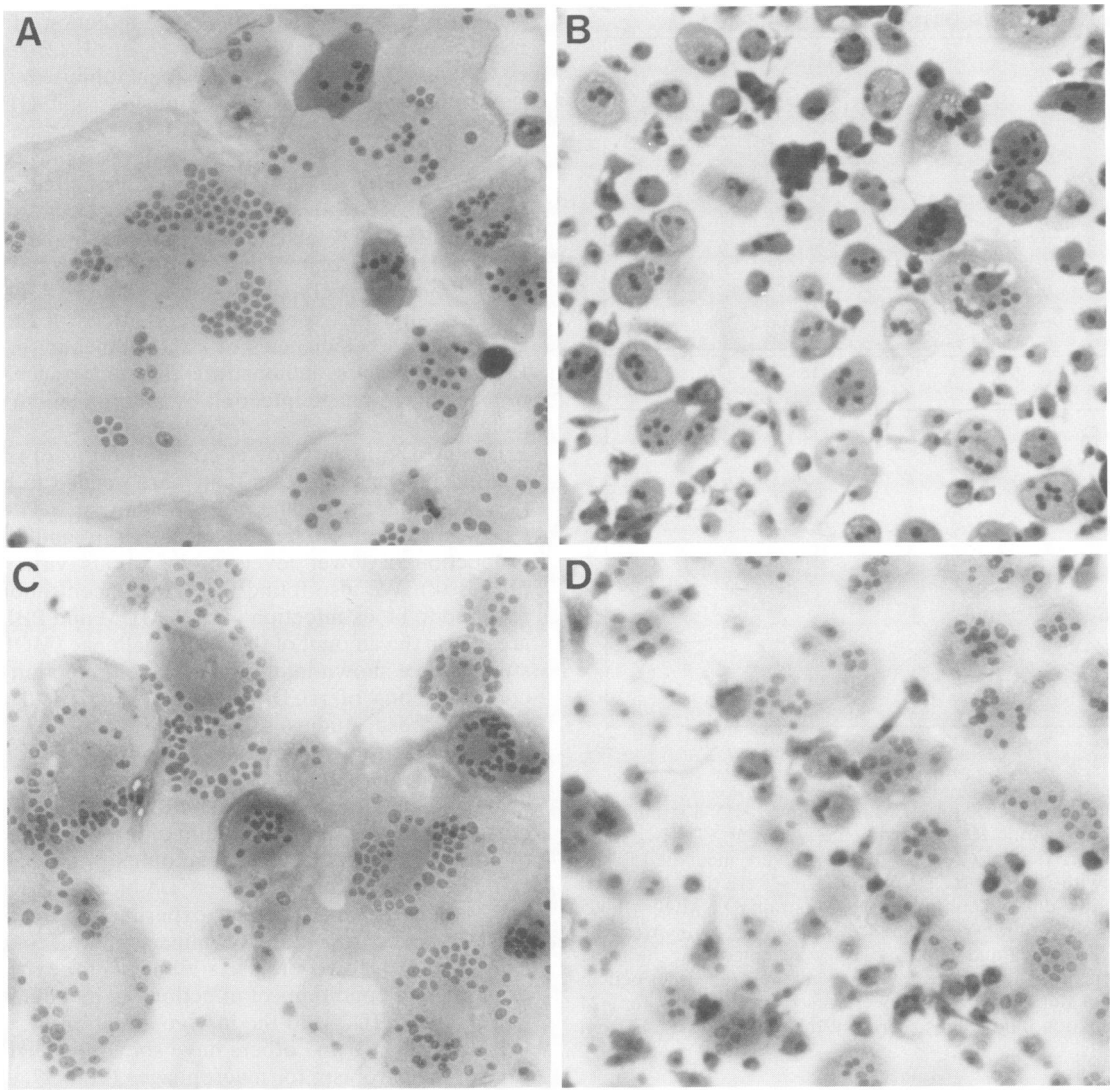

FIG. 8. Effect of rsCD4 and MAbs on cytopathic changes of HIV-1 infection. Seven-day-old cultures of MDM were infected with HIV-1 strain 89.6 (approximately 10^3 $TCID_{50}$s; MOI = 0.005 $TCID_{50}$ per cell), and 24 days later the cultures were fixed and examined by a modified Wright-Giemsa method. (A) MDM were infected in the absence of rsCD4 and MAb. (B) MDM were infected with virus which had been preincubated with 20 μg of rsCD4 per ml for 30 min prior to infection, and then rsCD4 was maintained in the culture medium. (C) MDM were incubated with 10 μg of MAb B33.1 per ml for 30 min prior to infection, and then B33.1 was maintained in the culture medium. (D) MDM were incubated with 10 μg of MAb Leu3a per ml for 30 min prior to infection, and then Leu3a was maintained in the culture medium. Magnification, ×100.

different biological character isolated in different geographic regions and thus most likely reflect a general phenomenon.

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LITERATURE CITED

1. **Asjo, B., I. Ivhed, M. Gidlund, S. Fuerstenberg, E. M. Fenyo, K. Nilsson, and H. Wigzell.** 1987. Susceptibility to infection by the human immunodeficiency virus (HIV) correlates with T4 expression in a parental monocytoid cell line and its subclones. Virology **157**:359–365.
2. **Capon, D. J., S. M. Chamow, J. Mordenti, S. A. Marsters, T. Gregory, H. Mitsuya, R. A. Byrn, C. Lucas, F. M. Wurm, J. E. Groopman, S. Broder, and D. H. Smith.** 1989. Designing CD4 immunoadhesions for AIDS therapy. Nature (London) **337**:525–531.
3. **Chayat, K. J., M. E. Harper, L. M. Marselle, E. B. Lewin, R. M. Rose, J. M. Oleske, L. G. Epstein, F. Wong-Staal, and R. C. Gallo.** 1986. Detection of HTLV-III RNA in lungs of patients with AIDS and pulmonary involvement. J. Am. Med. Assoc. **256**:2356–2359.
4. **Cheng-Mayer, C., and J. A. Levy.** 1988. Distinct biological and serological properties of human immunodeficiency viruses from the brain. Ann. Neurol. **23**(Suppl.):S58–S61.
5. **Cheng-Mayer, C., C. Weiss, S. Seto, and J. A. Levy.** 1989.

Isolates of human immunodeficiency virus type 1 from the brain may constitute a special group of the AIDS virus. Proc. Natl. Acad. Sci. USA **86**:8575–8579.

6. **Clapham, P. R., J. N. Weber, D. Whitby, K. McIntosh, A. G. Dalgleish, P. J. Maddon, K. C. Deen, R. W. Sweet, and R. A. Weiss.** 1989. Soluble CD4 blocks the infectivity of diverse strains of HIV and SIV for T cells and monocytes but not for brain and muscle cells. Nature (London) **337**:368–370.
7. **Clapham, P. R., R. A. Weiss, A. G. Dalgleish, M. Exley, D. Whitby, and N. Hogg.** 1987. Human immunodeficiency virus infection of monocytic and T-lymphocytic cells: receptor modulation and differentiation induced by phorbol ester. Virology **158**:44–51.
8. **Collman, R., N. F. Hassan, R. Walker, B. Godfrey, J. Cutilli, J. C. Hastings, H. Friedman, S. D. Douglas, and N. Nathanson.** 1989. Infection of monocyte-derived macrophages with human immunodeficiency virus type 1: monocyte-tropic and lymphocyte-tropic strains of HIV-1 show distinctive patterns of replication in a panel of cell types. J. Exp. Med. **170**:1149–1163.
9. **Cordonnier, A., L. Montagnier, and M. Emerman.** 1989. Single amino-acid changes in HIV envelope affect viral tropism and receptor binding. Nature (London) **340**:571–574.
10. **Crocker, P. R., W. A. Jefferies, S. J. Clark, L. P. Chung, and S. Gordon.** 1987. Species heterogeneity in macrophage expression of the CD4 antigen. J. Exp. Med. **166**:613–618.
11. **Crowe, S., J. Mills, and M. S. McGrath.** 1987. Quantitative immunocytofluorographic analysis of CD4 surface antigen expression and HIV infection of human peripheral blood monocyte/macrophages. AIDS Res. **3**:135–145.
12. **Dalgleish, A. G., P. C. L. Beverley, P. R. Clapham, D. H. Crawford, M. F. Greaves, and R. A. Weiss.** 1984. The CD4 (T4) antigen is an essential component of the receptor for the AIDS retrovirus. Nature (London) **312**:763–767.
13. **Deen, K. C., J. S. McDougal, R. Inacker, G. Folena-Wasserman, J. Arthos, J. Rosenberg, P. J. Maddon, R. Axel, and R. W. Sweet.** 1988. A soluble form of CD4 (T4) protein inhibits AIDS virus infection. Nature (London) **331**:82–84.
14. **Doyle, C., and J. L. Strominger.** 1987. Interaction between CD4 and class II MHC molecules mediates cell adhesion. Nature (London) **330**:256–259.
15. **Fazakerly, J. K., F. Gonzalez-Scarano, J. Strickler, B. Dietzschold, F. Karush, and N. Nathanson.** 1988. Organization of the middle RNA segment of snowshoe hare bunyavirus. Virology **197**:422–432.
16. **Fisher, R. A., J. M. Bertonis, W. Meier, V. A. Johnson, D. S. Costopoulos, T. Liu, R. Tizard, B. D. Walker, M. S. Hirsch, R. T. Schooley, and R. A. Flavell.** 1988. HIV infection is blocked in vitro by recombinant soluble CD4. Nature (London) **331**:76–78.
17. **Gartner, S., P. Markovits, D. M. Markovitz, M. H. Kaplan, R. C. Gallo, and M. Popovic.** 1986. The role of mononuclear phagocytes in HTLV-III/LAV infection. Science **233**:215–219.
18. **Gendelman, H. E., J. M. Orenstein, L. M. Baca, B. Weiser, H. Burger, D. C. Kalter, and M. S. Meltzer.** 1989. The macrophage in the persistence and pathogenesis of HIV infection. AIDS **3**:475–495.
19. **Gendelman, H. E., J. M. Orenstein, M. A. Martin, C. Ferrua, R. Mitra, T. Phipps, L. A. Wahl, H. C. Lane, A. S. Fauci, D. S. Burke, D. Skillman, and M. S. Meltzer.** 1988. Efficient isolation and propagation of human immunodeficiency virus on recombinant colony-stimulating factor-1 treated monocytes. J. Exp. Med. **167**:1428–1441.
20. **Haase, A. T.** 1986. Pathogenesis of lentivirus infections. Nature (London) **322**:130–136.
21. **Harbison, M. A., J. M. Gillis, P. Pinkston, R. A. Byrn, R. M. Rose, and S. M. Hammer.** 1990. Effects of recombinant soluble CD4 (rsCD4) on HIV-1 infection of monocyte/macrophages. J. Infect. Dis. **161**:1–6.
22. **Harouse, J. M., C. Kunsch, H. T. Hartle, M. A. Laughlin, J. A. Hoxie, B. Wigdahl, and F. Gonzalez-Scarano.** 1989. CD4-independent infection of human neural cells by human immunodeficiency virus type 1. J. Virol. **63**:2527–2533.
23. **Homsy, J., M. Meyer, M. Tateno, S. Clarkson, and J. A. Levy.** 1989. The Fc and not CD4 receptor mediates antibody enhancement of HIV infection in human cells. Science **244**:1357–1360.
24. **Hussey, R. E., N. E. Richardson, M. Kowalski, N. R. Brown, H. C. Chang, R. F. Siliciano, T. Dorfman, B. Walker, J. Sodroski, and E. L. Reinherz.** 1988. A soluble CD4 protein selectively inhibits HIV replication and syncytium formation. Nature (London) **331**:78–81.
25. **Jackson, J. B., R. W. Coombs, K. Sannerud, F. S. Rhame, and H. H. Balfour.** 1988. Rapid and sensitive viral culture method for human immunodeficiency virus type 1. J. Clin. Microbiol. **26**:1416–1418.
26. **Jouault, T., F. Chapuis, R. Oliver, C. Parravicini, E. Bahraoui, and J. C. Gluckman.** 1989. HIV infection of monocytic cells: role of antibody-mediated virus binding to Fc-gamma receptors. AIDS **3**:125–133.
27. **Kahn, J. O., J. D. Allan, T. L. Hodges, L. D. Kaplan, C. J. Arri, H. F. Fitch, A. E. Izu, J. Mordenti, S. A. Sherwin, J. E. Groopman, and P. Volberding.** 1990. The safety and pharmacokinetics of recombinant soluble CD4 (rCD4) in subjects with the acquired immunodeficiency syndrome (AIDS) and AIDS-related complex. Ann. Intern. Med. **112**:254–261.
28. **Kazazi, F., J. M. Mathijs, P. Foley, and A. L. Cunningham.** 1989. Variations in CD4 expression by human monocytes and macrophages and their relationship to infection with the human immunodeficiency virus. J. Gen. Virol. **70**:2661–2672.
29. **Klatzmann, K., E. Champagne, S. Chamaret, J. Gruest, D. Guetard, T. Hercent, J. Gluckman, and L. Montagnier.** 1984. T-lymphocyte T4 molecule behaves as the receptor for human retrovirus LAV. Nature (London) **312**:767–768.
30. **Koenig, S., H. E. Gendelman, J. M. Orenstein, M. C. Dal Canto, G. H. Pezeshkpour, M. Yungbluth, F. Janotta, A. Aksamit, M. A. Martin, and A. S. Fauci.** 1986. Detection of AIDS virus in macrophages in brain tissue from AIDS patients with encephalopathy. Science **233**:1089–1093.
31. **Koyanagi, Y., S. Miles, R. T. Mitsuyasu, J. E. Merrill, H. V. Vinters, and I. S. Y. Chen.** 1987. Dual infection of the central nervous system by AIDS viruses with distinct cellular tropisms. Science **236**:819–822.
32. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
33. **Manning, J. S., A. J. Hackett, and N. B. Darby, Jr.** 1971. Effect of polycations on sensitivity of BALB/3T3 cells to murine leukemia and sarcoma virus infectivity. Appl. Microbiol. **22**: 1162–1163.
34. **McDougal, J. S., M. S. Kennedy, J. M. Sligh, S. P. Cort, A. Mawle, and J. K. A. Nicholson.** 1986. Binding of HTLV-III/LAV to T4+ T cells by a complex of the 110K viral protein and the T4 molecule. Science **231**:382–385.
35. **McDougal, J. S., A. Mawle, S. P. Cort, J. A. Nicholson, G. D. Cross, J. A. Scheppler-Campbell, D. Hicks, and J. Sligh.** 1985. Cellular tropism of the human retrovirus HTLV-III/LAV. J. Immunol. **135**:3151–3162.
36. **Narayan, O., M. C. Zink, D. Huso, D. Sheffer, S. Crane, S. Kennedy-Stoskopf, P. E. Jolly, and J. E. Clements.** 1988. Lentiviruses of animals are biological models of the human immunodeficiency viruses. Microb. Pathog. **5**:149–157.
37. **Pauza, C. D.** 1988. HIV persistence in monocytes leads to pathogenesis and AIDS. Cell. Immunol. **112**:414–424.
38. **Pauza, C. D., and T. M. Price.** 1989. Human immunodeficiency virus infection of T cells and monocytes proceeds via receptor-mediated endocytosis. J. Cell Biol. **107**:959–968.
39. **Perussia, B., G. Trichieri, D. Lebman, J. Jankiewicz, B. Lange, and G. Rovera.** 1982. Monoclonal antibodies that detect differentiation surface antigens on human myelomonocytic cells. Blood **59**:382–392.
40. **Plata, F., B. Autran, L. P. Martins, S. Wain-Hobson, M. Raphael, C. Mayaud, M. Denis, J. M. Guillon, and P. Debre.** 1987. AIDS virus-specific cytotoxic T lymphocytes in lung disorders. Nature (London) **328**:348–351.
41. **Pomerantz, R. J., S. M. Monte, P. Donegan, T. R. Rota, M. W. Vogt, D. E. Craven, and M. S. Hirsch.** 1988. Human immunodeficiency virus (HIV) infection of the uterine cervix. Ann.

Intern. Med. **108**:321–327.
42. **Robinson, W. E., D. C. Montefiori, and W. M. Mitchell.** 1988. Antibody-dependent enhancement of human immunodeficiency virus type 1 infection. Lancet **i**:790–794.
43. **Sattentau, Q. J., A. G. Dalgleish, R. A. Weiss, and P. C. L. Beverley.** 1986. Epitopes of the CD4 antigen and HIV infection. Science **234**:1120–1123.
44. **Schooley, R. T., T. C. Merigan, P. Gaut, M. S. Hirsch, M. Holodniy, T. Flynn, S. Liu, R. E. Byington, S. Henochowicz, E. Gubish, D. Spriggs, D. Kufe, J. Schindler, A. Dawson, D. Thomas, D. G. Hanson, B. Letwin, T. Liu, J. Gulinello, S. Kennedy, R. Fisher, and D. Ho.** 1990. Recombinant soluble CD4 therapy in patients with the acquired immunodeficiency syndrome (AIDS) and AIDS-related complex. Ann. Intern. Med. **112**:247–253.
45. **Smith, D. H., R. A. Byrn, S. A. Marsters, T. Gregory, J. E. Groopman, and D. J. Capon.** 1987. Blocking of HIV-1 infectivity by a soluble, secreted form of the CD4 antigen. Science **238**: 1704–1707.
46. **Smith, S. D., M. Shatsky, P. S. Cohen, R. Warnke, M. P. Link, and B. E. Glader.** 1984. Monoclonal antibody and enzymatic profiles of human malignant T-lymphoid cells and derived cell lines. Cancer Res. **44**:5657–5660.
47. **Stoler, M. H., T. A. Eskin, S. Benn, R. Angerer, and L. M. Angerer.** 1986. Human T-cell lymphotropic virus type III infection of the central nervous system. J. Am. Med. Assoc. **256:** 2360–2364.
48. **Takeda, A., C. U. Tuazon, and F. A. Ennis.** 1988. Antibody-enhanced infection by HIV-1 via Fc receptor-mediated entry. Science **242**:580–583.
49. **Tateno, M., F. Gonzalez-Scarano, and J. A. Levy.** 1989. Human immunodeficiency virus can infect CD4-negative human fibroblastoid cells. Proc. Natl. Acad. Sci. USA **86**:4287–4290.
50. **Traunecker, A., W. Luke, and K. Karjalainen.** 1988. Soluble CD4 molecules neutralize human immunodeficiency virus type 1. Nature (London) **331**:84–86.
51. **Traunecker, A., J. Schneider, H. Kiefer, and K. Karjalainen.** 1989. Highly efficient neutralization of HIV with recombinant CD4-immunoglobulin molecules. Nature (London) **339**:68–70.
52. **Tschaler, E., V. Groh, M. Popovic, D. L. Mann, K. Konrad, B. Safi, L. Eron, F. diMarzo Veronese, K. Wolff, and G. Stingl.** 1987. Epidermal Langerhans cells—a target for HTLV-III/LAV infection. J. Invest. Dermatol. **88**:233–237.
53. **Watanabe, M., K. A. Reimann, P. A. DeLong, T. Liu, R. A. Fisher, and N. L. Letvin.** 1989. Effect of recombinant soluble CD4 in rhesus monkeys infected with simian immunodeficiency virus of macaques. Nature (London) **337**:267–270.
54. **Wood, G. S., N. L. Warner, and R. A. Warnke.** 1983. Anti-Leu-3/T4 antibodies react with cells of monocyte/macrophage and Langerhans lineage. J. Immunol. **131**:212–216.