Limited expression of R5-tropic HIV-1 in CCR5-positive type 1–polarized T cells explained by their ability to produce RANTES, MIP-1α, and MIP-1β

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Human T helper (Th) cells (Th1- or Th2-oriented memory T cells as well as Th1- or Th2-polarized naive T cells) were infected in vitro with an R5-tropic HIV-1 strain (BaL) and assessed for their profile of cytokine production, CCR5 receptor expression, and HIV-1 p24 antigen (p24 Ag) production. Higher p24 Ag production was found in CCR5-negative Th2-like memory T cells than in CCR5-positive Th1-like memory T cells. By contrast, p24 Ag production was higher in Th1-polarized activated naive T cells in the first 4 days after infection. However, p24 Ag production in Th1-polarized T cells became comparable or even lower than the production in Th2-polarized populations later in infection or when the cells were infected with HIV-1BaL after secondary stimulation. The higher levels of p24 Ag production by Th1-polarized naive T cells soon after infection reflected a higher virus entry, as assessed by the single round infection assay using the HIV–chloramphenicol acetyl transferase (HIV-CAT) R5-tropic virus that contains the envelope protein of HIV-1 YU2 strain. The limitation of viral spread in the Th1-polarized populations, despite the initial higher level of T-cell entry of R5-tropic strains, was due to the ability of Th1 cells to produce greater amounts of β-chemokines than Th2 cells. In fact, an inverse correlation was observed between Th1-polarized naive T cells and Th1-like memory-activated T cells in regards to p24 Ag production and the release of the following CCR5-binding chemokines: regulated on activation normal T expressed and secreted (RANTES), macrophage inflammatory protein–1α (MIP-1α), and MIP-1β. Moreover, infection with the HIV-1BaL strain of Th1-polarized T cells in the presence of a mixture of anti-RANTES, anti–MIP-1α, and anti–MIP-1β neutralizing antibodies resulted in a significant increase of HIV-1 expression. These findings suggest that Th1-type responses may favor CD4+ T-cell infection by R5-tropic HIV-1 strains, but HIV-1 spread in Th1 cells is limited by their ability to produce CCR5-binding chemokines. (Blood. 2000;95:1167-1174)

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Introduction

HIV-1 isolates exhibit marked differences in their ability to infect $CD4^+$ T cells. While all strains infect primary $CD4^+$ T cells, most primary isolates also infect macrophages (M tropic) but fail to infect transformed $CD4^+$ T cell lines.[1,2] Other isolates replicate well in $CD4^+$ T cell lines (T tropic) but fail to infect macrophages.[3-5] The underlying source of permissiveness for M and T tropic viruses has recently been recognized. In order for HIV-1 to infect lymphocytes or macrophages, one or more cofactors are required in conjunction with the CD4 molecule for virus/cell fusion to occur. CCR5 is a 7-transmembrane receptor for the following β-chemokines: regulated on activation normal T expressed and secreted (RANTES), macrophage inflammatory protein–1α (MIP-1α), and MIP-1β. CCR5 confers susceptibility to infection by certain M-tropic (R5-tropic) strains of HIV-1, whereas CXCR4, the 7-transmembrane receptor for the α-chemokine stromal cell–derived factor–1 (SDF-1), has been shown to serve as a cofactor for T tropic (X4-tropic) HIV-1 strains.[6-8]

Human $CD4^+$ T helper (Th) cells are heterogeneous in their cytokine production profile, but under certain conditions $CD4^+$ Th-cell–mediated immune responses can polarize into opposite pathways, which have been defined as type 1 (Th1) or type 2 (Th2). Th1 cells produce interferon-γ (IFN-γ) but not interleukin-4 (IL-4), whereas Th2 cells produce IL-4 as well as IL-5, IL-10, and IL-13 but not IFN-γ.[9] Interestingly, CCR5 has been shown to be preferentially expressed in Th1 cells,[10,11] whereas CXCR4, although constitutively present on the majority of naive T cells, is up-regulated by IL-4[12,13] and down-regulated by IFN-γ.[13] Recently we and others have shown that at least in vitro, IL-4 also favors the entry of X4-tropic HIV-1 strains in $CD4^+$ T cells.[13-15] Thus, the occurrence of Th2 responses may provide a selective pressure for the emergence of X4-tropic highly aggressive HIV-1 strains and consequently for a more rapid progression toward full-blown disease.[13-18]

In this study, the effect of in vitro infection of Th1-like or Th2-like $CD4^+$ T cells with an R5-tropic HIV-1 strain (BaL) was investigated. Surprisingly, comparable or even higher HIV-1 24 antigen (p24 Ag) production was found in Th2-p than in Th1-polarized populations infected with the same R5-tropic HIV-1 strain, despite the higher or even selective CCR5 expression in Th1 cells. This apparent paradox was explained by the observation that following activation, both Th1-like memory T cells and Th1-polarized activated naive T cells showed significantly higher

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Submitted April 1, 1999; accepted October 19, 1999.

Supported by grants from the Ministero delta Sanità (AIDS Project, 98), MPI, and Associazione Italiana Ricerca sul Cancro (AIRC).

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production of β-chemokines RANTES, MIP-1α, and MIP-1β than the corresponding Th2-like memory or naive T cells from the same donors. More importantly, infection of Th1-polarized T cells with R5-tropic HIV-1 strain in the presence of a mixture of anti-RANTES, anti–MIP-1α, and anti–MIP-1β antibodies resulted in a significant increase of p24 Ag production in Th1-polarized T cells. These findings suggest that Th1 responses may favor higher $CD4^+$ T-cell entry of R5-tropic strains, but HIV-1 spread among Th1 cells is limited by their ability to produce CCR5-binding chemokines.

Materials and methods

Subjects

Peripheral blood (PB) samples used in this study were obtained from informed atopic adult volunteers sensitive to *Dermatophagoides pteronyssinus* group 1 (Der p 1) and from umbilical cord blood (UCB) of newborns in accordance with the ethical standards of the responsible regional committee on human experimentation.

Reagents

The medium used throughout was RPMI 1640 (Seromed, Berlin, Germany) supplemented with 2 mmol/L L-glutamine, 1% nonessential amino acids, 1% pyruvate, 2×10^{-5} mol/L 2-mercaptoethanol (all from Gibco Life Technologies, Grand Island, NY), and 100 µg/mL kanamycin and 10 µg/mL gentamycin (Sigma, St Louis, MO). Other materials used included streptokinase (SK) Ag (Behring, L'Aquila, Italy); Der p 1 allergen (gift of Lofarma SpA, Milano, Italy); phytohemagglutinin (PHA) (Gibco, Milan, Italy); PMA, ionomycin, brefeldin A, and saponin (Sigma); human recombinant IL-2 (gift of Eurocetus, Milan, Italy); human recombinant IL-4 and IL-12 (R & D Systems, Minneapolis, MN); purified anti-CD8, anti-CD14, anti-CD20, anti-CD56, and anti-CD45R0 monoclonal antibodies (mAbs) (Becton Dickinson, San Jose, CA); antiglycophorin A and B mAbs (Sigma); and goat antimouse immunoglobulin G (IgG) conjugated with magnetic beads (Miltenyi Biotec, Bisley, Germany).

Clone names and isotypes neutralizing are noted in parentheses for the following materials that were used: neutralizing anti–MIP-1α, anti–MIP-1β (24 006.111, IgG2b), and anti-RANTES mAb (21445.1 IgG1) mAb (R & D Systems); phycoerythrin-conjugated (PE-conjugated) anti–IL-4 (3010.211, IgG1); fluorescein isothiocyanate–conjugated anti–interferon-γ (FITC-conjugated anti–INF-γ) (25 723.11, IgG2b); peridinin chlorophyll protein (Per CP)–conjugated anti-CD8 (SK1, IgG1) mAb, allophycocyanin–conjugated (APC-conjugated) anti-CD3 (SK7, IgG1) mAb, and APC-streptavidin (Becton Dickinson, San Jose, CA); biotin-conjugated anti–IL-4 (4D9, IgG1) mAb (gift from C. Heusser, Novartis, Basel, Switzerland); purified and fluorochrome-conjugated isotype control mAbs (Southern Biotechnology Associates, Birmingham, AL); PE–anti-CXCR4 (12G5, IgG2a), FITC–anti-CCR5 (2D7, IgG2a), and PE–anti–MIP-1α (11A3, IgG2a) mAbs (PharMingen, San Diego, CA); RANTES, MIP-1α and MIP-1β enzyme-linked immunosorbent assay (ELISA) kits (R & D Systems); and p24 Ag ELISA kit (HIVAG-1 monoclonal) (Abbott, Wiesbaden-Delkenheim, Germany).

Generation of short-term Ag-specific T cell lines from adult PB lymphocytes

Ag-specific $CD4^+$ T cell lines were generated as previously described.[13,19] Briefly, mononuclear cell (MNC) suspensions were obtained from PB of 8 atopic Der p 1–sensitive donors by centrifugation on Ficoll-Hypaque gradient and stimulated in RPMI medium containing 5% autologous serum in the presence of SK (100 units/mL) or Der p 1 (5 µg/mL) for 5 days. On day 5, activated T cells were expanded in the presence of recombinant IL-2 (20 units/mL).

Generation of Th1- and Th2-oriented lines from UCB lymphocytes

Polyclonal $CD4^+$ T cell lines were generated from UCB MNC suspensions of 12 newborns, as previously described.[13,19] Briefly, $CD4^+$ $CD45RA^+$ T cells were purified by negative magnetic selection using magnet-activated cell sorting (MACS) following a 2-step incubation with a mixture of anti-CD8, anti-CD14, anti-CD20, anti-CD56, anti-CD45R0, and anti-glycophorin A and B mAbs. This process was followed by incubation with conjugated goat antimouse IgG with magnetic beads. Recovered cells (more than 99% $CD3^+$ $CD4^+$ $CD45RA^+$) were then stimulated with 0.1% vol:vol PHA and 20 units/mL IL-2 in the absence or presence of 100 units/mL IL-4 or IL-12 in RPMI medium containing 10% heat-inactivated fetal calf serum (FCS) (primary stimulation). In some experiments, IL-12– or IL-4–conditioned naive $CD4^+$ T cells were subjected to a second round of stimulation by PHA/IL-2 in the presence of the same cytokine by which they had been previously conditioned (secondary stimulation).

Intracytofluorimetric analysis of cytokine production

Intracytofluorimetric analysis of IFN-γ, IL-4, and MIP-1α synthesis at single-cell level was performed as described.[13,19] Briefly, T-cell blasts were stimulated with 10 ng/mL PMA plus 1 µmol/L ionomycin for 4 hours; in the last 2 hours, the cells were stimulated in the presence of 5 µg/mL brefeldin A. After incubation, cells were washed twice with PBS (pH 7.2), fixed 15 minutes with formaldehyde (2% in PBS; pH 7.2), washed twice with 0.5% BSA in PBS (pH 7.2), permeabilized with PBS (pH 7.2) containing 0.5% BSA and 0.5% saponin, and then incubated with the specific mAb. Incubation with the biotin-labeled anti–IL-4 mAb was followed by a second incubation with APC-streptavidin. Cells were then analyzed on a fluorescence-activated cell sorter (FACSCalibur, CellQuest software; Becton Dickinson). The area of positivity was determined using an isotype-matched mAb. In all cytofluorimetric analyses, a total of 10^4 events for each sample was acquired.

Flow cytometric analysis of surface molecules

Detection of CXCR4 and CCR5 on the surface of T cells was performed by flow cytometry, as described.[13,19] Briefly, after saturation of nonspecific binding sites with total rabbit IgG, cells were incubated for 20 minutes at 4°C with specific or isotype control mAbs. Finally, cells were washed and analyzed (FACSCalibur, CellQuest; Becton Dickinson). In all cytofluorimetric analyses, a total of 10^4 events for each sample was acquired.

Generation of HIV-1 viral stocks

HIV-1BaL stocks were generated from supernatants of human monocyte-derived macrophages (MDM) and infected with a low-passage seed stock of the BaL virus. Briefly, MDM were obtained by plating human PBL in complete medium. After 7 days in culture, nonadherent cells were removed, while adherent cells were incubated overnight with HIV-1BaL at 37°C. After removing unbound virus, fresh medium was added, replaced every 2-3 days, and assayed for p24 Ag content. P24 Ag-positive supernatants were titrated by limiting dilution on PHA/IL-2–activated human PBMC.[20] HIV-1IIIB stocks were generated from supernatant of the H9 IIIB infected T cell line, and titrated by limiting dilution in the syncytia formation assay using the C8166 T-cell line, as previously described.[13]

In vitro infection with HIV-1IIIB and HIV-1BaL

Infection of Ag-specific short-term T cell lines or PHA-activated naive T cells with HIV-1IIIB or BaL strain was performed as previously described.[13] Briefly, on day 15, SK-specific or Der p 1–specific short-term T cell lines (more than 99% $CD3^+$ $CD4^+$) were incubated with either the HIV-1IIIB or HIV-1BaL strain for 2 hours at 37°C, then extensively washed to remove unbound virus, and plated at the final density of 10^6 cells per well in complete medium in which 20 units/mL IL-2 were added. The multiplicity of infection (MOI) used for the HIV-1IIIB strain was 1 virion to 1 cell (as determined on the C8166 T cell line),[13] while the MOI used for the HIV-1BaL strain was 1 virion to 10^4 cells, as determined on PHA/IL-2

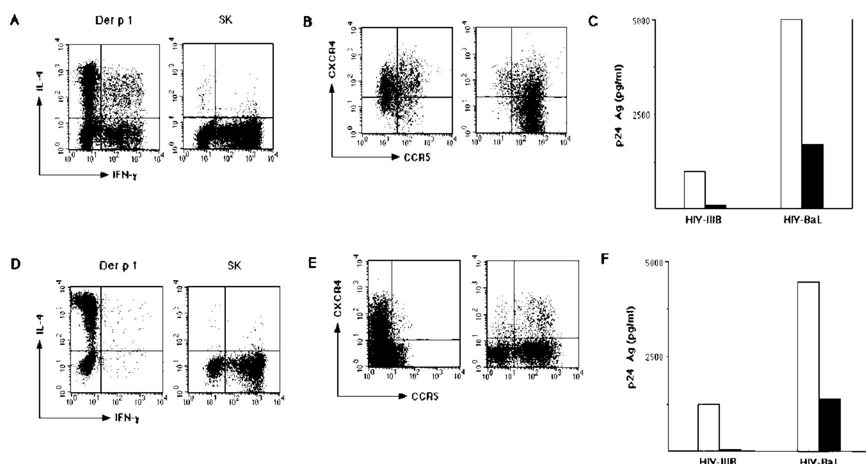

Figure 1. Higher expression of both X4-tropic and R5-tropic HIV-1 strains in Der p 1– and SK-specific short-term $CD4^+$ T cell lines. Intracytofluorimetric analysis of IL-4 and IFN-γ production (A and D) and cytofluorimetric detection of CXCR4 and CCR5 surface expression (B and E) by Der p 1– and SK-specific $CD4^+$ T cells from 2 different atopic donors, examined on day 15 of culture, as described in "Materials and Methods." Spontaneous release of p24 Ag on day 21 (day 6 after infection with the X4–HIV-1IIIB or R5-HIV-1BaL strains) by Der p 1–specific (open bars) and SK-specific (black bars) CD4+ T cells (C and F).

activated human PBMC.[20] After 6 days in culture, cell-free supernatants were assayed for p24 Ag as well as for MIP-1α, MIP-1β, and RANTES content. Three days after primary or secondary stimulation, IL-12– or IL-4–conditioned naive $CD4^+$ T cells were incubated with either the HIV-1IIIB or HIV-1BaL strain, as described above, and plated at the final density of 10^6 cells per well in complete medium in which 20 units/mL IL-2 were added. After 2, 4, and 6 days, cell-free culture supernatants were assayed for p24 Ag, RANTES, MIP-1α, and MIP-1β content.

Single-round infection with R5-tropic and X4-tropic recombinant HIV chloramphenicol acetyl transferase viruses and assays

Recombinant HIV-1 containing different envelope (env) proteins linked to the chloramphenicol acetyl transferase (*CAT*) reporter gene were generated by cotransfection of 10^6 293 cell[20] by the calcium phosphate method with 7 μg HIV-CAT reporter plasmid and 1 μg plasmid encoding the env proteins from a laboratory-adapted X4-tropic isolate (HXBc2) or an R5-tropic primary HIV-1 isolate (YU2). The HIV-CAT reporter plasmid contains an HIV-1 provirus deleted in the *env* gene in which the *nef* gene has been replaced with the *CAT* gene.[21] The transfected 293 cell supernatants containing the recombinant viruses were collected, filtered through 0.45 μm pore-size millipore filters, and assayed for reverse transcriptase (RT) activity. These viruses were then used at equal numbers of RT activity to infect UCB T cells or T cells from SK-specific or Der p 1–specific short-term T cell lines. At day 4 after infection, the cells were lysed, and cell extracts were normalized to total protein content and used for the measurement of CAT activity by thin layer chromatography. CAT activity was then evaluated by the conversion of chloramphenicol in its acetylated forms and expressed as the percentage of conversion.[22]

Cell viability and thymidine incorporation

Cell viability was determined in both naive and memory HIV-1–infected T cells by counting the number of trypan blue negative cells. The proliferation rate was determined by measuring ^{3}H-thymidine uptake. Briefly, at the indicated times, 10^5 viable cells were pulsed with 0.185 MBq (5 μCi/mL) of ^{3}H-thymidine for 16 hours. Cells were then harvested on glass fiber filters, and ^{3}H-thymidine uptake was determined by scintillation counting.

Evaluation of RANTES, MIP-1α, MIP-1β and p24 Ag production

Measurement of RANTES, MIP-1α, MIP-1β, and p24 Ag concentrations in cell-free culture supernatants was performed by appropriate ELISA kits according to manufacturers' instructions.

Statistical analysis

Statistical analysis of the results was performed by the Student *t* test or by linear regression analysis.

Results

Higher levels of HIV-1 p24 Ag production in CCR5-negative Th2-like than in CCR5-positive Th1-like memory T cells infected with an R50-tropic strain

To investigate the effects of the infection of Th1-like and Th2-like $CD4^+$ T cells with an R5-tropic HIV-1 strain, short-term T cell lines specific for SK or Der p 1 were generated from atopic Der p 1–sensitive individuals. The results of 2 representative experiments are shown in Figure 1. Following stimulation, most T-cell blasts from 1 of the 2 Der p 1–specific lines produced IL-4, but not IFN-γ (Th2-like) or both IL-4 and IFN-γ (designed as type 0 Th–like [Th0-like]), and a few of them produced only IFN-γ (Th1-like) (Figure 1A). In the other Der p 1–specific T cell line, only T-cell blasts producing IL-4 but not IFN-γ (Th2-like) were detected (Figure 1D). By contrast, virtually all T-cell blasts from SK-specific lines of both donors produced IFN-γ but not IL-4 (Th1-like) (Figure 1A and D). SK-specific and Der p 1–specific T cells also clearly differed in their expression of CCR5 and CXCR4 molecules; in both cases the great majority of SK-specific T-cell blasts showed CCR5 expression but little or no CXCR4 expression. By contrast, Der p 1–specific T-cell blasts showed high CXCR4 expression, whereas CCR5 expression was lower (Figure 1B and E). As expected, after infection with the X4-tropic HIV-1IIIB strain, Der p 1–specific lines (Th0/Th2-like, high CXCR4 expression) showed significantly higher p24 Ag production than SK-specific lines (Th1-like, low CXCR4 expression). Surprisingly, p24 Ag production was also higher in Der p 1–specific T cell lines than in SK-specific T cell lines infected with HIV-1BaL (Figure 1C and F). Table 1 summarizes the results obtained by measuring p24 Ag levels in 8 separate experiments. Mean values of p24 Ag production were significantly higher in Der p 1–specific T cell lines infected with HIV-1IIIB than in infected SK-specific T cell lines. Likewise, significantly higher p24 Ag production was found in supernatants of Der p1–specific T-cell cultures (Th0/Th2-like, low CCR5 expression) than in those of SK-specific (Th1-like, high CCR5 expression) T-cell cultures infected with HIV-1BaL.

p24 Ag production in Th1- and Th2-polarized naive T cells

The effects of infection with X4-tropic or R5-tropic HIV-1 strains of naive T cells were then investigated. To this end, UCB $CD45RA^+R0^-CD4^+$ T cells were stimulated with PHA and IL-2 in

Table 1. Production of p24 Ag by SK-specific and Der p 1–specific short-term T cell lines infected in vitro with the HIV-1BaL strain

Ag Specificity	No. of T Cell Lines	p24 Ag (pg/mL) HIV-1IIIB*	p24 Ag (pg/mL) HIV-1BaL
SK	8	100 ± 17	1,460 ± 210
Der p 1	8	783 ± 120	4,234 ± 635
P values		<.005	<.005

SK-specific and Der p 1–specific short-term T cell lines were generated from PBMC of 8 Der p 1–sensitive atopic donors, as described in Materials and Methods. On day 15, T cell lines were infected with the HIV-1IIIB or HIV-1BaL strains, and on day 6 after infection, they were assessed for p24 Ag production, as previously described.

*Values represent the mean ± SEM (standard error of the mean) of p24 Ag concentrations in cell-free supernatants.

the absence or in the presence of IL-12 or IL-4, which have been shown to polarize naive T cells toward the Th1 or the Th2 profile, respectively.[11,13,19] Both IL-12– and IL-4–conditioned naive T cells were infected with HIV-1IIIB or HIV-1BaL 3 days after infection, and p24 Ag production was evaluated in T-cell culture supernatants. Moreover, to establish whether possible differences in p24 Ag production reflected different levels of viral entry or were related to a different state of cell activation and/or proliferation, parallel cultures were infected with HIV-1–CAT viruses containing HXBc2 (X4-tropic) or YU2 (R5-tropic) env proteins, which are capable of only 1 cycle of infection, and CAT activity was assessed after infection. Finally, CD4, CD69, and CD25 expression; the numbers of viable cells; and the cell proliferation state in the same cultures were also evaluated. As expected, all IL-12–conditioned T cell lines showed high IFN-γ and little IL-4 intracellular synthesis, whereas IL-4–conditioned T cell lines exhibited high IL-4 and little IFN-γ synthesis (data not shown). At day 4 after infection with HIV-1IIIB, p24 Ag production was higher in Th2-polarized than in Th1-polarized T-cell cultures. However, in contrast with the results observed in memory T cell lines, p24 Ag production was higher in Th1-polarized than in Th2-polarized BaL-infected T cell cultures (Figure 2A). Accordingly, CAT activity in cell lysates was higher in Th2-polarized naive T cells infected with X4-tropic recombinant strain HXBc2 than in Th1-polarized naive T cells; whereas CAT activity was higher in Th1-polarized naive T cells infected with the R5-tropic recombinant strain YU2 than in the infected Th2-polarized naive T cells (Figure 2B).

As a control, SK-specific (Th1) and Der p 1–specific (Th2) short-term T cell lines were infected with HIV-1BaL or HIV-1IIIB and the recombinant YU2 and HXBc2 HIV-1 strains. The cell lines were then assessed for p24 Ag production and CAT activity, respectively. As expected, p24 Ag production was higher in Der p 1–specific than in SK-specific T cells infected with either HIV-1BaL or HIV-1IIIB strain (Figure 1, Figure 2D). Accordingly, CAT activity was higher in Der p 1–specific than SK-specific T cells infected with either YU2 or HXBc2 HIV-1 strains(Figure 2E). Of note, we did not observe differences in CD4, CD25, and CD69 expression (data not shown) nor differences in the numbers of viable cells or ^{3}H-thymidine incorporation between Th1- and Th2-polarized naive or memory T cell lines (Figure 2C and F). These findings strongly suggest that differences in the expression of X4-tropic or R5-tropic HIV-1 strains between Th1- and Th2-polarized T-cell cultures are mainly related to differences in viral entry rather than in levels of CD4 expression or cell activation, viability, or proliferation.

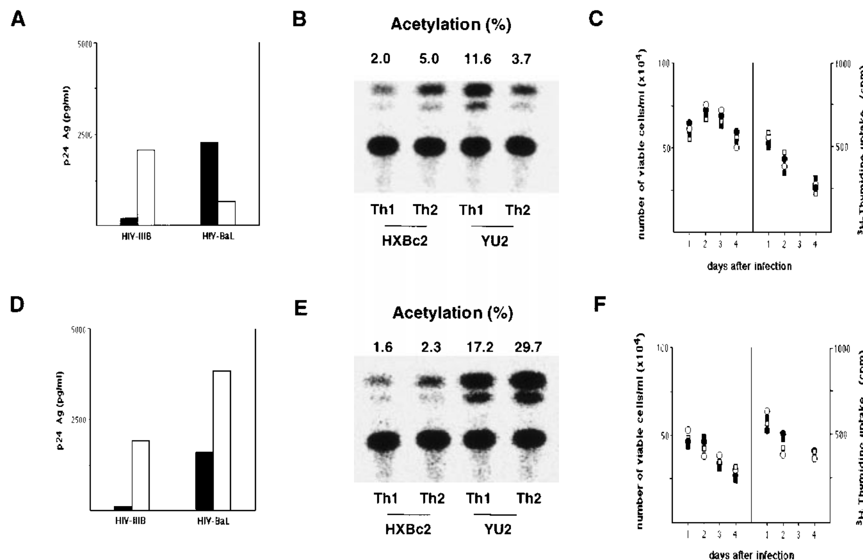

Figure 2. Differences in p24 Ag production by Th1-polarized versus Th2-polarized naive T cells are due to differences in viral entry and not in cell proliferation. Naive CD4^{+} T cells were activated with PHA and IL-2 in the presence of IL-12 (Th1-polarized) or IL-4 (Th2-polarized) and infected 3 days later with HIV-1IIIB (X4-tropic) or HIV-1BaL (R5-tropic). Parallel cultures were infected with recombinant HIV-1–CAT viruses containing HXBc2 or YU2 env proteins (X4-tropic and R5-tropic, respectively). SK-specific (Th1) and Der p 1–specific (Th2) T cell lines (Figure 1) were also infected with either HIV-1BaL, HIV-1IIIB, or HIV-1 strains YU2 or 1HXBc2. (A) Spontaneous p24 Ag release measured in supernatants of Th1-polarized (black columns) or Th2-polarized (white columns) cell cultures 4 days after infection with HIV-1IIIB or HIV-1BaL. (B) CAT activity in cell lysates from parallel cultures infected with X4-tropic or R5-tropic HIV-CAT viruses measured 4 days after infection, as described in "Materials and Methods." (C) Numbers of viable (trypan blue negative) cells in Th1-polarized (black symbols) or Th2-polarized (white symbols) T-cell cultures from day 1 to day 4 after infection with HXBc2 (circles) or YU2 (squares) HIV-CAT viruses (left panel) and levels of ^{3}H-thymidine uptake in the same culture (right panel). (D) Spontaneous p24 Ag release in supernatants of SK-specific (Th1; black bars) or Der p 1–specific (Th2; open bars) T cells 6 days after infection with HIV-1IIIB or HIV-1BaL. (E) CAT activity in cell lysates from a parallel culture infected with YU2 or HXBc2 HIV-1 strains. (F) Numbers of viable (trypan blue negative) cells in SK-polarized (black symbols) or Der p 1–polarized (white symbols) T-cell cultures from day 1 to day 4 after infection with HXBc2 (circles) or YU2 (squares) HIV-CAT viruses (left panel) and levels of ^{3}H-thymidine uptake in the same culture (right panel). A representative experiment is shown.

HIV-1 expression in Th1-like T cells is limited by the production of RANTES, MIP-1α, and MIP-1β

The kinetics of HIV-1 spread during the initial phases of Th1 or Th2 polarization of naive T cells was then analyzed in greater detail. To this end, naive T cells isolated from the UCB of 12 different donors were activated with PHA plus IL-2 in the presence of IL-12 or IL-4. Three days after activation, both IL-12– and IL-4–conditioned naive $CD4^+$ T cells were infected with the HIV-1BaL strain, and p24 Ag production was measured on days 2, 4, and 6 after infection. The results of these experiments are summarized in Figure 3A. As expected, there was a significantly higher production of p24 Ag by IL-12–conditioned (Th1-polarized) T cells than by IL-4–conditioned (Th2-polarized) T cells between days 0 and 2 and between days 2 and 4 after infection. However, between days 4 and 6, p24 Ag production was more increased in IL-4–conditioned cultures than in IL-12–conditioned cultures. Furthermore, when Th1-polarized and Th2-polarized $CD4^+$ T cell lines were infected with the HIV-1BaL strain 3 days after a secondary stimulation, p24 Ag production appeared to be significantly higher in Th2-polarized than in Th1-polarized populations (Figure 3B).

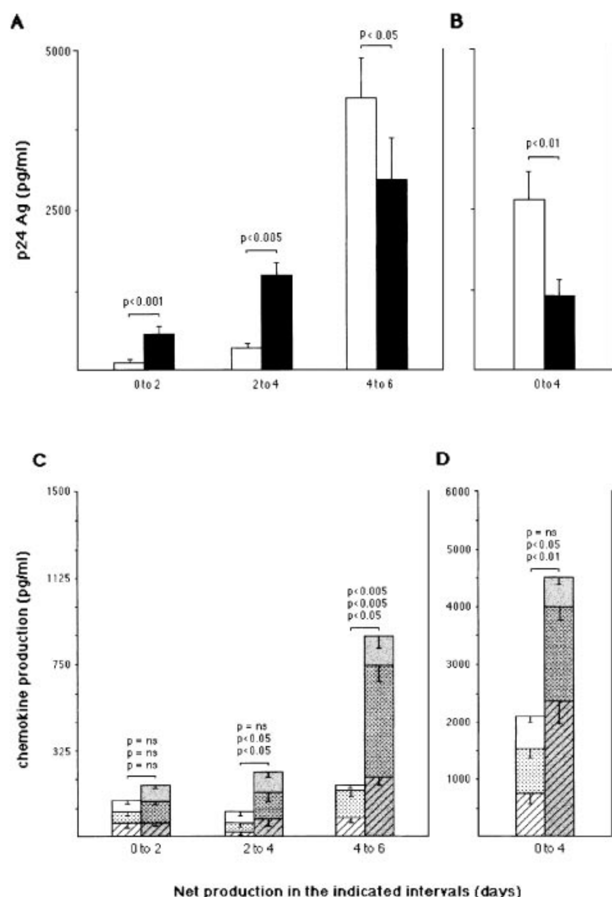

Figure 3. Kinetics of p24 Ag and β-chemokine production by Th1-polarized or Th2-polarized naive T cells infected with HIV-1BaL. Spontaneous release of p24 Ag and β-chemokines was assessed in the same supernatants of naive activated $CD4^+$ T cells infected with HIV-1BaL 3 days after primary (A and C) or secondary (B and D) stimulation. Open and closed columns represent p24 Ag concentrations found in supernatants from Th2-polarized or Th1-polarized T cells, respectively. White and gray columns represent concentrations of MIP-1α (hatched areas), MIP-1β (dotted areas), and RANTES (empty areas). Results are expressed as net production of p24 Ag or β-chemokines at the indicated days according to the following formula: concentration at $time_x$ − concentration at $time_{x-1}$. Mean values ± SE of 12 separate experiments (A and C) and 8 separate experiments (B and D) are reported.

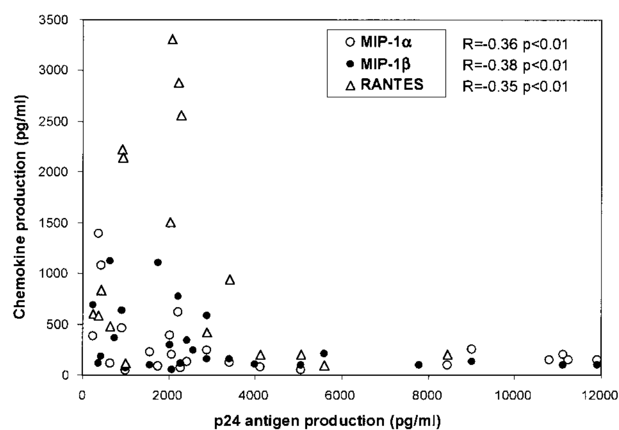

Figure 4. Inverse correlation between p24 Ag production and spontaneous release of RANTES, MIP-1α, or MIP-1β, by $CD4^+$ naive T cells infected with HIV-1BaL strain. R values were determined by linear regression analysis between p24 Ag and each chemokine concentration.

We then hypothesized that the ability of HIV-1 to spread in Th1-like T cells was limited by the production of β-chemokines RANTES, MIP-1α, and MIP-1β, which reduced the entry of R5-tropic HIV-1 strains by competing for the CCR5 coreceptor. To this end, RANTES, MIP-1α, and MIP-1β concentrations were measured in the supernatants of the same IL-12–conditioned and IL-4–conditioned activated naive $CD4^+$ T cells between days 0 and 2, days 2 and 4, and days 4 and 6 following infection with HIV-1BaL. As shown in Figure 3C, there were no differences in the production of the 3 β-chemokines between IL-12–conditioned and IL-4–conditioned T-cell cultures from days 0 to 2. However, the production of MIP-1α and MIP-1β was significantly higher in IL-12–conditioned cultures than in IL-4–conditioned cultures between days 2 and 4. Significantly higher concentrations of all the 3 β-chemokines were found in IL-12–conditioned T-cell cultures compared with IL-4–conditioned T-cell cultures between days 4 and 6 following infection (Figure 3C). After secondary stimulation, the β-chemokine content was also significantly higher in Th1-polarized than in Th2-polarized T-cell cultures (Figure 3D). On day 6, a significant inverse correlation between the amount of RANTES, MIP-1α, and MIP-1β and the concentration of p24 Ag present in the culture supernatants of the same HIV-1BaL-infected T cells could be observed (Figure 4).

To provide more direct evidence that limiting the spread of HIV-1BaL in IL-12–conditioned T cells was related to the ability of these cells to produce high amounts of β-chemokines, both IL-12–conditioned and IL-4–conditioned activated naive T cells were infected with HIV-1BaL in the absence or presence of a mixture of anti-RANTES, anti–MIP-1α, and anti–MIP-1β neutralizing mAbs. Production of p24 Ag was then measured in culture supernatants on days 4 and 6 after infection. As shown in Figure 5, the increase of HIV-1BaL expression in the presence of anti–β-chemokine antibodies was significantly higher in Th1-polarized cultures than in Th2-polarized cultures on days 4 and 6 after HIV-1 infection.

To support the concept that the lower p24 Ag production observed in Th1-oriented memory T cell lines compared with Th2-oriented memory T cell lines was also related to the higher ability of the former to produce β-chemokines, RANTES, MIP-1α

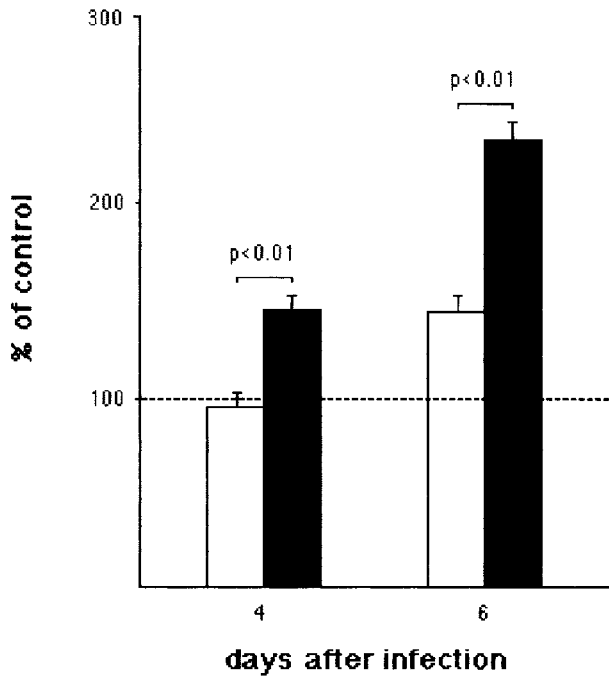

Figure 5. Increase of p24 Ag release by Th1-polarized naive CD4+ T cells infected with HIV-1BaL in the presence of anti-RANTES, anti-MIP-1α, and anti-MIP-1β neutralizing mAbs. Th1-polarized (black columns) and Th2-polarized (open columns) CD4+–activated naive T cells were infected with HIV-1BaL in the presence of a mixture of anti-RANTES, anti-MIP-1α, and anti-MIP-1β or of isotype-matched control mAbs. Values are expressed as the percent of p24 Ag released in the presence of antichemokine mAbs versus the percent of p24 Ag released in the presence of control mAbs. Columns represent mean values ± SE of 9 separate experiments.

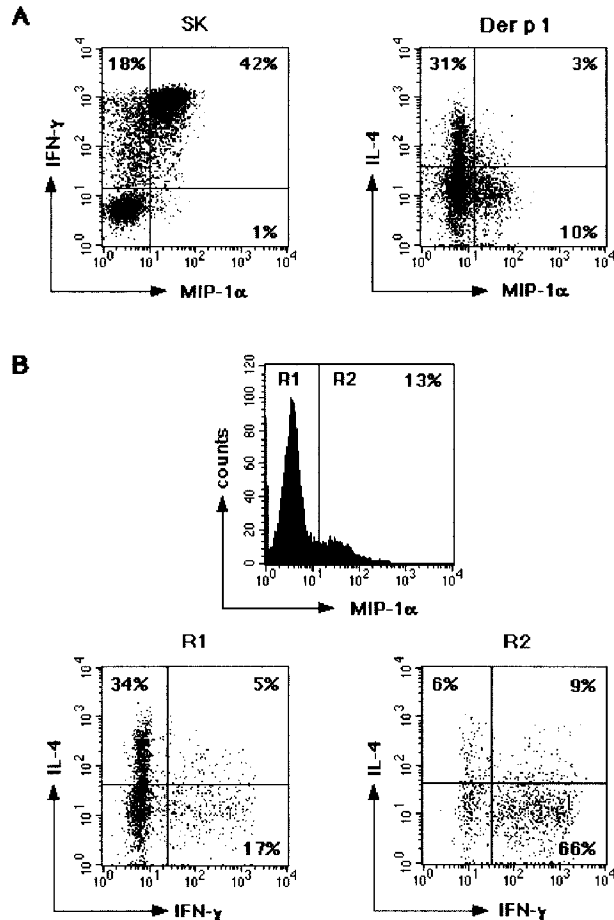

Figure 6. Intracellular detection of MIP-1α production by IFN-γ– producing Ag-specific CD4+ T cells but not by IL-4–producing antigen-specific CD4+ T cells. (A) T-cell blasts from SK-specific or Der p 1–specific short-term T cell lines were stimulated with PMA plus ionomycin on day 6 after infection with HIV-1BaL strain and simultaneously analyzed for MIP-1αb IFN-γ, and IL-4 content, as described in "Materials and Methods." (B) IL-4 and IFN-γ production in MIP-1α–negative (R1) and MIP-1α–positive (R2) Der p 1–specific CD4+ T cells shown in (A). The area of MIP-1α positivity was determined against an isotype-matched antibody.

and MIP-1β contents were measured in culture supernatants from both SK-specific (Th1-like) and Der p 1–specific (Th0/Th2-like) cell lines. As shown in Table 2, mean concentrations of the 3 β-chemokines, assessed on day 6 after infection, were significantly higher in cell-free supernatants from SK-specific T cell lines than in those from Der p 1–specific T cell lines. Furthermore, when MIP-1α synthesis was evaluated at single-cell level, a higher percentage of MIP-1α producing cells in SK-specific than in Der p 1-specific T cell lines was observed (Figure 6A). More interestingly, the great majority of MIP-1α–producing cells appeared to be contained within the population of IFN-γ–producing cells, whereas there were virtually no cells producing both IL-4 and MIP-1α (Figure 6B).

Table 2. Production of RANTES, MIP-1α, and MIP-1β by SK-specific and Der p 1–specific short-term T cell lines infected in vitro with the HIV-1 BaL strain

Ag Specificity	No. of T Cell Lines	Chemokine Concentration (pg/mL)		
		RANTES	MIP-1α	MIP-1β
SK	8	285 ± 84*	414 ± 109	2402 ± 1043
Der p 1	8	41 ± 9	102 ± 8	98 ± 30
P values		<.01	<.01	<.03

SK-specific and Der p 1–specific short-term T cell lines were generated from PBMC of 8 Der p 1–sensitive atopic donors, as described in Materials and Methods. On day 15, T cell lines were infected with the HIV-1BaL strain, and on day 6 after infection they were assessed for the production of RANTES, MIP-1α, and MIP-1β, as described in "Materials and Methods."

*Values represent the mean ± SE of chemokine concentrations in cell-free supernatants.

Discussion

It has been suggested that during HIV-1 infection there is a bias toward Th2-like responses and hence Th1 inhibition, which may contribute to the loss of control of the immune system over HIV-1 infection and result in progression to AIDS.[24] In subsequent studies, we[25] and others[26,27] were unable to support the concept of a general massive shift to a Th2 pattern in HIV-1–infected individuals. However, it was found that HIV-1 replicates more easily in Th2 and Th0 clones rather than in Th1 clones in vitro.[22] The latter finding was confirmed by some authors[28,29] but challenged by others.[30,31] The reasons for these discrepancies have become partially clearer after the demonstration that CCR5, the 7-transmembrane receptor for β-chemokines RANTES, MIP-1α, and MIP-1β that confers susceptibility to infection by M-tropic strains of HIV-1,[6,7] is predominantly or selectively expressed by activated Th1 cells.[10,11] On the other side, CXCR4, the 7-transmembrane receptor for the α-chemokine SDF-1, which serves as a cofactor for T-tropic HIV-1 strains,[8] is up-regulated by IL-4[12,13] and down-regulated by IFN-γ.[13] Accordingly, it has been shown that at least

in vitro, IL-4 also up-regulates the entry of X4-tropic HIV-1 strains in $CD4^+$ T cells.[13-15] Thus, at least one of the reasons for higher expression in Th2 cells of T-tropic (X4-tropic) HIV-1 strains is presently understood. More importantly, these findings provide evidence of why the occurrence of Th2 responses may result in a selective pressure for the emergence of X4-tropic, highly aggressive HIV-1 strains and consequently for a more rapid progression toward full-blown disease.[13-18]

The results reported here provide the explanation for the limited HIV-1 expression observed in Th1-polarized T cells infected with R5-tropic HIV-1 despite the higher expression of CCR5 by these cells.[10,11] In fact, soon after infection, HIV-1 entry is probably easier in Th1-polarized than in Th2-polarized $CD4^+$ naive T cells, which is suggested by the higher concentration of p24 Ag in supernatants of Th1-polarized T cells between days 0 and 4 after infection. This higher p24 Ag production by Th1-polarized T cells reflected a higher viral entry into T cells, as assessed by T-cell infection with defective viruses that do not go through repeated rounds of replication. By contrast, we did not observe significant differences in expression in CD4, T-cell activation markers CD69 and CD25, or in T-cell proliferation. This suggests that higher p24 Ag production in Th1-polarized T cells infected with R5-tropic HIV-1 strains in the initial phases after infection did not depend upon higher levels of replication subsequent to viral entry into T cells. However, further spread of R5-tropic strain among Th1 cells was limited. On day 6 after infection, there were no differences in p24 Ag production in T cells with prevalent Th1 or Th2 cytokine profile. More importantly, when naive $CD4^+$ T cells were infected with HIV-1BaL after secondary stimulation, p24 Ag production was even higher in Th2-polarized populations than in Th1-polarized populations, thus paralleling the results obtained in Th1-like and Th2-like memory T cells infected in vitro with the same viral strain.

The limitation of HIV-1 expression in Th1 cells appeared to be related, at least in part, to the ongoing production of RANTES, MIP-1α, and MIP-1β by the same cells. First, concentrations of these chemokines were much higher in the supernatants of both Th1-like memory T cells and Th1-polarized activated naive T cells than in those of memory Th0/Th2-like T cells and Th2-polarized activated naive T cells, respectively. Chemokine concentrations also appeared to be inversely related to the levels of p24 Ag released in the supernatants of the same T-cell cultures. In addition, the simultaneous detection at single-cell level of MIP-1α and IL-4 or IFN-γ revealed that the great majority of MIP-1α–producing cells was contained within the population of IFN-γ–producing cells, whereas there were virtually no cells that were able to produce IL-4, but not IFN-γ, that also produced MIP-1α. These findings are consistent with previous reports showing that synthesis of RANTES, MIP-1α, and MIP-1β is associated with type 1 immune response both in vitro[32] and in vivo.[33] Moreover, an inverse relationship between the spontaneous production of the 3 β-chemokines and p24 Ag by activated naive T cells infected with a R5-tropic HIV-1 strain was observed. Finally, and most importantly, infection with R5-tropic HIV-1BaL strain in the presence of anti–β-chemokine neutralizing Abs resulted in higher HIV-1 expression by Th1-polarized naive T cells, even in the more advanced phases of infection, thus paralleling or even overcoming the entity of HIV-1 expression seen at the same time in Th2-polarized T cells.

Taken together, the results of this study support the concept that the entry of R5-tropic HIV-1 strains into $CD4^+$ T cells is indeed favored by the occurrence of Th1-type effector responses via the preferential expression by Th1 cells of the CCR5 coreceptor. These findings parallel previous results demonstrating the preferential entry of X4-tropic strains in CXCR4-expressing Th0/Th2-like T cells.[13-16] However, they also indicate that the entry of R5-tropic HIV-1 strains in Th1 cells is rapidly counterbalanced by the production of chemokines that compete with the virus for its interaction with CCR5 coreceptor. This phenomenon may provide an important protective mechanism against viral spread to other cells present in the microenvironment during Th1-type effector responses. On the other hand, the possibility that other factors acting at post-entry level also influence the replication kinetics of different HIV-1 strains in Th1 and/or Th2 cells cannot be excluded. For example, triggering of the CD30 molecule, which is preferentially expressed by Th2 cells,[34] has been found to favor HIV-1 replication[35,36] through its ability to activate NFκB binding to HIV-1 LTRs.[36-38]

Thus, our findings conclude that the interaction between the type of HIV-1 infection and the pathway of the ongoing T-cell effector response, despite its complexity, may represent a crucial mechanism in determining the outcome of HIV-1 infection. These data stress the concept that the immune stimuli that preferentially evoke Th1 responses provide a minor risk of progression in HIV-infected individuals. By contrast, infections or vaccinations that preferentially stimulate Th2 responses and perhaps even the heavy exposure to innocuous allergens in atopic subjects can represent a major risk, and therefore they should possibly be avoided or reduced. The same concept must also be taken into account in the choice of vaccination protocols against HIV-1.

Acknowledgments

We thank Dr J Sodroski (Dana-Farber Cancer Institute) and Dr A Borsetti for kindly providing the HIV-CAT provirus and HXBc2 and YU2 env expressor plasmids and for helpful suggestions.

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